UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

DOCTORADO EN CIENCIAS BIOMEDICAS

"CONTROL DEL VOLUMEN CELULAR EN CONDICIONES ANISOSMOTICAS: VIAS DE MOVILIZACION DE OSMOLITOS Y CASCADAS DE SEÑALIZACION INVOLUCRADAS."

T E S I S

QUE PARA OBTENER EL TITULO DE:

DOCTOR EN CIENCIAS

P R E S E N T A :

R O D R I G O   F R A N C O   C R U Z

DIRECTOR DE TESIS:
DRA. HERMINIA PASANTES MORALES

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PRESENTA

RODRIGO FRANCO CRUZ

DIRECTOR DE TESIS

DRA. HERMINIA PASANTES MORALES

A mis dos Cristales,
a través de los cuales veo mi vida

Porque

"La ciencia más útil es aquella cuyo fruto es el más comunicable".
Leonardo Da Vinci. Artista y sabio renacentista italiano.

"La ciencia es el misticismo de los hechos; la verdad es que nadie
sabe nada". Leónidas Andreiev. Escritor ruso.

"La ciencia ha salvado mi vida". Carl Sagan. Astrofísico EE.UU.

"La ciencia apenas sirve para darnos una idea de la extensión de
nuestra ignorancia". Félicité Robert de Lamennais

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INDICE

2

ABSTRACT

Cell volume control is an important process for normal cell metabolism and homeostasis and it has been shown involved in the progression of apoptotic cell death, differentiation and migration. Cell volume adjustments involve several mechanisms from which osmolyte translocation has been shown as the primarily response following cell volume challenges. In the present PH. D. thesis, we study and characterize several mechanisms involved on the regulation of cell volume in response to hyposmotic conditions. The first part of this work shows that cells as individuals or as integrated tissues, from brain cell preparations, differentially respond to decreases in the extracellular osmolarity, regarding the reduction extent and rate. In response to sudden decreases in osmolarity, cells swelled and subsequently restore (totally or partially) their dimensions by a process named regulatory volume decrease (RVD), which was mediated by both organic (amino acids) and inorganic (K^+) release. On the other hand when cells are exposed to gradual reductions in osmolarity, they are able to either prevent or reduce the maximal swelling observed at the same hyposmotic stimulus by sudden decreases in osmolarity. This process named isovolumetric regulation (IVR) was observed mediated mainly by organic osmolyte amino acid release, in contrast to RVD. This process has important physiological implications for brain ionic homeostasis. On the second part of the thesis, we show the characterization of amino acid release in response to hyposmotic swelling. Our data shows the presence of different efflux pathways for both osmolyte amino acid taurine and neurotransmitter amino acids GABA and glutamate (followed as D-aspartate). While hyposmotic-induced taurine release (HTR), was mediated by a chloride channel blockers-sensitive pathway, modulated by tyrosine kinases and phosphatidyl inositide 3-phosphate kinase (PI3K), neurotransmitter release by hyposmotic conditions was modulated by protein kinase C (PKC) activity and dependent on cytoskeleton integrity. We proposed the possible participation of an exocytotic mechanism for neurotransmitter release by cell hyposmotic swelling and we discuss the importance of a different efflux pathway for neurotransmitter release that may be implicated in brain hyperexcitability during hyponatremia. At the last part of the present study we investigate the participation of transmembrane receptors with intrinsic tyrosine kinase activity on the osmotransduccion machinery that mediates HTR activation. HTR was dependent on the activation of the epidermal growth factor receptor (EGFR) induced by hyposmolarity. Moreover, EGFR activation by hyposmolarity mediates PI3K activation, suggesting a link between EGFR-PI3K and HTR. Other tyrosine kinases and/or RTK may also be implicated in this phenomenon. We cannot discard the participation of other signaling events on the modulation/activation of HTR, as to mention reactive oxygen species (ROS), phospholipaces (PLC and PLA_2) and $[Ca^{2+}]_i$.

RESUMEN

El volumen celular es un factor determinante en la homeostasis celular. Los cambios en el volumen celular están involucrados en el progreso de distintos fenómenos como son procesos metabólicos, excitabilidad neuronal, proliferación y diferenciación celular, así como muerte necrótica y apoptótica. En este trabajo se aborda el estudio y caracterización de distintos eventos involucrados en la regulación del volumen subsecuente a hinchamiento celular anisosmótico (condiciones hiposmóticas). Los resultados muestran la capacidad que tienen las células y tejidos de enfrentar disminuciones en la osmolaridad del medio extracelular (condiciones hiposmóticas) dependiendo de la magnitud y tasa de reducción de la osmolaridad. Mientras que condiciones súbitas de hiposmolaridad inducen importantes incrementos en volumen de manera transitoria, los cambios graduales permiten un mejor ajuste del contenido intracelular de osmolitos y por consiguiente del volumen. Esta condición puede inclusive prevenir por completo los cambios en volumen, debido al ajuste constante del mismo, o por lo menos reducir la magnitud de los cambios en volumen celular, debido a una mayor eficiencia en los procesos reguladores. Esto debido principalmente a una mayor reducción en el contenido de osmolitos orgánicos (aminoácidos) de manera preferencial en comparación con los cambios en el contenido de osmolitos inorgánicos, lo cual tiene implicaciones fisiológicas importantes para la homeostasis. La regulación del volumen en condiciones hiposmóticas involucra la translocación de solutos a través de la membrana para contrarrestar el gradiente osmótico y el flujo neto originado por el mismo. Esto debe involucrar mecanismos de detección de cambios en el volumen y de transducción de esa señal hacia la activación de vías específicas de salida de osmolitos. En este trabajo se observó que la liberación de taurina activada por hiposmolaridad o hinchamiento hiposmótico (LTAH), está mediada por una vía con características de un canal aniónico. Eventos de fosforilación en residuos de tirosina que involucran la activación del receptores tirosina cinasa y la activación de la fosfatidil inositol-3 cinasa (PI3K), están involucradas en la regulación y/o activación de la LTAH. Por otra parte, la salida de aminoácidos neurotransmisores como el glutamato y el GABA está mediada por un proceso regulado por la actividad de la proteína cinasa C (PKC) y dependiente de la integridad del citoesqueleto de actina, lo que sugiere la posible participación de un evento de fusión vesicular en este fenómeno. Esto claramente muestra la participación de distintas vías de salida de aminoácidos (una para la taurina y otra para aminoácidos neurotransmisores), lo que puede ser de alta relevancia en los cambios en la excitabilidad observados en condiciones de hiponatremia. En este trabajo también se reporta, por primera vez la participación de receptores con actividad de tirosina cinasa (RTK), como es el receptor al factor de crecimiento epidermal (EGFR), aunque no se descarte la participación de otros, como parte de la vía de transducción de señales que modula la LTAH. La participación de este receptor y otros RTK como parte del mecanismo de detección del cambio en volumen es una línea de investigación que queda abierta para ser esclarecida.

PREFACIO

En el presente trabajo de tesis doctoral, se estudiaron distintos aspectos relacionados con el control del volumen celular. Esto con el fin de esclarecer algunas de las preguntas que a lo largo del estudio del fenómeno de regulación del volumen han surgido, aspectos que a nuestro parecer, sería de gran importancia resolver, con el fin de entender este fenómeno de forma íntegra. Estas preguntas, de manera general, son: a. ¿cuál es la respuesta integral de un tejido a cambios en la osmolaridad extracelular?; b. si en condiciones fisio-patológicas en un organismo los cambios en la osmolaridad extracelular se dan de manera gradual, ¿cuáles son los mecanismos involucrados en el control del volumen en estas condiciones?; c ¿cuáles son las vías de movilización de osmolitos en condiciones hiposmóticas y cuáles los mecanismos de osmotransducción involucrados en la activación / modulación de estas vías?. En el curso de la investigación surgieron otros aspectos estudiados con el mismo objetivo mencionado.

Por el número de temas abordados, la tesis se estructura de la siguiente forma: una introducción al fenómeno del control del volumen celular, con el propósito de introducir de manera general, al proceso estudiado, incluyendo los objetivos generales del estudio. Posteriormente, y con base en los resultados obtenidos, el escrito se divide en tres secciones, cada una de las cuales presenta una introducción más definida de los procesos estudiados en relación a los objetivos específicos planteados en cada sección, y una discusión de los datos obtenidos. Asimismo se incluyen los artículos publicados con los resultados del trabajo. Finalmente, se incluye una conclusión general de las aportaciones hechas al área de estudio y su importancia. También se anexan dos apéndices con los artículos de revisión referidos en el texto y que también son producto del trabajo de tesis, así como las abreviaturas más comúnmente utilizadas. Esta organización es con el fin de dar una presentación lo suficientemente completa y comprensible del trabajo realizado.

INTRODUCCION

CONTROL DEL VOLUMEN CELULAR: IMPORTANCIA Y PARTICIPACION EN DISTINTOS PROCESOS FISIOLOGICOS

La capacidad de las células para regular su volumen es uno de los mecanismos homeostáticos más importantes y probablemente uno de los más antiguos, preservado a lo largo de la escala evolutiva. Un evento crucial en la evolución de la vida ocurrió cuando altas concentraciones de solutos no difusibles fueron encerrados dentro de una bicapa-lipídica semipermeable. Para llevar a cabo este paso, debieron de haberse desarrollado mecanismos para contrarrestar la presión osmótica dada por el equilibrio de las moléculas difusibles y el subsecuente incremento de la presión hidrostática dentro de la célula (Baslow, 1999). En el momento en que se volvió ventajoso para las moléculas primitivas rodearse con una membrana y controlar su medio, fue cuando cobró importancia el control y mantenimiento del volumen celular (Strange, 1994; Chamberlain y Strange, 1989).

Las células de los animales pluricelulares están expuestas, en condiciones fisiológicas, a un líquido intersticial isosmótico cuya composición es finamente regulada por mecanismos que controlan la ingestión y la excreción de agua y electrolitos. Para el caso de los mamíferos, se han desarrollado mecanismos, como las funciones renales, que regulan el fluido intersticial y el plasma circundante de los tejidos en condiciones normales. Sin embargo, algunos tipos de células especializadas se encuentran expuestas a medios anisosmóticos, como son las células epiteliales. Aunque la mayoría de las células de los vertebrados están en contacto con un líquido isosmótico, existen situaciones de riesgo en cuanto a su equilibrio osmótico, debido a cambios en la presión osmótica efectiva, por alteraciones en la osmolaridad o en el coeficiente de reflexión (Alvarez-Leefmans y Reus, 1996).

Aún en condiciones de isosmolaridad, la constancia en el volumen celular puede ser modificada por alteraciones en la concentración iónica extracelular, como son un incremento en la concentración de K^+ y $HCO3^-$ extracelular, así como por la presencia de aniones orgánicos tales como acetato, lactato o propionato. La acumulación de aminoácidos a partir del transporte acoplado a Na^+ también origina hinchamiento celular (Lang, et al., 1998; Waldegger, et al., 1998). En teoría, cualquier reacción metabólica que

origine cambios en la síntesis o degradación de moléculas, y por consiguiente cambios en la osmolaridad intracelular, originará cambios proporcionales en el volumen. Sin embargo, estos son inmediatamente contrarrestados por mecanismos de control del volumen celular en condiciones fisiológicas, impidiendo un cambio en volumen

Los efectos más obvios de cambios en el volumen celular son mecánicos, lo cual puede alterar la función celular. En las neuronas, por ejemplo, debido a que su función depende de su arquitectura, estratificación y la localización especializada de las sinapsis, esta puede verse seriamente comprometida por cambios en el volumen. A nivel celular, el aumento o disminución del volumen puede alterar el metabolismo debido al cambio en las concentraciones de enzimas y substratos, y modificar los niveles de moléculas que participen en la transducción de señales (O´Neill, 1999). Por otra parte, un cambio en el volumen citosólico puede provocar alteraciones en la función de organelos como resultado de fluctuaciones en el pH intracelular y las concentraciones de enzimas, cofactores, calcio y fuerza iónica. Cambios en el volumen de células individuales tiene implicaciones importantes no solo para su funcionalidad individual, sino para la del órgano y por consiguiente para organismo al que pertenece. Además de la necesidad de mantener constante la concentración intracelular de solutos, muchos de los cuales son parte de eventos complejos de señalización, se ha propuesto recientemente que el volumen celular participa de manera directa en el control del metabolismo y expresión de genes, como señal de proliferación, muerte y migración celular, así como "disparador" de mecanismos que originen la inserción de proteínas membranales, canales, receptores y transportadores (Pasantes-Morales, 1996). Debido a su alta concentración intracelular, las proteínas normalmente se encuentran cercanas unas con otras, lo que facilita y modula su interacción física y enzimática, por lo que cambios en el volumen celular y por ende en el volumen citosólico, pueden afectar la interacción y propiedades cinéticas de distintos procesos enzimáticos (Meijer, 2003). A su vez cambios en el volumen celular interfieren con el reciclamiento de receptores membranales, y modifican la liberación de hormonas y neurotransmisores.

Regulación del volumen en el cerebro. Edema cerebral.

Para el caso de las neuronas, este tipo celular se encuentra continuamente sometido a alteraciones en el contenido de solutos intracelulares, debido a la acción de

neurotransmisores, hormonas y trenes de impulsos nerviosos, los cuales inducen cambios incesantes en los flujos netos de solutos y agua a través de su membrana plasmática. La activación de canales de Na^+ y catiónicos no selectivos, por aminoácidos neurotransmisores excitadores como el glutamato tendería a hinchar a las neuronas, mientras que la activación de canales de K^+ o canales aniónicos por neurotransmisores inhibidores como el GABA puede producir encogimiento. Sin embargo, estos cambios en volumen se contrarrestan de inmediato por mecanismos de control crónico del volumen. Los procesos fisiológicos involucrados en el control del volumen en el cerebro tienen implicaciones clínicas importantes, ya que cambios pequeños en el volumen pueden producir profundas secuelas clínicas. Por ello, la regulación de volumen en este órgano ha sido ampliamente estudiada.

El edema cerebral está dado por un incremento en la concentración de agua intracerebral, y es una reacción del cerebro a muchos cuadros patológicos. El edema cerebral representa un cuadro clínico muy severo. Se ha reportado que un incremento por arriba del 5% del volumen cerebral puede originar mortalidad y morbilidad substancial (Trachtman, 1992). Durante el edema cerebral, el volumen del parénquima cerebral (espacio intra o intercelular) se incrementa en detrimento del volumen sanguíneo y del líquido cefalorraquídeo, lo que causa un desbalance entre el volumen intracraneal y la presión intracraneal. La alteración o interrupción del metabolismo energético en las células representa el inicio del mecanismo patogénico en el edema cerebral. Esto es seguido por un daño mayor y la extensión de este cuadro a zonas adyacentes, lo que excede las capacidades locales de compensación. La disminución del líquido cerebroespinal y la pérdida de volumen sanguíneo conllevarían finalmente a daño cerebral y profundas secuelas clínicas. Mientras la presión intracraneal se eleva rápidamente, el desplazamiento caudal del parénquima del cerebro a través del foramen magnum puede causar la muerte por paro cardiaco o respiratorio debido a la opresión de los núcleos del tallo cerebral. El edema cerebral se puede clasificar según el compartimento involucrado en el incremento en volumen o contenido de agua. El edema vasogénico es aquel que se da por incremento en el contenido de agua en el espacio intersticial, principalmente por un aumento en el contenido de agua total del cerebro por alteración de la permeabilidad de la barrera hematoencefálica, mientras que el edema citotóxico es aquel que involucra un incremento en el volumen de los componentes

celulares del tejido cerebral. En este caso, nos interesa más el edema citotóxico que involucra un aumento en el volumen celular.

El edema celular o citotóxico es el incremento en el volumen de los elementos celulares del parénquima cerebral: inicialmente los elementos gliales, neuronas y después las células endoteliales. Este es un edema de tipo osmótico, originado por alteraciones en la presión osmótica, sin embargo se origina por diferentes factores que alteran el intercambio membranal e incrementos en la presión osmótica y por ende el incremento en el volumen de agua intracelular. El hinchamiento celular y la disminución del área interglial ocurren primero y continúa con la disminución del líquido cefalorraquídeo y el colapso vascular. El edema celular por si solo (sin la combinación del edema vasogénico) ocurre simplemente por un cambio en la distribución de agua del espacio intracelular y extracelular con un decremento concomitante de este último, pero sin un incremento en el volumen total del cerebro y por lo mismo sin un incremento en la presión intracraneal. El control del volumen celular en el cerebro tiene implicaciones funcionales relacionadas a su topología y a las alteraciones en las dimensiones del espacio extracelular. Se ha reportado que el espacio extracelular puede disminuir de su valor normal de 20% del volumen total, hasta un 5% en condiciones de isquemia, principalmente por un incremento en el volumen de los astrocitos (Kimelberg, 2000). El hinchamiento celular afecta la difusión de metabolitos, substratos y neurotransmisores, e incrementa la concentración localizada de sustancias y mensajeros así como la resistencia eléctrica extracelular (Kimelberg, 1995; 2000; Sykova, 2001)

Los cambios en el volumen celular en el cerebro pueden ser consecuencia de alteraciones en la osmolaridad de los fluidos externos o debido a cambios en la distribución de iones y agua durante condiciones isomóticas, ambos ocasionando un desbalance en la presión osmótica (Ballany y Grafe, 1988). El desbalance en la presión osmótica intra e intercelular es causado por: A) Incremento en la presión osmótica intracelular en condiciones isosmóticas (hiperosmosis intracelular o aumento en la concentración intracelular de solutos), en cuadros de hipoxia-anoxia, isquemia, encefalopatía hepática, excitotoxicidad y situaciones de estrés oxidativo, y por el bloqueo de los mecanismos de transporte fisiológicos; síndromes de desbalance osmótico (cetoacidosis diabética, hemodiálisis). Y B) por hiposmolaridad extracelular, en donde el edema celular anisosmótico ocasionado por alteraciones en la hipotonicidad del plasma,

ocurre sin daño celular primario y está asociado a patologías como hiponatremia, síndrome de secreción inapropiada de hormona antidiurética, intoxicación por agua, uso inapropiado de diuréticos, deshidratación, diabetes mellitus, diabetes insípidus, y probablemente, un componente hiposmótico en la isquemia.

MECANISMOS DE REGULACION DEL VOLUMEN

Los mecanismos fisiológicos involucrados en la regulación del volumen difieren a lo largo de la escala evolutiva. Las células animales carecen de paredes celulares rígidas y no pueden soportar gradientes de presión altos a través de sus membranas; por esto, la regulación de su volumen está dada por cambios en el contenido intracelular de solutos en la dirección necesaria para corregir el desequilibrio osmótico. Estos cambios incluyen transporte de iones y osmolitos orgánicos a través de la membrana así como la acumulación o síntesis de los mismos (Häussinger, 1996; Kenneth, 1994; Pasantes-Morales, 1996). Recientemente se ha descrito otro mecanismo importante en el control del volumen como es el bombeo activo de agua en contra de su gradiente de concentración, a través de cotransportadores de osmolitos (Baslow, 1999, Loo, 2002). Por otra parte existen mecanismos estáticos que presentan ciertos tipos celulares que les permiten enfrentar cambios en la presión osmótica como son una permeabilidad reducida al agua. En general, cada tipo celular utiliza varios mecanismos de control del volumen. La diversidad de todos estos mecanismos reguladores, permite a las células mantener la constancia del volumen celular frente a una gran variedad de situaciones.

Osmolitos involucrados en el control del volumen celular

La identidad y concentraciones intracelulares de los principales solutos varían entre los diferentes tipos de células, así como con las condiciones a las cuales las células están expuestas (Kirk, 1997). Las dos principales clases de solutos en las células son: 1) compuestos impermeantes orgánicos como proteínas en su mayoría; y 2) compuestos permeantes de dos tipos: compuestos inorgánicos, iones que contribuyen a determinar la osmolaridad tanto intracelular como extracelular; y compuestos orgánicos (denominados también osmolitos orgánicos o compatibles), como son ciertos compuestos metilados (metilaminas: glicerofosforilcolina y betaína), polialcoholes (sorbitol o inositol), urea y aminoácidos libres y sus derivados. Los osmolitos son aquellos solutos en el medio intra o

extracelular, cuya presencia contribuye a la osmolaridad del medio, i.e. a los cuales la célula es impermeable o cuyo coeficiente de reflexión es muy bajo (compuestos osmóticamente activos) (Yancey, 1994).

Los aminoácidos en particular, son los osmolítos orgánicos que parecen estar más involucrados en la regulación del volumen, tanto en células de organismos multicelulares como unicelulares, siendo la alanina el aminoácido más abundante (30-70% de la poza total intracelular) para el caso de los protozoarios y eucariontes unicelulares. Para el caso de células de organismos mas complejos, la taurina tiene un papel predominante. La taurina (NH_2-CH_2-CH_2-SO_3) es un aminoácido azufrado que no forma parte de las proteínas y, con excepción de la síntesis del ácido taurocólico en el hígado, no participa en ninguna función en el organismo. La taurina se acumula en las células por un transportador dependiente de Na^+. Este aminoácido se encuentra presente en todos los tejidos animales y en algunos, particularmente en los tejidos excitables, alcanza concentraciones muy altas. Así, la retina contiene niveles de taurina superiores a 40 mM mientras que en el corazón y el músculo estriado la concentración es del orden de 10-40 mM. La taurina tiene un pK_2 de 8.82, y por lo tanto se encuentra libre en el citosol de forma zwitterionica principalmente. Estas características de la taurina, que durante mucho tiempo no se les atribuía una función en específica, adquieren significado cuando se piensa en una función como osmolito. De hecho, la taurina puede considerarse como el osmolito ideal, ya que debido a su inercia metabólica puede moverse dentro y fuera de la célula modificando el contenido de agua sin afectar el metabolismo celular. Se ha sugerido que la taurina podría estar actuando como osmolito no sólo a nivel celular sino en el mantenimiento del volumen de espacios intracelulares, tales como los del retículo sarcoplásmico en el músculo, los canalículos secretores en las glándulas y los espacios discales en los fotorreceptores (Pasantes et al., 1998).

Además de la taurina, otros aminoácidos como glutamina, glutamato, glicina, prolina, serina, treonina, β-alanina, aspartato y GABA pueden también funcionar como osmolitos. Aunque la concentración intracelular de algunos de estos aminoácidos sea baja, la suma de todos ellos contribuye de manera significativa a la respuesta a cambios en el volumen celular, mediante la activación de mecanismos de transporte y/o cambios en las tasas de síntesis y proteolisis, de manera que la concentración intracelular de aminoácidos se incrementa o disminuye en respuesta a encogimiento o hinchamiento de

la célula. Los aminoácidos tienen un papel importante durante la adaptación a pequeños cambios en la osmolaridad extracelular; sin embargo en algunos casos, su contribución parece ser menor en la adaptación a osmolaridades excesivas como en el caso de médula del riñón. Esto queda a discusión más adelante para el caso de la hiponatremia asociada a edema cerebral.

Control crónico del volumen celular

Como ya se mencionó, en condiciones fisiológicas normales el volumen de cada tipo celular se ve alterado por el gradiente osmótico impuesto por la presencia de moléculas no difusibles (necesarias para mantener sus funciones metabólicas) tanto en el espacio intracelular como en el extracelular. La presencia de aniones polivalentes (proteínas) trae como consecuencia que la presión coloidosmótica intracelular sea mayor, por lo que la célula se hincharía sin la necesaria existencia de mecanismos de control del volumen en estas condiciones. La regulación crónica del volumen celular es aquella que se realiza de manera constante en condiciones fisiológicas. La regulación crónica del volumen se da principalmente por modificaciones en los flujos activos y pasivos de iones, y por las permeabilidades transmembranales y gradientes electroquímicos de los mismos. Se ha descrito a este tipo de regulación como un balance entre el transporte activo por la bomba o ATP-asa de Na^+/K^+, y fugas pasivas a través de diferentes vías (Tosteson y Hoffmann, 1960). La regulación crónica del volumen celular depende por lo tanto del bombeo activo de Na^+ como de la permeabilidad pasiva al K^+ (Jakobsson, 1980). Debido a esta acción de la bomba de Na^+/K^+, el Na^+ que entra a la célula por electrodifusión (a través de canales de Na^+) o por sistemas de transporte secundario (cotransportadores de Na^+/H^+ y de Na^+/Ca^{2+}, cotransportadores de $Na^+/K^+/2Cl^-$, o los cotransportadores de Na^+ y aminoácidos), es transportado hacia el exterior, de tal manera que la membrana se comporta como si fuese impermeable al Na^+, por lo menos en el estado de reposo. La regulación crónica del volumen celular también puede involucrar síntesis y degradación de macromoléculas. Es importante mencionar la importancia de distintas bombas iónicas que funcionan de manera constante en el restablecimiento de los gradientes iónicos de los compartimentos extracelular e intracelular, posterior a una alteración inicial de los mismos por distintos procesos metabólicos. Recientemente se ha propuesto que la regulación crónica del volumen celular debe estar bajo control genético. Este tipo de

regulación es de vital importancia para la sobrevivencia y buen funcionamiento de la célula (Figura 1).
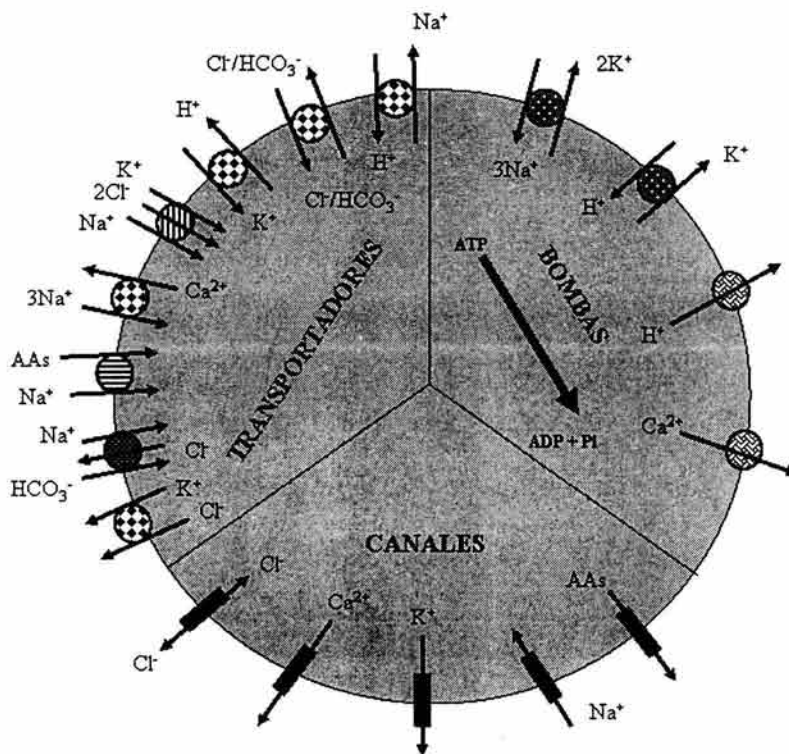
Figura 1. Control del volumen celular en condiciones fisiológicas (regulación crónica). El control del volumen se da principalmente por el equilibrio originado por la conjunción de distintos sistemas de transporte de iones y solutos orgánicos a través de la membrana por transportadores, canales y bombas. La teoría de bomba y fuga involucra la participación de la ATPasa Na^+-K^+ y de la permeabilidad pasiva del K^+. Es importante destacar la función constante de distintas bombas que mantienen los gradientes iónicos.

Regulación aguda del volumen celular

Además de poseer la capacidad de mantener su volumen constante, las células animales están dotadas de mecanismos que les permiten ajustar su volumen en condiciones anisosmóticas. Como ya se mencionó antes, las membranas celulares son altamente permeables al agua. Con excepción de plantas y bacterias, en las cuales la rigidez de la pared celular impide cambios en el volumen, las diferencias en la concentración de solutos entre el espacio intracelular y el extracelular debido a alteraciones en uno o ambos compartimentos (condiciones anisosmóticas), originan el flujo neto de agua a través de la membrana. Esto debido principalmente a gradientes de presión osmótica con el objetivo de corregir el desbalance originado, modificándose por consiguiente el volumen celular (Macknight, 1988). Sin embargo las células no se

comportan siempre como osmómetros perfectos, ya que posterior al cambio en volumen originado por la entrada o salida de agua, existe un proceso regulador de volumen que permite a las células recuperar, aunque sea de forma parcial, sus dimensiones normales (Hallows y Knauf, 1994). La regulación aguda del volumen celular es un proceso discontinuo que involucra principalmente la activación de vías de movilización latentes. Este tipo de regulación es muy importante para células expuestas a medios sujetos a amplios rangos de osmolaridad o cuando la osmolaridad normal se pierde ya sea durante un proceso fisiológico "normal" (como la captura de Na^+ dependiente de nutrientes) o por un proceso patológico; por lo que mediante este proceso de regulación activa se puede compensar desbalances ocasionales entre flujos de bombeo y fuga (Figura 2).

Cuando las células animales se exponen agudamente a medios anisosmóticos, inicialmente se comportan como osmómetros perfectos, sufriendo cambios en su volumen debido a movimientos pasivos de agua a través de la membrana (Kenneth, 1994); sin embargo; transcurrido un tiempo determinado, se observa que las células son capaces de regular su volumen hasta recuperar de manera parcial sus dimensiones originales. La regulación del volumen celular ha sido estudiada tanto en organismos procariontes (Chamberlain y Strange, 1989) como en organismos eucariontes, desde invertebrados hasta vertebrados así como en las plantas (Morgan, 1984). En mamíferos, el estudio se ha realizado en distintos tipos celulares como son eritrocitos, hepatocitos, linfocitos, fibroblastos, etc. (Lang et al., 1998). Para una mejor revisión sobre la diversidad de mecanismos involucrados en el control del volumen celular en distintos tipos celulares, referirse a Lang, et al., 1998b.

Decremento Regulador del Volumen (DRV)

En un medio hiposmótico las células inicialmente se hinchan, para después llevar a cabo una fase de encogimiento compensatorio, mediante la pérdida de solutos junto con agua osmoticamente obligada. A este proceso se le denomina Decremento Regulador del Volumen (DRV) (Figura 3). En células de vertebrados superiores, los iones tales como K^+, Na^+, Cl^- y HCO_3^-, comprenden la mayor parte de los solutos osmóticamente activos, siendo el K^+ y el Cl^- los de mayor concentración intracelular, por lo que el DRV se da por una pérdida neta de K^+ y Cl^-. Esto puede ocurrir mediante la activación del cotransporte

de K^+ - Cl^-, o la activación por separado de canales de K^+ y Cl^-, (Knauf, 1986; Knoblauch, 1989) que pueden o no, ser interdependientes.
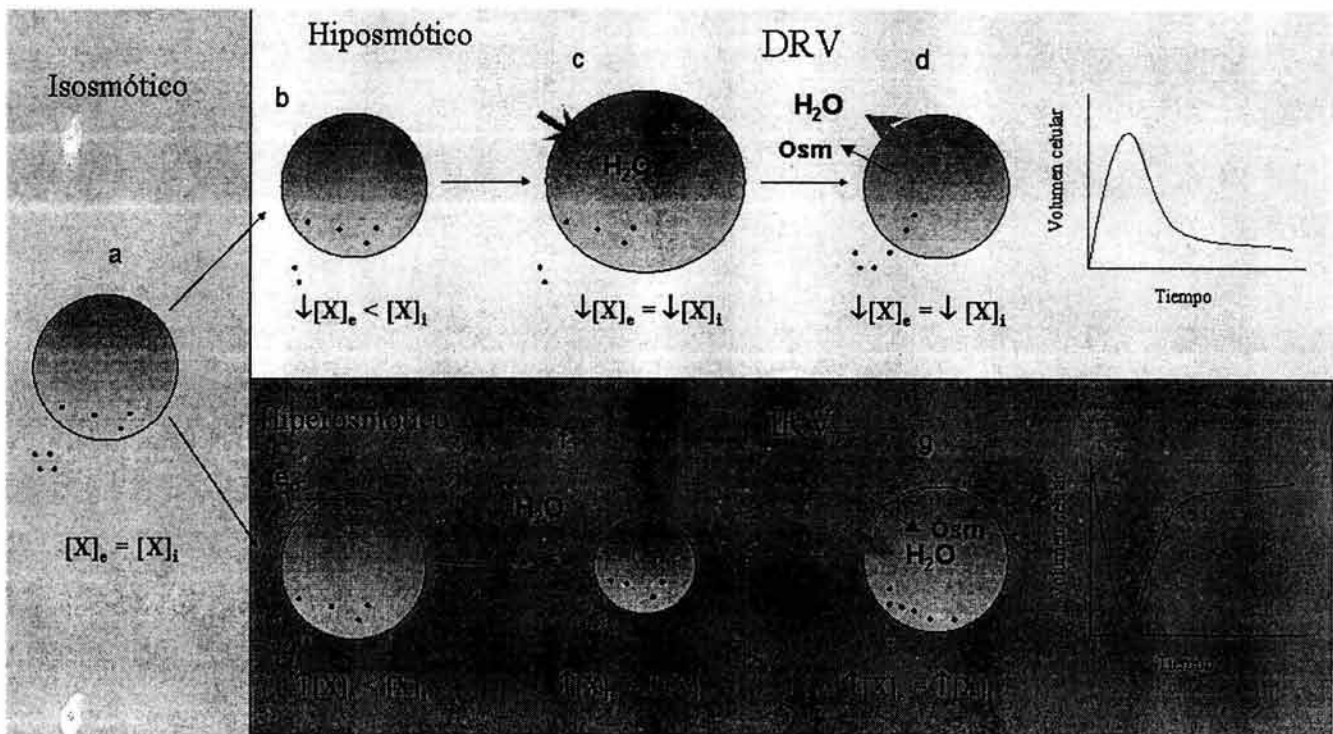
Figura 2. Control agudo del volumen celular en condiciones anisosmóticas. En condiciones isosmóticas (a) la concentración de solutos en ambos compartimentos, extracelular $[X]_e$, e intracelular $[X]_i$ es la misma (no así la identidad de los mismos), por lo que la presión osmótica en ambos compartimentos esta equilibrada. En condiciones hiposmóticas (b), o hiperosmóticas (e), cuando $[X]_e$ diminuye o incrementa según sea el caso respecto a $[X]_i$, se origina el flujo neto de agua debido al gradiente osmótico impuesto, hasta que $[X]_i$ alcanza un nuevo equilibrio respecto a $[X]_e$ (c y f), lo que modifica inicialmente el volumen celular (ver gráficos insertados a la izquierda). Posteriormente y persistiendo la anisosmolaridad (d y e) la célula es capaz de recuperar de manera parcial o total su volumen inicial mediante la activación de distintas vías de translocación de osmolitos que conllevan al movimiento de agua osmoticamente obligada sin alterar el equilibrio entre $[X]_e$ y $[X]_i$. A estos procesos se les denomina decremento regulador del volumen DRV e incremento regulador del volumen IRV, respectivamente.

Los canales aniónicos activados por hinchamiento no son selectivos, permitiendo el paso no sólo de Cl^- sino también de HCO_3^-, así como de aniones orgánicos y osmolitos orgánicos neutros. Adicionalmente el cotransportador Na^+/HCO_3^- puede estar participando en el DRV. Entre otras vías utilizadas para la movilización de K^+ y Cl^- están el cotransporte electroneutro de K^+/Cl^-, el cual se ha visto preferentemente activado por hinchamiento isotónico en algunas células. Otro mecanismos para liberar KCl, es la activación paralela del intercambiador K^+/H^+ y el intercambiador Cl^-/HCO_3^-, el H^+ y el HCO_3^- en el interior forman CO_2, el cual difunde posteriormente al exterior, por lo que no es osmóticamente activo. En condiciones hiposmóticas en algunos tipos celulares cuya concentración de Na^+ intracelular es alta se observa la extrusión de Na^+ por la acción

15

reversa del intercambiador Na^+/Ca^{++} con una salida de Ca^{++} paralela por la ATPasa de Ca^{++}, así como la activación de una ATPasa de Na^+ y una ATPasa de Na^+-K^+ insensible a ouabaina. Así mismo la entrada de Ca^{++} a través de canales catiónicos no selectivos activa la salida de K^+ a través de canales de K^+ sensibles a Ca^{++}. Los osmolitos orgánicos también participan de manera importante en el DRV; la disminución en el contenido intracelular de estos se lleva a cabo mediante fenómenos de liberación y/o incrmento en la síntesis de macromoléculas. Así, se ha visto que la movilización de aminoácidos (principalmente la taurina) esta involucrada en el DRV en varios tipos celulares de vertebrados, así como la liberación de sorbitol, mio-inositol y betaina (Ballantyne y Chamberlain, 1994; Yancey, 1994; Lang et al, 1998; 1998b).
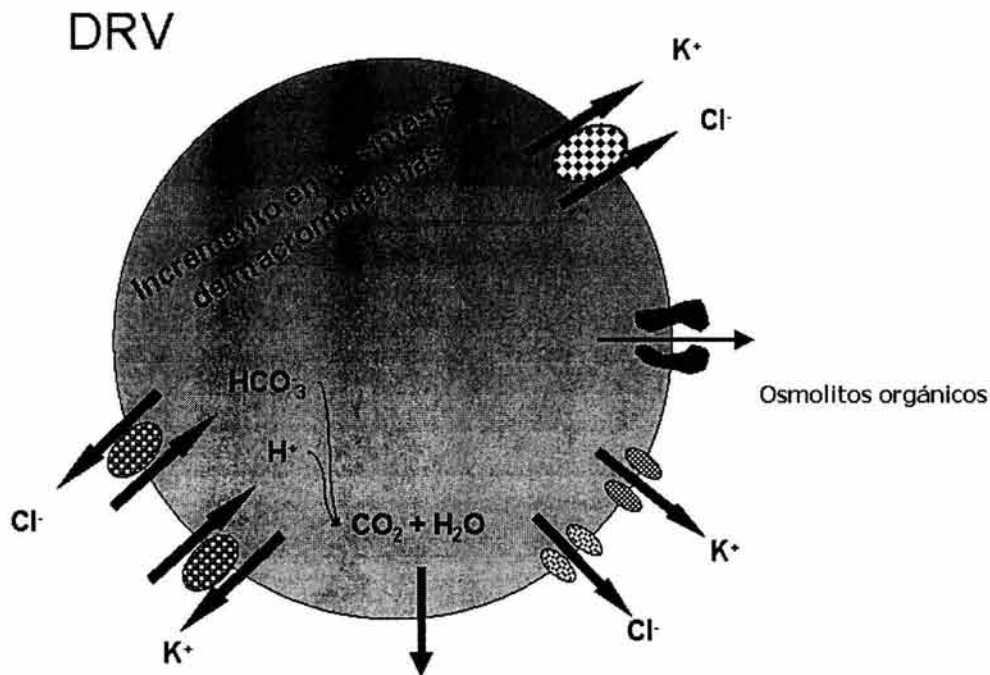
Figura 3. El decremento regulador del volumen (DRV) se da por la activación de mecanismos de salida de osmolitos como son el cotransportador K^+/Cl^-, los intercambiadores $Cl^-/HCO3^-$ y K^+/H^+, principalmente vías difusionales independientes de salida de K^+, Cl^- y osmolitos orgánicos.

El control de volumen como cualquier proceso homeostático, esta dado por la interacción de tres parámetros como son un receptor, un centro regulador o codificador y un efector. La regulación del volumen debe involucrar mecanismos que le permitan detectar o sensar cambios en el volumen (receptor) ya sean transitorios o prolongados.

Estos a su vez deben de transducir la señal en una vía de señalización (centro codificador) que module o active la movilización de osmolitos (efector) para contrarrestar el cambio en volumen. La mayoría de los trabajos en cuanto a la caracterización de los procesos involucrados en el control del volumen se han enfocado en el estudio de los efectores o vías de movilización de osmolitos en respuesta a cambios en volumen, sin embargo en muchos casos las identidades específicas de los mismos no han sido del todo esclarecidas. Para el segundo caso referente a las vías de señalización involucradas, el esfuerzo reciente ha generado una gran cantidad de resultados los cuales involucran a un sin número de vías como son: cinasas de proteínas, cinasas de fosfolípidos, fosfatasas, fosfolipasas y proteínas G. Lo que sugiere una especialización en este sentido referente al tipo de vía de movilización activada y osmolito involucrado, así como tipo de preparación (tejido o cultivo celular), o tipo celular estudiado. Los mecanismos involucrados en la detección inicial del cambio en volumen han sido poco estudiados a la fecha.

OBJETIVO GENERAL

El objetivo general de este proyecto fue caracterizar los procesos involucrados en la regulación del volumen celular subsecuente a hinchamiento hiposmótico. Particularmente se estudiaron la participación de los aminoácidos como osmolitos orgánicos involucrados en este fenómeno. El trabajo está dividido en tres partes:

1. Primera parte.   Caracterización de la liberación de osmolitos y cambios en el volumen celular en condiciones anisosmóticas: Respuesta a cambios graduales y súbitos de osmolaridad.

2. Segunda parte. Estudio de la vía de movilización de osmolitos activada por hiposmolaridad. Caracterización de algunos de los elementos de las vías de señalización involucradas en la activación y/o modulación de las vías de translocación.

3. Tercera parte. Participación de receptores transmembranales como sensores de cambios en el volumen celular o como mecanismos de amplificación de la vía de transducción

PRIMERA PARTE

REGULACION ISOVOLUMETRICA EN PREPARACIONES DE CELULAS NERVIOSAS

ANTECEDENTES

La regulación del volumen se ha estudiado principalmente en modelos de cambios súbitos de osmolaridad que, por ende, inducen cambios abruptos de volumen, lo que no refleja en muchos casos, los cambios de volumen que ocurren bajo distintas condiciones como la hiponatremia. En estas condiciones los cambios en la osmolaridad del medio extracelular son pequeños y graduales, aún en condiciones de hiponatremia crónica. El estudio de los cambios en volumen *in vitro* bajo condiciones de cambios de osmolaridad gradual se comenzó a caracterizar por el grupo de Lohr y Grantham en 1986, lo que ha generado el surgimiento de distintos trabajos bajo el mismo paradigma experimental, que han aportado información muy importante al respecto, sin embargo, no siendo muchos estos trabajos, preguntas como cuáles son los mecanismos de movilización de osmolitos y las vías de señalización involucradas siguen sin ser respondidas.

Regulación Isovolumétrica (RIV)

En un estudio realizado por Lohr y Grantham (1986) en células renales se observó que estas células tenían la capacidad de mantener su volumen constante en medios anisosmóticos, siempre que el cambio en osmolaridad se diera en forma gradual y a una tasa determinada. Este fenómeno se denominó Regulación Isovolumétrica (RIV), cuyo significado no involucra la ausencia de cambios en volumen, sino una regulación *pari pasu* de la concentración de osmolitos respecto al gradiente osmótico generado, siempre y cuando el cambio gradual en volumen sea a una tasa que le permita a la célula activar los mecanismos de transporte de osmolitos necesarios. Esta regulación, si los cambios osmóticos son crecientes (el medio se hace hiperosmótico), se lleva a cabo mediante la captura de solutos y acumulación de electrolitos como resultado de alteraciones en los mecanismos de intercambio iónico en la membrana o mediante la ATPasa Na^+-K^+ y el cotransportador $Na^+/K^+/2Cl^-$ (Lohr et al.,1989; Mountian y Van Driessche, 1997). Si los cambios en la osmolaridad son decrecientes (el medio se hace hiposmótico) la regulación
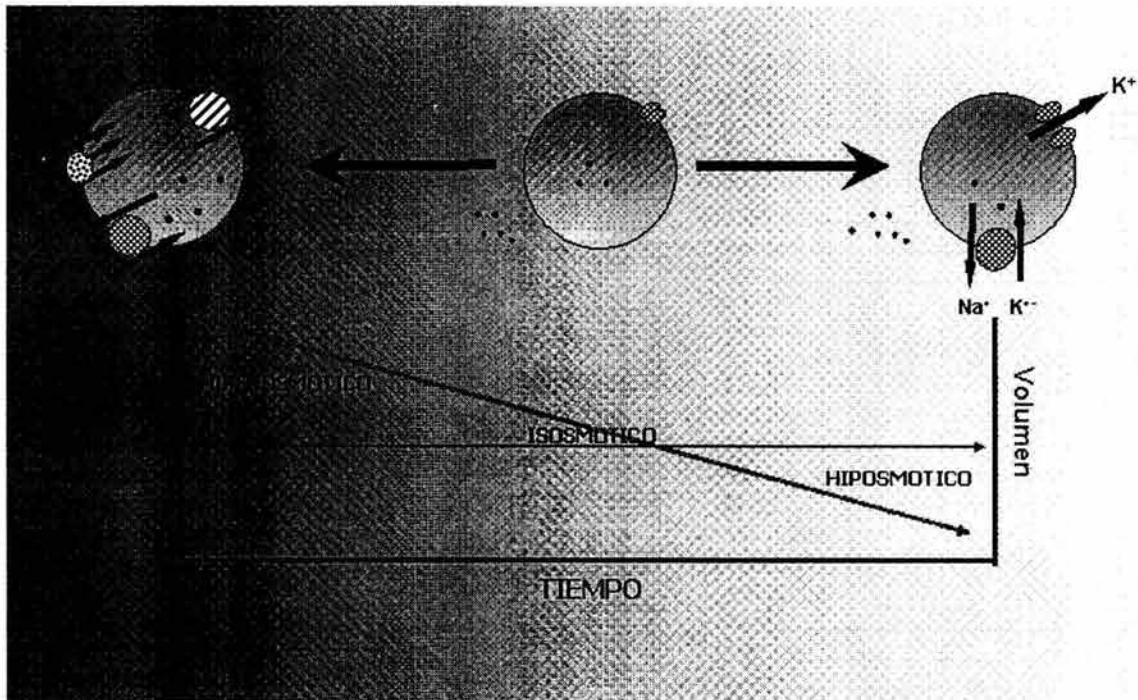
Figura 4. Regulación isovolumétrica (RIV). En condiciones de cambios graduales de osmolaridad algunos tipos celulares son capaces de mantener su volumen inicial (o dentro de ciertos valores) mediante la regulación constante del contenido intracelular de osmolitos, esto en relación tanto a la tasa como a la magnitud de cambio en la osmolaridad del medio.

se consigue mediante la pérdida aparente de solutos intracelulares por mecanismos que no están totalmente esclarecidos (Lohr, 1990; Van Driessche et al., 1997) (Figura 4). Sin embargo, no todas las células expuestas a cambios graduales de osmolaridad tienen la capacidad de llevar a cabo RIV. En un estudio reciente se demostró que los eritrocitos expuestos a cambios graduales de osmolaridad no son capaces de regular su volumen de manera eficiente debido a que la pérdida intracelular de osmolitos (K^+ y taurina), no es suficiente para contrarrestar el flujo neto de agua originado por el gradiente osmótico impuesto (Godart, 1999). Aunque en el DRV y el IRV se tienen bien caracterizados los principales mecanismos de regulación del volumen, en la RIV no se han caracterizado a fondo. De hecho actualmente sólo existen estudios en una línea celular de nefrona (A6) y en túbulos renales proximales (Lohr et al., 1990; Van Driessche et al., 1997). En tejido nervioso, la regulación isovolumétrica sólo se ha estudiado en glioma de rata C6 en condiciones hipertónicas (Mountian y Van Driessche, 1997), donde se ha encontrado que el aumento gradual en la osmolaridad del medio conlleva a una captura de electrolitos por

la bomba de Na^+/K^+ y el cotransportador $Na^+-K^+-2Cl^-$. La contribución de los osmolitos al RIV en células nerviosas no se conoce.

Hiponatremia

El Na^+ es el electrolito extracelular principal y el catión osmoticamente activo más importante. El cuerpo regula la concentración de Na^+ en el plasma, al ajustar el contenido de agua de los fluidos extracelulares y manteniendo el contenido total de Na^+ en el organismo dentro de un rango controlado. Sin embargo, alteraciones en la concentración extracelular de este ión en condiciones patológicas (hipernatremia si aumenta la concentración extracelular, e hiponatremia si disminuye), induce cambios en el volumen celular. La hiponatremia puede ser definida como una baja anormal en la concentración de sales de Na^+ y refleja un exceso de agua, relativa a la concentración del Na^+, ya sea por la pérdida de este ión, o por incremento en el contenido de agua en el organismo. Aunque el riñón es un órgano importante en la patogénesis, el órgano blanco de los cambios que producen morbilidad y mortalidad es el cerebro. En algunos casos, la hiponatremia no refleja necesariamente una disminución en la osmolaridad del medio extracelular (Oster y Singer, 1999).

El edema cerebral asociado con la hiponatremia puede originar severas secuelas clínicas de manera secundaria como edema pulmonar, infarto cerebral, ceguera cortical, estado vegetativo persistente, paro respiratorio y coma. La hiponatremia es el desorden clínico de electrolitos más común dentro de los hospitales. La hipotonicidad del plasma, asociada a hiponatremia, es observada más frecuentemente a nivel clínico que la hipertonicidad (hipernatremia) (Al-Salman, 2002; Fall, 2000). En pacientes en periodo postoperatorio se pueden presentar cuadros de hiponatremia debido a las altas concentraciones (5%) de dextrosa que se administra en el agua y a que los niveles de vasopresina se aumentan debido estrés (Kugler y Hustead, 2000). El desarrollo de signos y síntomas durante la hiponatremia depende de la velocidad y grado de desarrollo de la misma. La tasa de disminución del Na^+ en el plasma es importante en el desarrollo de cuadros neurológicos. En la hiponatremia aguda (que ocurre en menos de 48 h) se produce edema cerebral y severas alteraciones neurológicas. Los síntomas neurológicos asociados varían desde cuadros de confusión, hasta coma. La aparición de cuadros epilépticos se asocia a un incremento en la mortalidad del 50%. En general las

manifestaciones neurológicas están fuertemente asociadas al edema cerebral ocasionado (Verbalis, 1998). Por el contrario, la hiponatremia crónica se desarrolla de manera incidental en un periodo desconocido de tiempo (más de 48 h) y con frecuencia los pacientes permanecen asintomáticos. La hiponatremia crónica (concentración de Na^+ en el plasma de 126-135 mmol/l) ocurre en el 14% de los pacientes en hospitales, mientras que la hiponatremia aguda (concentración de Na^+ sanguíneo menor a 125 mmol/l) se ha reportado en el 1% de los pacientes, en su mayoría en personas adultas. En general, la hiponatremia se da en su forma crónica, pero la hiponatremia aguda está asociada con altos índices de mortalidad, siendo 60 veces más alta en pacientes sintomáticos.

La adaptación del cerebro a la disminución de la osmolaridad del plasma previene el desarrollo del edema cerebral. La respuesta adaptativa inicial incluye la pérdida de fluido cerebral, seguido por la extrusión de Na^+ y K^+. Posteriormente, existe una reducción importante en la cantidad de solutos orgánicos (Thurston, et al., 1992). Este cambio sustancial en el contenido de osmolitos, puede llevar a situaciones de riesgo si se corrige la hiponatremia en forma aguda (Verbalis y Gullans 1993), como son cuadros de mielinolisis central pontina (Ashrafian y Davey, 2001; Gross , 2001; Lampl y Yazdi, 2002).

Bajo las condiciones arriba mencionadas, las células cerebrales han desarrollado mecanismos de respuesta adaptativos, diseñados para modular el contenido citosólico de los solutos osmóticamente activos en respuesta a alteraciones en la osmolaridad del fluído extracelular. Las células del cerebro reaccionan ante diversas patologías asociadas a hinchamiento celular como la hiponatremia, activando vías de salida de electrolitos y osmolitos orgánicos como son taurina, glutamato, aspartato, glutamina y GABA dentro de los aminoácidos, así como también myo-inositol, creatinina y N-acetil aspartato, siendo la contribución de los electrolitos de un 60%-70% y la de los osmolitos orgánicos de un 30%-40% para el DRV (Lien et al., 1991; Thurston et al., 1987; Trachtman et al., 1990). Los mecanismos de regulación del volumen celular, estudiados en células nerviosas en modelos *in vitro*, pueden estar actuando *in vivo*, y ser responsables de los cambios en los niveles de osmolitos observados. Se ha visto que en respuesta a condiciones hiponatrémicas, las células nerviosas reaccionan activando el flujo de osmolitos orgánicos e inorgánicos. Durante la hiponatremia, existe una fase dentro de las primeras 3-24 h en la que se observa una disminución del 10-30% en las concentraciones de de Na^+, K^+ y Cl^- (Melton, 1987); en una segunda fase (aproximadamente de 2 días) se origina una

disminución (50-80%) en el contenido de osmolitos orgánicos, principalmente aminoácidos (taurina, glutamato, aspartato, glutamina y GABA), myo-inositol, creatinina y N-actyl aspartato (Bedford et al., 1993; Sterns et al., 1993; Thurston et al., 1980; Verbalis et al., 1991). Debido a que el contenido de osmolitos inorgánicos es superior al de los orgánicos, su contribución al DRV es mayor (60%-70%) que la de los orgánicos (30-40%). Este proceso se ha visto regulado por hormonas incluyendo la arginina vasopresina y las hormonas sexuales (Lien et al., 1991).

OBJETIVOS PARTICULARES

La caracterización de los mecanismos involucrados en la regulación del volumen se ha llevado a cabo principalmente en respuesta a cambios súbitos de osmolaridad; sin embargo, como ya se mencionó en la introducción en condiciones patológicas como la hiponatremia los cambios en la osmolaridad externa del medio no suceden de manera súbita. Por esto el estudio de los mecanismos de regulación de volumen en respuesta a cambios graduales de osmolaridad puede significar un acercamiento importante a la condición fisiológica y aún a situaciones patológicas. El objetivo del presente trabajo es estudiar los mecanismos de regulación de volumen involucrados y la movilización de osmolitos orgánicos (aminoácidos osmóticamente activos) e inorgánicos (iones como el K^+) en respuesta a cambios de osmolaridad comparando dos paradigmas experimentales diferentes: cambios súbitos y cambios graduales de osmolaridad, tanto en rebanadas de hipocampo de rata cuya interacción neurona glia permanece intacta, como en cultivos primarios de astrocitos para caracterizar la participación de los distintos componentes celulares en el tejido, comparando los resultados con otros trabajos en el laboratorio.

RESULTADOS:

Los resultados de esta sección se anexan a continuación en el formato de los artículos correspondientes ya publicados, los cuales contienen, una breve introducción la tema, la metodología utilizada, los datos y la discusión la cual se amplia en la siguiente sección.

Efflux of Osmolyte Amino Acids During Isovolumic Regulation in Hippocampal Slices

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The efflux of potassium (K^+) and amino acids from hippocampal slices was measured after sudden exposure to 10% (270 mOsm), 25% (225 mOsm) or 50% (150 mOsm) hyposmotic solutions or after gradual decrease (−2.5 mOsm/min) in external osmolarity. In slices suddenly exposed to 50% hyposmotic solutions, swelling was followed by partial (74%) cell volume recovery, suggesting regulatory volume decrease (RVD). With gradual hyposmotic changes, no increase in cell water content was observed even when the solution at the end of the experiment was 50% hyposmotic, showing the occurrence of isovolumic regulation (IVR). The gradual decrease in osmolarity elicited the efflux of ^3H-taurine with a threshold at −5 mOsm and D-[^3H]aspartate (as marker for glutamate) and at −20 mOsm for [^3H]GABA. The efflux rate of [^3H]taurine was always notably higher than those of [^3H]GABA and D-[^3H]aspartate, with a maximal increase over the isosmotic efflux of about 7-fold for [^3H]taurine and 3- and 2-fold for [^3H]GABA and D-[^3H]aspartate, respectively. The amino acid content in slices exposed to 50% hyposmotic solutions (abrupt change) during 20 min decreased by 50.6% and 62.6% (gradual change). Taurine and glutamate showed the largest decrease. An enhancement in ^{86}Rb efflux and a corresponding decrease in K^+ tissue content was seen in association with RVD but not with IVR. These results demonstrate the contribution of amino acids to IVR and indicate their involvement in this mechanism of cell volume control. J. Neurosci. Res. 61:701–711, 2000.
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Key words: taurine; cell swelling; K efflux; regulatory volume decrease; hyposmolarity

Cell volume control is maintained by the transmembrane fluxes of osmotically active solutes in the necessary direction to counteract water movements caused by changes in external osmolarity or by osmotic gradients originated during the normal cell functioning (Hoffmann and Dunham, 1995; Häussinger, 1996; Lang et al., 1998). Osmolyte translocation subsequent to hyposmotic cell swelling has been extensively investigated in a variety of preparations (Pasantes-Morales, 1996; Strange et al., 1996;

Kirk, 1997; Nilius et al., 1997). Most of these studies refer to osmolyte fluxes activated in response to abrupt and large decreases in external osmolarity whereas less is known about the osmolyte movements after isosmotic swelling or after small and gradual reductions in external osmolarity, an approach more closely resembling pathophysiological situations (Kimelberg and Ransom, 1986; McManus and Churchwell, 1994; Fraser and Arieff, 1997). An early study in renal proximal tubules (Lohr and Grantham, 1986) demonstrated that cells maintain their normal size within a large range of external osmolarities if the change occurs slowly and gradually. This phenomenon has been named isovolumetric or isovolumic regulation (IVR). This term may not mean that the cell volume is not increasing but rather that it is maintained by microadjustments accomplished by the efflux of intracellular osmolytes. This is suggested by the fact that cells gradually exposed to increasingly hyposmotic external solutions, shrink when suddenly returned to isosmotic conditions (Lohr and Grantham, 1986). Information about the osmolytes involved in this process and their mechanisms of translocation is scarce. In the distal nephron cell line (A6), IVR leads to an efflux of K^+ but no other osmolytes have been identified yet (Van Driessche et al., 1997). In the present work, we addressed to this point in hippocampal slices measuring the efflux of K^+ and some amino acids known to play a role as osmolytes, i.e., glutamate, GABA and taurine, in response to gradual and continuous reductions (2.5 mOsm/min) in external osmolarity. The threshold and rate of release of the different osmolytes was compared to that elicited by acute, large changes in osmolarity. To our knowledge, this is the first time that changes in osmolyte amino acids during IVR have been reported.

MATERIALS AND METHODS

Materials

5-Nitro-[3-phenylpropylamino]benzoic acid (NPPB) was from Research Biochemicals Inc. (Natick, MA); 1,9, dideoxyforskolin (DDF) and niflumic acid were from Sigma Co. (St. Louis, MO). All radiolabeled compounds were from New England Nuclear (Boston, MA).

Hippocampal Slices

Male adult Wistar rats (200–250 g body weight) were used throughout the study. Animals were killed by decapitation and brains rapidly removed and submerged into cold Krebs-Hepes medium that contained (in mM): 135 NaCl, 1.0 $CaCl_2$, 1.17 $MgCl_2$, 1.7 KH_2PO_4, 5 KCl, 5 dextrose and 10 HEPES (300 mOsm/l, isosmotic, pH 7.4). Hippocampus from both hemispheres were dissected and transverse slices (400 μm thick) obtained with a McIlwain tissue chopper. Slices were immediately submerged into isosmotic medium continuously bubbled with 0_2 and kept at room temperature at least for half an hour before the assays.

Experimental Solutions

Hyposmotic solutions [270 mOsm/l (10%); 225 mOsm/l (25%); 150 mOsm/l (50%)], were prepared by reducing the concentration of NaCl accordingly. Final osmolarities were verified with a freezing-point osmometer (Osmette A, Precision Systems Inc. Natick, MA). To obtain continuous and gradual changes in osmolarity, a gradient-generating system was constructed as described in detail by Van Driessche et al. (1997). Briefly, the system consisted of two identical glass cylinders interconnected at their bases by a tube with an interrupting valve. The first container was filled with isosmotic medium and the second with the same volume of 50% hyposmotic medium. Media were kept at 37°C placing the cylinders on a temperature-controlled hot plate with stirring. Perfusion medium was pulled from the first container with a peristaltic pump, producing that 50% hyposmotic medium begin to enter this cylinder, mixing gradually and continuously with the isosmotic medium. In this way, an osmotic gradient is generated with a rate of change of −2.5 mOsm/min. At the end of the experiment (60 min later) the superfusion medium reached 150 mOsm/l (50% hyposmotic).

Tissue Water Content

Changes in hippocampal slice volume were indirectly estimated by quantification of the tissue water content and [^{14}C]inulin distribution. For measuring tissue water content, previously weighted slices kept in isosmotic media at 37°C were either, incubated in media of decreased osmolarity during the time indicated at each experiment or superfused with an increasingly hyposmotic medium (osmotic gradient), until the external osmolarity reached 150 mOsm (50% hyposmotic). At the end of experiments, slices were recovered, blotted, dried in oven for 24 hr at 90°C, and re-weighted to obtain the dry weight. Results are expressed as μl of water per mg of tissue dry weight. To estimate the changes of the intracellular volume the distribution of ^{14}C-inulin was assessed after the procedure of Fishman et al. (1977). Slices were incubated with 18.5 MBq/ml of

[^{14}C]inulin during 60 min. At the end of this period, slices were blotted and transferred to the experimental (isosmotic or hyposmotic) media containing the same concentration of [^{14}C]inulin. At the time indicated in each experiment, the slices were removed from the medium, blotted and the retained radioactivity and radioactivity in the medium, measured in a scintillation spectrometer. Extracellular space in rat hippocampal slices was estimated as 17% of total water content in isosmotic conditions, while the percentage change of intracellular volume was calculated from the [^{14}C]inulin data and expressed as μl of water/mg dry weight.

Labeled Amino Acids and ^{86}Rb Efflux

Hippocampal slices were incubated in isosmotic medium with the labeled amino acids, [^{3}H]taurine (60 min, 55.5 MBq/ml), [^{3}H]GABA or D-[^{3}H]aspartate (as a metabolically inert analogue of glutamate) (30 min, 37 MBq/ml) or with ^{86}Rb as a tracer for K^+ (30 min, 74 MBq/ml). After the loading period, slices were transferred into perfusion chambers (0.4 ml vol) and washed by perfusion with warmed (37°C), isosmotic medium at a rate of 1 ml/min during 15 min. From hereafter, samples were collected every minute and after 5–10 min of basal release, the osmolarity of the perfusion medium was suddenly or gradually decreased as follows. In the first case, the perfusion medium (isosmotic) was switched to one of reduced osmolarity (either 10, 25 or 50 % hyposmotic) and samples were collected during 25 min. In the second case, slices were superfused with the osmotic gradient and samples were collected every min up to 60 min. When the effect of Cl^- channel blockers was examined, drugs were preincubated during 15 min and were present through the whole experiment. Controls contained the corresponding vehicle. The release of labeled amino acids and that of ^{86}Rb was expressed as efflux rate constants, i.e., the radioactivity released at any given time as percent of total radioactivity present in the cells at that time.

Measurement of Amino Acid and K^+ Content in Slices

After perfusing tissue with the experimental solutions indicated, individual slices were homogenized in 500 μl of water. Amino acids were extracted with 70% ethanol, mixed with o-phthaldialdehyde and quantified by standard reverse phase HPLC. Endogenous K^+ content was measured in slices previously exposed to one of the experimental paradigms. Slices were digested in boiling HCl (0.1 M, 30 min), then filtered and the K^+ content determined by atomic absorption spectrometry.

RESULTS

Changes in Water Content

Figure 1A shows the water content of hippocampal slices exposed to 10%, 25% and 50% hyposmotic solutions during 5 min. An increase in water content paralleled the decrease in external osmolarity. As compared to the isosmotic condition, the water content increased by 22.1, 41.6 and 63.2% in 10%, 25% and 50% hyposmotic solutions, respectively. In all these experiments, results are expressed as μl of water per mg of dry weight. The interstitial space assessed by [^{14}C]inulin retention (17%) was subtracted in all cases to obtain the
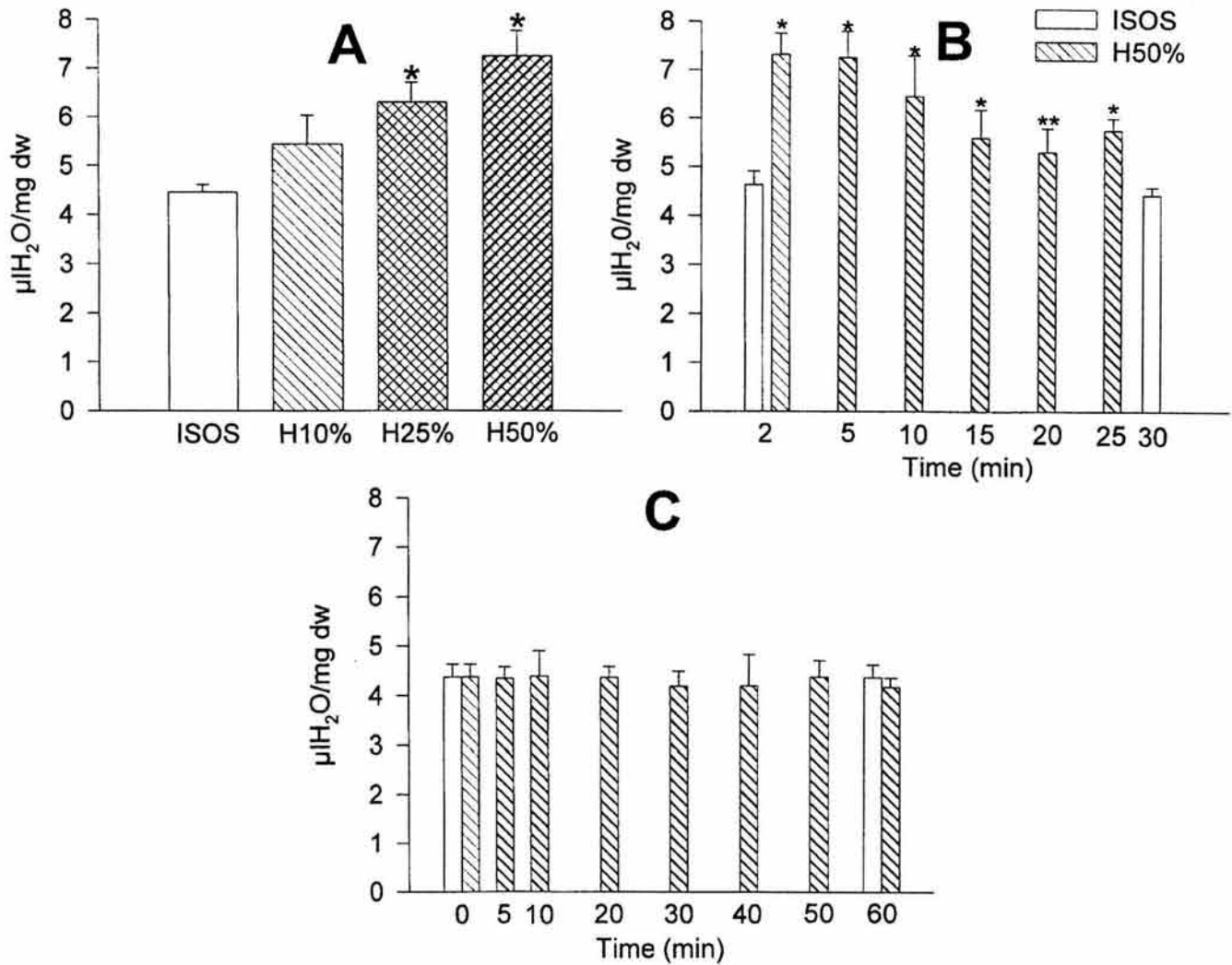
Fig. 1. Changes in water content of hippocampal slices suddenly or gradually exposed to hyposmotic media. (A) After a 10 min period in isosmotic medium, hippocampal slices were exposed to solutions of reduced osmolarity (10%, 25% or 50% hyposmotic) during 5 min. Control slices were kept in isosmotic medium (isos). (B) Slice water content was measured after 2 min incubation in isosmotic medium (empty bar at left), after exposure to 50% hyposmotic medium during the times indicated (dashed bars) or 5 min after return to isosmotic medium (empty bar at right). (C) Slices were superfused with an osmotic gradient (dashed bars) (rate of osmolarity change: -2.5 mOsm/min) and recovered for water content determination at the indicated times. Control slices (empty bars) were superfused 60 min with isosmotic medium and water content determined at the beginning and at the end of the superfusion. Data are expressed as μl of H_2O per mg of tissue dry weight (dw) and are means ± SE. n = A: 8–10; B: 4–8; C: 4–8. *$P < 0.005$, **$P < 0.05$ as compared to the corresponding isosmotic condition.

net change in intracellular water space. To examine the ability of cell volume recovery in the slices, the change in intracellular water content was followed at different times after exposure to 50% hyposmotic medium (Fig. 1B). Two min after the stimulus, cell volume has increased 58% above cell volume in isosmotic medium and remained unchanged up to 5 min. Thereafter, cell volume progressively decreased and after 20 min, cells have recovered 74.5% of their original volume (Fig. 1B), suggesting regulatory volume decrease. Cell vol-

ume recovery was not completed within 30 min of exposure (not shown), but returning the slices to isosmotic medium (Fig. 1B) rapidly attained the original volume. When the change in external osmolarity was slow and gradual (2.5 mOsm/min), swelling did not occur within the 60 min of exposure to the osmotic gradient, despite the marked decrease in external osmolarity, which at that time has been reduced to 150 mOsm (Fig. 1C). These results indicate the occurrence of IVR in hippocampal slices.

Fig. 2. Amino acid release from rat hippocampal slices suddenly exposed to 50% hyposmotic medium. Slices preloaded with D-[³H]aspartate (A), [³H]GABA (B) or [³H]-taurine (C) were superfused 5 min with isosmotic medium. At the time pointed by the arrow, the medium was replaced by 50% hyposmotic solution. One min fractions were collected during 20 min. Data are expressed as efflux rate constants (min⁻¹) and are means ± SE (n = 6–10). The insets show the percentage release ± SE of the corresponding labeled amino acid during the time of exposure to the hyposmotic solution (min 6–25).

Amino Acid Release After Sudden Exposure to Hyposmotic Solutions

Figure 2 shows the release of [³H]taurine, D-[³H]aspartate and [³H]GABA from hippocampal slices in response to sudden exposure to 50% hyposmotic solutions. The efflux of D-[³H]aspartate and [³H]GABA was activated immediately after the stimulus and also rapidly inactivated. The time course of release showed some irregularities that may suggest the occurrence of more than one component in the efflux pattern. The release curves, however, fitted well to a single exponential decay with $r^2 = 0.950$ for D-[³H]aspartate and 0.976 for [³H]GABA. During the time of exposure to the hyposmotic medium the release of [³H]GABA and D-[³H]aspartate accounted

for 52% and 77%, respectively, of the total labeled amino acid incorporated during loading (Fig. 2A,B). The efflux of [³H]taurine elicited by 50% hyposmotic medium increased slowly, reaching the maximal rate release only at 7 min after the stimulus (Fig. 2C). More than 80% of the labeled taurine in the preparation was released during the time of exposure to the hyposmotic medium (Fig. 2C). Hyposmotic 25% and 10% solutions still evoked a significant increase in the efflux of the three amino acids (Fig. 3A–C).

To minimize possible effects of reuptake or diffusion through the layers of the slice on the amino acid fluxes, experiments were carried out increasing the perfusion rate to 2 ml per min. This maneuver did not change neither
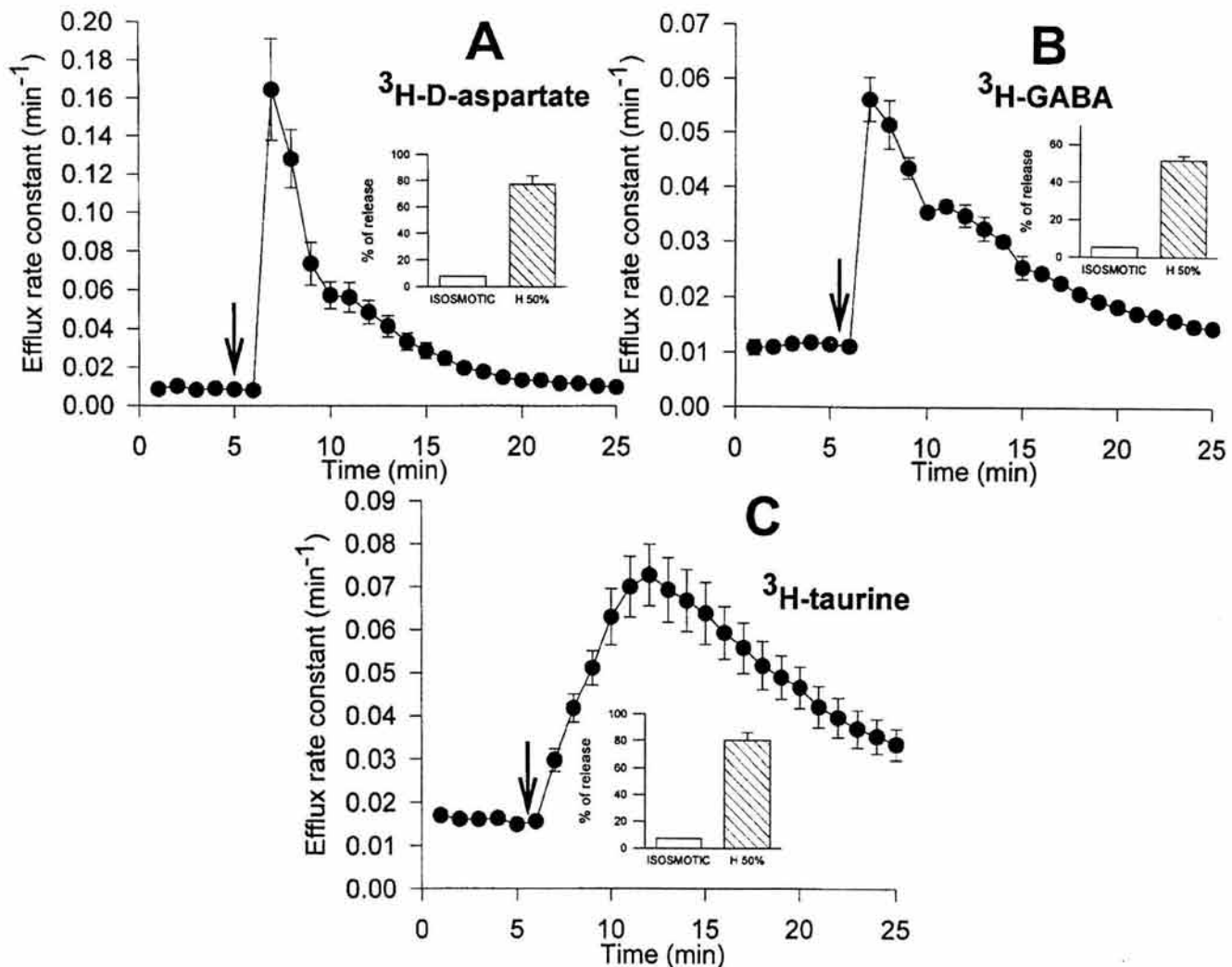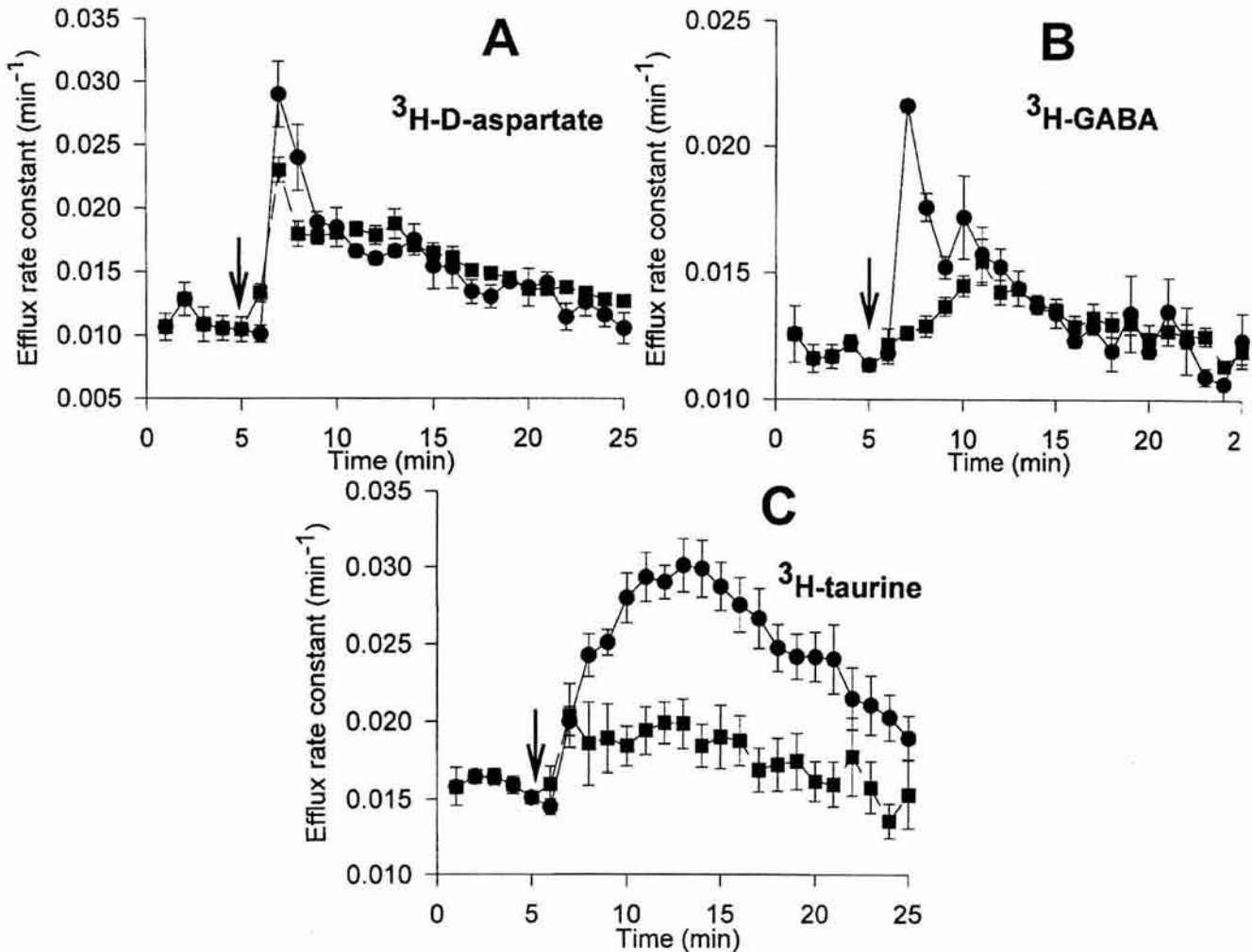
Fig. 3. Amino acid release from rat hippocampal slices suddenly exposed to 10% and 25% hyposmotic media. Slices preloaded with D-[³H]aspartate (A), [³H]GABA (B) or [³H]-taurine (C) were treated as described in Figure 2; 10% (■) or 25% (●). Data are expressed as efflux rate constants (min⁻¹) and are means ± SE (n = 6–10). The points in hyposmotic 25% were significantly different from basal release (P < 0.05) from 7–16 min for D-[³H]aspartate, 7–23 for [³H]GABA and 6–25 for [³H]-taurine. In 10% hyposmotic medium data were significant from 7–23 for D-[³H]aspartate, 7–17 for [³H]GABA and 10–16 for [³H]-taurine.

the time course nor the magnitude of efflux for any of the amino acids as compared to experiments with superfusion rate of 1 ml/min (not shown). Solutions containing the same low NaCl concentration as in 50% hyposmotic solutions but made isosmotic with sucrose did not elicit any efflux of amino acids (not shown).

Amino Acid Release After Exposure to Gradual Decreases in Osmolarity

Figure 4 shows the efflux of [³H]taurine, [³H]GABA and D-[³H]aspartate from hippocampal slices exposed to medium of osmolarity decreased at a change rate of 2.5 mOsm/min. Slices exposure to the osmotic gradient enhanced the release of [³H]taurine and D-[³H]aspartate as early as 2 min after the stimulus, corresponding to a decrease in osmo-

larity of 5 mOsm. Amino acid release at this time was significantly higher (P < 0.005) than in isosmotic medium. The efflux of [³H]GABA exhibited a higher threshold with significant increases observed at −20 mOsm (P < 0.005) (Fig. 4). The efflux rate of [³H]taurine was always notably higher than that of [³H]GABA and D-[³H]aspartate, with maximal increases over the isosmotic efflux of about 7-fold for [³H]taurine and 3- and 2-fold for [³H]GABA and D-[³H]aspartate, respectively.

Effect of Cl⁻ Channel Blockers

NPPB, DDF and niflumic acid, known blockers of the osmosensitive Cl⁻/amino acid pathway were tested on [³H]taurine efflux stimulated by sudden or gradual changes
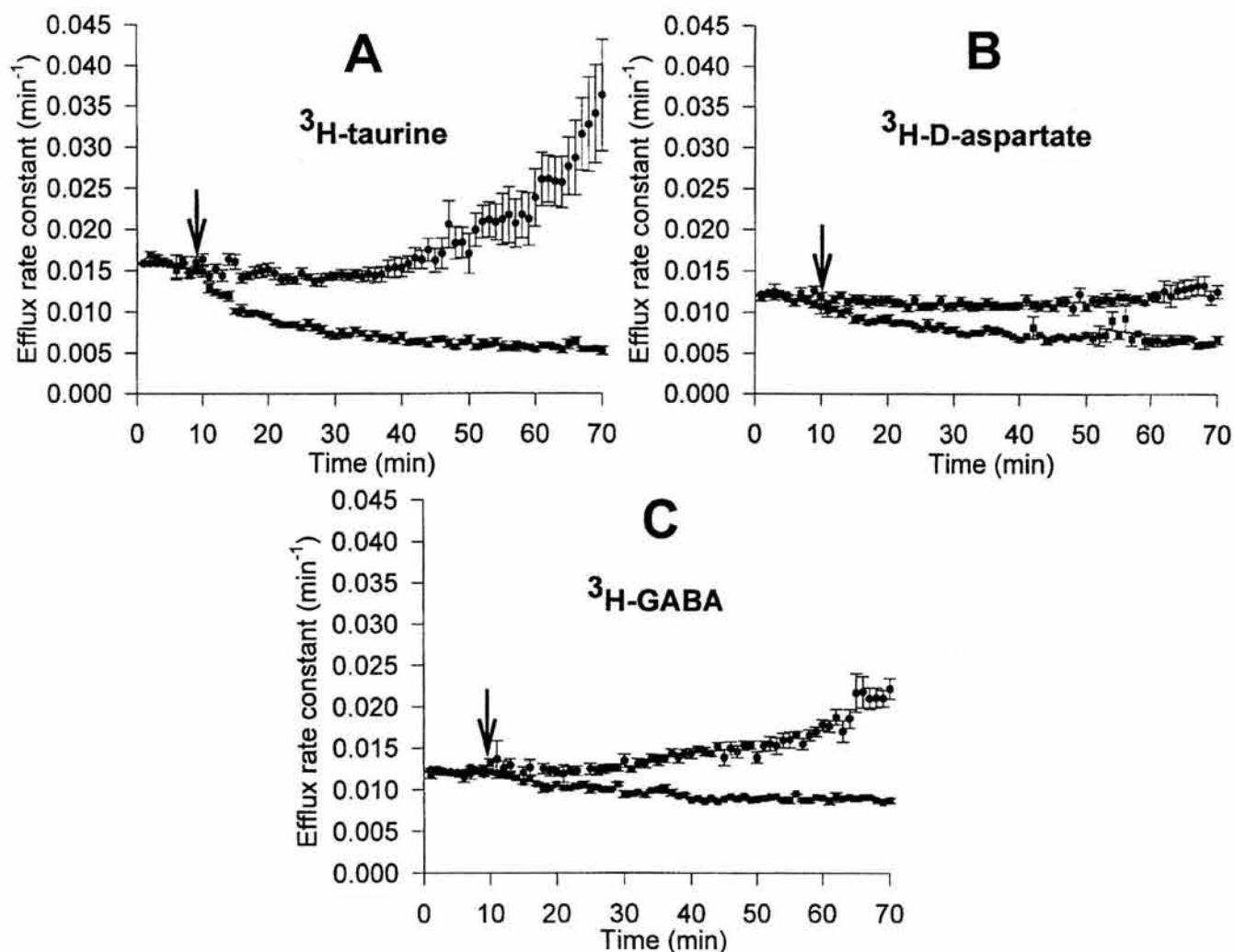
Fig. 4. Amino acid release from hippocampal slices exposed to gradual and progressive reductions in external osmolarity. Slices preloaded with [³H]-taurine (A), D-[³H]aspartate (B), or [³H]GABA (C), were super-fused 10 min with isosmotic medium. At the time pointed by the arrow in (●) the external osmolarity was continuously decreased at a rate of −2.5 mOsm/min until the medium osmolarity reached 150 mOsm (50% hyposmotic). Controls (■) were superfused with isosmotic medium. Data are expressed as efflux rate constants (min⁻¹) and are means ± SE (n = 8–10). Significant differences (P < 0.005) between basal release and experimental condition were found from 12th fraction and onward for taurine and D-[³H]aspartate (A, B), and from the 18th fraction for [³H]GABA (C).

in osmolarity. The effect of agents which reduced the volume-sensitive taurine release in cultured brain cells, DDF, NPPB and niflumic acid (Sánchez-Olea et al., 1996), was tested on the efflux of [³H]taurine from hippocampal slices during the 20 min of exposure to 50% hyposmotic solution (min 5–25, Fig. 5A). DDF (100 μM) did not significantly affected this release, whereas NPPB (100 μM) and niflumic acid (600 μM) decreased it markedly (Fig. 5A). The total [³H]-taurine efflux calculated as % release as in Figure 2 (inset) was reduced by 58% (NPPB) and 69.6% (niflumic acid). The effect of niflumic acid was then tested on [³H]tau-rine efflux during IVR. As shown in Figure 5B this agent exhibited the same notable inhibitory action.

Decrease in Amino Acid Concentration by Hyposmotic Solutions or After IVR

The decrease in the total free amino acid content in the hippocampal slices exposed to 50% hyposmotic solution or to IVR conditions, is shown in Figure 6A. A reduction of 50.6% (50% hyposmotic) and 62.6% (IVR) was observed. Amino acids showing the largest decrease in the two conditions were glutamate (52% and 65% in hyposmotic and isovolumic treatment, respectively) and taurine (64% and 78% in the same conditions) (Fig. 6B,C). Results are expressed as nmol/mg wet weight. In this condition, some dilution of amino acid levels is expected when the treatment results in a change in cell volume

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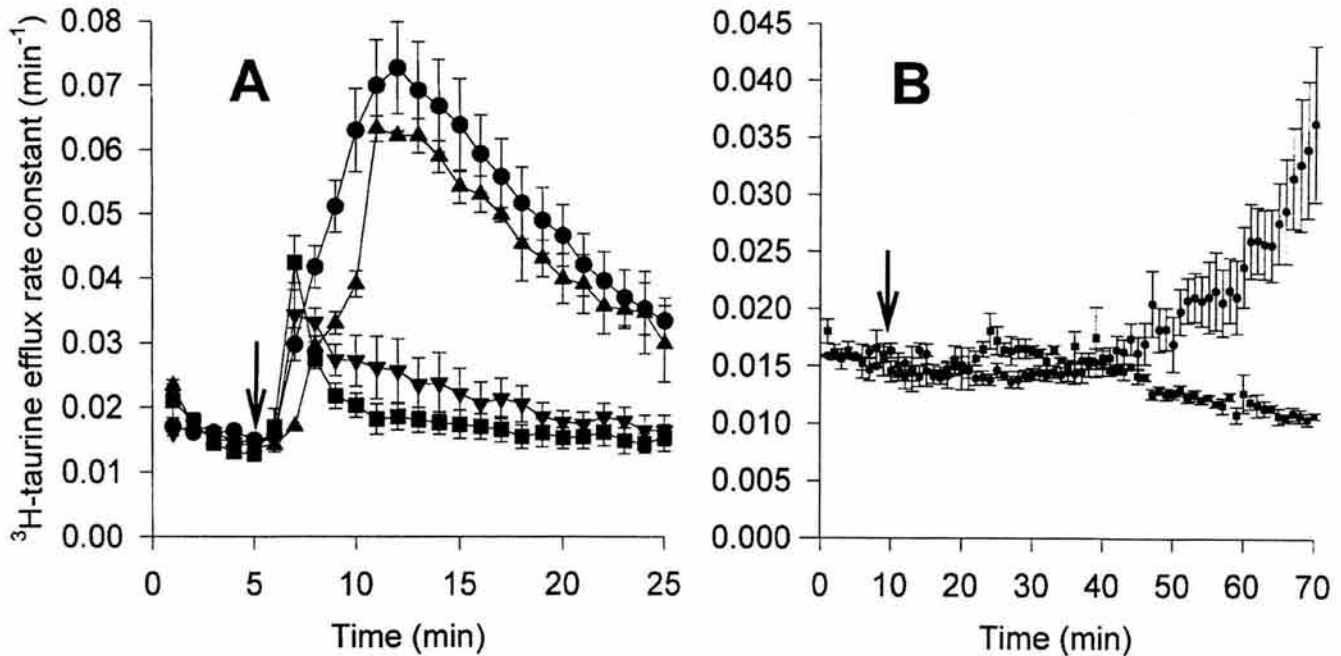
Fig. 5. Effect of anion/amino acid channel blockers on the osmosensitive [³H]-taurine efflux from hippocampal slices. (A) Slices preloaded with [³H]-taurine were preincubated during 15 min in isosmotic medium without additions (control: ●), or plus 100 μM DDF (▲), 100 μM NPPB (▼) or 600 μM niflumic acid (■) and then superfused with isosmotic medium and 50% hyposmotic medium (arrow). Blockers were present in all solutions used. Significant differences (P < 0.005) were found between control condition and niflumic acid or NPPB, from the 9th fraction. (B) Slices preloaded with [³H]-taurine were exposed to the osmotic gradient in the absence (control, ●) or presence (■) of 600 μM niflumic acid. Control data are the same shown in Figure 4A. Data are expressed as efflux rate constants (min⁻¹) and are means ± SE (n = 4–8).

(50% hyposmotic). Because the samples for estimation of amino acid content were taken at min 20, the volume in slices exposed to the hyposmotic medium is still 14.7% higher than in isosmotic medium (Fig. 1B). Therefore, a dilution of about 15% is contributing to the observed decrease in the amino acid content under this condition. This does not occur in the IVR paradigm in which there is no volume change.

⁸⁶Rb Release and Changes in K⁺ Levels During RVD and IVR

Hippocampal slices loaded with ⁸⁶Rb and suddenly exposed to 10%, 25% and 50% hyposmotic solutions responded with a fast and transient release of the labeled tracer. The time course release was similar in all solutions and the rate release increased as the external osmolarity was reduced (Fig. 7A). In contrast, exposure to gradual changes in osmolarity did not elicit any significant ⁸⁶Rb efflux (Fig. 7B). Figure 7C shows the K⁺ content of slices treated as in ⁸⁶Rb efflux experiments. In 10% and 25% hyposmotic solutions, K⁺ concentration did not significantly decreased. A reduction of about 40% in K⁺ concentration was observed in the slices exposed to 50% hyposmotic solutions. Figure 7D shows the K⁺ content of slices superfused during the indicated times with isosmotic medium (empty bars) or with the gradually diluted medium (dashed bars). Essentially no changes in K⁺ levels were observed through all the superfusion period with isosmotic medium. Also no significant changes were detected after superfusion with the gradually diluted medium (Fig. 7D).

DISCUSSION

The results of the present work suggest the occurrence of RVD in hippocampal slices after swelling elicited by hyposmotic solutions. This is still a controversial matter in this preparation. In a study measuring changes in cell volume in CA1 neurons, no indication of volume regulation could be found (Andrew et al., 1997) whereas, similar to our results, another report describes partial cell volume recovery in the whole slice (Chebabo et al., 1995). This discrepancy may be attributable in part, to differences in the cell population in the areas examined or to the magnitude of the hyposmotic challenge. Variations have been reported in the extent of swelling and of volume recovery within the various brain cell types and even within regions of the same cell (Andrew and McVicar, 1994; Chebabo et al., 1995; Baraban et al., 1997). More efficient volume regulation seems to occur associated with stronger osmotic stress (Chebabo et al., 1995).

Amino acid release from brain cells as part of the mechanism of RVD is well documented in a variety of
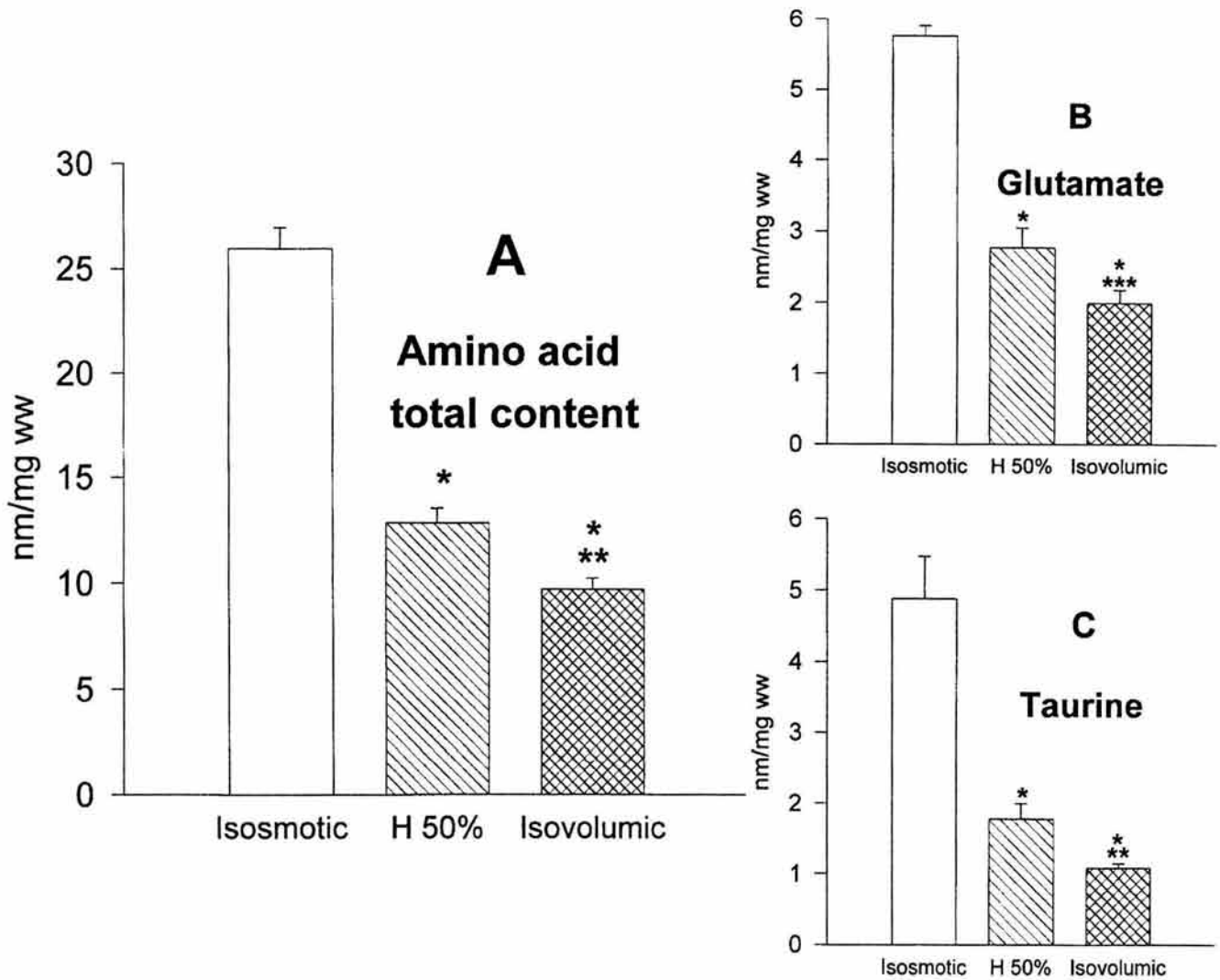
Fig. 6. Amino acid content in rat hippocampal slices exposed to sudden or gradual decreases in external osmolarity. Slices were perfused during 10 min with isosmotic medium (empty bars) or with 50% hyposmotic medium (dashed bars) or with an osmotic gradient (isovolumic) for 60 min (crossed bars). At the end of the experiments amino acid content was analyzed by HPLC. (A) Total content. (B) Glutamate content. (C) Taurine content. Data are expressed as nmol/mg wet weight and represent the mean of 8 experiments ± SE. Asterisks indicate significant differences *$P < 0.001$; **$P < 0.02$; ***$P < 0.05$ vs. control.

brain tissue preparations, including cultured astrocytes and neurons (Kimelberg et al., 1990; Pasantes-Morales et al., 1993), brain cortex slices (Law, 1994, 1996) and in brain in vivo (Solís et al., 1988; Estevez et al., 1999). This also occurs in hippocampal slices as shown in the present work. Amino acids are part of the pool of organic osmolytes that contribute to counteract the changes in brain water content during hyponatremia (Lien et al., 1991). The contribution of organic osmolytes to this compensatory response of brain cells has been calculated in about 29%, with the pool of amino acids accounting for approximately 15% (Lien et al., 1991). It is noteworthy that the amino acids preferentially released in response to changes in cell vol-

ume, are also neuroactive compounds, and they could consequently affect neuronal excitability. For instance, the efflux of glutamate here observed may contribute to the consistently reported effects of hyposmolarity increasing neuronal firing rate in hippocampal slices (Roper et al., 1992; Huang et al., 1997).

Although the experimental model of sudden and marked decreases in osmolarity had rendered valuable information to elucidate some basic mechanisms of cell volume control, such changes probably never occur in brain under physiological conditions. Even during pathological situations such as chronic hyponatremia, water intoxication or the inappropriate handling of antidiuretic
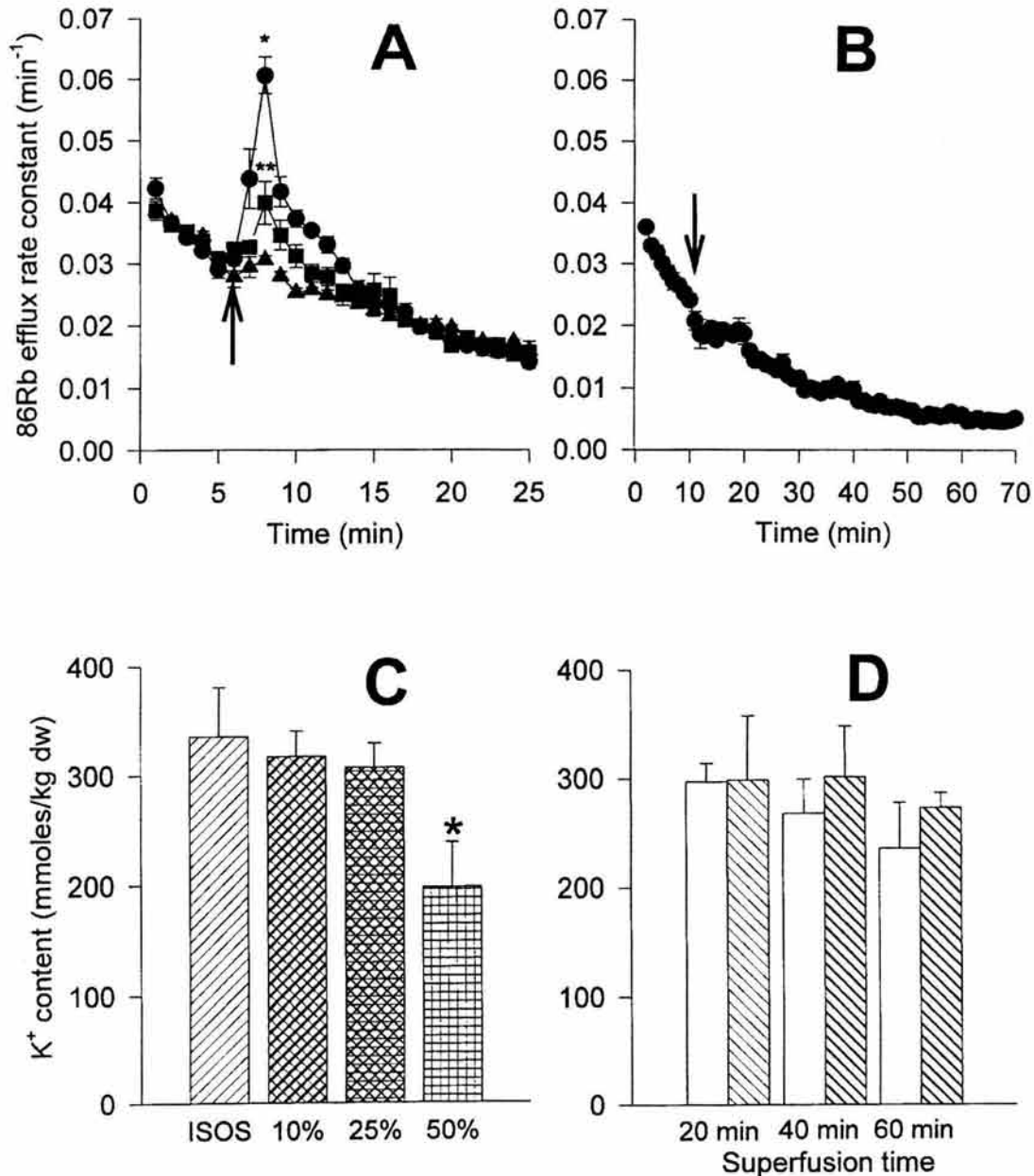
Fig. 7. ^{86}Rb efflux and K^+ content in hippocampal slices exposed to sudden or gradual reductions in external osmolarity. (A) Slices pre-loaded with ^{86}Rb were superfused 10 min with isosmotic medium and then, at the arrow, with solutions of decreased osmolarity: 10% (▲), 25% (■) or 50% (●), for 20 min. (B) Same as in A, but at min 10 (arrow), the external osmolarity decreased at a rate of 2.5 mOsm/min. Data are expressed as efflux rate constants (min^{-1}) and are means ± SE (n = 4–6). *$P < 0.001$, **$P < 0.05$, as compared with the last basal efflux point. (C) After superfusion with either isosmotic medium or hyposmotic solutions (10%, 25%, or 50%) for 20 min, slices were digested with HCl and the K^+ content measured by atomic absorption spectrometry. (D) Slices superfused with isosmotic medium (empty bars) or exposed to the osmotic gradient (dashed bars) were recovered at different times (20, 40, or 60 min) for determination of K^+ content. Results are expressed as mmol of K^+/Kg of dry weight. Data are means ± SE (n = 3–4). *$P < 0.05$, as compared to the isosmotic condition.

hormone, the osmolarity changes in the interstitial space in brain, occur most likely in a gradual manner as the osmotic challenge from plasma progressively surpasses the brain

homeostatic resistances (Trachtman, 1991; McManus and Churchwell, 1994; Fraser and Arieff, 1997). Thus, the experimental approach of the present work, having as

31

stimulus a gradual and slow decrease in external osmolarity, reflects more accurately the pathophysiological conditions. This paradigm has been previously used in the distal nephron cell line A6 (Van Driessche et al., 1997) and in renal proximal tubules (Lohr and Grantham, 1986; Lohr, 1990), where it was first described that cell volume remains stable over a broad range of osmolarities, provided that the rate of change is gradual. This constancy in cell volume, named IVR seems to result from an active process of volume control accomplished by the adjustment of osmolyte intracellular content, as evidenced by the shrinkage observed when cells previously exposed to gradual hyposmotic changes are suddenly returned to isosmotic medium (Lohr and Grantham, 1986). The osmolytes involved in IVR are not well known. Studies on renal cells implicate K^+ as an active osmolyte during this process (Lohr, 1990; Van Driessche et al., 1997). In the present work we now showed the contribution of amino acids, preferentially taurine, GABA and glutamate. The efflux of both, taurine and glutamate was responsive to very small changes in osmolarity, of about 5 mOsm. The efflux of taurine, however, was always significantly higher than those of GABA and glutamate, and also taurine exhibited the largest decrease in the intracellular content. Several possible explanations could account for the observed differences between the three amino acids: i) a difference in the diffusion through the layers of the slice; ii) a different permeability coefficient through the osmosensitive pathway; and iii) the relative availability of amino acid pools to be released upon hyposmolarity. A distinct washout rate due to different diffusional barriers for each amino acid seems unlikely, because the amino acid efflux during IVR in our experiments was compared, point to point, with the efflux in isosmotic conditions. This would correct any possible difference related to diffusion. In addition, the amino acid efflux pattern during RVD, was opposite to that in IVR, with the release of D-[^{3}H]aspartate being the highest and most rapid and that of [^{3}H]taurine the lowest and most delayed. This is observed even when the superfusion rate in the two paradigms was identical. Finally, in studies on IVR in cultured astrocytes and neurons in monolayer (unpublished results), where the diffusion factor is minimized, still the release of taurine clearly surpasses that of GABA and D-aspartate. From all these results, it is likely that the preferential release of taurine during IVR is related either to higher permeation through the osmosensitive pathway or to more availability of the intracellular taurine pools. A similar early release of taurine as compared to other amino acids, has been found in studies in rat brain in vivo upon perfusion by microdialysis with media of decreased osmolarity (Solís et al., 1988; Estevez et al., 1999;). All this suggests an important role for taurine in the mechanisms of cell volume control in brain. It is as yet unclear whether the mechanism of amino acid release during IVR and RVD are similar, although the inhibitory effect of niflumic acid points into that direction.

As previously mentioned, K^+ is clearly implicated in IVR in renal cells (Lohr, 1990; Van Driessche et al., 1997).

In A6 cells, K^+ efflux (traced by radiolabeled ^{86}Rb) increases during IVR with a threshold activation at 210 mOsm (about 30% hyposmotic). When the external osmolarity has dropped to 150 mOsm (50% hyposmotic), cells have lost 29% of the intracellular K^+. A similar decrease occurs after sudden exposure to 50% hyposmotic solutions (Van Driessche et al., 1997). In contrast, in hippocampal slices whereas K^+ is clearly mobilized during RVD, no ^{86}Rb efflux nor change in K^+ occurred during IVR. This is an unexpected result because K^+ is a key osmolyte in essentially all cell types. It should be noticed, however, that unlike in cells in culture, in the hippocampal slices having an intact cytoarchitecture, buffering of extracellular K^+ by the efficient mechanisms known to exist in brain tissue, could mask an osmosensitive release occurring gradually as during IVR. Due to the key role played by K^+ in nervous excitability, its extracellular levels in brain have to be kept under strict control.

The decrease in amino acids content occurring during IVR is clearly insufficient to compensate for the change in external osmolarity and therefore, other factors have to be considered to explain the maintenance of cell volume under these conditions. One or several of the following possibilities may be considered: 1) swelling is overall restricted when the osmolarity change is small and gradual, 2) other organic osmolytes, such as creatine, myo-inositol, sorbitol or N-acetyl aspartate, are also contributing to counteract the external osmolarity, and altogether compensate for the initial phase of hyposmotic stress, when K^+ release has not yet still activated, 3) swelling occurs in some but not in all cells, and the decrease in amino acids and other osmolytes is required to compensate the change in cell volume only in a minor population of cells (Andrew and McVicar, 1994; Aitken et al., 1998), 4) a redistribution of osmolyte amino acids between different types of cells i.e., neurons and astrocytes, as has been observed in the cerebellum (Nagelhus et al., 1993), even when it may importantly contribute to regulate cell volume in specific types of cells, it may not result in a large net efflux, 5) amino acids, particularly taurine, may serve a signaling role, instead of acting directly as an osmolyte (Hussy et al., 1997), and 6) rapid metabolic changes such as synthesis of macromolecules, i.e., glycogen, may contribute to reduce the intracellular osmolyte pool necessary to reach the osmotic equilibrium (Häussinger, 1996).

Results of the present work and those in renal cells (Lohr and Grantham, 1986; Van Driessche et al., 1997) contrast with those recently reported in trout erythrocytes, that do not exhibit IVR (Godart et al., 1999). In these cells, gradual changes in osmolarity failed to significantly increase the efflux of intracellular osmotically active solutes including K^+ and taurine (Godart et al., 1999). Clearly, studies on the occurrence and features of IVR in different cell types are essential for a better understanding of the physiological significance of this mechanism of volume regulation.

REFERENCES

Aitken PG, Borgdorff AJ, Juta AJA, Kiehart DP, Somjen GG, Wadman WJ. 1998. Volume changes induced by osmotic stress in freshly isolated rat hippocampal neurons. Pflugers Arch 436:991–998.

Andrew RD, Lobinowich ME, Osehobo EP. 1997. Evidence against volume regulation by cortical brain cells during acute osmotic stress. Exp Neurol 143:300–312.

Andrew RD, MacVicar BA. 1994. Imaging cell volume changes and neuronal excitation in the hippocampal slice. Neuroscience 62:371–383.

Baraban SC, Bellingham MC, Berger AJ, Schwartzkroin PA. 1997. Osmolarity modulates K^+ channel function on rat hippocampal interneurons but not CA1 pyramidal neurons. J Physiol 498:679–689.

Chebabo SR, Hester MA, Jing J, Aitken PG, Somjen GG. 1995. Interstitial space, electrical resistance and ion concentrations during hypotonia of rat hippocampal slices. J Physiol 487:685–697.

Estevez AY, O'Regan MH, Song D, Phillis JW. 1999. Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. Neurochem Res 24:447–452.

Fishman RA, Reiner M, Chan PH. 1977. Metabolic changes associated with isosmotic regulation in brain cortex. J Neurochem 28:1061–1067.

Fraser CL, Arieff AI. 1997. Epidemiology, pathophysiology, and management of hyponatremic encephalopathy. Am J Med 102:67–77.

Godart H, Ellory JC, Motais R. 1999. Regulatory volume response of erythrocytes exposed to a gradual and slow decrease in medium osmolarity. Pflugers Arch 437:776–779.

Häussinger D. 1996. The role of cellular hydration for the regulation of cell function. Biochem J 313:697–710.

Hoffmann EK, Dunham PB. 1995. Membrane mechanisms and intracellular signaling in cell volume regulation. Int Rev Cytol 161:173–262.

Huang R, Bossut DF, Somjen GG. 1997. Enhancement of whole cell synaptic currents by low osmolarity and by low [NaCl] in rat hippocampal slices. J Neurophysiol 77:2349–2359.

Hussy N, Deleuze C, Pantaloni A, Desarménien MG, Moos F. 1997. Agonist action of taurine on glycine receptors in rat supraoptic magnocellular neurons: possible role in osmoregulation. J Physiol (London) 502:609–621.

Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA. 1990. Swelling induced release of glutamate, aspartate, and taurine from astrocyte cultures. J Neurosci 10:1583–1591.

Kimelberg HK, Ransom BR. 1986. Physiological aspects of astrocytic swelling. In: Fedoroff S, Vernadakis A, editors. Astrocytes, Vol 3. San Diego: Academic Press. p 129–166.

Kirk K. 1997. Swelling-activated organic osmolyte channels. J Membr Biol 158:1–6.

Lang F, Busch GL, Ritter M, Völkl H, Waldegger S, Gulbins E, Häussinger D. 1998. Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:47–306.

Law RO. 1994. Taurine efflux and the regulation of cell volume in incubated slices of rat cerebral cortex. Biochim Biophys Acta 1221:21–28.

Law RO. 1996. Volume regulation and the efflux of amino acids from cells in incubated slices of rat cerebral cortex I. Characteristics of transport mechanisms. Biochim Biophys Acta 1314:34–42.

Lien YH, Shapiro JI, Chan L. 1991. Study of brain electrolytes and organic osmolytes during correction of chronic hyponatremia. J Clin Invest 88:303–309.

Lohr JW. 1990. Isovolumetric regulation of renal proximal tubules in hypotonic medium. Ren Physiol Biochem 13:233–240.

Lohr JW, Grantham JJ. 1986. Isovolumetric regulation of isolated S_2 proximal tubules in anisotonic media. J Clin Invest 78:1165–1172.

McManus ML, Churchwell KB. 1994. Clinical significance of cellular osmoregulation. In: Strange K, editor. Cellular and molecular physiology of cell volume regulation. London: CRC Press. p 63–80.

Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G. 1997. Properties of volume-regulated anion channels in mammalian cells. Prog Biophys Mol Biol 68:69–119.

Nagelhus EA, Lehmann A, Ottersen OP. 1993. Neuronal exchange of taurine during hypo-osmotic stress: a combined immunocytochemical and biochemical analysis in rat cerebellar cortex. Neuroscience 54:615–631.

Pasantes-Morales H. 1996. Volume regulation in brain cells: cellular and molecular mechanisms. Metab Brain Dis 11:187–204.

Pasantes-Morales H, Alavéz S, Sánchez-Olea R, Morán J. 1993. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem Res 18:445–452.

Roper SN, Obenaus A, Dudek FE. 1992. Osmolality and nonsynaptic epileptiform bursts in rat CA1 and dentate gyrus. Ann Neurol 31:81–85.

Sánchez-Olea R, Morales M, García O, Pasantes-Morales H. 1996. Cl^- channel blockers inhibit the volume-activated efflux of Cl^- and taurine in cultured neurons. Am J Physiol 270:C1703–C1708.

Solís JM, Herranz AS, Herras O, Lerma J, Del Río RM. 1988. Does taurine act as an osmoregulatory substance in the rat brain? Neurosci Lett 91:53–58.

Strange K, Emma F, Jackson PS. 1996. Cellular and molecular physiology of volume-sensitive anion channels. Am J Physiol 270:C711–C730.

Trachtman H. 1991. Cell volume regulation: a review of cerebral adaptive mechanisms and implications for clinical treatment of osmolal disturbances II. Ped Nephrol 5:743–750.

Van Driessche W, de Smet P, Li J, Allen S, Zizi M, Mountian I. 1997. Isovolumetric regulation in a distal nephron cell line (A6). Am J Physiol 272:C1890–C1898.

Osmolytes and Mechanisms Involved in Regulatory Volume Decrease Under Conditions of Sudden or Gradual Osmolarity Decrease*

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A decrease in external osmolarity results in cell swelling and the immediate activation of a mechanism to restore cell volume, known as regulatory volume decrease (RVD). When exposed to a gradual osmolarity decrease (GODE), some cells do not swell. This reflects the operation of an active regulatory process known as isovolumetric regulation (IVR). The mechanisms underlying IVR appear similar to those activated during RVD, namely the extrusion of K^+, Cl^-, amino acids. and other organic molecules. A previous study has documented IVR in cerebellar granule neurons, parallel to an early efflux of taurine and Cl^-, whereas K^- efflux is delayed. In this work we briefly review the importance of amino acids in the mechanisms of cell volume control in the brain, with emphasis on IVR. We also present experiments showing the response to GODE in cerebellar astrocytes. The currents activated during GODE, recorded in the whole-cell configuration of the patch clamp technique, indicate the early activation of an anion current, followed by a more delayed cation current. A correlation between the time course of amino acid efflux during GODE and the occurrence or not of IVR in various cell types, suggest the importance of these osmolytes in the volume regulatory process in this model.

INTRODUCTION

The maintenance of a constant cell volume is a homeostatic imperative in animal cells. Changes in cell water content, by affecting the concentration of messenger molecules impair the complex signaling network, crucial for cell functioning and for intercellular communication. It is then not surprising that the ability to regulate cell volume is an ancient conserved trait present in essentially all species throughout evolution (1). Cell volume is challenged by changes in external osmolarity in aquatic species and in a few cell types in terrestrial animals, but also in pathological conditions associated with hyponatremia (1,2). Cell volume increase may also occur in isosmotic conditions as consequence of changes in ion distribution either in pathological or in physiological conditions. Most studies at present have focused on the mechanisms of cell volume adjustment in hyposmotic conditions.

Cell membrane is highly permeable to water, and therefore any difference in osmolarity across the membrane results in net water movements in the necessary direction to reach the osmotic equilibrium. In face of

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65

a decrease in external osmolarity, cells initially behave as almost perfect osmometers and swell with a magnitude proportional to the osmolarity reduction. Immediately after, an active volume correction initiates, based on the extrusion of intracellular solutes together with osmotically obligated water, which tends to reduce the osmotic difference and to normalize cell volume. This homeostatic mechanism is known as regulatory volume decrease (RVD). The time necessary to fully activate RVD and to regain cell volume is variable in different cell types. In brain cells *in vitro*, RVD occurs rapidly, with a 70%–80% recovery reached within few minutes, as result of osmolyte efflux activation (3). RVD is a complex chain of events requiring a sensor to detect transient changes in cell volume, a signaling cascade to transduce the information about the volume change into the activation of pathways for osmolyte extrusion, and a "memory" of the original cell volume that sets the timing for inactivation of the regulatory process. Most efforts have been directed to identifying and characterizing the osmolyte efflux pathways, and it is only recently that interest has risen in understanding the osmotransduction mechanisms. There is so far only sparse information about the nature of the volume-sensing mechanisms.

K^+ and Cl^- extrusion during RVD occurs through separate volume-sensitive K^+ and Cl^- channels whose properties have been characterized in some detail but for which the molecular identity remains unknown. While the volume-sensitive Cl^- channel exhibits similar properties in most cells (4), the K^+ channel exhibits differences according to the cell types studied (5). The osmosensitive efflux of taurine and possibly other amino acids occurs via a leak pathway with essentially no involvement of energy-dependent carriers. Taurine translocation in response to swelling is passive, directed only by the concentration gradient (6). The unexpected sensitivity of the hyposmotic taurine release to Cl^- channel blockers raised the possibility of a common pathway for translocation of Cl^-, taurine, and possibly other organic osmolytes (7), a notion that has not yet been conclusively confirmed nor discarded. The Cl^- channel involved in RVD exhibits a broad permeability spectrum, allowing the passage of large anions and organic anions, including glutamate, aspartate, and taurine in the anionic form, showing that at least the size of the pore is sufficiently large to permeate amino acids (8).

The transduction signaling elements ultimately leading to activation of pathways for osmolyte extrusion are not well known at present. Hyposmotic swelling leads to changes in the concentration of second messengers, such as Ca^{2+}, cAMP, IP3, and arachidonic acid, but their influence on the effector pathways is poorly defined (9). Tyrosine kinase phosphorylation modulates Cl^- and taurine but not glutamate osmosensitive fluxes (10,11).

DISCUSSION

Cell Swelling in Brain

Cell volume perturbation is a challenge for homeostasis in all animal organs, but it has particularly dramatic consequences in the brain. The limits to expansion imposed by the rigid skull, give narrow margins for the buffering of intracranial volume changes. As expansion occurs, the constraining of small vessels generates episodes of ischemia, infarct, excitotoxicity and neuronal death. In extreme conditions, caudal herniation of the brain parenchyma through the foramen magnum affects brain stem nuclei, resulting in death by respiratory and cardiac arrest. Besides these extreme effects, brain cell swelling may also lead to hyperexcitability and excitotoxicity (12,13).

Early studies in chronic hyponatremia showed that brain does not behave as a perfect osmometer, and the initial swelling is followed by progressive water loss until almost complete normalization, despite the persistence of hyponatremia. The observed electrolyte decrease was not sufficient to compensate the loss of water, and evidence was then obtained about a significant contribution of organic osmolytes (14), including the most abundant amino acids, as well as of N-acetylaspartate, *myo*-inositol, creatine, phosphocreatine, phosphoethanolamine, and glycerophosphoryl choline (15). In rodents, taurine is the most important organic osmolyte, because it is highly concentrated and shows the largest reduction during hyponatremia. In other species with lower brain taurine content, compounds such as N-acetylaspartate, may have an important role (16). The estimation of organic osmolyte change in all these studies does not discriminate neither the regional variation within the brain nor possible differences in the cell type. Cells in culture exposed to hyposmotic media represent a convenient model to address to these questions.

RVD has been studied in detail in astrocytes and neurons from primary cultures (17–19), and in neuroblastoma and glioma cells lines (20,21). In cultured astrocytes and neurons, hyposmosis leads to rapid swelling followed by typical RVD. The efflux pattern of amino acids closely parallels the time course of the change in cell volume in contrast to Cl^- and K^+ fluxes which are faster (Cl^-) or slower (K^+) than the change in cell volume (22). Osmosensitive efflux of amino acids

has also been reported in hippocampal and cortical slices (23,24) and *in vivo* during continuous superfusion of cerebral cortex (24) or by microdialysis (25). In all these preparations, taurine is the most sensitive to the osmotic perturbation, with the lowest release threshold and the largest amount released (23–26).

Brain cell edema also occurs in isosmotic conditions (called cytotoxic edema) associated with ischemic stroke, head trauma and hepatic encephalopathy (2). Swelling also occurs in excitotoxicity and seizures (12). This condition conveys more risks than hyposmotic swelling, because in cytotoxic swelling there is no clear evidence of efficient cell volume correction. The mechanisms generating swelling may be somewhat different in each pathology, but in all cases the influx of anions (Cl^- or/and bicarbonate) is a consistent causal factor. This may limit or suppress the possibility of Cl^- and K^+ participating in the compensatory mechanisms to regulate swelling, and the contribution of organic osmolytes may not be sufficient to regulate cell volume. Amino acid efflux during cytotoxic swelling has been documented in experimental models of ischemia and hyperammonemia and in conditions of oxidative stress. Amino acid release is sensitive to Cl^- channel blockers, thus suggesting a mechanism of efflux similar to that characterized in hyposmotic swelling. There is little information about possible signaling cascades for activation of mechanisms of cell volume control in isosmotic swelling (27).

Isovolumetric Regulation

Most studies about the mechanisms of cell volume regulation after hyposmotic swelling in brain cells and in other cell types have been carried out by cell exposure to large and sudden osmolarity (SODE) decreases. Although these studies have rendered valuable information about the basic mechanisms of cell volume control, such changes probably never occur in brain under physiological or pathological conditions. A paradigm closer to the *in vivo* situation was first devised by Lohr and Grantham (28) in renal proximal tubules, in which cells were exposed to small and gradual osmolarity decreases (GODE). Under these conditions, cell volume remains stable over a broad range of osmolarities, even when the osmolarity drops to 50%. This constancy in cell volume was named "isovolumetric regulation" (IVR), which has the implicit idea of an active mechanism of volume adjustment rather than the absence of swelling. This notion is based on the shrinkage observed in cells returned to an isosmotic medium, due to the loss of intra-

cellular osmolytes (28). After this early report, IVR has been observed in the renal cell line A6 (29), cerebellar granule neurons (30), and hippocampal slices (31). In C6 glioma cells, IVR is observed only at very small reductions in osmolarity, lower than 0.4 mOsm/min (32). Cultured myocytes (33) exposed to GODE, even when they do not exhibit IVR, they swell consistently less than after SODE, suggesting more efficient mechanisms of volume regulation in GODE. The same type of response has been observed in cultured astrocytes, as next described. In the trout erythrocytes, cells swell the same, regardless of the experimental paradigm used (34).

Volume Changes in Cerebellar Cultured Astrocytes During GODE

In this work we examined the response of cultured rat cerebellar astrocytes, and some of its features were compared to those previously reported in cerebellar granule neurons and renal cells. For this study, primary cell cultures of cerebellar astrocytes from 8-day-old rat cerebellum were prepared as previously described (18). A superfusion medium with a linear reduction in osmolarity of −1.8 mOsm/min was formed, as described (28). The dilution linearity was verified in a freezing point osmometer. The procedure for measuring changes in cell volume and osmolyte release were made following the method described in reference (35) and is indicated in the corresponding figures.

Figure 1 shows that astrocytes exposed to GODE (−1.8 mOsm/min) exhibited a constant increase in cell volume (Fig. 1A), which was lower than that attained when cells are challenged by SODE of the same magnitude (cell volume in 15%, 30% and 50% hyposmotic solutions shown in the upper line of Fig. 1B). When cells were returned to isosmotic conditions, cell volume overshooed basal levels and cell shrinkage was observed indicating the loss of intracellular osmolytes (Fig 1A).

This response is similar to that exhibited by glioma C6 cells and myocytes in the same conditions of osmolarity decrease and could be interpreted as an absence of IVR in these cells (32,33), in clear contrast to IVR occurrence in neurons and renal cells (28–30). However, cell volume control in conditions of GODE, seems to be more rapid and efficient than in the model of SODE, preventing maxium cell swelling elicited by hyposmolarity. As shown in Fig. 1B, astrocytes in GODE swell about 24%–54% less than those in SODE. In this later condition, astrocytes will eventually counteract swelling by the typical RVD, but the time required is longer. Therefore, when osmolarity is gradually reduced, less
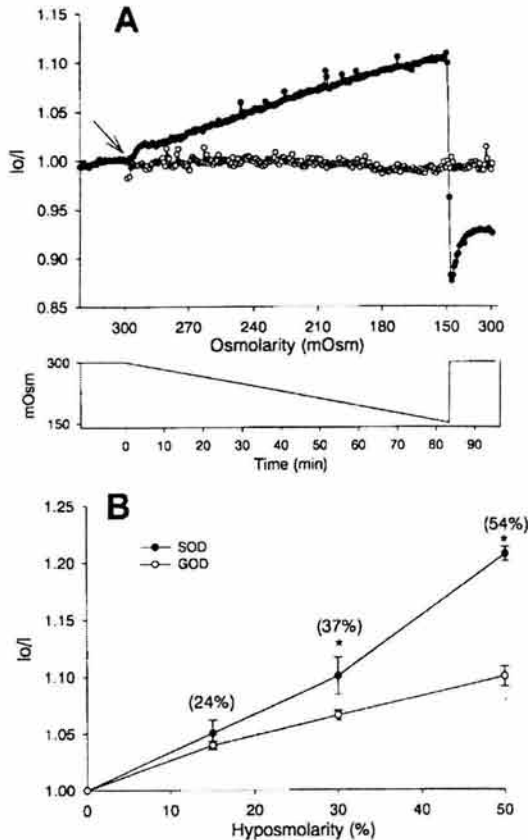
Fig. 1. Cell volume changes of cerebellar astrocytes exposed to gradual changes in external osmolarity. Estimation of relative cell volume was performed using large-angle light scattering as described by McManus et al.. (35). Cells seeded on rectangular coverslips were placed on a superfusion chamber on an Aminco-Bowman luminescence spectrometer (series 2, SLM AMINCO, Rochester, NY, USA). Excitation and emission wavelength were 585 and 581, respectively. Data are given as the inverse of the emission, as emitted light intensity inversely correlates with cell volume. A, Upper panel: a representative experiment of cell volume changes during GODE (●). Cells were superfused (rate 2.0 ml/min at 37°C.) during 10 min with isosmotic medium. At the indicated time (arrow), astrocytes were exposed to the osmotic gradient (−1.8 mOsm/min) during 83 min (lower panel), when the osmolarity has decreased 50% (150 mOsm). At this point cells were returned to isosmotic medium (300 mOsm). Data are plotted against the change in extracellular osmolarity. (○) Continuous superfusion in isosmotic medium. B, Cell volume in astrocytes at −15%, −30%, and −50% hyposmotic medium, reached in conditions of sudden changes in osmolarity (●) or during the exposure to the osmotic gradient (○). The numbers in parenthesis indicate the difference (in %) of GODE against SODE. Points are means ± SE. n = 4. *P* < .05 with respect to sudden changes in osmolarity.

swelling is expected to occur in brain, thus making hyposmolarity a less stressing situation. It is noteworthy that in the GODE model, astrocyte swelling was more

effectively counteracted at larger decreases in osmolarity. Cell volume observed when the osmolarity attained H-15%, H-30%, or H-50% was 24%, 37%, and 54% lower, respectively, than the volume reached after an abrupt reduction in osmolarity of the same magnitude. This may be due to a more substantial contribution of organic osmolytes as the external osmolarity is reduced, as next shown.

Amino Acid Release and Whole Cell Currents Activated by GODE in Astrocytes

The efflux of amino acid evoked by GODE is shown in Fig. 2. An activation threshold of H-15%, H-20%, and H-16% was found for taurine, D-aspartate, and glycine, respectively. As osmolarity reduction progresses, the efflux rate for amino acids was markedly and progressively enhanced (Fig. 2). D-Aspartate exhibited the highest rate release, followed by taurine, while glycine rate efflux was lower (Fig. 2). At the end of the experiment, when external osmolarity has been reduced 50%, astrocytes have released about 60%, 72%, and 50% of the total radioactivity loaded for taurine, D-aspartate, and glycine, respectively (Fig. 2, insets). When these results are compared to those in neurons, it is apparent that the release of taurine and D-aspartate activate earlier in neurons than in astrocytes (Table I), suggesting a higher sensitivity of the pathway for the efflux of these amino acids and possibly of other organic osmolytes as well. This might explain the more efficient volume control in neurons.

K^+ and Cl^- are prominent elements of cell volume adjustment in the SODE model, and they likely are also playing a role during regulation in GODE. Figure 3 shows the currents activated by the gradual osmolarity decrease in cultured astrocytes exposed to GODE recorded in the whole-cell configuration of the patch clamp technique. The figure illustrates the current activation in response to GODE recorded at 264, 219, and 174 mOsm, corresponding to osmolarity decreases of 12%, 27%, and 42%, respectively. As shown in Fig. 3, the outward current was more prominent than the inward current, even though the time course was similar in both cases. As a consequence of this current activation, cells markedly depolarize from the resting potential of −69 mV to −45 mV, detected only after 20 min of the gradient onset. At 40 min of gradient exposure the membrane potential has changed to −47 mV and at min 70 to −50 mV (Fig. 3). The time course of the membrane potential change elicited by the osmotic gradient suggests that, at small decreases in osmolarity, a Cl^-
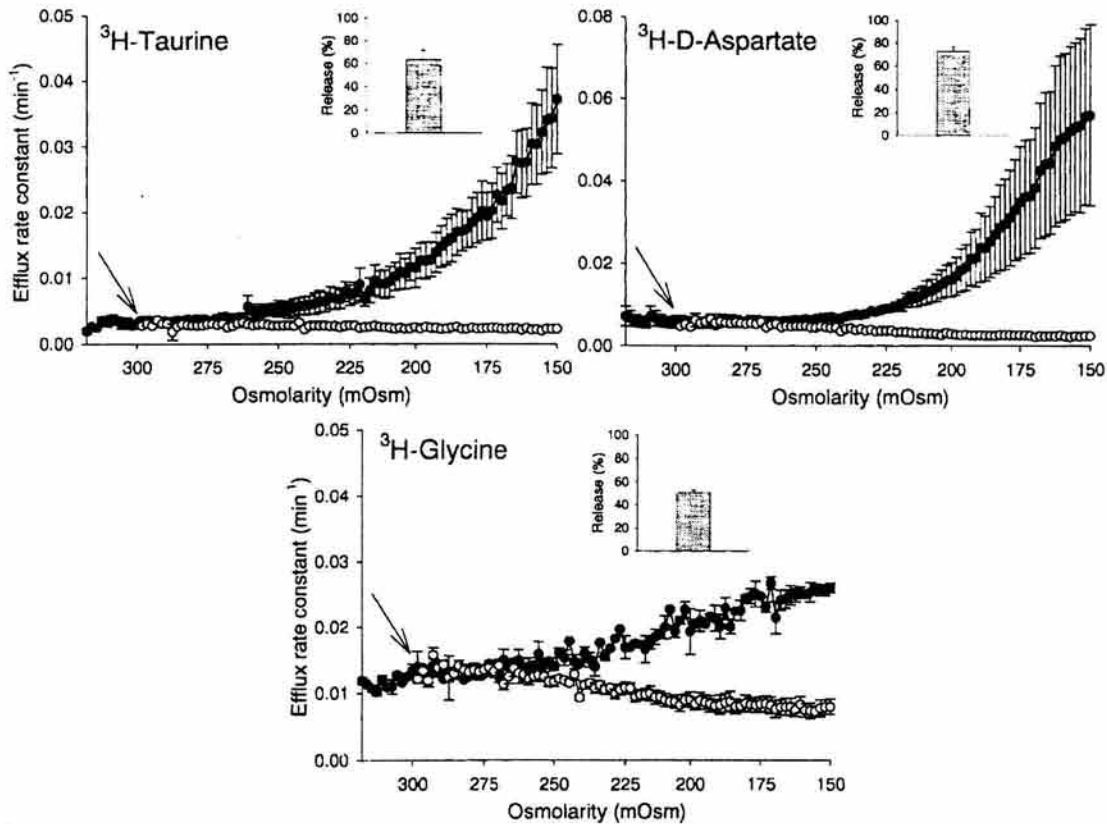
Fig. 2. Amino acid release from cerebellar astrocytes exposed to gradual reductions in external osmolarity. Cells were preloaded with ^{3}H-taurine, ^{3}H-D-aspartate, and ^{3}H-glycine as described in Tuz et al. (30) After the loading period, cells were washed and superfused (rate 2.0 ml/min) during 20 min with isosmotic medium. At the indicated time (arrow) in (●), superfusion continued with the osmotic gradient generated as in Fig. 1 (−1.8 mOsm/min). In (○), superfusion continued with isosmotic medium. Fractions were collected every min. Experiments were performed at 37°C. Data are expressed as efflux rate constants (min^{-1}) and are means ± SE of four experiments and are plotted against the change in the extracellular osmolarity. Insets in each case show the amount of radioactivity released at the end of the experiments.

current is primarily activated, followed by a cation (most likely K$^+$) current, which prevents further depolarization toward the Cl$^-$ equilibrium potential (−38 mV). We did not explore the specific ion carried by these currents, but they likely correspond to Cl$^-$ and K$^+$. Electrophysiological studies in cultured astrocytes in conditions of GODE have shown also marked initial depolarization with a change in membrane potential of −30 to −40 mV, consistent with activation of an early Cl$^-$ current (36,37).

In neurons and renal cells, GODE elicits ^{86}Rb efflux with threshold activation at H-27% for neurons and H-20% for renal cells (Table I). In astrocytes, in contrast, we could not detect any efflux of K$^-$ (^{86}Rb). This result is similar to that observed in hippocampal slices, where no efflux of K$^+$ could be observed (Table I). Also, in a study in cultured astrocytes chronically exposed to hyposmotic conditions, the cell content of taurine is markedly and progressively reduced, while that of K$^+$ was unchanged (38). The special ability of astrocytes to efficiently accumulate external K$^+$ could counteract the efflux activated by hyposmolarity. This feature may contribute to a less effective volume regulation in astrocytes, compared to neurons. It is well known that astrocytes swell more than neurons in most swelling generating conditions.

Table I. Hyposmotic (H) Activation Threshold of ^{86}Rb and Labeled Amino acids from Different Preparations in GODE conditions

	Amino acids		
	Taurine	Glutamate*	K$^+$ (^{86}Rb)
Cerebellar granule neurons[30]	H-2% 294 mOsm	H-19% 243 mOsm	H-29% 213 mOsm
Cerebellar astrocytes	H-13% 261 mOsm	H-18% 246 mOsm	ND
Hippocampal slices[31]	H-1.6% 295 mOsm	H-1.6% 295 mOsm	ND
A6 cell line[29]	NE	NE	H-19% 210 mOsm

Note: Data are expressed as the necessary reduction in osmolarity (hyposmolarity, H; and mOsm), at which osmolyte release attains significance with respect to isosmotic basal release.
Superscript numbers indicate references. * Glutamate release was followed as ^{3}H-D-aspartate efflux. ND, no detectable difference between ^{86}Rb efflux in isosmotic and hyposmotic gradient conditions; NE, not examined.

Why IVR Is Present in Some Cells and Absent in Others?

According to their behavior facing GODE, three types of cell response have been so far observed. In the first one, cells like renal cells and cerebellar granule neurons respond showing IVR (28–30). Another type of response is that of glioma C6 cells, cultured myocytes (32,33), and astrocytes (present work), in which GODE elicits a continuous volume increase, which although being lower than that evoked by the sudden hyposmolarity decrease, as discussed above, cannot be considered as IVR. The third type of cell response, so far found only in trout erythrocytes (34), is that in which cells swell to the same extent in both, SODE and GODE.

The volume corrective mechanisms during GODE may or may not be identical to those operating in RVD.
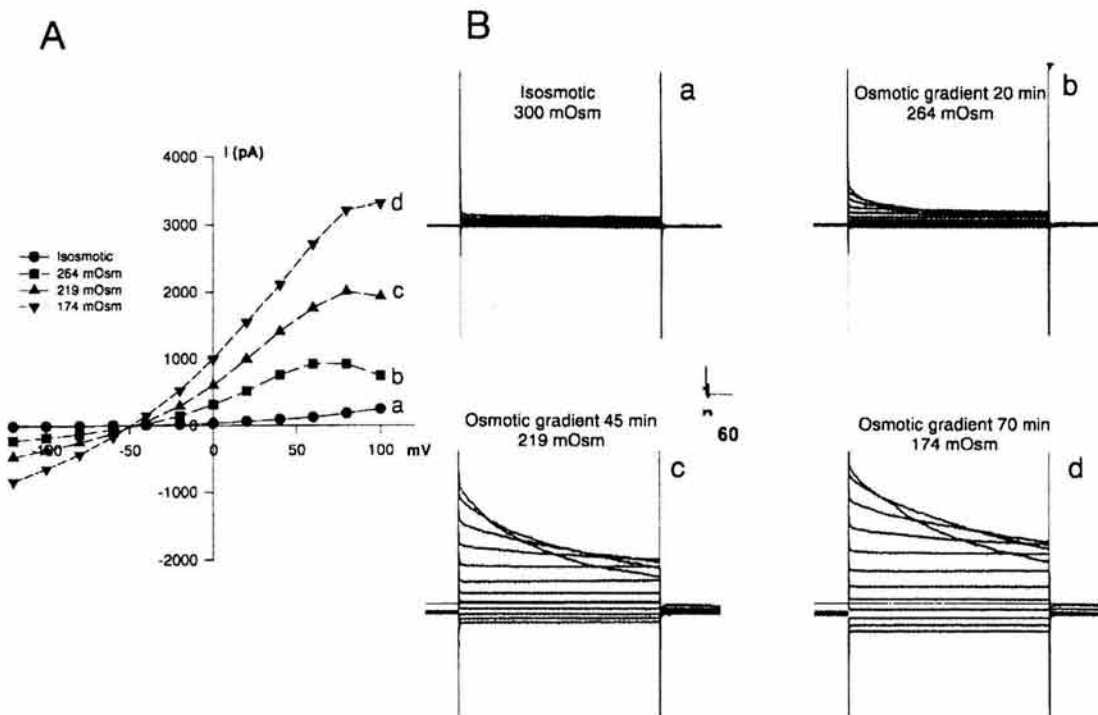
Fig. 3. Osmotic gradient–induced activation of whole-cell currents in cerebellar astrocytes. Astrocytes were seeded in 35-mm petri dishes at a low density of 0.3 mill/ml. Currents were monitored with Axopatch 200 patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell membrane currents of cultured astrocytes were measured using ruptured patches. The time course of whole cell currents were obtained by potential ranging from −120 to +100 mV in 20-mV increments for 300 ms from a holding potential of −70mV. Patch electrodes were prepared from 1.5 mm OD, 1.5 mm D borosilicate glass (World Precision Instruments, Inc., Sarasota, FL) with 3–5 MΩ when filled with pipette solution. The standard pipette solution contained (mM): 110 K$^+$ aspartate, 30 KCl, 1 MgCl$_2$, 10 HEPES, 5 EGTA, 5 MgATP, pH 7.4 adjusted with KOH (300 mOsm). Cells were superfused (2 ml/min) and osmotic gradient (−1.8 mOsm/min) was performed as in Fig. 1, at 37°C. In A, current-voltage (I–V) relationship obtained from whole-cell currents recorded in isosmotic (300 mOsm) conditions (a), and when the extracellular medium reached 264 (b), 219 (c), and 174 mOsm (d) during the osmotic gradient. In B, whole-cell currents recorded at points a, b, c, and d in A. Membrane potential changed in these conditions from −69 mV in isosmotic conditions (a), to −45 (b), −47 (c) and −50 mV (d) during the osmotic gradient. A representative experiment of n = 4.

The osmolytes involved seems the same in the two conditions, namely K^+, Cl^-, and organic molecules, but the activation threshold and the efficacy of the osmolyte translocation pathways might be different. Such differences could explain why some cells exhibit IVR and some others do not. Unfortunately, the information available about such mechanisms in the GODE model is in most cases, only scarce. The response to GODE in A6 cells, in cerebellar granule neurons and in myocytes, involves K^+ extrusion (29,30,33). In astrocytes the change in membrane potential observed during GODE, suggests a contribution of both anionic and cationic currents, the latter likely corresponding to K^+ currents. In the trout erythrocytes, in contrast, there is essentially no efflux of K^+ (34). The contribution of amino acids (taurine) has been examined in myocytes (33), cerebellar granule neurons, and hippocampal slices (30,31), but the time course and the osmolarity threshold of amino acid release has been shown only in main cell preparations. A comparison between taurine and D-aspartate efflux in neurons and astrocytes shows a higher sensitivity of this release in neurons, as compared to astrocytes (30 and present results). This may account, at least in part, to the more efficient regulation observed in neurons in the GODE model. In fact, blockade of Cl^- and amino acid efflux fully prevents IVR in neurons. In the trout erythrocytes, no efflux of taurine occurs during GODE (34). These results, together with those in astrocytes, showing a more effective cell volume control at the time when amino acid efflux occurs, stress the importance of these compounds in conditions of hyposmolarity changes closer to the physiological situation. It should be mentioned in this respect, that in chronic hyponatremia, changes in osmolarity are small but persist during several hours or even days, and the adaptive mechanisms of the brain in the long term do not rely on inorganic ions but on organic osmolytes, including importantly taurine and other amino acids (14).

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REFERENCES

1. Lang, F., Busch, G. L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E., and Haussinger, D. 1998. Functional significance of cell volume regulatory mechanisms. Physiol. Rev. 78:247–306.
2. Kimelberg, H. K. 1995. Current concepts of brain edema: Review of laboratory investigations. J. Neurosurg. 83:1051–1059.
3. Pasantes-Morales, H. 1996. Volume regulation in brain cells: Cellular and molecular mechanisms. Metab. Brain. Dis. 11: 187–204.
4. Okada, Y. 1997. Volume expansion-sensing outward-rectifier Cl channel: Fresh start to the molecular identity and volume sensor. Am. J. Physiol. 273:C755–C789.
5. Pasantes-Morales, H. and Morales-Mulia, S. 2000. Influence of calcium on regulatory volume decrease: Role of potassium channels. Nephron 86:414–427.
6. Sanchez-Olea, R., Moran, J., Schousboe, A., and Pasantes-Morales, H. 1991 Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. Neurosci. Lett. 130:233–236.
7. Junankar, P. R. and Kirk, K. 2000. Organic osmolyte channels: A comparative view. Cell. Physiol. Biochem. 10:355–360.
8. Strange, K., Emma, F., and Jackson, P. S. 1996. Cellular and molecular physiology of volume-sensitive anion channels. Am. J. Physiol. 270:C711–C730.
9. Mongin, A. A. and Orlov, S. N. 2001. Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology. 8:77–88.
10. Pasantes-Morales, H. and Franco, R. 2002. Influence of protein tyrosine kinases on taurine release. Cerebellum. 1:103–109.
11. Pasantes-Morales, H., Franco, R., Ochoa, L., and Ordaz, B. 2002. Osmosensitive release of neurotransmitter amino acids: Relevance and mechanisms. Neurochem. Res. 27:59–65.
12. Andrew, R. D. 1991. Seizure and acute osmotic change: Clinical and neurophysiological aspects. J. Neurol. Sci. 101:7–18.
13. Schwartzkroin, P. A., Baraban, S. C., and Hochman, D. W. 1998. Osmolarity, ionic flux, and changes in brain excitability. Epilepsy Res. 32:275–285.
14. Verbalis, J. G. and Gullans, S. R. 1991. Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res. 567:274–282.
15. Sterns, R. H, Baer, J., Ebersol, S., Thomas, D., Lohr, J. W., and Kamm, D. E. 1993. Organic osmolytes in acute hyponatremia. Am. J. Physiol. 264:F833–F836.
16. Taylor, D. L., Davies, S. E., Obrenovitch, T. P., Doheny, M. H., Patsalos, P. N., Clark, J. B., and Symon, L. 1995. Investigation into the role of N-acetylaspartate in cerebral osmoregulation. J. Neurochem. 65:275–281.
17. Pasantes-Morales, H., Maar T. E., and Moran, J. 1993. Cell volume regulation in cultured cerebellar granule neurons. J. Neurosci. Res. 34:219–224.
18. Pasantes-Morales, H., Murray, R. A., Lilja, L., and Moran, J. 1994. Regulatory volume decrease in cultured astrocytes: I. Potassium- and chloride-activated permeability. Am. J. Physiol. 266:C165–C171.
19. Pasantes-Morales, H., Murray, R. A., Sanchez-Olea, R., and Moran. J. 1996. Regulatory volume decrease in cultured astrocytes: II. Permeability pathway to amino acids and polyols. Am. J. Physiol. 266:C172–C178.
20. Basavappa, S., Huang, C. C., Mangel, A. W., Lebedev, D. V., Knauf, P. A., and Ellory, J. C. 1996. Swelling-activated amino acid efflux in the human neuroblastoma cell line CHP-100. J. Neurophysiol. 76:764–769.
21. Strange, K. and Morrison, R. 1992. Volume regulation during recovery from chronic hypertonicity in brain glial cells. Am. J. Physiol. 263:C412–C419.
22. Pasantes-Morales, H., Cardin, V., Morales-Mulia, S., and Quesada, O. 1998. Regulatory volume decrease in cultured brain cells: Rate limiting factors. Pages 43–56, in Okada, Y. (ed.), Cell volume regulation: The molecular mechanism and volume sensing machinery. Amsterdam: Elsevier Science.
23. Law. R. O. 1994. Taurine efflux and the regulation of cell volume in incubated slices of rat cerebral cortex. Biochim. Biophys. Acta. 1221:21–28.
24. Bothwell, J. H., Rae, C., Dixon, R. M., Styles, P., and Bhakoo, K. K. 2001. Hypo-osmotic swelling-activated release of organic osmolytes in brain slices: Implications for brain oedema in vivo. J. Neurochem. 77:1632–1640.

25. Estevez, A. Y., O'Regan, M. H., Song, D., and Phillis, J. W. 1999. Effects of anion channel blockers on hyposmotically induced amino acid release from the *in vivo* rat cerebral cortex. Neurochem. Res. 24:447–452.
26. Solis, J. M., Herranz, A. S., Herreras, O., Lerma, J., and Martin del Rio, R. 1988. Does taurine act as an osmoregulatory substance in the rat brain? Neurosci. Lett. 91:53–58.
27. Pasantes-Morales, H., Franco, R., Torres-Marquez, M. E., Hernandez-Fonseca, K., and Ortega, A. 2000. Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: Contribution and mechanisms. Cell. Physiol. Biochem. 10:361–370.
28. Lohr, J. W. and Grantham J. J. 1986. Isovolumetric regulation of isolated S2 proximal tubules in anisotonic media. J. Clin. Invest. 78:1165–1172.
29. Van Driessche, W., De Smet, P., Li, J., Allen, S., Zizi, M., and Mountian, I. 1997. Isovolumetric regulation in a distal nephron cell line (A6). Am. J. Physiol. 272:C1890–C1898.
30. Tuz, K., Ordaz, B., Vaca, L., Quesada, O., and Pasantes-Morales, H. 2001. Isovolumetric regulation mechanisms in cultured cerebellar granule neurons. J. Neurochem. 79:143–151.
31. Franco, R., Quesada, O., and Pasantes-Morales, H. 2000. Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J. Neurosci. Res. 61:701–711.
32. Lohr, J. W. and Yohe. L. 2000. Isovolumetric regulation of rat glial cells during development and correction of hypo-osmolality. Neurosci. Lett. 286:5–8.
33. Souza, M. M., Boyle, R. T., and Lieberman. M. 2000. Different physiological mechanisms control isovolumetric regulation and regulatory volume decrease in chick embryo cardiomyocytes. Cell. Biol. Int. 24:713–721.
34. Godart, H., Ellory, J. C., and Motais, R. 1999. Regulatory volume response of erythrocytes exposed to a gradual and slow decrease in medium osmolality. Pflugers Arch. 437:776–779.
35. McManus, M., Fischbarg, J., Sun, A., Hebert, S., and Strange, K. 1993. Laser light-scattering system for studying cell volume regulation and membrane transport processes. Am. J. Physiol. 265:C562–C570.
36. Olson, J. E. and Li, G. Z. 1997. Increased potassium, chloride, and taurine conductances in astrocytes during hypoosmotic swelling. Glia. 20:254–261.
37. Kimelberg, H. K., Anderson, E., and Kettenmann, H. 1990. Swelling-induced changes in electrophysiological properties of cultured astrocytes and oligodendrocytes: II. Whole-cell currents. Brain Res. 529:262–268.
38. Olson, J. E. 1999. Osmolyte contents of cultured astrocytes grown in hypoosmotic medium. Biochim. Biophys. Acta. 1453:175–179.

DISCUSION

En este primera parte, se estudió la existencia de mecanismos de regulación del volumen comparando dos modelos de estrés hiposmótico, como son el de cambios súbitos de osmolaridad y cambios graduales. La capacidad que presentaron las rebanadas de hipocampo de mantener su volumen constante en condiciones de cambios graduales de osmolaridad que se demostró en el presente trabajo, indica la presencia de regulación isovolumétrica en esta preparación. Este mecanismo adaptativo al momento de publicación del trabajo sólo había sido descrito previamente en células renales, sin que se hubiesen estudiado con detalle los mecanismos responsables de esta respuesta (Lohr y Grantam, 1986; Lohr, et al., 1986; Lohr, 1990; Van Driessche, 1997). Estudios posteriores han demostrado la presencia de RIV en neuronas granulares de cerebelo de rata (Tuz, et al., 2001) y han confirmado su presencia tanto en la rebanada integra del hipocampo de rata como en neuronas piramidales aisladas del hipocampo (Zhang y Bourque, 2003). Sin embargo, no todos los tipos de células nerviosas lo presentan, ya que neuronas aisladas del núcleo supraóptico, no mostraron alguna presencia de RIV, lo que puede estar en función de su participación como células osmosensoras. Esto les permitiría codificar los distintos estímulos osmóticos mediante la activación de canales inactivados por estiramiento, para la regulación de la excitabilidad y posterior liberación de hormonas involucradas en el control de la osmolaridad del plasma como es la vasopresina (Voisin y Bourque, 2002). En otros tipos celulares como son cardiomiocitos del embrión de pollo y células de glioma C6, sólo se observa una RIV de manera parcial dentro de pequeños rangos de osmolaridad (Lohr y Yohe, et al., 2000; Souza, et al., 2000).

En el caso de los astrocitos, no se observó RIV, sin embargo, el control del volumen en condiciones de cambios graduales de osmolaridad fue más eficiente que en cambios súbitos. La ausencia de RIV puede relacionarse a la mayor susceptibilidad de los astrocitos a cambios de volumen comparado con las neuronas, fenómeno observado en distintas condiciones de edema celular como en el traumatismo craneoencefálico. Este fenómeno puede deberse a la relativa alta expresión de acuaporinas (canales de agua) que se ha reportado para el caso de los astrocitos en comparación con las neuronas (Venero, et al., 1997; Badaut, et al., 2002; Papadopoulos, et al., 2002). Es importante mencionar que es probable que la tasa necesaria para alcanzar RIV deba ser mucho menor para el caso de los astrocitos que para las neuronas (1.8 mOsm/min utilizada en el

presente trabajo y en Tuz, et al., 2001), como lo observado para el caso de la línea celular de glioma C6, en donde una tasa menor a la utilizada en el presente trabajo permite un RIV parcial (Lohr y Yohe, 2000).

En condiciones de cambios graduales en la osmolaridad, la liberación de aminoácidos muestra umbrales de activación diferentes para los distintos aminoácidos. En el caso de la rebanada de hipocampo, la taurina es el aminoácido más sensible, mostrando un umbral más bajo y una velocidad de salida más alta. Para el caso de los astrocitos, el umbral de activación de la taurina respecto a lo observado tanto en la rebanada de hipocampo, como en neuronas en cultivo (Tuz, et al., 2001) es mucho más tardío lo que explicaría las diferencias observadas en cuanto a RIV, así mismo la tasa de activación es menor que la del D-aspartato. Como ya se mencionó en la introducción, la taurina es un aminoácido metabólicamente inerte, por lo que puede moverse dentro y fuera de la célula modificando el contenido de agua sin afectar el metabolismo celular. En cambio, en el caso de los aminoácidos neuroactivos como el GABA y en especial el glutamato, su acumulación en el espacio extracelular en el tejido nervioso puede llevar a profundas alteraciones en la excitabilidad neuronal. Además de su mayor movilidad observada en los experimentos usando el trazador radioactivo, la taurina es el aminoácido que durante la RIV presenta un mayor decremento en la concentración endógena no solo en las rebanadas, sino también en neuronas en cultivo (Tuz K, et al., 2001).

En la rebanada de hipocampo, los datos obtenidos en condiciones de RIV contrastan con lo observado en condiciones de DRV en donde la liberación de K^+ parece jugar un papel importante como osmolito inorgánico en la regulación del volumen. Estudios realizados en células renales (Lohr, 1990; Van Driessche et al., 1997), y posteriormente en neuronas en cultivo, implican al K^+ como un osmolito activo durante el proceso de RIV, siendo un paso limitante para la constancia del volumen característica. En estos estudios, se observa una liberación de Rb^{86} en condiciones de RIV con un umbral de activación de 20 y 30% de hiposmolaridad. Estos resultados contrastan con los observados en el presente trabajo en donde no se pudo observar un incremento en la liberación de Rb^{86} ni una disminución en los niveles endógenos de K^+ para la rebanada de hipocampo, los mecanismos de amortiguamiento del K^+ que existen en el tejido nervioso íntegro, pueden enmascarar la liberación sensible a cambios graduales en la osmolaridad del medio durante RIV, mediante la redistribución del K^+ liberado a través de la glía

(Ameder et al., 1997; Laming, 2000; Wolfgang, 2000). En los astrocitos en cultivo, expuestos a cambios graduales de osmolaridad se activa una conductancia probablemente acarreada por Cl^- y K^+ como se explica en el artículo correspondiente. Sin embargo al no observar ningún flujo de Rb^{86}, se puede sugerir una recaptura importante del K^+ liberado al medio (lo que enmascararía el flujo del trazador radioactivo) a través de vías como cotransportadores ($Na^+/K^+/2Cl^-$) o canales de K^+ los cuales se ha demostrado participan en la captura de K^+ en estas células en condiciones de altos niveles de K^+ extracelular e hinchamiento celular (Gnatenco, et al., 2002; MacVicar, et al., 2002; Su, et al., 2002). Por lo tanto en los astrocitos bajo estas condiciones no habría una pérdida neta del contenido de K^+ intracelular. Esto sustentaría la hipótesis de que en la rebanada el amortiguamiento de K^+ por células gliales limita la pérdida intracelular de K^+, así como el hecho de que una reducción en movilización de K^+ en los astrocitos impediría una RIV más eficiente, enfatizando la importancia de la movilización de osmolitos orgánicos en estas condiciones.

Importancia

Los mecanismos de regulación isovolumétrica estudiados en el presente trabajo, podrían representar aquellos que se activan *in vivo* durante condiciones de hiponatremia crónica. En un estudio de hiponatremia crónica experimental en la rata, el contenido de agua en el cerebro se mantiene constante durante periodos de hiponatremia prolongada (días) (Gullans y Verbalis, 1993; Verbalis y Gullans, 1991). Igualmente en ratas hiponatrémicas, el contenido de agua en rebanadas corticales permanece sin cambio, probablemente debido a que la entrada de agua originada por el gradiente osmótico ocurre de manera gradual y es contrarrestada con la pérdida lenta de solutos intracelulares (Law, 1999a; 1999b). Estos resultados difieren de lo observado en casos de hiponatremia aguda, en donde sí se presenta un incremento en el contenido de agua en el cerebro (Trachtman, 1992). Es importante recalcar la importancia del control del volumen en los niveles fisiológicos normales ya que existen trabajos en los que se reportan severas alteraciones neurológicas cuando el volumen se incrementa 10% como son el origen de cuadros epilépticos (Trachtman, 1992; Schwartzkroin, et al 1998).

Durante un estado crónico de hiponatremia existe una reducción sostenida en el contenido cerebral de electrolitos de 10% a 30%, mientras que el contenido de agua sólo

aumenta 0.6% (Verbalis y Drutarosky, 1988). Por otra parte, el contenido de osmolitos orgánicos como taurina, inositol, myo-inositol, creatinina y glutamina disminuye 60% a 84% después de dos días de hiponatremia (Sterns 1993; Verbalis y Gullans, 1990; 1991). La participación de los osmolitos orgánicos y en particular de los aminoácidos en el control del contenido de agua cerebral es muy importante. El análisis del cambio en el contenido de osmolitos orgánicos e inorgánicos en los estudios de Verbalis y Gullans (1991) y de Thurnston y colaboradores (1980), muestran que la contribución de las moléculas orgánicas (aminoácidos y otros osmolitos) es cuantitativamente mayor que la del K^+ y se mantiene durante un tiempo más prolongado (Verbalis y Gullans, 1991). Proporcionalmente, la taurina es el aminoácido que tiene una mayor contribución ya que sus concentraciones celulares son las más altas y su decremento frente a la hiponatremia es muy importante (67%) (Thurston et al., 1980). No sólo en este, sino en otros trabajos acerca de hiponatremia se observa que los aminoácidos que disminuyen en mayor grado su concentración en el cerebro son la taurina (60-85%) y el glutamato (38.5-69%), seguidos del GABA (56%), la glicina (55%) y la alanina (52%) (Bedford et al., 1993; Sterns, 1993; Thurston y Hauhart, 1987b; Verbalis, 1991; 1993). En estos trabajos se reporta que el contenido total de aminoácidos disminuye hasta un 61%.

Estos resultados concuerdan con los obtenidos en nuestro modelo experimental, los cuales sugieren que en condiciones de RIV, el K^+ no parece tener un papel importante en el mantenimiento del volumen en preparaciones de tejido íntegro. Esto puede tener relación con el papel crítico que tiene este ión en el mantenimiento de la excitabilidad neuronal normal. Aumentos en los niveles de K^+ extracelular de 5 mM por arriba de las concentraciones fisiológicas normales, incrementan la frecuencia en las descargas epilépticas sincronizadas así como la excitabilidad neuronal en el hipocampo (Leschinger et al., 1993; Stringer y Lothman, 1988; Tancredi y Avoli, 1987). Asimismo, cambios pequeños en la concentración de K^+ extracelular alteran la transmisión sináptica en la región CA1 del hipocampo (Lux et al., 1986; Rausche et al., 1990). En estas condiciones, la contribución de los aminoácidos como osmolitos orgánicos puede representar una ventaja clara para contrarrestar el cambio en volumen sin afectar notablemente las condiciones normales de excitabilidad. En este sentido, de nuevo la taurina puede tener una implicación preferente debida en parte a su inercia metabólica y también a sus acciones inhibitorias a nivel sináptico. Se ha descrito que la taurina a concentraciones altas puede tener un efecto inhibidor mediado probablemente a través de la interacción

45

con los receptores postsinápticos del GABA y la glicina (Kontro y Oja, 1983; 1985; 1987; López-Colomé y Pasantes-Morales, 1981; López-Colomé, 1981). Tal acción podría contrarrestar los efectos del K^+ y de los aminoácidos neurotransmisores como el glutamato, involucrados en la excitabilidad neuronal. Es importante mencionar que en nuestros resultados, la magnitud en la reducción del contenido de aminoácidos no alcanza a contrarrestar los cambios en la osmolaridad del medio, por lo que se requiere necesariamente la implicación, ya sea de otros osmolitos y/o de hipótesis alternativas. En relación con la participación de otros osmolitos orgánicos, se sabe que compuestos como la creatinina, los polialcoholes, el acido N-acetil aspartato, pueden también participar en el control del volumen en el cerebro (Lien et al., 1991; Thurnston et al., 1987). Otra posible alternativa es la que sugiere que los movimientos de los aminoácidos pueden estar circunscritos a regiones específicas o aún más, a tipos celulares específicos. En estudios de inmunohistoquímica se ha demostrado que en el cerebelo en condiciones isosmóticas, la taurina se encuentra localizada preferentemente en las neuronas y ausente en los astrocitos. En respuesta a un estímulo hiponatrémico hay un cambio dramático en la distribución del aminoácido, que aparece ahora acumulado en los astrocitos y ausente en las neuronas (Nagelhus et al 1993).

Para una mayor discusión respecto a la importancia de la hiponatremia, participación de osmolitos orgánicos y la relación con el modelo de regulación isovolumétrica, referirse a los artículos de revisión 1, 2 y 3 en el Apéndice I.

SEGUNDA PARTE

VIAS DE MOVILIZACION DE AMINOACIDOS EN CONDICIONES HIPOSMOTICAS Y VIAS DE SEÑALIZACION INVOLUCRADAS

ANTECEDENTES

Vías de movilización de los osmolitos orgánicos en condiciones hiposmóticas

La translocación de osmolitos subsecuente a hinchamiento hiposmótico ha sido investigada en diferentes preparaciones incluyendo bacterias, algas, protozoarios, plantas, invertebrados, y vertebrados (Kirk, 1997; Nilius et al., 1997). Por las características observadas a la fecha se ha postulado que en su mayoría, los distintos osmolitos orgánicos involucrados, se movilizan a través de una vía común, por lo que en general, los datos obtenidos para el caso de un osmolito (como la taurina) se han extrapolado de manera general no solo a otros miembros de la misma clase de osmolito (aminoácidos), sino también a la liberación de otro tipo de moléculas poco relacionadas estructuralmente (como el inositol). Esto en muchos casos ha demostrado no ser lo correcto, y ha generado controversia, pues parece depender también del modelo estudiado. En este apartado se hace una revisión de las características de la liberación de los aminoácidos en condiciones hiposmóticas, cuyo estudio se ha enfocado principalmente en la taurina como osmolito ideal.

La vía de movilización de osmolitos orgánicos presenta ciertas características similares como son: a) capacidad de permitir flujo bidireccional determinado por el gradiente electroquímico que prevalezca (vía difusional); b) transporte no saturable, no susceptible a trans-estimulación, independiente de Na^+ y energía; c) inhibida por una gran variedad de bloqueadores de canales de cloro. Esto sugiere la participación de un canal aniónico como vía de movilización de osmolitos orgánicos, probablemente el mismo .nvolucrado en la salida de Cl⁻ activada por hiposmolaridad denominado VSOAC (Strange y Jackson, 1995). Esta hipótesis ha ganado fuerza debido a evidencia experimental que demuestra la existencia de corrientes de aspartato, glutamato y taurina (en forma aniónica a pH alcalino) activadas por hiposmolaridad, mediadas por un canal rectificador saliente, con las mismas características del canal de Cl⁻ sensible a volumen, lo que demuestra que

47

estos canales tienen una permeabilidad significativa a estos aminoácidos y otros osmolitos orgánicos (Roy, 1994; 1995; Roy y Banderali, 1994). En otros reportes se ha observado que concentraciones altas de osmolitos como el sorbitol y el mio-inositol interfieren con la conductancia de Cl^- activada por volumen. Con base en la permeabilidad a distintos solutos se ha postulado que el diámetro del poro del VSOAC es de 8-9 °A, con preferencia al paso de solutos hidrofóbicos sobre los hidrofílicos (Junankar y Kirk, 2000; Kirk y Strange, 1998).

La liberación de taurina en respuesta al cambio de volumen, es independiente de la presencia de Na^+ en el medio extracelular y de la temperatura, lo cual descarta la participación del transportador del aminoácido dependiente de Na^+ como mecanismo de liberación inducida por hiposmolaridad. En cambio, el movimiento de taurina en respuesta al aumento en el volumen celular, se da a través de vías difusionales en los que el movimiento de este aminoácido está dirigido por su gradiente de concentración (Sánchez-Olea et al., 1991). Una característica de los mecanismos de movilización de la taurina asociada a cambios en el volumen celular es que se inhibe por bloqueadores de canales de Cl^- como DIDS, dipiridamol, NPPB, DDF, ácidos grasos poli-insaturados y ácido niflúmico (Pasantes-Morales et al., 1994; Sánchez-Olea et al., 1993; 1996). Esto ha llevado a sugerir 1) que el transporte de este osmolito se realiza a través de un canal aniónico con características tales que permitan el paso de osmolitos zwiteriones como son los aminoácidos neutros, 2) de una señal de activación común a las dos vías que se afecta con los fármacos empleados; o bien, 3) que existe una interrelación de manera muy estrecha, de modo que la supresión de uno de los flujos lleva al bloqueo del otro. Estas interrogantes no son fáciles de aclarar, ya que por una parte, los inhibidores existentes hasta la fecha no permiten discriminar entre los distintos tipos de canales de Cl^- y por otra, los canales de Cl^- involucrados no se han identificado plenamente a nivel molecular.

Vías de transducción de señales involucradas en la movilización de osmolitos en respuesta a cambios en el volumen celular

Las células reaccionan a cambios en el medio ambiente, el cual perciben a través de señales extracelulares. Estas señales pueden ser físicas o químicas. Las células son capaces de detectar estos cambios y transducirlos en señales específicas. En condiciones de hinchamiento celular se ha reportado la activación de un sin número de cascadas de

señalización o vías de transducción de señales. Para muchas de estas vías no se ha determinado claramente el o los papeles que juegan dentro de la respuesta integra de la célula al hinchamiento celular. En muchos casos esta respuesta puede darse de manera tardía involucrando la expresión de distintos genes; en otros casos no se descarta la simple activación de estas señales como un epifenómeno concurrente el estrés generado por el cambio en volumen celular. Para el tema que concierne a este trabajo, el enfoque de este apartado se centra en las vías de señalización involucradas en la activación y/o modulación de la movilización de osmolitos orgánicos en respuesta a hiposmolaridad. Particularmente nos enfocamos en las vías de señalización que se ha reportado que modulan y/o activan la conductancia aniónica sensible a volumen (I_{Clvol}) y la liberación de taurina activada por hiposmolaridad (LTAH) tomando en cuenta la hipótesis discutida antes de una vía común de translocación de estos osmolitos en respuesta a hinchamiento anisosmótico. Para una extensa y mejor revisión al respecto de las vías de señalización activadas por estrés osmótico y su relación con el control del volumen celular, referirse a las siguientes revisiones (Eggermont, et al., 2001; Hoffmann, 2000; Jakab, et al., 2003; Mongin y Orlov, 2001; Pasantes-Morales, et al., 2000; Schliess y Haussinger, 2000; Van der Wijk, et al., 2000; Weiergraber y Haussinger, 2000;).

Como ya se ha mencionado, el hinchamiento hiposmótico induce en la mayoría de los tipos celulares un incremento en la concentración de Ca^{++} intracelular. Sin embargo, con excepción de algunos casos (ver Pasantes-Morales y Morales-Mulia 2000; y Pasantes-Morales, et al., 2000), la activación de I_{Clvol} y la LTAH es independiente de Ca^{++}. Existe evidencia que muestra que la activación de la salida de estos osmolitos requiere una concentración basal mínima de Ca^{++} (~40 a 50 nM), no así un incremento inducido por hiposmolaridad a lo que se denomino requerimiento de "calcio permisivo" (Szucs, et al., 1996; Mongin, et al., 1999, Huang et al., 2001).

En algunos tipos celulares se ha observado, que en condiciones hiposmóticas se incrementan los niveles de diacilglicerol (DAG), el cual activa a la PKC. En un estudio realizado en células aisladas del tubo colector, hepatoma HTC, células de colangiocarcinoma Mz-ChA-1 y células de cancer cervical HT-3, se caracterizó la translocación de distintas isoformas específicas de PKC en respuesta a un estrés hiposmótico, de las cuales PKCα, PKCε y PKCζ se translocan del citosol a la membrana plasmática y perinuclear, lo que sugiere la participación de estas enzimas en la regulación

de distintas cascadas en respuesta a estrés hiposmótico (Chou, et al., 1998; Liu, et al., 2003; Roman, et al., 1998). A la fecha se han reportado diferentes resultados sobre la participación de PKC en la activación de I_{Clvol} (Boese, et al., 2000; Chou, et al., 1998; Furst, et al., 2002; Moran, et al., 2001; Nilius, et al., 1997; Okada, 1997; Robson y Hunter, 1994; Shi, et al., 2002; Verdon, et al., 1995; Wei, et al., 2003; Zhong et al., 2002). La liberación de aminoácidos por hiposmolaridad no se modula por la actividad de PKC en neuronas granulares de cerebelo (Morales-Mulia, et al., 2001), células de eritroleucemia (Huang, et al., 2001), células sanguíneas de raja (Hubert, et al., 2000) en el núcleo supraóptico de la rata (Deleuze, et al., 2000) y en rebanadas de corteza (Estevez, et al., 1999b) (en esta última preparación, el efecto de PKC solo es potenciador). Sin embargo en células cardiacas y células de cáncer cervical humano, la liberación osmosensible de aminoácidos es sensible a inhibidores de PKC (Shen et al., 2001; Song, et al., 1998).

El hinchamiento anisosmótico induce la activación de varias fosfolipasas, incluyendo PLA_2. La activación de PLA_2 regula la activación de I_{Clvol} y la LTAH por hiposmolaridad en algunos tipos celulares (Basavappa, et al., 1998; Hoffmann, 2000; Mitchell, et al., 1997; Thoroed, et al., 1997; Von Weikersthal et al., 1997). Para algunos tipos celulares se ha reportado que el inhibidor específico de la PLA_2 de 85kDa (citosólica dependiente de Ca^{++}), cuya translocación al núcleo es inducida por hiposmolaridad (Pedersen, et al., 2000), inhibe la liberación de ácido araquidónico en condiciones hiposmóticas, así como la activación de I_{Clvol} y de la LTAH (Basavappa, et al., 1998). Estos mecanismos de señalización parecen ser célula específica ya que en otros estudios no se han observado los mismos resultados (Estevez, et al., 1999b). Un estudio reciente sugiere la participación de la $iPLA_2$ (citosólica independiente de Ca^{++}) en la LTAH en fibroblastos (Lambert, 2003)

La activación de las MAP cinasas ERK1/ERK2 por hiposmolaridad es un fenómeno ampliamente reportado (Haussinger y Schliess, 1999). Los mecanismos de activación de esta vía se han descrito perfectamente para el caso de levaduras (Kultz y Burg, 1998, Widmann, et al., 1999). A pesar de la consistencia en la activación de la vía de las MAP cinasas por hiposmolaridad, ésta no parece estar involucrada en la regulación de la salida de osmolitos. Pocos estudios a la fecha sustentan la participación de ERK1/ERK2 en la activación de I_{Clvol} y de la LTAH (Crepel et al., 1998; Shen, et al., 2001). En general estos fenómenos son independientes de la actividad de ERK/1/ERK2 inducida por

hiposmolaridad. La activación de las SAP cinasas p38 y JNK por hiposmolaridad también ha sido reportada ampliamente en distintos tipos celulares, sin embargo en ninguno de estos modelos se ha correlacionado esta activación con la de I_{Clvol} y la LTAH (Tilly, et al., 1996b).

El hinchamiento hiposmótico induce un aumento en los eventos de fosforilación de residuos de tirosina (Tilly, et al., 1993). A la fecha se ha reportado la activación de distintas cinasas de tirosina como son p125FAK (de la Paz, et al., 2002; Tilly, et al., 1996), p56lck (Lepple-Wienhues, et al., 1998), p72SYK y p56LYN (Musch, et al., 1999). Es muy probable que existan otras cinasas de tirosina activadas por hiposmolaridad, no descritas a la fecha. En contraste con lo observado para otras vías de señalización, la actividad de cinasas de tirosina, inducida por hiposmolaridad parece ser esencial en la activación de I_{Clvol} y de la LTAH, y por consiguiente para el progreso del DRV. En la mayoría de los tipos celulares estudiados hasta el momento, distintos inhibidores de cinasas de tirosina, bloquean la activación de I_{Clvol}, mientras que inhibidores de fosfatasas de tirosina potencian su activación (Crepel, et al., 1998; Lepple-Wienhues, et al., 1998; Shi, et al., 2003; Shuba, et al., 2000; Sorota, 1995; Thoroed, et al., 1999; Voets, et al., 1998; Wei, et al., 2003;), aunque existen algunas excepciones (Gosling, et al., 1995; Doroshenko, 1998, Thoroed, et al., 1999). Inhibidores específicos de cinasas de tipo src previenen la activación de I_{Clvol} en linfocitos y miocitos ventriculares (Lepple-Wienhues, et al., 1998; Browe y Baumgarten, 2003). Sin embargo, es solo para el caso de los linfocitos que se ha determinado la identidad molecular de la cinasa de tirosina involucrada (p56lck) (Lepple-Wienhues, et al., 1998; Lepple-Wienhues, et al., 2000).

Trabajos recientes han reportado que la LTAH se potencia por inhibidores de fosfatasas de tirosina y se reduce de manera importante por inhibidores de cinasas de tirosina (Deleuze, et al., 2000; Mongin, et al., 1999b; Morales-Mulia, et al., 2001). En relación a las identidades moleculares involucradas, sólo se ha identificado a p72SYK y p56LYN, como enzimas involucradas en la fosforilación de la banda 3 que regula la LTAH en eritrocitos de raja (Musch, et al., 1999).

La mayoría de los estudios relacionados con la caracterización de la liberación de osmolitos en condiciones hiposmóticas se han hecho para el caso de I_{Clvol} y de la LTAH, debido a la hipótesis del VSOAC como vía común de movilización de estos osmolitos. Los

resultados obtenidos, en muchos casos, se han extrapolado a otros osmolitos orgánicos dentro del mismo grupo (como serían aminoácidos) o inclusive osmolitos no relacionados estructuralmente (mio-inositol). Sin embargo, tal generalización no es correcta en muchos casos puede. A la fecha, poco se sabe de las vías de movilización y de las vías de señalización involucradas en la liberación de otro tipo de aminoácidos como son aminoácidos neurotransmisores, los cuales pueden tener un papel tanto como osmolitos involucrados en el control del volumen celular, o como neurotransmisores involucrados en cambios en la excitabilidad celular para el caso de células excitables.

OBJETIVOS PARTICULARES

Los objetivos de esta segunda parte del proyecto se enfocan en la caracterización de la vía de movilización de osmolitos aminoácidos en respuesta a hinchamiento hiposmótico y las vías de señalización involucradas. Para esto se compararon las características farmacológicas de la taurina y aminoácidos neurotransmisores (glutamato y GABA), para esclarecer si comparten tanto la vía de translocación como los elementos de señalización. Este trabajo se realizó en rebanadas de tejido nervioso, hipocampo y corteza cerebral.

RESULTADOS

Los resultados de esta sección se anexan a continuación en el formato de los artículos correspondientes ya publicados, los cuales contienen, una breve introducción la tema, la metodología utilizada, los datos y la discusión la cual se amplia en la siguiente sección.

ORIGINAL ARTICLE

R. Franco · M. E. Torres-Márquez
H. Pasantes-Morales

Evidence for two mechanisms of amino acid osmolyte release from hippocampal slices

Abstract A 30% decrease in osmolarity stimulated ^{3}H-taurine, ^{3}H-GABA and glutamate (followed as ^{3}H-D-aspartate) efflux from rat hippocampal slices. ^{3}H-taurine efflux was activated rapidly but inactivated slowly. It was decreased markedly by 100 μM 5-nitro-(3-phenyl-propylamino)benzoic acid (NPPB) and 600 μM niflumic acid and inhibited strongly by tyrphostins AG18, AG879 and AG112 (25–100 μM), suggesting a tyrosine kinase-mediated mechanism. Hyposmolarity activated the mitogen-activated protein kinases (MAPK) extracellular-signal-related kinase-1/2 (ERK1/ERK2) and p38, but blockade of this reaction did not affect ^{3}H-taurine efflux. Hyposmosis also activated phosphatidylinositide 3-kinase (PI3K) and its prevention by wortmannin (100 nM) essentially abolished ^{3}H-taurine efflux. ^{3}H-taurine efflux was insensitive to the protein kinase C (PKC) blocker chelerythrine (2.5 μM) or to cytochalasin E (3 μM). The release of ^{3}H-GABA and ^{3}H-D-aspartate occurred by a different mechanism, characterized by rapid activation and inactivation, insensitivity to NPPB, niflumic acid, tyrphostins or wortmannin. ^{3}H-GABA and ^{3}H-D-aspartate efflux was not due to external [NaCl] decrease, cytosolic Ca^{2+} increase or depolarization, or to reverse operation of the carrier. This novel mechanism of amino acid release may be mediated by Ca^{2+}-independent exocytosis and modulated by PKC and actin cytoskeleton disruption, as suggested by its inhibition by chelerythrine and potentiation by 100 nM phorbol-12-myristate-13 acetate (PMA) and cytochalasin E. GABA and glutamate osmosensitive efflux may explain the hyposmolarity-elicited increase in amplitude of inhibitory and excitatory post-synaptic potentials in hippocampal slices as well as the hyperexcitability associated with hyponatraemia.

Keywords Taurine · Hyposmolarity · Swelling · Regulatory volume decrease

Introduction

Amino acids are part of the organic osmolyte pool contributing to regulatory volume decrease (RVD) in most cells [34]. Among them, taurine has been studied in detail mainly because of its metabolic inertness and is often considered to represent osmolyte amino acids. In cultured cells, taurine is released upon hyposmosis through a leak pathway, with essentially no contribution of energy-dependent carriers [15, 35]. Remarkably, osmosensitive taurine release is sensitive to Cl$^-$ channel blockers, suggesting a common pathway for efflux of Cl$^-$ and amino acids, and possibly other organic osmolytes as well [22, 36, 39]. This pathway may be an anion channel-like molecule of broad specificity. Even though the electrophysiological properties and pharmacological profile of this pathway have been characterized in detail, its molecular identity is still unknown [30, 31]. The activation signal and the transduction intermediates are not well defined. Ca^{2+} appears to be a good candidate as messenger in transduction because, in most cells, swelling elicits an increase in cytosolic Ca^{2+} [32]. Moreover, recent evidence for volume-associated phosphorylation of tyrosine kinases and the sensitivity of osmolyte translocation pathways to tyrosine kinase blockers [43] has indicated the involvement of such kinases in the modulation of the volume-activated anion channel. Most reports have focussed on the volume-sensitive Cl$^-$ channel with less information available for osmosensitive taurine fluxes. Essentially no studies have addressed the features of the release of other organic osmolytes, including amino acids, which, similar to taurine, are released from brain cells in response to hyposmolarity [21, 33]. In the present study in hippocampal slices, we compared some of

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the properties of osmosensitive efflux of ³H-taurine, ³H-GABA and glutamate (followed as ³H-D-aspartate), including the time course of release activation and inactivation, the effect of Cl⁻ channel blockers, the involvement of tyrosine kinases, in particular the mitogen-activated protein kinases (MAPK) extracellular-signal-related kinase-1/2 (ERK1/2) and MAPK-p38, and of phosphatidylinositide 3-kinase (PI3K) and the effect of phospholipase blockers. The efflux of taurine had properties remarkably different from those of GABA and glutamate. These may represent either a different mechanism of osmosensitive release or a response not related directly to the volume regulatory process but rather to epiphenomena associated with the complex mechanism of swelling and volume regulation.

Materials and methods

Materials

Anti-MAPK-p38 and anti-ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA), p-Akt antibody was from Cell Signaling Technology (Beverly, Mass., USA), ATF2 (activating transcription factor 2, recombinant protein fusion of residues 19–96 and glutathione S-transferase) was from New England Biotechnology (Beverly, Mass., USA) and okadaic acid was from GIBCO (Gaithersburg, Md., USA). 5-Nitro-(3-phenylpropylamino)benzoic acid (NPPB), BAPTA-AM and wortmannin were from RBI (Natick, Mass., USA); tyrphostins (AG18, AG112 and AG879), lavendustin A, herbimycin A, dephostatin, phorbol-12-myristate-13 acetate (PMA), LY294002, PD98059, SB202190, arachydonyltrifluoromethyl ketone (AACOCF3), methyl arachidonyl fluorophosphonate (MAFP), 7,7-dimethyl-5,8-eicosadienoic acid (DEDA), chelerythrine, cytochalasin E and EGTA-AM were from Calbiochem-Novabiochem (San Diego, Calif., USA). [³H]-taurine, [³H]-GABA and [³H]-D-aspartate were from New England Nuclear (Boston, Mass., USA). [γ³²-P]ATP was from Amersham Pharmacia Biotech. Dihydrokainic acid was from Tocris Cookson (Ballwin, Mo., USA). Niflumic acid, 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS), 4-bromophenacylbromide (4BpB), 1-(2-{[(diphenylmetilene)-imino]oxy}etil)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO-711), o-vanadate and all other reagents were of analytical grade and from Sigma (St. Louis, Mo., USA).

Hippocampal slices

Male adults Wistar rats (weighing 200–250 g) were used throughout the study. Animals were killed by decapitation and brains rapidly removed and submerged in cold Krebs-HEPES medium containing (in mM): 135 NaCl, 1.0 CaCl₂, 1.17 MgCl₂, 1.7 KH₂PO₄, 5 KCl, 5 dextrose and 10 HEPES (300 mOsm/l, isosmotic, pH 7.4). Hippocampal slices, 400 μm thick, obtained as earlier described [11] were submerged immediately in isosmotic medium continuously bubbled with O₂ and kept at room temperature at least for 30 min before the assays. The hyposmotic solution medium (osmolarity reduced by 30% to 210 mOsm/l), was prepared by reducing the [NaCl]. Final osmolarities were verified with a freezing-point osmometer (Osmette A, Precision Systems, Natick, Mass., USA).

Labelled amino acid efflux

Hippocampal slices were incubated in isosmotic medium with the labelled amino acids ³H-taurine (60 min, 55.5 MBq/ml), ³H-GABA or ³H-D-aspartate (as a metabolically inert analogue of glutamate,

30 min, 37 MBq/ml). Then, slices were transferred into perfusion chambers (0.4 ml volume) and washed by perfusion with warmed (37 °C), isosmotic medium at 1 ml/min for 15 min. Thereafter, samples were collected every minute. After 5–10 min of basal release, the osmolarity of the perfusion medium was changed suddenly to 30% hyposmotic medium and samples were collected for 25 min. Preincubation times with drugs varied depending upon the experiment and are indicated in the corresponding figure legend. Controls contained the corresponding vehicle. Amino acid release data are expressed as radioactivity released per minute as a percentage of the total incorporated. When amino acid release was calculated as efflux rate constants, i.e. the radioactivity released at any given time as percentage of total radioactivity present in the tissue at that time, the same kinetic profile was obtained [35].

MAPK-p38 Kinase Assay

MAPK-p38 kinase activity was measured by an immune complex kinase assay using ATF2 as substrate. Briefly, hippocampal slices were incubated for 5 min in isosmotic or hyposmotic medium and, where indicated, 50 μM SB202190 added. The reactions were terminated by addition of lysis buffer containing 20 mM TRIS pH 7.5, 1% Triton X-100, 150 mM NaCl, 20 mM NaF, 0.2 mM sodium o-vanadate, 1 mM EDTA, 1 mM EGTA and 5 mM phenylmethylsulphonyl fluoride. Samples were homogenized and sonicated. Following centrifugation at 15,000 g for 15 min at 4 °C, cleared lysates (approximately 1.5 mg protein) were incubated with 4 μl/sample anti-p38 antibody plus protein A-sepharose beads (3:1 v/v) and incubated for 90 min at 4 °C to precipitate the immune complexes. Samples were centrifuged at 15,000 g for 10 min and the beads then washed twice in kinase buffer containing 25 mM 3-(N-morpholino)propanesulphonic acid (MOPS), 25 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol and 0.1 mM sodium o-vanadate. The kinase assay was initiated by the addition of 20 μl kinase buffer containing 10 μCi [γ³²P]ATP and 50 ng ATF2 to the washed beads. Reactions were incubated for 20 min at 30 °C and then terminated by the addition of 8 μl 5×Laemmli SDS sample buffer. Samples were boiled and centrifuged briefly and the products resolved by 15% SDS-PAGE. The incorporation of ³²P was visualized by autoradiography and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif., USA). Western blots for immunoprecipitated MAPK-p38 were made and developed with enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

ERK1/2 assay

ERK1/2 activity was followed by myelin basic protein (MBP) phosphorylation as described elsewhere [29].

PI3K activity

PI3K activity was determined indirectly by phosphorylation of Akt protein. Samples were prepared as described above, 2 mg of protein dissolved in lysis buffer was immunoprecipitated with a phospho-specific antibody that recognizes Akt phosphorylated at Ser-473. The phosphorylated form of Akt was detected by immunoblotting using the same antibody. These commercially available reagents were used according to the manufacturers' recommendations.
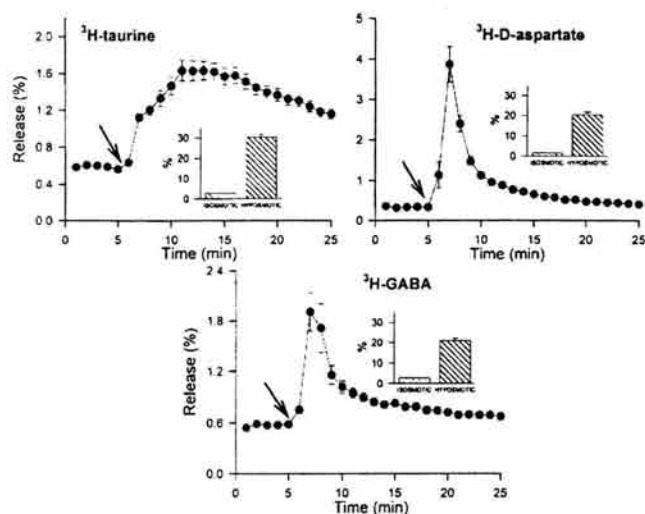
Results

Release pattern and effect of ion replacement

Figure 1 shows the time course of ³H-taurine, ³H-GABA and ³H-D-aspartate release from hippocampal slices in

response to 30% hyposmotic solutions. Marked differences were observed in the efflux pattern among the three amino acids. While the release of ^{3}H-D-aspartate and ^{3}H-GABA was rapidly activated and inactivated, release of ^{3}H-taurine was delayed, reaching a peak only 6–7 min after the stimulus and essentially no inactivation was observed up to 20 min. At the end of the experiment, 20 min after the stimulus, the total amount of ^{3}H-taurine released corresponded to 50% of the label accumulated in the slice, while for ^{3}H-GABA and ^{3}H-D-aspartate total release corresponded to 25% and 35%, respectively (Fig. 1). Hyposmotic solutions were made by decreasing the [NaCl] in the medium. To investigate whether this decrease, rather than the hyposmolarity itself, was the trigger for amino acid release, slices were exposed to media containing a decreased amount of

NaCl as in the hyposmotic solution, but made isosmotic again with mannitol. Under these conditions no increase in amino acid efflux over basal levels was detected, indicating a specific response to hyposmolarity. We next examined the effect of ion replacement in the hyposmotic solution. Replacing all the Na^+ in the medium by choline did not affect the release of ^{3}H-taurine but delayed the inactivation phase of ^{3}H-D-aspartate and ^{3}H-GABA efflux, resulting in increased percentage release (Table 1). Replacing Cl^- by gluconates did not affect the efflux of any of the amino acids (Table 1). Omission of Ca^{2+} and addition of 0.5 mM EGTA had no effect on the fluxes of any of the amino acids. Reducing cytosolic Ca^{2+} by treatment with BAPTA-AM did not affect the efflux of ^{3}H-taurine or ^{3}H-GABA but increased that of ^{3}H-D-aspartate (Table 1).

The effect of Cl- channel blockers

The osmosensitive release of ^{3}H-taurine is sensitive to Cl^- channel blockers in a variety of cell types, including cultured neurons and astrocytes, in the supraoptic nucleus, and in vivo in a cortical cup preparation [4, 8, 18, 36, 37]. Consistent with these results, niflumic acid and NPPB (100 μM) essentially abolished ^{3}H-taurine efflux in hippocampal slices (Fig. 2). DIDS reduced taurine release only at a high concentration (600 μM). Interestingly, the efflux of ^{3}H-GABA and ^{3}H-D-aspartate was notably less sensitive to these agents. In fact, only DIDS exerted a significant inhibitory effect, but niflumic acid and NPPB, in clear contrast to ^{3}H-taurine efflux, were without effect (Fig. 2). Noteworthy in these experiments was an early ^{3}H-taurine release fraction that was resistant to the Cl^- channel blockers and showed an efflux pattern similar to those of ^{3}H-GABA and ^{3}H-D-aspartate, i.e. rapid activation and inactivation phases (Fig. 2).

Fig. 1 Amino acid release from rat hippocampal slices exposed to 30% hyposmotic medium. Slices preloaded with ^{3}H-taurine, ^{3}H-D-aspartate or ^{3}H-GABA were superfused for 5 min with isosmotic medium. Thereafter (arrow), the medium was replaced by 30% hyposmotic solution. One-min fractions were collected for 20 min. Data represent the radioactivity released per minute expressed as percentage of the total incorporated and are means±SE. The insets show the percentage (mean±SE) of total release of the corresponding labelled amino acid during the period of exposure to the hyposmotic solution (min 6–25) and during the basal collection period (min 1–5)

The involvement of tyrosine kinases

The osmosensitive efflux of taurine is influenced notably by manipulation of tyrosine kinase activity in cultured brain cells [28, 29], in the supraoptic nucleus [7] and in skate red cells [17]. In these preparations, taurine efflux

Table 1 Effect of ionic replacement and Ca^{2+} dependency of amino acid osmosensitive release from hippocampal slices. Slices were loaded with labelled amino acids and superfused as described in Materials and methods. Data indicate the release (expressed as the percentage of the total label incorporated) during a 20-min exposure to medium made 30% hyposmotic by decreasing [NaCl]. In Cl-- and Na+-free media, Cl- was replaced by gluconates and Na+ by choline, respectively. In Ca^{2-}-free medium, Ca^{2+} was omitted and 0.5 mM EGTA was added to the solution. To chelate intracellular Ca^{2+}, slices were treated with 30 μM EGTA-AM or BAPTA-AM for 20 min prior to superfusion. Means±SE, $n=4$–16 experiments

Condition	^{3}H-taurine release (%)	^{3}H-D-aspartate release (%)	^{3}H-GABA release (%)
Control (NaCl)	30.3±1.41	20.9±1.20	0.9±1.61
Cl--free	31.0±1.26	19.5±1.40	25.9±0.76
Na+-free	30.9±1.03	30.4±2.90	25.9±0.76
Ca2+-free	29.5±1.06	22.9±1.23	20.7±1.10
EGTA-AM	30.9±1.32	24.7±2.93	21.7±1.56
BAPTA-AM	32.5±1.72	24.5±1.50	23.5±1.56
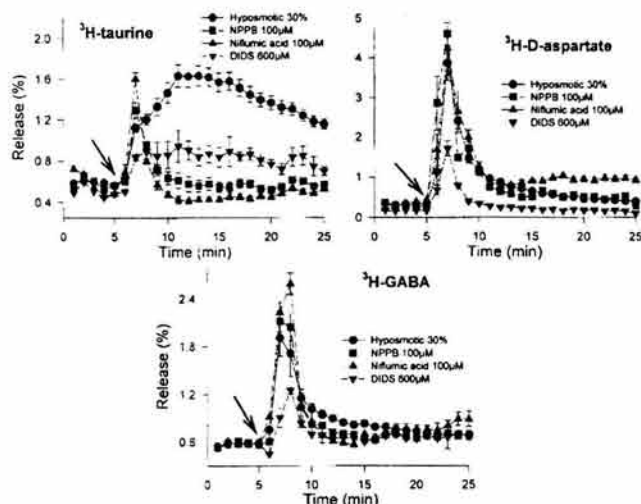
Fig. 2 Effect of anion channel blockers on amino acid release from rat hippocampal slices. Slices preloaded with amino acid tracers were preincubated for 15 min in isosmotic medium with 100 μM NPPB (■), 100 μM niflumic acid (▲) or 600 μM DIDS (▼) (vehicle controls ●). Thereafter, slices were superfused and data presented as in Fig. 1. Blockers were present in all solutions used throughout the experiment. Means±SE, n=4–10. *P<0.001 in fractions 10–25 for all blockers in taurine efflux curves; **P<0.05 in fractions 7–25 for the effect of DIDS on D-aspartate efflux curve and for fractions 7–16 for GABA efflux curves

is inhibited markedly by tyrphostins, which block tyrosine kinases, and potentiated by o-vanadate, which maintains tyrosine phosphorylation. In hippocampal slices, tyrphostins AG18 (50 μM), AG879 (25 μM) and AG112 (100 μM) all inhibited the release of ³H-taurine potently (Fig. 3). Again, there was an early component of the efflux curve that, as with the Cl⁻ channel blockers, was unaffected by tyrphostins (Fig. 3). The non-functional analogue of tyrphostin AG18, AG9 was without effect. The other tyrosine kinase blockers tested, genistein (up to 100 μM), herbimycin A (1 μM) and lavendustin A (10 μM), failed to modify ³H-taurine release (Table 2). In contrast to observations in cell lines and in primary cell cultures, no effect on ³H-taurine release was observed with the tyrosine kinase blockers o-vanadate (up to 1 mM) and dephostatin (20 μM) (Table 2). The effect of AG18 was tested on the release of ³H-GABA and ³H-D-aspartate. In contrast to its potent inhibition of ³H-taurine efflux, this blocker did not affect the release of ³H-GABA and ³H-D-aspartate.

The effect of tyrphostins on ³H-taurine osmosensitive efflux is suggestive of one or several steps mediated by tyrosine kinases, as part of the signalling cascade for activation or operation of the taurine translocation pathway. To contribute to the identification of the kinases involved, we examined the effect of hyposmolarity on the activity of the MAPK ERK1/2 and p38 and on the tyrosine-kinase-activated PI3K, as well as the consequences of inhibiting their activity on the osmosensitive ³H-taurine efflux. These kinases are activated in response to hyposmolarity in a variety of cell types (review in [13]).
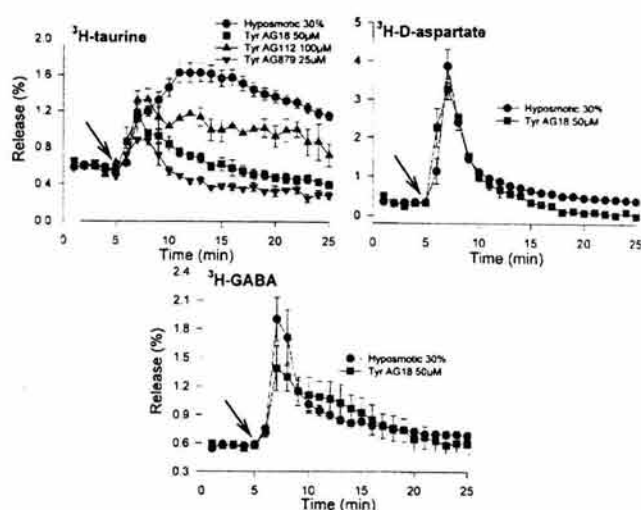
Fig. 3 Effect of tyrphostins on hyposmolarity-induced amino acid release from rat hippocampal slices. Slices preloaded with amino acid tracers were preincubated for 1 h in isosmotic medium without (●) or with 50 μM tyrphostin AG18 (■), 100 μM AG112 (▲) or 25 μM AG879 (▼). Thereafter, slices were superfused and data presented as in Fig. 1. Tyrphostins were present in all solutions throughout the experiment. Means±SE, n=4–10

Table 2 Effect of tyrosine kinase and tyrosine phosphatase blockers on the osmosensitive efflux of taurine. Hippocampal slices were loaded with ³H-taurine and preincubated with the drugs for 60 min. The slices were the washed and superfused with isosmotic medium and with 30% hyposmotic medium, containing the same concentration of the drugs tested. Data represent the release (percentage of total incorporated label) during the 20-min exposure to hyposmotic medium minus the isosmotic release in the same period. Means±SE, n=4–16 experiments

Drug	Concentration (μM)	3H-taurine release (%)
Control		23.31±1.41
Tyrosine kinase blockers		
Tyr AG18	50	8.93±1.30*
Tyr AG112	100	16.52±1.82*
Tyr AG879	25	5.14±1.10*
Genistein	100	26.34±2.11
Herbimycin A	1	28.68±4.95
Lavendustin A	10	23.86±1.34
Tyrosine phosphatase blockers		
o-Vanadate	1000	24.52±1.31
Dephostatin	20	26.69±1.14

*P<0.001, **P<0.005 vs. control

Figure 4 shows the effect of swelling on ERK1/2 phosphorylation in hippocampal slices. In contrast to cells in culture, the hyposmotic stimulus activated ERK1/2 only marginally in our preparation. PD98059 is used widely to prevent ERK1/2 activation and its effect was tested on taurine efflux in the hippocampal slices. Despite a slight effect of this blocker reducing ERK1/2 activity in hyposmotic conditions to levels slightly below the isosmotic activity, PD98059 (50 μM), had no influence on ³H-taurine efflux (Fig. 4). Another tyrosine kinase seemingly

A

MBP →

	I	H	H + PD
%	100	110	98

ERK1 →
ERK2 →

B
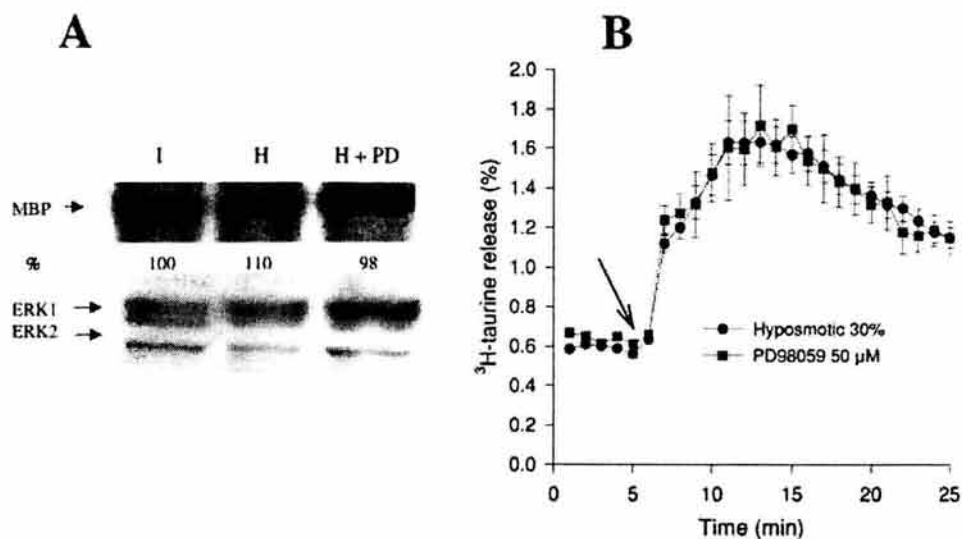
Fig. 4 A Extracellular signal-related kinase 1/2 (ERK1/2) activity in response to hyposmolarity in hippocampal slices. Slices were exposed to media: isosmotic (*I*), 30% hyposmotic (*H*), or 30% hyposmotic+50 μM PD98059 (*H+PD*) for 5 min. Representative results of three independent experiments are shown. Myelin basic protein (*MBP*) phosphorylation (*upper panel*) was assayed using the immune-complex against antibody ERK1/2. The effect of hyposmolarity with or without PD98059 is shown as percentage of isosmotic activity. A Western blot of ERK1/2 for the corresponding assay is shown in *lower panel*. **B** Slices preloaded with ^{3}H-taurine were preincubated for 1 h in isosmotic medium without (●) or with 50 μM PD98059 (■). Thereafter, slices were superfused and data presented as in Fig. 1. PD98059 was present in all solutions throughout the experiment. Means±SE, *n*=6

A

ATF2 →

	I	H + SB	H
%	100	50	200

p38 →

B
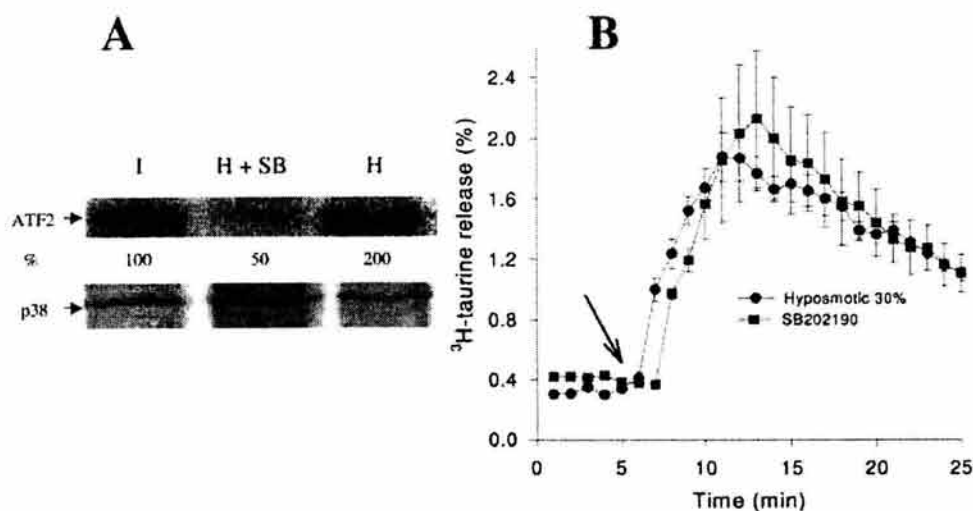
Fig. 5 A Mitogen-activated protein kinase-p38 (MAPK-p38) activity in response to hyposmolarity in hippocampal slices. Slices were exposed to media: isosmotic (*I*), 30% hyposmotic+50 μM SB202190 (*H+SB*) or 30% hyposmotic (*H*) for 5 min. Representative results of three independent experiments are shown. Activating transcription factor 2 (*ATF2*) phosphorylation (*upper panel*) was assayed using the immune-complex against MAPK-p38 antibody. The effect of hyposmolarity with or without SB202190 is shown as percentage of isosmotic activity. A Western blot of p38 for the corresponding assay is shown in the *lower panel*. **B** Slices preloaded with ^{3}H-taurine were preincubated for 1 h in isosmotic medium without (●) or with 50 μM SB202190 (■). Thereafter, slices were superfused and data presented as in Fig. 1. SB202190 was present in all solutions throughout the experiment. Means± SE, *n*=6

involved in cell response to hyposmosis is MAPK-p38. Figure 5 shows a clear activation of p38 following the hyposmotic stimulus. This effect was efficiently prevented by the blocker SB202190 (50 μM), which however, did not decrease ^{3}H-taurine efflux.

Activation of PI3K by hyposmosis has been reported in intestinal 407 and hepatoma cells and in cholangiocytes [10, 24, 40]. In the present study PI3K activity was assessed by measuring the phosphorylation of Akt. Figure 6 shows the clear enhancement of PI3K activity upon hyposmotic stimulus. This reaction was blocked by wortmannin (100 nM) and, to a lesser extent, by LY294002 (25 μM). Wortmannin was a very potent blocker of ^{3}H-taurine efflux. As shown in Fig. 6, the magnitude of the inhibition by this agent was almost the
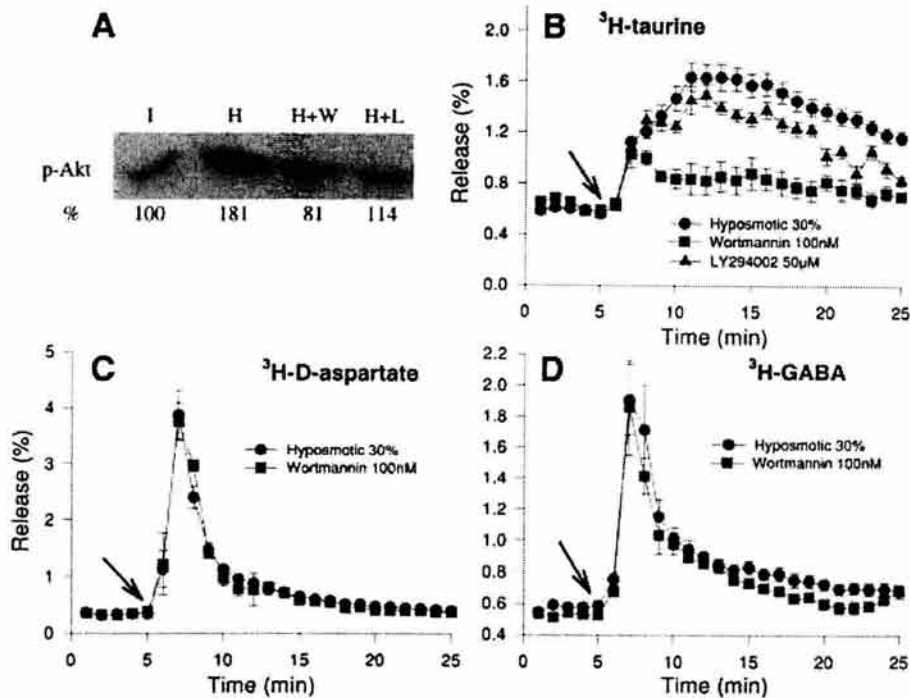
Fig. 6 A–D Phosphatidylinositide 3-kinase (PI3K) activity in response to hyposmotic solution in hippocampal slices. **A** Slices were exposed to media: isosmotic (*I*), 30% hyposmotic (*H*), 30% hyposmotic+100 nM wortmannin (*H+W*) or 30% hyposmotic +25 μM LY294002 (*H+LY*) for 5 min. Representative results of three independent experiments are shown. PI3K activity was quantified by detection of the phosphorylated form of Akt by immunoblotting with a phospho-specific antibody that recognizes Akt phosphorylated at Ser-473. **B** Slices preloaded with ³H-taurine were preincubated for 1 h in isosmotic medium without (●) or with 100 nM wortmannin (■) or 25 μM LY294002 (▲). **C**, **D** Slices loaded with ³H-D-aspartate and ³H-GABA treated with wortmannin as in **B**. Thereafter, slices were superfused and data presented as in Fig. 1. Blockers were present in all solutions throughout the experiment. Means±SE, *n*=6. *P<0.001 from fractions 9–25; **P<0.05 in fractions 13–19; ***P<0.01 in fractions 20–25

same as that of tyrphostin. Similar to the effect of the Cl⁻ channel blockers and of tyrphostin, an early peak of taurine release was insensitive to wortmannin (Fig. 6). LY294002 was clearly less effective than wortmannin in decreasing ³H-taurine efflux. Similar to tyrphostins, the fluxes of ³H-D-aspartate and ³H-GABA were essentially unaffected by wortmannin (Fig. 6).

Phospholipase blockers

Involvement of phospholipases in the osmosensitive ³H-taurine flux from hippocampal slices was examined using the general blockers 4BpB and DEDA, as well as AACOCF3 and MAFP, agents more specific for the cytosolic form of phospholipase A2, for which a role in ³H-taurine efflux in CHP-100 neuroblastoma cells has been demonstrated clearly [3]. Only 4BpB (20 μM) significantly reduced ³H-taurine fluxes (control 30.31±1.41;

4BpB 18.103±0.78; *n*=4, *P*<0.001). None of the other blockers influenced this release: MAPF (20 μM) 31.46±1.99, *n*=4; AACOCF3 (50 μM) 30.57±2.9, *n*=4. The latter was also tested at up to 150 μM without any effect.

Possible mechanisms for GABA and D-aspartate release

The above results clearly show different mechanisms for the release of ³H-GABA and ³H-D-aspartate as compared with taurine. We explored two alternatives. One was that ³H-GABA and ³H-D-aspartate fluxes occur by the reverse operation of Na⁺-dependent transporters. This was assessed by the effect of the carrier blockers NO-711 (GABA) and dihydrokainic acid (glutamate), agents known to block amino acid transport in hippocampal slices effectively [25]. The release of ³H-D-aspartate was unaffected by 1 mM dihydrokainic acid (control 20.5±1.20; dihydrokainic acid 19.51±2.29 *n*=4). Treatment with NO-711 (20μM) delayed the inactivation phase of ³H-GABA efflux, thus increasing the total amount released (control 20.98±1.61; NO-711 26.3± 2.15, *n*=4), but the peak release and activation time were unaffected.

Another possibility for the mechanism of amino acid release addressed here was that of exocytosis, mediated by a change in the actin cytoskeleton modulated by PKC. For this purpose, the effect of PKC modulation and cytoskeletal disruption was examined for ³H-taurine and ³H-D-aspartate release. Figure 7 shows a decrease of ³H-D-aspartate release by the PKC blocker chelerythrine and potentiation by up-regulation with PMA. None of these treatments affected ³H-taurine efflux (Fig. 7). Disruption of the actin cytoskeleton with cytochalasin E
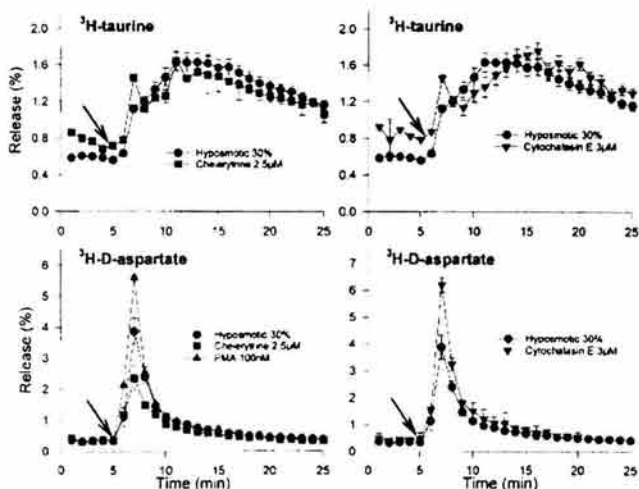
Fig. 7 Effect of modulation of protein kinase C (PKC) activity and of cytochalasin E on ³H-taurine and ³H-D-aspartate release from rat hippocampal slices exposed to 30% hyposmotic medium. Slices were loaded with the labelled amino acids and then treated for 30 min with 2.5 µM chelerythrine (■) or 100 nM PMA (▲) or for 2 h with 3 µM cytochalasin E (▼) (vehicle controls ●). Thereafter, slices were superfused and data presented as in Fig. 1. Drugs were present in all solutions throughout the experiment. Means±SE, $n=4-6$; *$P<0.001$

also failed to affect ³H-taurine efflux but potentiated the release of ³H-D-aspartate (Fig. 7).

Discussion

The present results showed the prompt release of ³H-taurine, ³H-GABA and glutamate (followed as ³H-D-aspartate) from hippocampal slices in response to hyposmolarity. These amino acids are those released preferentially under hyposmotic conditions from hippocampus, both in vitro and in vivo preparations [12, 27, 38]. The characterization of the amino acid osmosensitive release carried out in the present work, showed that while taurine efflux exhibited, in general, the features typically described for most cells, the mechanism of GABA and glutamate release was notably different. This interesting observation raises a number of questions about the source and the function of this release.

Taurine efflux

The hyposmolarity-induced taurine efflux from hippocampal slices showed the general pattern and pharmacological profile previously described for brain cells [36, 37]. Taurine release was inhibited by niflumic acid, NPPB and DIDS and was sensitive to blockers of protein tyrosine kinases. Involvement of tyrosine kinases in the mechanism of taurine release in response to hyposmotic stress has been suggested in various cell types, including brain cells, on the basis of the effects of tyrosine kinase

and phosphatase blockers [7, 17, 19, 26, 28, 29]. Not all blockers exert the same effect, particularly in brain cells where tyrphostins are more potent than genistein. Astrocytes and cerebellar granule neurons [28, 29], and the hippocampal slices here reported, are particularly sensitive to tyrphostins, clearly more so than skate erythrocytes [17]. Herbimycin and lavendustin have no effect on taurine efflux in hippocampal slices, as is the case in Jurkat T-lymphocytes [26]. The meaning of these differences is still obscure, but may be related to the involvement of tyrosine kinases with different sensitivity to the blockers in the different cell types. Inhibition of tyrosine phosphatases by o-vanadate potentiates osmosensitive taurine efflux in cultured cerebellar granule neurons and in rat supraoptic nucleus [7, 29]. This effect was not observed here in hippocampal slices or in trout red blood cells [19], despite the inhibitory effect of tyrosine kinase blockers in this preparation. It is unclear so far whether this is due to permeability restriction in hippocampal slices compared with cell monolayers or to a different sensitivity of the phosphatases involved.

While a role for tyrosine kinases in the mechanisms of hyposmolarity-induced taurine efflux seems well supported, the specific enzymes involved are not yet fully identified. Some possibilities were examined in the present study. The MAPK ERK1/2 were among the first kinases found to be activated by hyposmolarity in a variety of cell types, including epithelial cells, brain cells and myocytes [13]. However, this reaction seems to be unrelated to the mechanisms of osmolyte extrusion, since its prevention by specific MAPK/ERK-kinase (MEK) blockers such as PD98059 consistently fails to modify Cl⁻ and taurine osmosensitive pathways [29, 42] (an exception is the volume-activated Cl⁻ currents in cortical astrocytes that are decreased by PD98059 [6]. Much the same can be concluded for MAPK-p38 that, despite being activated by hyposmolarity, appears to be unrelated to the volume-activated Cl⁻ pathway [41]. This lack of connection was also observed in the present study in hippocampal slices in which ERK1/2 and particularly p38 were activated by swelling, but had no effect on taurine fluxes. These observations suggest that the activation of these MAPK may be epiphenomena, probably associated with some of the numerous events concurrent with cell volume changes, such as stress, adhesion and cytoskeletal rearrangements, but clearly unrelated to the activation or operation of osmolyte extrusion pathways. In contrast, the tyrosine-activated kinase PI3K appears to play a key role in the signalling cascades leading to osmotransduction. Activation by hyposmolarity of PI3K and its prevention by wortmannin have been reported in Intestine 407 and hepatoma cells and in cholangiocytes [10, 24, 40]. In these cells wortmannin also blocks the volume-activated Cl⁻ currents [10, 40]. In hippocampal slices a robust activation of PI3K by hyposmolarity was observed, which was essentially abolished by wortmannin, and this blockade led to a dramatic inhibition of taurine efflux. Previously, we have observed potent inhibition by wortmannin in cultured cerebellar granule neurons [29].

LY294002 also decreased PI3K stimulation by hyposmosis, with lower potency, and, accordingly, had a weaker effect in decreasing taurine efflux. These results underline the essential role of this enzyme in the osmosensitive taurine pathway. The step within the taurine efflux transduction cascade where PI3K functions and its place in the hierarchy of signalling remain to be defined.

The involvement of phospholipases on taurine efflux activated by swelling was also examined in the present study. The most compelling evidence connecting phospholipases to taurine efflux comes from a study in CHP-100 neuroblastoma cells, showing a concomitant hyposmolarity-induced release of arachidonic acid and taurine, both inhibited by AACOCF3, a specific blocker of the 85-kDa PLA2 [3]. This agent was ineffective in hippocampal slices and the more potent, irreversible blocker of this phospholipase, MAPF, was similarly ineffective. The two general blockers of phospholipases also tested, DEDA and 4BpB, had different effects. DEDA was ineffective whilst 4BpB was very potent. Considering the insensitivity of taurine fluxes to all other phospholipase blockers, the effect of 4BpB suggests an action different from phospholipase inhibition and allows the conclusion that, in our preparation, these enzymes do not participate in the taurine efflux activation. Similarly, in the brain in vivo, taurine efflux is insensitive to any of the phospholipase blockers, with exception of marginal inhibition by AACOCF3 [9].

The modulation by PKC, in connection with membrane actin cytoskeleton as a possible mechanism for amino acid release, was also examined in the present work. In hippocampal slices, taurine efflux was not influenced by PKC blockers or by actin cytoskeleton disruption, consistent with results in various cell types. These results further emphasize the marked differences here reported between the osmosensitive fluxes of taurine in comparison to those of GABA and glutamate.

An interesting finding of the present study was the detection of an early component of taurine efflux, uncovered by its resistance to both Cl⁻ channel blockers and tyrosine kinase inhibitors. This fraction, accounting for about 17% of the total amount of taurine released by hyposmolarity, activated and inactivated within the first 1–3 min after the stimulus and seemed, in general, similar to the efflux of GABA and D-aspartate, the features of which are discussed below.

GABA and glutamate (D-aspartate) efflux

Hyposmolarity elicited a rapid efflux of GABA and D–aspartate from hippocampal slices, with very rapid activation and inactivation. This time course contrasts with the delayed and sustained efflux of ³H-taurine. The insensitivity of D-aspartate and GABA release to Cl⁻ channel blockers, which consistently inhibit the osmosensitive leak pathway for organic osmolytes, including taurine, suggests a different pathway for translocation of

GABA and glutamate. Furthermore, this mechanism is not modulated by tyrosine kinases, which are clearly involved in the volume-activated osmolyte pathway in most cell types so far examined. A report on cultured astrocytes has also documented the insensitivity of the osmolarity-sensitive efflux of glutamate to tyrphostins [28]. Altogether, these results clearly indicate different translocation mechanisms for the osmolarity-evoked release of GABA and glutamate and possibly also different activation signals. A possibility explored in the present study was the reverse operation of the carrier. However, neither the ion dependence nor the sensitivity to specific carrier blockers supported this hypothesis. Regarding the activation signals, GABA and glutamate release may respond to other stimuli associated with events concurrent with swelling or/and volume regulation. The efflux may occur subsequent to an increase in cytosolic Ca²⁺ or to swelling-associated depolarization, known to occur in various cell types, including astrocytes [20, 32]. However, this response is likely to be due to rapid Cl⁻ extrusion through the volume-activated pathway and, consequently, should be prevented by niflumic acid or NPPB. Thus, the insensitivity of GABA/glutamate release to these blockers is evidence against the notion of depolarization as the trigger for this release. In addition, there is no evidence of depolarization linked to swelling in hippocampal slices [5].

A further possibility is an exocytosis-mediated mechanism of release for GABA and glutamate, since hyposmotic stimulation leads to a robust increase in the rate of both endo- and exocytosis [43]. In support of this option are the present results suggesting that PKC and cytoskeleton could be effectors of the osmosensitive efflux of these amino acids (but not for taurine release). Much evidence indicates that the membrane actin network may act as a barrier to exocytotic phenomena, and that its disassembly dynamics are modulated by PKC. The Ca²⁺ independence of amino acid release here observed would argue against the possibility of vesicular exocytosis (typically Ca²⁺ dependent), as the mechanism of osmosensitive release. However, there is increasing evidence showing stimulation of exocytosis by phosphorylation reactions (PKC) without a Ca²⁺ signal, either directly releasing the brake for exocytosis, or increasing the Ca²⁺ sensitivity for exocytotic release [14, 23]. In this way, residual cytosolic Ca²⁺ may be a sufficient trigger for release in connection with phosphorylation reactions. Ca²⁺-independent, PKC-mediated exocytosis, occurring via small GTPases has also been suggested as an alternate mechanism. These are interesting avenues for future research aimed to clarify the mechanisms of the osmosensitive release of some amino acids. In the meantime, the present results may contribute to explaining the effects of hyposmolarity on synaptic currents in hippocampal slices. Lowering osmolarity causes reversible, osmolarity-dependent enhancement of up to 400% of excitatory postsynaptic currents and of about 180% of the inhibitory postsynaptic currents [16]. These effects may be due in part to the enhanced efflux of glutamate and GABA re-

ported in the present study. These results may also contribute to explain the increased seizure susceptibility observed during hyponatraemia [1, 2].

References

1. Andrew RD (1991) Seizure and acute osmotic change: clinical and neurophysiological aspects. J Neurol Sci 101:7–182
2. Arieff AI (1986) Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. N Engl J Med 314:1529–1535
3. Basavappa S, Pedersen SF, Jorgensen NK, Ellory JC, Hoffmann EK (1998) Swelling-induced arachidonic acid release via the 85-kDa cPLA2 in human neuroblastoma cells. J Neurophysiol 79:1441–1449
4. Bres V, Hurbin A, Duvoid A, Orcel H, Moos FC, Rabie A, Hussy N (2000) Pharmacological characterization of volume-sensitive, taurine permeable anion channels in rat supraoptic glial cells. Br J Pharmacol130:1976–1982
5. Chebabo SR, Hester MA, Aitken PG, Somjen GG (1995) Hypotonic exposure enhances synaptic transmission and triggers spreading depression in rat hippocampal tissue slices. Brain Res 695:203–216
6. Crepel V, Panenka W, Kelly ME, MacVicar BA (1998) Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. J Neurosci 18:11196–11206
7. Deleuze C, Duvoid A, Moos FC, Hussy N (2000) Tyrosine phosphorylation modulates the osmosensitivity of volume-dependent taurine efflux from glial cells in the rat supraoptic nucleus. J Physiol (Lond) 523:291–299
8. Estevez AY, O'Regan MH, Song D, Phillis JW (1999) Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. Neurochem Res 24:447–452
9. Estevez AY, O'Regan MH, Song D, Phillis JW (1999) Hyposmotically induced amino acid release from the rat cerebral cortex: role of phospholipases and protein kinases. Brain Res 844:1–9
10. Feranchak AP, Roman RM, Doctor RB, Salter KD, Toker A, Fitz JG (1999) The lipid products of phosphoinositide 3-kinase contribute to regulation of cholangiocyte ATP and chloride transport. J Biol Chem 274:30979–30986
11. Franco R, Quesada O, Pasantes-Morales H (2000) Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J Neurosci Res 61:701–711
12. Haugstad T S, Langmoen IA (1996) Release of brain amino acids during hyposmolar stress and energy deprivation. J Neurosurg Anesthesiol 8:159–168
13. Haussinger D, Schliess F (1999) Osmotic induction of signaling cascades: role in regulation of cell function. Biochem Biophys Res Commun 255:551–555
14. Hille B, Billiard J, Babcock DF, Nguyen T, Koh DS (1999) Stimulation of exocytosis without a calcium signal. J Physiol (Lond)520:23–31
15. Hoffmann EK, Lambert IH, Simonsen LO (1988) Mechanisms in volume regulation in Ehrlich ascites tumor cells. Renal Physiol Biochem 11:221–247
16. Huang R, Bossut DF, Somjen GG (1997) Enhancement of whole cell synaptic currents by low osmolarity and by low [NaCl] in rat hippocampal slices. J Neurophysiol 77:2349–2359
17. Hubert EM, Musch MW, Goldstein L (2000) Inhibition of volume-stimulated taurine efflux and tyrosine kinase activity in the skate red blood cell. Pflügers Arch 440:132–139
18. Jackson PS, Strange K (1993) Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. Am J Physiol 265:C1489–C1500
19. Kiessling K, Ellory JC, Cossins AR (2000) The relationship between hypotonically-induced taurine and K fluxes in trout red blood cells. Pflügers Arch 440:467–475
20. Kimelberg HK, O'Connor E (1988) Swelling of astrocytes causes membrane potential depolarization. Glia 1:219–224
21. Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA (1990) Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. J Neurosci 10:1583–1591
22. Kirk K (1997) Swelling-activated organic osmolyte channels. J Membr Biol 158:1–16
23. Knight DE, Grafenstein H von, Athayde CM (1989) Calcium-dependent and calcium-independent exocytosis. Trends Neurol Sci 12:451–458
24. Krause U, Rider MH, Hue L (1996) Protein kinase signaling pathway triggered by cell swelling and involved in the activation of glycogen synthase and acetyl-CoA carboxylase in isolated rat hepatocytes. J Biol Chem 271:16668–16673
25. Kuwahara O, Mitsumoto Y, Chiba K, Mohri T (1992) Characterization of D-aspartic acid uptake by rat hippocampal slices and effect of ischemic conditions. J Neurochem 59:616–621
26. Lang F, Madlung J, Siemen D, Ellory C, Lepple-Wienhues A, Gulbins E (2000) The involvement of caspases in the CD95 (Fas/Apo-1)- but not swelling-induced cellular taurine release from Jurkat T-lymphocytes. Pflügers Arch 440:93–99
27. Lehmann A, Carlstrom C, Nagelhus EA, Ottersen OP (1991) Elevation of taurine in hippocampal extracellular fluid and cerebrospinal fluid of acutely hypoosmotic rats: contribution by influx from blood? J Neurochem 56:690–697
28. Mongin AA, Reddi JM, Charniga C, Kimelberg HK (1999) [3H]taurine and D-[3H]aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. Am J Physiol 276:C1226–C1230
29. Morales-Mulia S, Cardin V, Torres-Marquez ME, Crevenna A, Pasantes-Morales H (2001) Influence of protein kinases on the osmosensitive release of taurine from cerebellar granule neurons. Neurochem Int 38:153–161
30. Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G (1997) Properties of volume-regulated anion channels in mammal cells. Prog Biophys Mol Biol 68:69–119
31. Okada Y (1997) Volume expansion-sensing outwarD-rectifier Cl- channel: fresh star to the molecular identity and volume sensor Am J Physiol 273:C755–C789
32. Pasantes-Morales H, Morales Mulia S (2000) Influence of calcium on regulatory volume decrease: role of potassium channels. Nephron 86:414–427
33. Pasantes-Morales H, Alavez S, Sanchez Olea R, Moran J (1993) Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem Res 18:445–452
34. Pasantes-Morales H, Franco R, Torres-Marquez ME, Hernandez-Fonseca K, Ortega A (2000) Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: contribution and mechanisms. Cell Physiol Biochem 10:361–370
35. Sanchez-Olea R, Moran J, Schousboe A, Pasantes-Morales H (1991) Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. Neurosci Lett 130:233–236
36. Sanchez-Olea R, Pena C, Moran J, Pasantes-Morales H (1993) Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl-transport in cultured astrocytes. Neurosci Lett 156:141–144
37. Sanchez-Olea R, Morales M, Garcia O, Pasantes-Morales H (1996) Cl channel blockers inhibit the volume-activated efflux of Cl and taurine in cultured neurons. Am J Physiol 270: C1703–C1708

38. Solís JM, Herranz AS, Herraz O, Lerma J, Del Rio RM (1988) Does taurine acts as an osmoregulatory substance in the rat brain? Neurosci. Lett 91:53–58
39. Strange K, Jackson PS (1995) Swelling-activated organic osmolyte efflux: a new role for anion channels. Kidney Int 48: 994–1003
40. Tilly BC, Edixhoven MJ, Tertoolen LG, Morii N, Saitoh Y, Narumiya S, Jonge HR de (1996) Activation of the osmo-sensitive chloride conductance involves P21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. Mol Biol Cell 7: 1419–1427
41. Tilly BC, Gaestel M, Engel K, Edixhoven MJ, Jonge HR de (1996) Hypo-osmotic cell swelling activates the p38 MAP kinase signalling cascade. FEBS Lett 395:133–136
42. Wijk T van der, Jonge HR de, Tilly BC (1999) Osmotic cell swelling-induced ATP release mediates the activation of extracellular signal-regulated protein kinase (Erk)-1/2 but not the activation of osmo-sensitive anion channels. Biochem J 343: 579–586
43. Wijk T van der, Tomassen SF, Jonge HR de, Tilly BC (2000) Signalling mechanisms involved in volume regulation of intestinal epithelial cells. Cell Physiol Biochem 10:289–296

Osmosensitive Taurine Release
Does Taurine Share the Same Efflux Pathway With Chloride and Other Amino Acid Osmolytes?

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1.   INTRODUCTION

Swelling subsequent to hyposmotic conditions activates a process of volume regulation present in most cell types. This volume adjustment is accomplished by osmolyte translocation towards the extracellular space to reach a new osmotic equilibrium. Molecules involved in this homeostatic mechanism have been broadly classified into two categories: organic and inorganic osmolytes. Inorganic osmolytes comprise mainly the intracellular ions K^+ and Cl^-. Cell swelling-induced activation of separate K^+ and Cl^- channels has been described in most preparations. Organic osmolytes are grouped in three categories: amino acids, polyalcohols and methylamines. These osmolytes, particularly taurine, are present in high intracellular concentrations and may also play a role as cytoprotectants[1]. Amino acids are part of the organic osmolyte pool contributing to RVD in most cells[1,2]. Among them, taurine has been studied in detail mainly due to its metabolic inertness, and it is often considered as representative of all osmolyte amino acids.

1.1   Volume Sensitive Organic Osmolyte and Anion Pathway

Volume regulatory loss of organic osmolytes has been characterized in a wide range of cell types. Efflux of these osmolytes seems to be mediated by passive concentration gradient-driven pathways, which do not exhibit saturation in their efflux profile and are not susceptible to trans-stimulation[3]. It is now generally accepted that swelling-activated organic osmolyte release is achieved

by diffusion through membrane pores, rather than by carrier transport. Transport pathways for organic osmolytes are in general Na^+-independent and non-stereoselective. A particular feature of organic osmolyte release is its sensitivity to general anion channel blockers[4-6]. This has raised the question of whether the volume-sensitive Cl^- channel may be the permeation pathway for these osmolytes. Electrophysiological evidence has shown that amino acids and some other organic osmolytes permeate through the volume-sensitive anion/Cl^- channel (VSAC), which has broad permeability, and the necessary size pore $(8-9 \text{ Å})^{2,7}$ to allow translocation of amino acids as large as glutamate, as well as other structurally unrelated osmolytes. This channel was named volume-sensitive organic osmolyte/anion channel (VSOAC) by Strange and coworkers[8]. Experimental evidence for the existence of this common pathway is not conclusive so far, and as the characterization of the volume-sensitive Cl^- channel and the organic osmolyte fluxes progresses, evidence against the notion of a common Cl^-/osmolyte pathway becomes less consistent. In this review, we address basic questions that remain unanswered: 1) may the volume-sensitive Cl^- channel be a common pathway for both Cl^- and taurine?; and 2) are taurine and other amino acid osmolytes translocated through the same efflux pathway? We present here results pertaining to these two possibilities and discuss the current state of the field.

2.   EVIDENCE AGAINST A COMMON PATHWAY FOR SWELLING-ACTIVATED TAURINE AND Cl^- RELEASE.

Taurine is found at concentrations of up to tens of millimolar in vertebrate cells under physiological conditions. It has a pK2 of 8.82 and is therefore present in the cytosol predominantly as an electroneutral zwitterion[9]. Swelling-activated taurine release (SATR) has been observed in almost all preparations studied to date. SATR occurs via a non-saturable Na^+-independent transport pathway, inhibited by both anion exchanger blockers (DIDS, SITS, niflumic acid and pyridoxal phosphate), and chloride channel blockers (NPPB, dideoxyforskolin and tamoxifen), and modulated by tyrosine kinases and PI3K activity[for rev. see 10 and 11], as has been also reported for VSAC[12-14].

There is recent evidence against a common pathway for both osmolytes. In Ehlrich ascites cells SATR and VSAC fluxes are pharmacological distinct[15], the former being inhibited by DIDS and niflumic acid, and stimulated by arachidonic acid and LTD4, while the later is inhibited by arachidonic acid, tamoxifen and insensitive to DIDS and niflumic acid[16]. Studies in rat mammary gland and skate erythrocytes demonstrate SATR release without an activation of VSAC[17-20], and the opposite was observed in human biliary cell line[21]. In skate erythrocytes, the osmosensitive taurine and Cl^- fluxes appear mediated by different pathways,

taurine through a channel and Cl⁻ by cotransporter pathways[20]. Different pathways for SATR and VSAC were also suggested in HeLa cells[22], where SATR was more sensitive to DIDS than I^{125} efflux. In this study SATR and I^{125} efflux elicited differences in their kinetic activation and inactivation profiles. Differences between VSAC and SATR in their sensitivity to Cl⁻ channel blockers have been found in NIH3T3 and CHO cells[23,24]. In NIH3T3 cells, the small G-protein Rho A modulates the Cl⁻ channel conductance while SATR remains almost unaffected[25]. SATR, but not swelling-activated Cl⁻ conductance, occurs in Xenopus laevis oocytes [26].

3. THE SWELLING-ACTIVATED TAURINE AND AMINO ACID RELEASE

Taurine is convenient for studies aimed to characterize osmolyte fluxes, because it is abundant in cells and tissues and is essentially metabolically inert, and has been often considered as representative of amino acids and other organic osmolytes. Besides taurine, other amino acids translocate in response to hyposmotic swelling. In the same way, glycine, β-alanine, GABA, leucine, glutamine, aspartate, and glutamate permeate through the swelling-activated Cl⁻ /anion pathway with Paa/Pcl ranging from 0.25 to 0.78 in cell types including MDCK cells, inner medullary collecting duct IMCD cells and glial cells[27-31]. Amino acid release as for the case of taurine is inhibited by Cl⁻ channel blockers (SITS, DIDS, L644-711, niflumic acid, NPPB, dideoxyforskolin and tamoxifen, furosemide, 9-AC and dipyridamole) in different preparations including primary cortical and cerebellar astrocytes, cerebellar granule neurons and cultured cortical neurons, neuroblastoma CHP-100, cortex, hippocampal slices, chick retina, NIH3T3 cell and endothelial cells[23,32-31]. Hyposmotic-induced N-acetylaspartate release has been studied in rat striatum preparations, hippocampal slices *and in vivo* studies; similar to SATR, N-acetylaspartate release was also inhibited by Cl⁻ channel blockers[40,43-46]. These results may suggest a common efflux pathway for SATR and other amino acids and would, in principle, allow the extrapolation of SATR results to all other osmolyte amino acids.

Recent results from our and other laboratories indicate that the osmosensitive taurine efflux properties clearly differ from those of amino acids such as GABA and glutamate which may act as neurotransmitters. The efflux rate of taurine, glutamate and D-aspartate were similar in cultured astrocytes regarding the time course and the effect of Cl-channel inhibitors, but are differentially modulated by tyrosine kinase blockers[34-37,47]. More important differences between SATR and other amino acids were found in hippocampal[39,48] and cortex slices (Figure 1) including: 1) The osmosensitive amino acid neurotransmitter release shows a kinetic release profile notably different from SATR. Taurine efflux exhibits delayed activation and inactivation while that of glutamate and GABA fully activate immediately after the stimulus and also rapidly inactivate, 2) GABA

and glutamate efflux are insensitive to Cl⁻ channel blockers, which typically inhibit SATR, 3) SATR is modulated by signaling cascades involving tyrosine phosphorylation events, including an important role for the tyrosine kinase target PI3K; while GABA and glutamate fluxes are not responsive to tyrosine phosphorylation state nor to the influence of PI3K activity, and 4) swelling-activated neurotransmitter release, but not SATR, is influenced by the cytoskeleton depolymerization and manipulation of PKC activity.
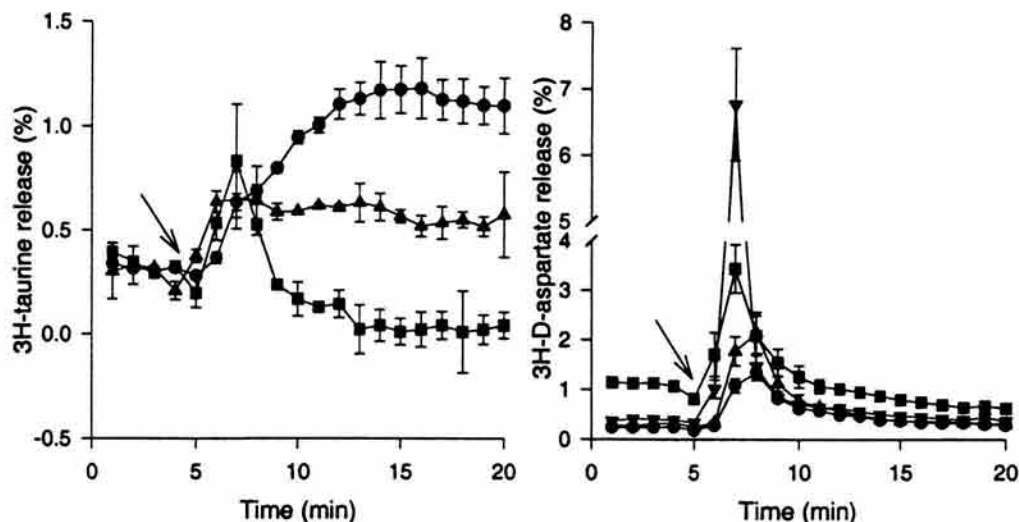
Figure 1. Amino acid release from cortex slices exposed to 30% hyposmotic medium. Slices preloaded with ³H-taurine or ³H-D-aspartate were superfused 5 min with isosmotic medium. Thereafter (arrow), the medium was replaced by 30% hyposmotic solution. One-min fractions were collected during 20 min. ³H-Taurine release was inhibited by tyrosine kinase blockers (50μM AG18 (■)) and PI3K inhibitors (wortmannin 100nM (▲)). In contrast, glutamate release (followed as ³H-D-aspartate) was insensitive to these agents but potentiated by PKC activation (100nM PMA (▼)). (●) Controls with vehicle. Data represent the radioactivity released per min expressed as percentage of the total incorporated and are means ± SE (n=4-6).

In the same way, in rat cerebral cortex dialysates and bullfrog sympathetic ganglia different effects were obtained between SATR and SAAAC in the presence of several Cl⁻ channel blockers, SATR being the most widely sensitive and that of GABA, D-aspartate and glutamate rather inhibited in the presence of these agents[49,50]. Moreover, *in vivo* studies show that glutamate and aspartate are preferentially released during acute hyponatremia while taurine release is sensitive to both chronic and acute hyponatremia[51]. In cultured neurons taurine efflux is more sensitive to osmolarity perturbations than glutamate and GABA [52]. Hyposmotic-induced amino acid neurotransmitter release in excitable cells has been suggested to involve an exocytotic mechanism elicited in response to cell depolarization by either Cl⁻ release or by activation of non-selective cation channels; or by calcium-independent vesicle fusion mechanisms[reviewed in 53].

4. CONCLUDING REMARKS

The molecular identification and characterization of the swelling-activated efflux pathway for organic osmolytes release has been attempted by several research groups in the field. During their efforts there was initial evidence that suggested a common efflux pathway named VSOAC for both swelling activated-organic osmolyte release and VSAC. This led to the common extrapolation of experimental results from one osmolyte release (either taurine or chloride) to others (like amines, amino acids or polyalcohols). In this review, we summarize increasing evidence that points to different efflux pathways for SATR with respect to the VSAC. We also show that for the organic osmolyte group of amino acids, care must be taken in the further extrapolation of SATR to other swelling-activated release of amino acids at least for the case of neurotransmitter amino acids in excitable cells. In this case, it is clear that in brain preparations, different pathways mediate the release of amino acid neurotransmitter release (like GABA and glutamate) with respect to that of SATR. In brain preparations, the different regulatory mechanisms and efflux pathways involved in the swelling-activated amino acid neurotransmitter release with respect to that of taurine may be of physiological relevance because high extracellular concentrations of these neurotransmitters may lead to exocytotic insults, during conditions of cell swelling. It must be taken into account that several efflux pathways may be activated during cell swelling conditions for different osmolytes or even for the same one[54-56]. All this data challenge the hypothesis of VSOAC as the common efflux pathway for the release of organic osmolytes and anion conductance elicited by cell swelling conditions, and even for a common pathway for taurine and other amino acid osmolytes in excitable cells.

ACKNOWLEDGEMENTS

REFERENCES

1. Lang, F., Busch, G.L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E., and Haussinger, D., 1998, Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* 78:247-306.
2. Pasantes-Morales, H., 1996, Volume regulation in brain cells: cellular and molecular mechanisms. *Metab. Brain Dis.* 11:187-204.
3. Kirk, K., and Strange, K., 1998, Functional properties and physiological roles of organic solute channels. *Annu. Rev. Physiol.* 60:719-739.
4. Kirk, K., Ellory, J.C., and Young, J.D., 1992, Transport of organic substrates via a volume-activated channel. *J. Biol. Chem.* 267:23475-23478.

2. Pasantes-Morales, H., 1996, Volume regulation in brain cells: cellular and molecular mechanisms. *Metab. Brain Dis.* 11:187-204.
3. Kirk, K., and Strange, K., 1998, Functional properties and physiological roles of organic solute channels. *Annu. Rev. Physiol.* 60:719-739.
4. Kirk, K., Ellory, J.C., and Young, J.D., 1992, Transport of organic substrates via a volume-activated channel. *J. Biol. Chem.* 267:23475-23478.
5. Junankar, P.R., and Kirk, K., 2000, Organic osmolyte channels: a comparative view. *Cell. Physiol. Biochem.* 10:355-360.
6. Kirk, K., 1997, Swelling-activated organic osmolyte channels. *J. Membr. Biol.* 158:1-16.
7. Strange, K., and Jackson, P.S., 1995, Swelling-activated organic osmolyte efflux: a new role for anion channels. *Kidney Int.* 48:994-1003.
8. Strange, K., Emma, F., and Jackson, P.S., 1996, Cellular and molecular physiology of volume-sensitive anion channels. *Am. J. Physiol.* 270:C711-C730.
9. Huxtable, RJ., 1992, Physiological actions of taurine. *Physiol. Rev.* 72:101-163.
10. Pasantes-Morales, H., Franco, R., Torres-Marquez, M.E., Hernandez-Fonseca, K., and Ortega, A., 2000, Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: contribution and mechanisms. *Cell. Physiol. Biochem.* 10:361-370.
11. Pasantes-Morales, H., and Franco, R., 2002, Influence of protein tyrosine kinases on cell volume change-induced taurine release. *The Cerebellum.* 1:103-109.
12. Lepple-Wienhues, A., Szabo, I., Wieland, U., Heil, L., Gulbins, E., and Lang, F., 2000, Tyrosine kinases open lymphocyte chloride channels. *Cell. Physiol. Biochem.* 10:307-312.
13. Feranchak, A.P., Roman, R.M., Doctor, R.B., Salter, K.D., Toker, A., and Fitz, J.G., 1999, The lipid products of phosphoinositide 3-kinase contribute to regulation of cholangiocyte ATP and chloride transport. *J. Biol. Chem.* 274:30979-30986.
14. Voets, T., Manolopoulos, V., Eggermont, J., Ellory, C., Droogmans, G., and Nilius, B., 1998, Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. *J. Physiol.* 506:341-352.
15. Lambert, I.H., and Hoffmann, E.K., 1994, Cell swelling activates separate taurine and chloride channels in Ehrlich mouse ascites tumor cells. *J. Membr. Biol.* 142:289-298.
16. Hoffmann, E.K., 2000, Intracellular signalling involved in volume regulatory decrease. *Cell. Physiol. Biochem.* 10:273-288.
17. Shennan, D.B., and Thomson, J., 2000, Further evidence for the existence of a volume-activated taurine efflux pathway in rat mammary tissue independent from volume-sensitive Cl- channels. *Acta Physiol. Scand.* 168:295-299.
18. Shennan, D.B., Cliff, M.J., and Hawkins, P., 1996, Volume-sensitive taurine efflux from mammary tissue is not obliged to utilize volume-activated anion channels. *Biosci. Rep.* 16:459-465.
19. Shennan, D.B., McNeillie, S.A., and Curran, D.E., 1994, The effect of a hyposmotic shock on amino acid efflux from lactating rat mammary tissue: stimulation of taurine and glycine efflux via a pathway distinct from anion exchange and volume-activated anion channels. *Exp. Physiol.* 79:797-808.
20. Davis-Amaral, E.M., Musch, M.W., and Goldstein, L., 1996, Chloride and taurine effluxes occur by different pathways in skate erythrocytes. *Am. J. Physiol.* 271:R1544-R1549.
21. Roman, R.M., Wang, Y., and Fitz, J.G., 1996, Regulation of cell volume in a human biliary cell line: activation of K+ and Cl- currents. *Am. J. Physiol.* 1996 271:G239-G248.
22. Stutzin, A., Eguiguren, A.L., Cid, L.P., and Sepulveda, F.V., 1997, Modulation by extracellular Cl- of volume-activated organic osmolyte and halide permeabilities in HeLa cells. *Am. J. Physiol.* 273:C999-C1007.
23.. Moran, J., Miranda, D., Pena-Segura, C., and Pasantes-Morales, H., 1997, Volume regulation in NIH/3T3 cells not expressing P-glycoprotein. II. Chloride and amino acid fluxes. *Am. J. Physiol.* 272:C1804-C1809.
24. Sanchez-Olea, R., Fuller, C., Benos, D., and Pasantes-Morales, H., 1995, Volume-associated osmolyte fluxes in cell lines with or without the anion exchanger. *Am. J. Physiol.* 269:C1280-C1286.

25. Pedersen, S.F., Beisner, K.H., Hougaard, C., Willumsen, B.M., Lambert, I.H., and Hoffmann, E.K., 2002, Rho family GTP binding proteins are involved in the regulatory volume decrease process in NIH3T3 mouse fibroblasts. *J. Physiol.* 541:779-796.
26. Stegen, C., Matskevich, I., Wagner, C.A., Paulmichl, M., Lang, F., and Broer, S., 2000, Swelling-induced taurine release without chloride channel activity in Xenopus laevis oocytes expressing anion channels and transporters. *Biochim. Biophys. Acta.* 1467:91-100.
27. Banderali, U., and Roy, G., 1992, Anion channels for amino acids in MDCK cells. *Am. J. Physiol.* 263:C1200-C1207.
28. Roy, G., and Banderali, U., 1994, Channels for ions and amino acids in kidney cultured cells (MDCK) during volume regulation. *J. Exp. Zool.* 268:121-126.
29. Boese, S.H., Wehner, F., and Kinne, R.K., 1996, Taurine permeation through swelling-activated anion conductance in rat IMCD cells in primary culture. *Am. J. Physiol.* 271:F498-F507.
30. Roy, G., 1995, Amino acid current through anion channels in cultured human glial cells. *J. Membr. Biol.* 147:35-44.
31. Olson, J.E., and Li, G.Z., 1997, Increased potassium, chloride, and taurine conductances in astrocytes during hypoosmotic swelling. *Glia.* 20:254-61.
32. Pasantes-Morales, H., Chacon, E., Murray, R.A., and Moran, J., 1994, Properties of osmolyte fluxes activated during regulatory volume decrease in cultured cerebellar granule neurons. *J. Neurosci. Res.* 37:720-727.
33. Sanchez-Olea, R., Morales, M., Garcia, O., and Pasantes-Morales, H., 1996, Cl channel blockers inhibit the volume-activated efflux of Cl and taurine in cultured neurons. *Am. J. Physiol.* 270:C1703-C1708.
34. Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S., and Waniewski, R.A., 1990. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J. Neurosci.* 10:1583-1591.
35. Sanchez-Olea, R., Pena, C., Moran, J., and Pasantes-Morales, H., 1993, Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl- transport in cultured astrocytes. *Neurosci. Lett.* 156:141-144.
36. Pasantes-Morales, H., Murray, R.A., Sanchez-Olea, R., and Moran, J., 1994, Regulatory volume decrease in cultured astrocytes. II. Permeability pathway to amino acids and polyols. *Am. J. Physiol.* 266:C172-C178.
37. Rutledge, E.M., Aschner, M., and Kimelberg, HK., 1998, Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. *Am. J. Physiol.* 274:C1511-C1520.
38. Basavappa, S., Huang, C.C., Mangel, A.W., Lebedev, D.V., Knauf, P.A., and Ellory, J.C., 1996, Swelling-activated amino acid efflux in the human neuroblastoma cell line CHP-100. *J. Neurophysiol.* 76:764-769.
39. Franco, R., Quesada, O., and Pasantes-Morales, H., 2000, Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. *J. Neurosci. Res.* 61:701-711.
40. Bothwell, J.H., Rae, C., Dixon, R.M., Styles, P., and Bhakoo, K.K., 2001, Hypo-osmotic swelling-activated release of organic osmolytes in brain slices: implications for brain oedema in vivo. *J. Neurochem.* 77:1632-1640.
41. Pasantes-Morales, H., Ochoa de la Paz, L.D., Sepulveda, J., and Quesada, O., 1999, Amino acids as osmolytes in the retina. *Neurochem. Res.* 24:1339-1346.
42. Manolopoulos, V.G., Voets, T., Declercq, P.E., Droogmans, G., and Nilius, B., 1997, Swelling-activated efflux of taurine and other organic osmolytes in endothelial cells. *Am. J. Physiol.* 273:C214-C222.
43. Sterns, R.H., Baer, J., Ebersol, S., Thomas, D., Lohr J.W., and Kamm, D.E., 1993, Organic osmolytes in acute hyponatremia. *Am. J. Physiol.* 264:F833-F836.
44. Taylor, D.L., Davies, S.E., Obrenovitch, T.P., Doheny, M.H., Patsalos, P.N., Clark, J.B., and Symon, L., 1995, Investigation into the role of N-acetylaspartate in cerebral osmoregulation. *J. Neurochem.* 65:275-281.

45. Davies, S.E., Gotoh, M., Richards, D.A., and Obrenovitch, T.P., 1998, Hypoosmolarity induces an increase of extracellular N-acetylaspartate concentration in the rat striatum. *Neurochem. Res.* 23:1021-1025.
46. Baslow, M.H., 2002 Evidence supporting a role for N-acetyl-L-aspartate as a molecular water pump in myelinated neurons in the central nervous system. An analytical review. *Neurochem. Int.* 40:295-300.
47. Mongin, A.A., Reddi, J.M., Charniga, C., and Kimelberg, H.K., 1999, [3H]taurine and D-[3H]aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. *Am. J. Physiol.* 276:C1226-C1230.
48. Franco, R., Torres-Marquez, M.E., and Pasantes-Morales, H., 2001, Evidence for two mechanisms of amino acid osmolyte release from hippocampal slices. *Pflugers Arch.* 442:791-800.
49. Sakai, S., and Tosaka, T., 1999, Analysis of hyposmolarity-induced taurine efflux pathways in the bullfrog sympathetic ganglia. *Neurochem. Int.* 34:203-212.
50. Estevez, A.Y., O'Regan, M.H., Song, D., and Phillis, J.W., 1999, Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. *Neurochem. Res.* 24:447-452.
51. Verbalis, J.G., and Gullans, S.R., 1991, Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. *Brain Res.* 567:274-282.
52. Pasantes-Morales, H., Alavez, S., Sanchez-Olea, R., and Moran, J., 1993, Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. *Neurochem. Res.* 18:445-452.
53. Pasantes-Morales, H., Franco, R., Ochoa, L., and Ordaz, B., 2002, Osmosensitive release of neurotransmitter amino acids: relevance and mechanisms. *Neurochem. Res.* 27:59-65.
54. Zhang, J.J., and Jacob, T.J., 1997, Three different Cl- channels in the bovine ciliary epithelium activated by hypotonic stress. *J. Physiol.* 499:379-389.
55. Staines, H.M., Godfrey, E.M., Lapaix, F., Egee S., Thomas, S., and Ellory, J.C., 2002, Two functionally distinct organic osmolyte pathways in Plasmodium gallinaceum-infected chicken red blood cells. *Biochim. Biophys. Acta.* 1561:98-108.
56. Banderali, U., and Ehrenfeld, J., 1996, Heterogeneity of volume-sensitive chloride channels in basolateral membranes of A6 epithelial cells in culture. *J. Membr. Biol.* 154:23-33.

Datos no incluidos en los artículos.
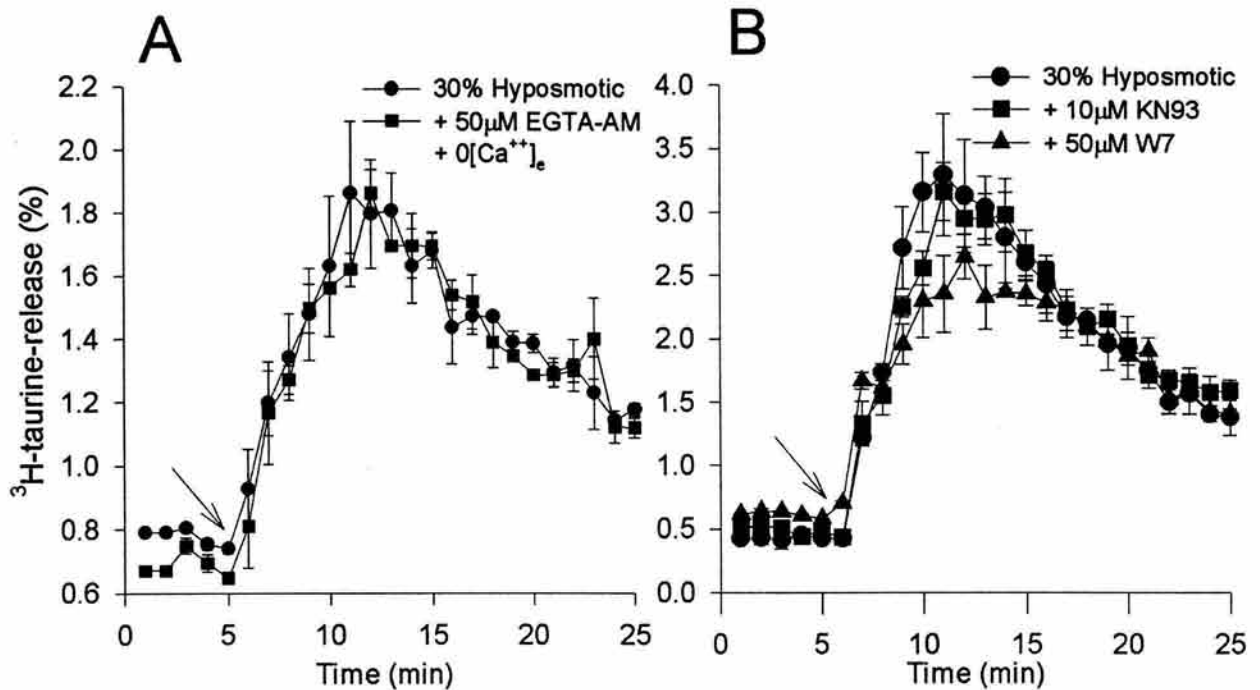
Figura 5. Dependencia de calcio de la LTAH. Las rebanadas de hipocampo se preincuban con la marca radioactiva como se explica en 7.3.1. **A.** Las rebanadas fueron preincubadas 30 min con 50μM EGTA-AM. Posteriormente fueron expuestas al sistema de perfusión ya descrito, en ausencia de Ca^{++} extracelular y 0.5mM EGTA en las soluciones experimentales $(0[Ca^{++}]_e)$. **B.** Las rebanadas fueron preincubadas con 50μM W7 o 10μM KN93 durante 30 min y después perfundidas como en **A.** W7 y KN93 estuvieron presentes durante todo el experimento. La flecha indica el cambio de medio isosmótico a 30% hiposmótico. Los datos se expresan como % de liberación y son promedios ± ES de n = 4. Controles (●) con el mismo tratamiento con el vehículo. Los datos están comparados con los controles (ausencia de tratamiento o fármaco) del día

Como se mencionó en la introducción, la activación de I_{Clvol} y la LTAH se ha reportado modulada por distintas vías de señalización incluyendo Ca^{++}, PKC, PLA2. En este trabajo se trató de confirmar o descartar la participación de estas vías en la LTAH en el hipocampo. En la figura 5, las rebanadas de hipocampo fueron preincubadas con EGTA-AM (quelante intracelular de Ca^{++}), y expuestas a un medio hiposmótico en ausencia de calcio extracelular. En estas condiciones la LTAH no se vio afectada, lo que sugiere que es independiente de Ca^{++} intracelular. Por otra parte, la LTAH no se vio inhibida por antagonistas de la calmodulina (W7) ni por inhibidores de la cinasa dependiente de calmodulina tipo II CAMKII (KN93), lo que descarta la participación de estas enzimas en la activación de LTAH.
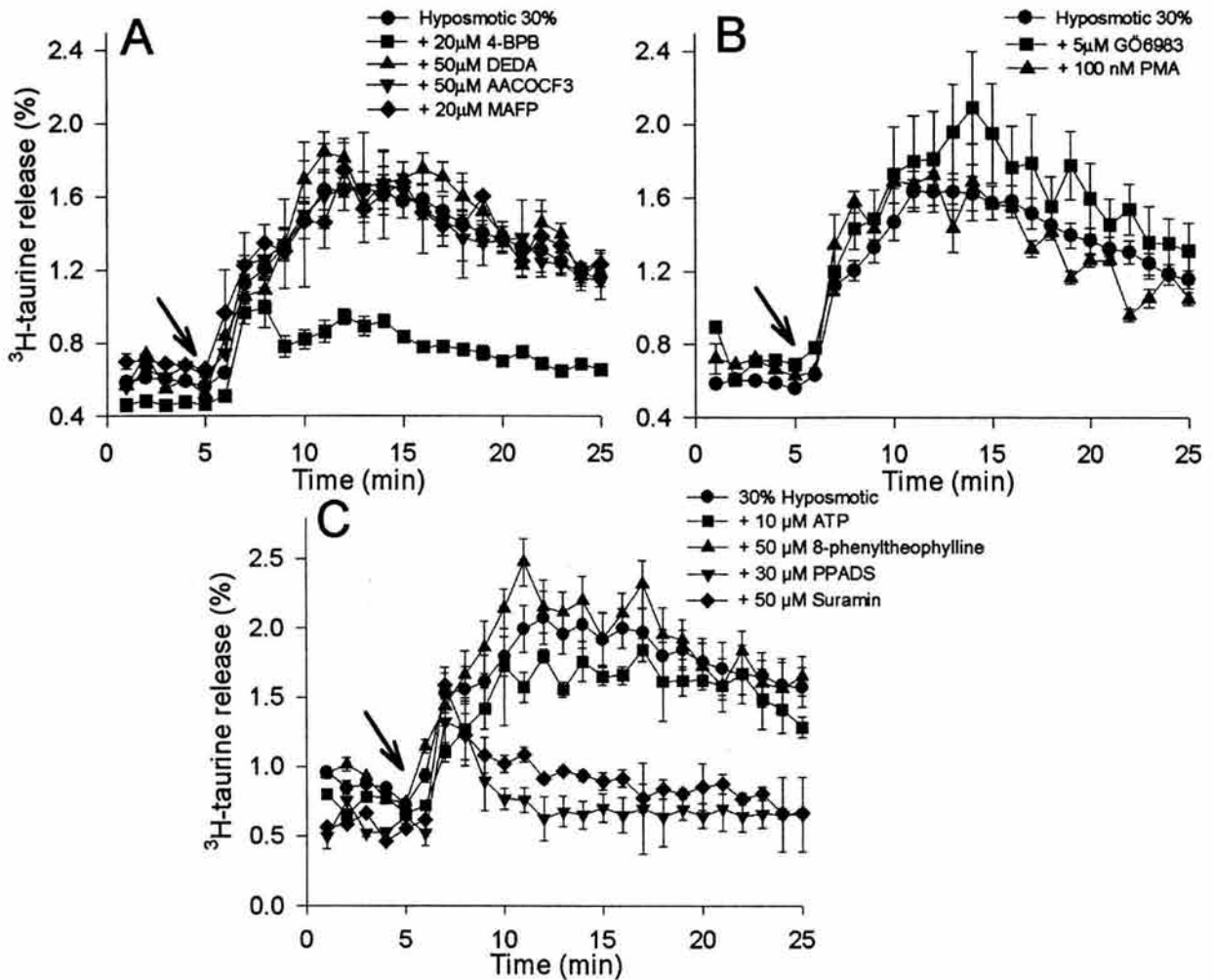
Figura 6. Participación de distintas vías de señalización en la LTAH. Las rebanadas de hipocampo se preincuban con la marca radioactiva como se explica en 7.3.1. El tejido fue preincubado con los fármacos indicados durante 1 h (al menos que otra cosa se indique) y estuvieron presentes durante todo el experimento. Posteriormente el tejido fue expuesto al sistema de perfusión ya descrito. **A.** Efecto de inhibidores de generales de PLA_2, DEDA (acido 7,7-dimetil-5,8-eicosadienoico.) y 4-BPB (4-bromofenacilbromuro), e inhibidores de $cPLA_2$ AACOCF3 (arachidoniltrifluorometil cetona) y MAFP (metil arachidonil fluorofosfonato). **B.** Efecto de la activación de PKC con PMA (acetato de forbol-12-miristato-13; 15 min de preincubación) y del inhibidor de PKC GÖ6983 sobre la LTAH. **C.** Efecto de la modulación de receptores purinérgicos sobre la LTAH. El agonista de los receptores purinérgicos, ATP (adenosine 5-trifosfato), se adicionó al momento del estímulo hiposmótico y estuvo presente durante todo el estímulo. Los antagonistas de los receptores purinérgicos (suramina, 8-fenilteofilina y PPADS), se preincubaron 15 minutos y estuvieron durante toda la perfusión. Los datos se expresan como % de liberación y son promedios ± ES de n = 4. Controles (●) con el mismo tratamiento con el vehículo. Los datos están comparados con los controles (en ausencia de tratamiento o fármaco) del día

En la Figura 6A. Se muestra la participación de distintos inhibidores generales de PLA_2 (DEDA, y 4-BPB) y de inhibidores específicos de $cPLA_2$, las cuales se han visto regulan la LTAH en algunos tipos celulares. De estos agentes solo el 4-BPB, tuvo una

inhibición significativa sobre la LTAH, aunque no se descarta un efecto inespecífico de este fármaco, debido a que el DEDA, otro inhibidor general de PLA_2 no tuvo el mismo efecto. Es probable que el efecto del 4-BPB, esté relacionado con la participación de la $iPLA_2$, la cual se ha reportado modula la LTAH en fibroblastos (Lambert, 2003). Sin embargo es necesario corroborar esto mediante el uso de inhibidores específicos de iPLA2 como la bromoenol-lactona (BEL).

A

B
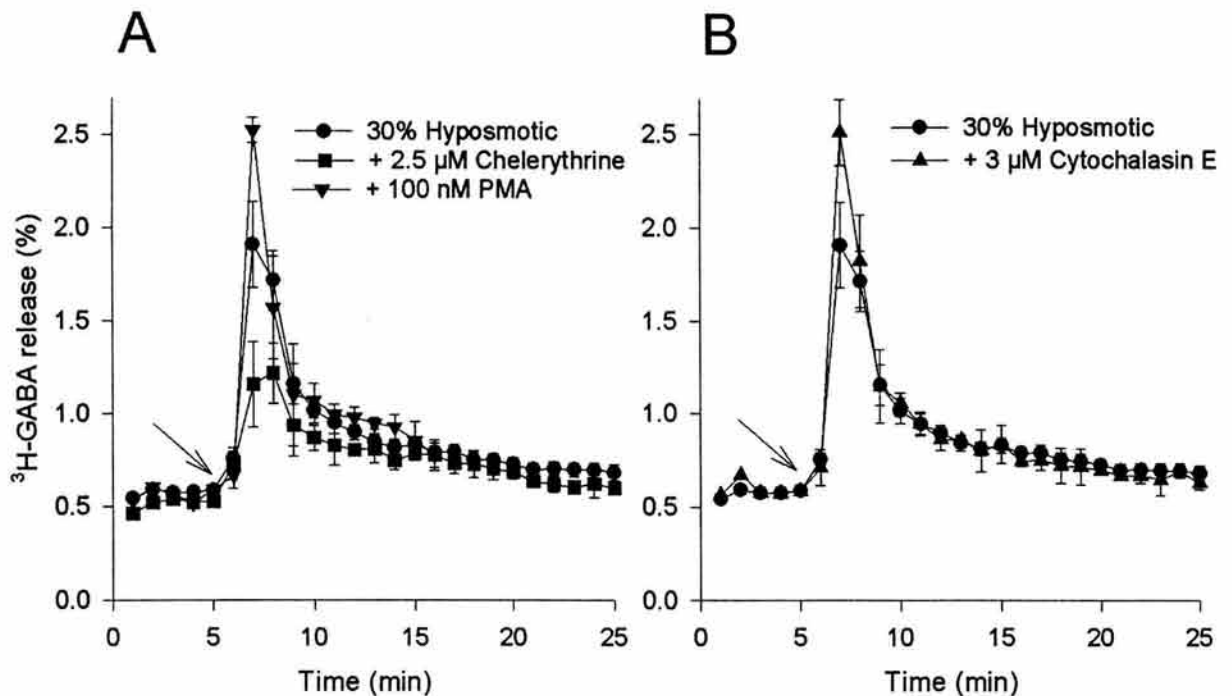
Figura 7. Efecto de la modulación de la actividad de la proteína cinasa C (PKC), y efecto de la citocalasina E, sobre la liberación de ^{3}H-GABA activada por hiposmolaridad en rebanadas de hipocampo de rata expuestas a un medio 30% hiposmótico. La perfusión se realizó como se explica en 7.3.1. Las rebanadas fueron preincubadas 30 min con queleritrina o PMA; o 2 hr con citocalasina E. Los agentes estuvieron presente durante toda la perfusión La flecha indica el cambio de medio isosmótico a 30% hiposmótico. Los datos se expresan como % de liberación y son promedios ± ES de n = 4. Controles (●) con el mismo tratamiento con el vehículo.

La Figura 6B muestra el efecto de la adición de esteres de forbol (PMA) y de la inhibición de PKC sobre la LTAH. Como se menciona en el artículo 3 de esta sección, la LTAH no se modula por la actividad de PKC. Por otra parte, observamos que algunos antagonistas de receptores purinérgicos, los cuales se han postulado que pueden funcionar como un sistema parácrino de modulación del DRV, inhiben la LTAH, sin embargo, la adición de ATP extracelular no tuvo efecto alguno sobre la misma. El efecto de los antagonistas sobre la LTAH puede deberse a un bloqueo directo de la vía de

liberación de este osmolito como ha sido reportado en otros estudios (Galietta, et al., 1997).

Como se mencionó en el artículo 3, la liberación de glutamato por hiposmolaridad se ve modulada por PKC y por el citoesqueleto de actina. En la Figura 7 se muestra el efecto de la despolimerización del citoesqueleto de actina y de la modulación de la actividad de PKC por medio de esteres de forbol (PMA) y queleritrina, sobre la liberación de GABA inducida por hiposmolaridad. De forma similar a lo observado para el glutamato, la despolimerización del citoesqueleto de actina con citocalasina E y la activación de PKC con PMA inducen una potenciación de la liberación de GABA, mientras que el inhibidor general de PKC, queleritrina, disminuye significativamente la liberación de este aminoácido. Esto corrobora la participación de una sola vía de liberación de aminoácidos neurotransmisores (GABA y glutamato) distinta a la observada para la taurina (Ver articulo 3).

Para descartar la participación de un sistema de transporte en reversa como mecanismo involucrado en la liberación de aminoácidos neurotransmisores, se probó el efecto de bloqueadores del transporte membranal de estos aminoácidos sobre la liberación activada por hinchamiento hiposmótico (ver Articulo 3 para una mejor explicación). El ácido dihidrokaínico (inhibidor del transporte de glutamato) y el NO-711 (inhibidor del transportador de GABA), no tienen efecto sobre la liberación de estos neurotransmisores por hiposmolaridad (Figura 8A y C).

Como se sugirió anteriormente, la liberación de neurotransmisores por hiposmolaridad puede estar siendo mediada por un evento de fusión vesicular (exocitosis). La exocitosis de neurotransmisores es dependiente de Ca^{++} intracelular, por lo que a continuación estudiamos la dependencia de Ca^{++} de esté fenómeno. La Figura 8B y D muestra que la liberación de neurotransmisores en estas condiciones es independiente de Ca^{++} (aunque como se menciona para el caso de la taurina, no se puede descartar un incremento en Ca^{++} intracelular en nuestra condición experimental, debido a la ausencia de mediciones de cambios en el contenido de Ca^{++} en el citosol, así como la posible participación de microdominios del mismo catión). Sin embargo esto, como se explica más adelante, no descarta la posible participación de un evento de fusión

vesicular como mecanismo de liberación de estos aminoácidos en condiciones hiposmóticas.
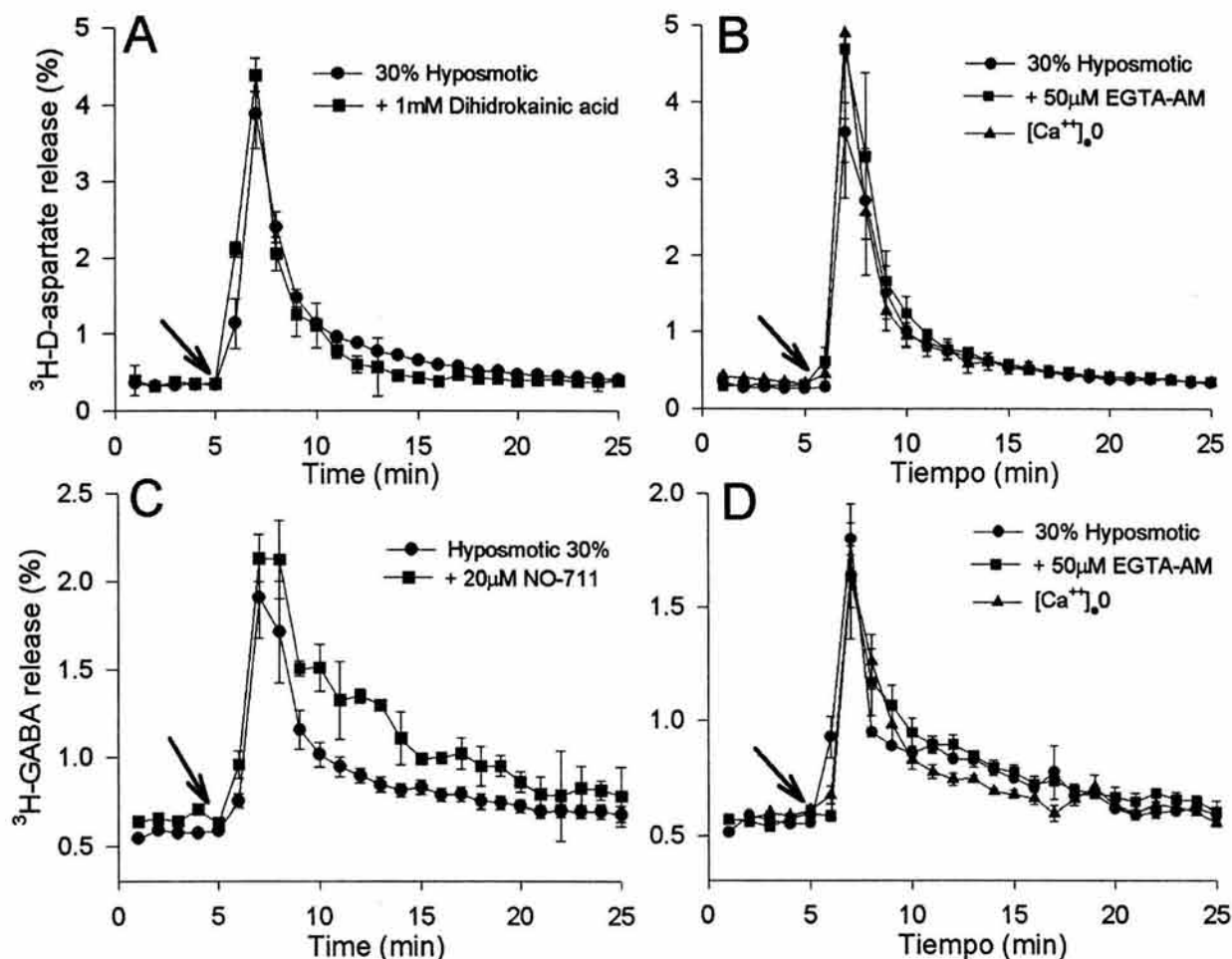
Figura 8. Dependencia de Ca^{++} y efecto de la inhibición los transportadores membranales de neurotransmisores, sobre la liberación de ^{3}H-GABA y ^{3}H-D-aspartato activada por hiposmolaridad en rebanadas de hipocampo de rata expuestas a un medio 30% hiposmótico. La perfusión se realizó como se explica en 7.3.1. **A.** y **C.** Las rebanadas fueron preincubadas 30 min con bloqueadores del transporte de glutamato y GABA, ácido dihidrokaínico y NO-711 respectivamente. Los agentes estuvieron presentes durante toda la perfusión. **B.** y **D.** Las rebanadas fueron preincubadas 30 min con 50µM EGTA_AM. Posteriormente fueron expuestas al sistema de perfusión ya descrito, en ausencia de Ca^{++} extracelular y 0.5mM EGTA en las soluciones experimentales (0[Ca^{++}]$_e$). La flecha indica el cambio de medio isosmótico a 30% hiposmótico. Los datos se expresan como % de liberación y son promedios ± ES de n = 4. Controles (●) con el mismo tratamiento con el vehículo. Los datos están comparados con los controles (en ausencia de tratamiento o fármaco) del día

DISCUSION

Los aminoácidos juegan un papel importante como osmolitos orgánicos involucrados en el control del volumen celular. La liberación de aminoácidos en respuesta a cambios en volumen es un componente importante dentro de la respuesta adaptativa al incremento en volumen celular. En esta parte del trabajo se caracterizaron las vías de translocación de aminoácidos activadas por hiposmolaridad, y las cascadas de señalización que la regulan, en este caso en preparaciones de tejido nervioso. Los resultados claramente evidencian la participación de distintas vías en la movilización de aminoácidos, probablemente relacionado al papel neurotransmisor de los mismos. Como ya se mencionó, existe la hipótesis de una vía común de movilización de osmolitos (VSOAC), lo cual no es cierto en muchos casos, incluyendo este estudio realizado en preparaciones de tejido nervioso.

Liberación de taurina por hiposmolaridad

Se postula que el movimiento de taurina en respuesta al aumento en el volumen celular, se da a través de vías difusionales en las que el movimiento de este aminoácido está dirigido por su gradiente de concentración (Sánchez-Olea et al., 1991). En astrocitos y neuronas en cultivo, se observa que la liberación de taurina disminuye notablemente en presencia de los inhibidores de canales de Cl⁻, lo que ha llevado a sugerir una posible vía común, que podría ser un canal aniónico poco selectivo. Aunque con distinta potencia, estos inhibidores son todos efectivos para reducir la liberación osmosensible de taurina (Sanchez-Olea et al., 1996; Pasantes-Morales, 1996; Pasantes-Morales et al., 1999). En nuestros resultados observamos que en condiciones de hinchamiento hiposmótico la movilización de taurina esta dada por una vía con características de canal de aniones/Cl⁻ por su inhibición por bloqueadores típicos de canales de cloro como son el NPPB, ácido niflúmico y DIDS. Esto sugiere que la movilización de taurina podría estar dada por aquella que media la conductancia a Cl⁻ sensible a hinchamiento (I$_{clvol}$, VDAC, I$_{swell}$), denominada VSOAC, la cual se postula es compartida con otro tipo de osmolitos orgánicos.

La liberación de taurina por hiposmolaridad es, al igual que la conductancia a Cl⁻ activada en estas condiciones, modulada por distintas vías de señalización dependiendo

del tipo celular estudiado (ver Pasantes-Morales, et al., 2000b para una revisión del tema). Nuestros datos muestran que en preparaciones de tejido nervioso expuestas a medio hiposmótico, la liberación de taurina se reduce de manera importante en presencia de inhibidores de cinasas de tirosina. A la fecha la participación de cinasas de tirosina en la regulación/activación de la liberación de taurina por hinchamiento anisosmótico se ha reportado en distintos tipos celulares incluyendo preparaciones de tejido nervioso (Mongin, et al., 1999b; Cardin V, et al., 2003; de la Paz LD, et al., 2002; Pedersen, et al., 2002; Huang, et al., 2001; Shen, et al., 2001; ver artículo 5 de revisión en el Apéndice II para profundizar en el tema). Sin embargo la sensibilidad a bloqueadores de cinasas de tirosina estáa restringida sólo a tirfostinas, análogos del ATP diseñados para competir por los sitios específicos de unión a ATP de receptores membranales con actividad intrínseca de cinasa de tirosina (receptores a factores de crecimiento o RTKs) (Al-Obeidi, et al., 1998; Fry, et al., 2003; Gazit, et al., 1991; Levitzki, 1990; 1992; Levitzki y Gazit, 1995; Levitzki y Bohmer, 1998; Osherov, et al., 1993), lo que podría estar sugiriendo la participación de estos receptores en la activación de la LTA, en contraste con la relativa insensibilidad a inhibidores de cinasas de tirosina general los cuales son menos específicos para los RTKs (El-Zarruk y Van den Berg, 1999: Fang, et al., 1999; Haas, et al., 2002). Sólo en algunos tipos celulares se ha visto que la LTAH se inhibe por otros bloqueadores de cinasas de tirosina distintos a las tirfostina (Huang, et al., 2001; Pedersen, et al., 2002). Los inhibidores de cinasas tipo src, no tienen efecto sobre la LTAH en muchos tipos celulares (Deleuze, et al., 2000; ver tercera sección de esta tesis). Así mismo, en todos los modelos estudiados, se ha visto la conjunción de otras vías de señalización además de cinasas de tirosina en la regulación de la movilización de taurina.

La identidad molecular de las cinasas de tirosina y/o aquellas reguladas por fosforilaciones en residuos de tirosina, involucradas en la movilización de taurina es escasa. En algunos casos se ha postulado la participación de MAPK (cinasas activadas por mitógenos), ERK1 y ERK2 (Shen, et al., 2001). Sin embargo en nuestros resultados y otros estudios a la fecha, la liberación de taurina activada por hiposmolaridad no depende de la actividad de estas cinasas, la cual se incrementa durante este fenómeno (Morales-Mulia, et al., 2001; de la Paz, et al., 2002; Deleuze, et al., 2000). Esto no quiere decir que la activación de MAP cinasas y de las SAP cinasas, no participe en el control del volumen celular en respuesta a condiciones hiposmóticas, de hecho existen reportes en donde se ha relacionado el incremento en la actividad de estas enzimas con otros mecanismos

involucrados en la regulación del volumen en condiciones hiposmóticas como son cambios en la tasa de proteólisis (Haussinger, et al., 1999); en la expresión de factores de transcripción de genes tardíos que pueden estar regulando la expresión de distintos mecanismos de regulación del contenido de osmolitos que estén asociados a este proceso homeostático (Finkenzeller, et al., 1994; Sadoshima, et al., 1996; Zhang, et al., 1998); e inclusive en la activación de conductancias de K^+, Cl^- y Na^+ sensibles a volumen (Crepel, et al., 1998; Feranchak, et al., 2001; Vom Dahl, et al., 2000). En el hígado, la activación de las MAP cinasas están involucrada en la estimulación del transporte de ácido biliar de los canalículos por hiposmolaridad, así como la alcalinización de compartimentos endosomales en las células parenquimatosas (Haussinger y Schliess, 1999b).

Por otra parte, en este trabajo se demostró que la hiposmolaridad incrementa la activación de la fosfatidil inositol-3 cinasa (PI3K), cuya inhibición disminuye de manera importante la liberación de taurina en estas condiciones. La participación de PI3K en la liberación de taurina (de la Paz LD, et al., 2002; Morales-Mulia, et al., 2001; Cardin, et al., 2003), y en la conductancia de Cl^- activada por hiposmolaridad (Shi, et al., 2002; Feranchak, et al., 1998; 1999) ha sido reportada, a la fecha, en otros sistemas. Sin embargo en algunos tipos celulares, los inhibidores de la PI3K no tienen efecto sobre I_{Clvol} y la LTAH. (Szucs, et al., 1996b; Pedersen, et al., 2002). El incremento en la actividad de PI3K por hiposmolaridad, se ha asociado también con la modulación de la translocación de otros osmolitos (intercambio Na^+/H^+, transporte de Na^+, transporte de glutamina) y a la regulación de distintas vías metabólicas (actividad de la sintasa de glicógeno y la carboxilasa de acetil-CoA) e inducción de la proliferación celular (Kim, et al., 2001). Esto sugiere que probablemente PI3K funciona como un centro de osmotransducción importante para distintos procesos involucrados en el DRV. No obstante quedan preguntas muy importantes por responder.

A la fecha se han descrito distintos subtipos de PI3K, las cuales difieren en su conformación estructural, y por lo tanto en su mecanismo de activación y las cascadas de señalización activadas. Las hipótesis que a continuación se postulan en relación al vía de activación de la PI3K por hiposmolaridad y el mecanismo involucrado en la regulación de la LTAH, se centran en las características y estudios descritos a la fecha sobre la PI3K de la Clase IA. Esto debido principalmente a que es la isoforma que más se ha estudiado a la

fecha y de la cual se tiene mayor conocimiento sobre los mecanismos de activación y señalización involucrados, los cuales, como se vera mas adelante, pueden estar participando en la regulación de la LTAH. Sin embargo, no se descarta la posible participación de otras isoformas en este fenómeno.
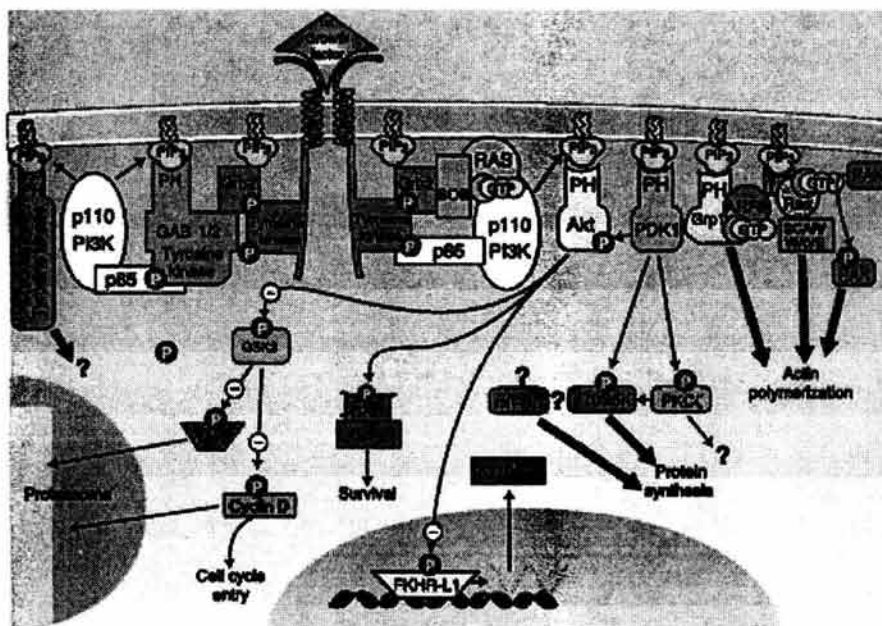
Figura 9. Activación de la fosfatidil inositol-3 cinasa (PI3K) por receptores con actividad de cinasa de tirosina. La subunidad p85 (reguladora) se une a residuos de tirosina autofosforilados en el receptor. Esto permite el acercamiento de la subunidad p110 (catalítica) a los fosfoinositidos de membrana, que al ser fosforilados activan diferentes vías de señalización (tomado de Cantley, 2002)

¿Cómo es que PI3K podría estar siendo activada por estrés hiposmótico? En su estructura, PI3K tiene dominios de unión a regiones ricas en prolina (SH3), a proteínas G pequeñas de la familia de p21Rho y Ras (regiones BH), y a residuos fosforilados de tirosina (SH2) (Djordjevic y Driscoll, 2002; Fruman, et al., 1998). De manera general, PI3K puede ser activada por el reconocimiento de residuos de tirosina fosforilados en receptores membranales con actividad de cinasa de tirosina (RTK), con su subsecuente fosforilación; por la unión a proteínas de andamiaje como Shc a los mismos receptores; vía Ras o Rho; por subunidades βγ de proteínas G heterotriméricas; o por unión de regiones ricas en prolina a cinasas tipo src y JAK, y su posterior fosforilación (Leevers, et al., 1999, Vanhaesebroek, et al., 2001) (ver Figura 9). De esta forma PI3K puede ser activada por distintos tipos de receptores tipo RTK (receptores a factores de crecimiento), asociados a cinasas de tirosina (integrinas, receptores a trombina), o acoplados a

proteínas G (receptores a ácido lisofosfatídico, ATP). PI3K, esta conformada por una subunidad reguladora (p85) y una catalítica (p110). En un esquema general, la activación de receptores membranales induce el reclutamiento de la subunidad p110 a la membrana por medio de la interacción de la p85, la cual tiene todos los dominios mencionados, con distintas proteínas mediante los distintos sitios de reconocimiento (Wymann y Pirola, 1998). Esto podría explicar por qué la presencia de estos agonistas es capaz de activar y/o potenciar a I_{Clvol}, y a la LTAH, y a su vez sugeriría la posible participación de estos receptores como vías de señalización activadas por hiposmolaridad, como parte de la cascada de osmotransducción, o involucrados en la detección del cambio en volumen (ver tercera sección de esta tesis). Por otra parte, se ha observado el aumento en la actividad de la PI3K al aumentar la curvatura de la membrana, lo que abre la posibilidad de que la tensión o curvamiento membranal actúe directamente sobre PI3K aumentando su actividad en condiciones hiposmóticas, sin un intermediario, esto a su vez, sugeriría la participación de PI3K como posible sensor de volumen (Hubner, et al., 1998).
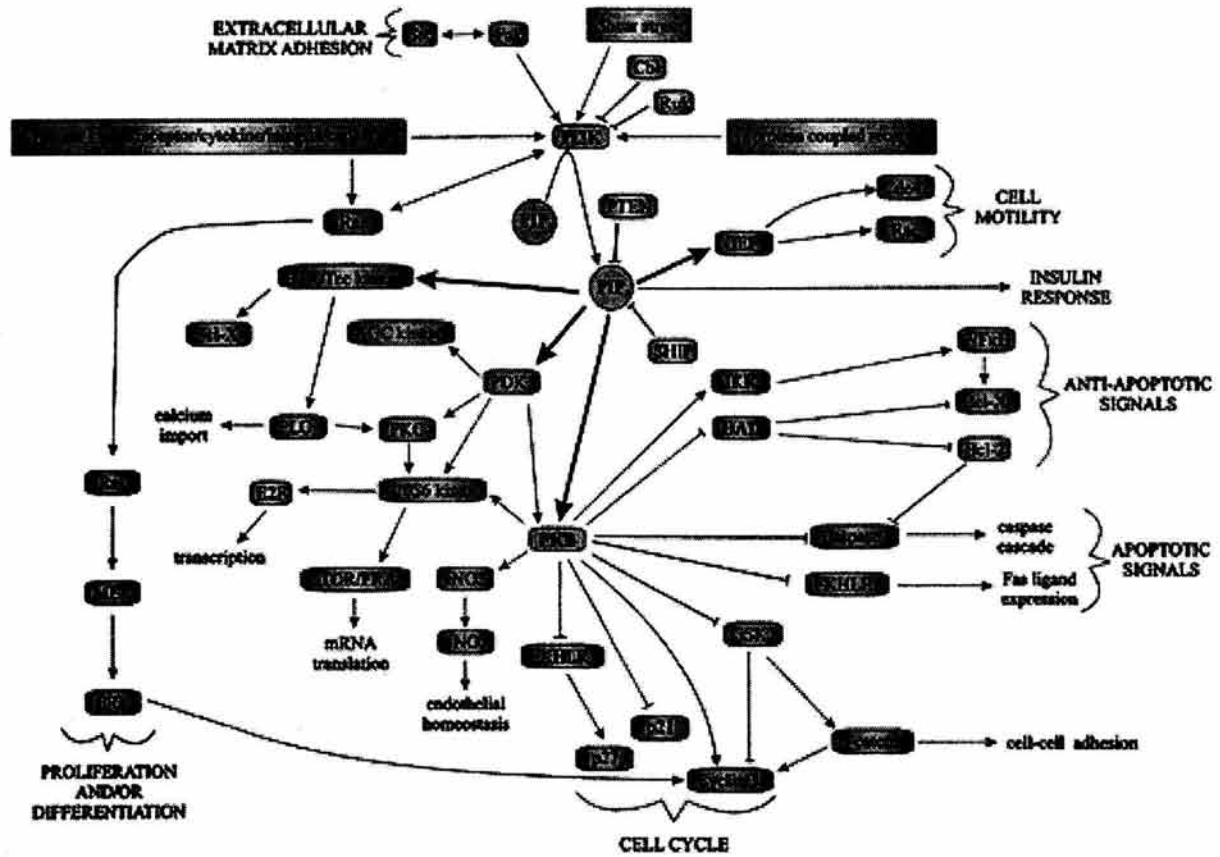
Figura 10. Vías de señalización activadas a partir de la activación de PI3K y la generación de PIP$_3$ (fosfoinositidos trifosfato) a partir de PIP$_2$ (fosfoinositidos difosfato). Tomado de (Payrastre, et al., 2001)

¿Cómo es que PI3K podría estar regulando la activación de I_{Clvol}, o la LTAH? PI3K es una cinasa de lípidos cuya activación, induce su translocación a la membrana plasmática de manera que tenga acceso a los substratos lipídicos membranales. Una vez en membrana, cataliza la transferencia del fosfato en posición γ del ATP a la posición D3 de los fosfoinosítidos (Cantley, 2002; Katso, et al., 2001). Esta fosforilación genera 3 tipos de fosfoinosítidos que pueden funcionar como segundos mensajeros que son PtdIns(3)P, PtdIns(3,4)P_2 y PtdIns(3,4,5)P_3, los cuales pueden estar regulando la activación de diferentes enzimas (Payrastre, et al., 2001; ver Figura 10). En células COS-7, expuestas a hipotonicidad se ha reportado la formación de fosfoinosítidos PtdIns(3)P, PtdIns(3,4)P_2 , PtdIns(3,5)P y PtdIns(3,4,5)P_2 (Dove, et al., 1997). La inhibición de la actividad de AKT/PKB (uno de los principales blancos de la activación de PI3K), de manera directa por un inhibidor específico, no altera la LTAH, lo que descarta la participación de ésta vía (Ver Figura 10C, en la tercera sección). La activación de factores intercambiadores de GTP (GEF) por PI3K, los cuales modulan la actividad de Rac y Cdc42, podría estar involucrada en la modulación del citoesqueleto cortical de actina inducida por hiposmolaridad que se ha reportado está regulada por estas proteínas (Carton, et al., 2003; Takenawa y Itoh, 2001), que a su vez podrían participar en la regulación de la LTAH. Es importante mencionar que el papel del citoesqueleto cortical de actina en la LTAH, no puede ser descartado por los resultados obtenidos con citocalasinas, ya que en muchos casos éstos fármacos pueden no alteran la composición del mismo, sino sólo las fibras de actina a lo largo del citosol o pueden tener efectos inespecíficos (Cassimeris, et al., 1990; Lunn, et al., 2000; Mills, et al., 2000). Otro de los blancos importantes de la actividad de PI3K, incluyen al grupo de PKC nuevas o atípicas, ε, η, ζ, λ,, MAP cinasas y a la PLCγ (Toker, 2000; Vanhaesebroeck y Waterfield, 1999). Sin embargo, la ausencia de efecto de los inhibidores de PKC y MAP cinasas, sobre la LTAH, y su independencia de Ca^{++}, descartan éstas posibilidades. La participación de otras cinasas como p70S6K (cinasa ribosomal), PAK, cinasas de tirosina de la familia TEC o la GSK-3 (cinasa inducible por glucocorticoides), las cuales se modulan por la actividad de PI3K (Cantrell, 2003), siendo algunas activadas por hiposmolaridad (Krause, et al., 1996), queda todavía por resolver.

Recientemente se ha reportado la actividad de PI3K como cinasa de proteínas, y como proteína de andamiaje dentro de distintas cascadas de señalización (Wymann, et al., 1999; Okkenhaug y Vanhaesebroeck, 2001), pero a la fecha no existen muchos

estudios al respecto que pudiesen sugerir la participación de estos procesos en el control de los flujos de osmolitos activados por hiposmolaridad. Por otra parte no se descarta la posible regulación de manera directa de PI3K, a través de los fosfoinosítidos generados sobre las vías de translocación involucradas en I_{Clvol}, o en la LTAH, la información respecto a canales iónicos regulados de esta forma es escasa. Feranchak y colaboradores (1998; 1999) proponen un sistema parácrino de regulación del volumen celular en células de hepatoma, en las cuales, PI3K regula la liberación de ATP por hiposmolaridad, el cual funciona como segundo mensajero que activa a I_{Clvol}, nadie ha reportado los mismos resultados en otros tipos celulares. Liberación de aminoácidos neurotransmisores por hiposmolaridad.

Liberación de aminoácidos neurotransmisores por hiposmolaridad

La liberación de aminoácidos neurotransmisores ha sido estudiada en distintos tipos de preparaciones nerviosas incluyendo células en cultivo. La importancia del estudio de este fenómeno radica en las alteraciones en la excitabilidad celular que conllevan incrementos en la concentración de sustancias neuroactivas no solo por el incremento en su concentración extracelular sino por la reducción en las dimensiones del mismo. En astrocitos en cultivo expuestos a condiciones hiposmóticas, se observa un incremento en la liberación de glutamato y aspartato, la cual disminuye parcialmente, en presencia de bloqueadores de canales de Cl⁻ (Kimelberg, et al., 1990; Pasantes-Morales, et al., 1993; Rutledge, et al., 1998). El incremento de la concentración de glutamato y aspartato puede deberse en parte a una reducción en los mecanismos de captura en células gliales (Kimelberg et al., 1995b). En rebanadas de cerebro, y estudios *in vivo* en corteza cerebral, se ha observado que en condiciones hiposmóticas, la liberación de aspartato, GABA, y glutamato es parcialmente sensible a los bloqueadores de canales de Cl⁻ NPPB, tamoxifen, ácido niflúmico, DIDS (Bothwell, et al., 2001; Estevez, et al., 1999a; Haugstad et al., 1997; Law, 1994b; 1996a; 1996b; Oja y Saransaari, 1996;Saransaari y Oja, 1992). En todos estos modelos de estudio la liberación osmosensible de taurina se inhibe en mayor grado por bloqueadores de canales de Cl⁻ respecto a lo observado para aminoácidos neurotransmisores. En general la liberación de taurina es más sensible a decrementos en la osmolaridad del medio, así como a la presencia de bloqueadores de canales de Cl⁻, en comparación con la liberación de otros aminoácidos, aún en células no excitables (Basavappa, et la., 1996; Grant, et al., 2000; Haynes y Goldstein, 1993; Miyata

et al., 1997; Pasantes-Morales et al., 1999; Sanchez-Olea, et al., 1991;). Disminuciones en la concentración de glutamato en respuesta a hiposmolaridad en otras preparaciones, también ha sido reportada (Bajnath, et al., 1997; Bursell, et al., 1996; Rasmusson, et al., 1993; Sanchez-Olea, et al., 1995; Shennan y McNeillie, 1995; Song, et al., 1998a; 1998b).

La liberación de glutamato a través de canales sensibles a volumen se ha reportado en otras condiciones asociadas a edema celular como son altas concentraciones de K^+ extracelular, isquemia y durante la depresión progresiva de la excitabilidad neuronal (spreading depression) (Aschner, et al., 2001; Basarsky, et al., 1999; Phillis, et al., 1999; Rutledge y Kimelberg, 1996; Saransaari y Oja, 1999; Sundstrom y Mo, 2002). Esta liberación es sensible a bloqueadores de canales de Cl⁻ (Basarsky, et al., 1999; Estevez, et al., 2000; Phillis, et al., 1997; 1998; Sanchez-Olea, et al., 1993;). Sin embargo en estas condiciones existe un componente importante mediado por otros mecanismos de liberación como es el funcionamiento del transportador en reversa (Djali y Dawson, 2001; Rossi, et al., 2000; Rutledge y Kimelberg, 1996; Seki, et al., 1999).

En nuestros resultados, observamos que la liberación de aminoácidos neurotransmisores presenta características distintas respecto de las observadas para la taurina. En condiciones hiposmóticas, la liberación de GABA y glutamato presenta una cinética de liberación de activación e inactivación rápida, mientras que la liberación de taurina es más lenta y sostenida. La movilización de GABA y glutamato fue insensible a bloqueadores de canales de Cl⁻. Las vías de señalización involucradas en la liberación de aminoácidos neurotransmisores por hiposmolaridad también difieren de lo reportado para la liberación de taurina. En estas condiciones, la salida de GABA y glutamato fue insensible a inhibidores de cinasa de tirosina y de PI3K. A la fecha se ha reportado que en condiciones hiposmóticas, la liberación de aspartato es insensible en una fracción muy grande a inhibidores de cinasas de tirosina (de la Paz LD, et al., 2002; Mongin, et al., 1999). Estas observaciones sugieren una vía de movilización de aminoácidos neurotransmisores distinta con respecto a la hipótesis del VSOAC, regulada por otras vías de señalización.

La operación en reversa del transportador de glutamato como mecanismo de extrusión al espacio extracelular ha sido reportada sólo para el caso de condiciones de hinchamiento celular debido a un incremento en la concentración de solutos en el espacio

intracelular (condiciones de alto K^+ extracelular, inhibición metabólica, encefalopatía hepática e isquemia), no para el caso de hinchamiento hiposmótico (Kimelberg, et al., 1993; Rossi, et al., 2000). En nuestros resultados, inhibidores del transportador a glutamato y aspartato no disminuyen la salida de estos aminoácidos en condiciones hiposmóticas, por lo que ésta posibilidad queda descartada como vía osmosensible de salida de aminoácidos neurotransmisores. Sin embargo, datos recientes sugieren que este mecanismo podría estar funcionando parcialmente en terminales nerviosas aisladas (Tuz, et al., 2004. en prensa)

Existen fenómenos concurrentes con el hinchamiento osmótico como consecuencia de reducciones en la osmolaridad del medio y de la subsecuente alteración en la concentración iónica de las células, como son cambios en el potencial de membrana, incrementos en las concentraciones de Ca^{++} citosólico y fenómenos de tensión membranal, que a su vez podrían inducir eventos de liberación vesiculada, o exocitosis, de neurotransmisores en estas condiciones. El hinchamiento hiposmótico puede inducir incrementos en los procesos de fusión vesicular a la membrana plasmática en muchos tipos celulares, incluyendo la liberación vesiculada de varias hormonas (Strbak y Greer, 2000). Por otra parte, la hipertonicidad inhibe el proceso de exocitosis (Rizoli, et al., 2000). Esto sugiere que los cambios en el volumen celular pueden estar fuertemente ligados a la modulación de las tasas de exocitosis/endocitosis. Es probable que la liberación de GABA y glutamato de las rebanadas de hipocampo en condiciones hiposmóticas esté mediada por exocitosis.

La liberación de GABA por exocitosis es un fenómeno ampliamente reportado en distintos modelos incluyendo la rebanada de hipocampo. Para el caso del D-aspartato, puede existir controversia en cuanto a su posible localización en vesículas sinápticas. En éste estudio, inicialmente se utilizó al D-aspartato, por ser considerado de manera general, como un análogo no metabolizable del glutamato, lo que nos permitiría no perder la marca radioactiva de mismo (seguido como liberación de D-aspartato), al ser incorporado en alguna reacción metabólica o síntesis de macromoléculas. Los transportadores membranales de glutamato captan ambos isómeros (L- y D-) tanto de glutamato como de aspartato, de manera eficiente. Esto permite que la marcar la poza citosólica libre de glutamato mediante el uso de ^3H-D-aspartato. Para el caso del transporte vesicular de glutamato, en un inicio se consideraba, que este no reconocía ni al

L- ni al D-aspartato, debido reportes en donde no se detectaba la presencia de estos en vesículas sinápticas purificada, lo que argüía en contra, de su posible papel como aminoácido neurotransmisor en el cerebro (Gasnier, 2000; Naito y Ueda, 1985; Winter y Ueda, 1993). Sin embargo, esto ha sido recientemente puesto a discusión, debido a la evidencia reciente que indican una colocalización de L- o D-aspartato con glutamato en terminales excitadoras en el hipocampo de rata (Gundersen, et al., 1998), modelo utilizado en este trabajo, y en sinaptosomas de cerebro de rata (Fleck, et al., 1001). Por otra parte, se ha reportado que ambos isómeros se liberan de manera exocitótica en preparaciones de hipocampo de rata, pinealocitos neuroendócrinos (Yatsushiro, et al., 1997), células PC12 y células granulares de cerebelo (Cousin, et al., 1997). Así mismo, la liberación de aspartato tanto en sinaptosomas de la región CA1 del hipocampo (Zhou, et al., 1995), como en vías excitadoras *in vivo* (Girault, et al., 1986; Paulsen y Fonnum, 1989) y rebanadas de cerebro (incluyendo al hipocampo) (Gundersen, et al., 1998; Klancknick, et al., 1992; Nadler, et al., 1976), es dependiente de Ca^{++}, y se inhibe por toxina tetánica y botulínica (Mahon, et al., 1992), las cuales inhiben la liberación vesicular de neurotransmisores mediante el rompimiento de proteínas del complejo de fusión de vesículas sinápticas con la membrana plasmática. Estos antecedentes soportan la hipótesis de una liberación exocitótica de D-aspartato por hiposmolaridad en la rebanada de hipocampo. Aunque el presente trabajo no tiene los argumentos necesarios para sustentar completamente esta hipótesis, la participación de este fenómeno se sugiere con base en las siguientes observaciones:

1) En condiciones hiposmóticas se ha reportado un incremento en la concentración de Ca^{++} en casi todos los tipos celulares estudiados (para una revisión, ver Jakab, et al., 2003; Tinel, et al., 2000; Pasantes-Morales y Morales Mulia, 2000), lo cual podría activar procesos de exocitosis en estas condiciones. Sin embargo, en nuestros resultados la liberación de aminoácidos neurotransmisores es independiente de Ca^{++} lo que argumenta en contra de un posible mecanismo de liberación sináptica típica. Datos recientes del laboratorio muestran que en terminales sinápticas, la liberación de aminoácidos es parcialmente dependiente de los niveles basales de Ca^{++}, por lo que la dependencia a este ión se sugiere solamente localizada en las terminales (Tuz, et al., 2004). En nuestro modelo de rebanadas, no cuantificamos los cambios en la concentración de Ca^{++} en condiciones hiposmóticas ni la poza de procedencia, por lo que no podemos descartar la participación de este ión ya sea a concentraciones basales, por incrementos residuales en

los niveles intracelulares, o en microdominios localizados. En contraste con la mayoría de los procesos exocitóticos, los procesos de fusión y liberación del contenido vesicular en condiciones hiposmóticas, no requieren un incremento en la concentración intracelular de Ca^{++}.

2) En nuestros resultados observamos que la liberación de neurotransmisores por hiposmolaridad se ve regulada por la actividad de PKC. La proteina cinasa C (PKC) es una cinasa de serina treonina cuya actividad modula la liberación de neurotransmisores en muchas preparaciones. En el sistema nervioso central se ha descrito la presencia de todos los subtipos de PKC, siendo los de mayor expresión PKCγ y PKCε (Nishizuka, 1995). La activación de PKC por esteres de forbol (PMA), induce un aumento en la liberación de neurotransmisores de casi todos los tipos de neuronas incluyendo colinérgicas, dopaminérgicas, noradrenérgicas, GABAérgicas, glutamatérgicas y serotoninérgicas (Majewski y Ilanazo, 1998; Nicholls, 1998). Las formas de modulación por las que PKC puede estar actuando y que se han reportado a la fecha, son las siguientes: a) Modulación de receptores presinápticos; b) Modulación de la conductancia de canales iónicos; c) Rompimiento de filamentos de actina por la fosforilación de proteínas asociadas al citoesqueleto, lo que facilita que las vesículas ubicadas en el reservorio del citoesqueleto se adosen a la membrana presinnáptica, incrementando el número de vesiculas disponibles para su liberación (Iannazo, 2001); y d) por la acción sobre distintos substratos intracelulares que a su vez modulan el proceso de exocitosis (GAP-43, MARCKS) (Vaughan, et al., 1999).

La asociación directa de PKC con proteínas del citoesqueleto modula la actividad de diferentes subtipos de PKC (Keenan y Kelleher, 1998). Cambios en la estructura del citoesqueleto inducen cambios enzimáticos en estas proteínas (Jaken y Parker, 2000; Toker, 1998). Esto podría explicar el efecto de la despolimerización del citoesqueleto en la liberación de neurotransmisores por hiposmolaridad. Este punto se discutirá más adelante. En rebanadas de hipocampo se ha reportado que los potenciales espontáneos postsinápticos miniatura excitadores e inhibidores, son potenciados por la activación de PKC por esteres de forbol de una manera independiente de Ca^{++}, esta potenciación es resistente a toxina tetánica y a la presencia de Cd^{++}. A su vez estos eventos requieren de los filamentos de actina. Este fenómeno, probablemente esta mediado por PKCε. El mecanismo de modulación por PKC puede involucrar ya sea un aumento en la poza

disponible de vesículas para ser liberadas, o un incremento en la sensibilidad al Ca^{++}, de la maquinaria de fusión (Bouron, 2001; Thomson, 2000)

3) En nuestros resultados también observamos que la liberación de GABA y glutamato depende de la integridad del citoesqueleto de actina. El papel del citoesqueleto en la liberación de neurotransmisores por exocitosis tiene una amplia evidencia experimental. Se ha observado que en el momento de la exocitosis se origina una despolimerización de los filamentos de actina. Los procesos de exocitosis y endocitosis se acompañan por cambios locales en la estructura del citoesqueleto de actina. Los agentes que perturban la polimerización y despolimerización del citoesqueleto alteran la tasa de exocitosis y endocitosis. Se ha demostrado que la exocitosis no se puede llevar a cabo sin un remodelamiento de la estructura de los filamentos de actina (Valentijn, et al., 1999). Los filamentos de actina permiten mantener la poza de reserva de vesículas cargadas de neurotransmisores lejos de la zona activa(mediante la unión a proteínas específicas como la sinapsina, cuya fosforilación permite que las vesículas se liberen del citoesqueleto) (Dresbach, et a., 2001), por lo que al despolimerizarse el citoesquleto, más vesículas se adosan a la presinapsis aumentando la cantidad de vesículas listas para ser liberadas (Doussau y Augustine, 2000).

Como ya se mencionó, los datos obtenidos no descartan la participación de un proceso de exocitosis de neurotransmisores en condiciones de hiposmolaridad; sin embargo son necesarios más datos para corroborar esta hipótesis para el caso de la rebanada de hipocampo. Resultados recientes en el laboratorio, sustentan la posible participación de este fenómeno en terminales nerviosas aisladas, en las cuales la liberación de aminoácidos es inhibida por toxinas (toxina tetánica), que desacoplan el complejo de fusión y por lo tanto impiden el proceso de exocitosis. El disparador de este fenómeno parece estar involucrando eventos de despolarización por la entrada de Na^+ a través de canales activados por estiramiento y dependencia de los niveles de Ca^{++} intracelular (Tuz, et al., 2004).

Diferentes vías de movilización de aminoácidos en preparaciones de células excitables. Importancia

Los datos obtenidos en el presente trabajo establecen una marcada diferencia entre la liberación de taurina activada por hiposmolaridad y la de aminoácidos neurotransmisores con base en los parámetros de la Tabla 1. El diferente manejo de ambos tipos de aminoácidos podría significar un mecanismo adaptativo presente solamente en las preparaciones de tejido nervioso. En el tejido nervioso sería más conveniente, que bajo condiciones de hinchamiento celular, el proceso de DRV involucre la movilización de osmolitos que alteren lo menos posible el metabolismo y sobre todo la excitabilidad del tejido, por lo que la existencia de varias vías de salida de estos aminoácidos con mecanismos de regulación distinta pueden significar una ventaja.

Como ya se mencionó, la liberación de aminoácidos neurotransmisores, así como la reducción en el espacio extracelular y sobre todo en el espacio sináptico, puede originar, alteraciones importantes en la excitabilidad del tejido nervioso. Por una parte la liberación de aminoácidos excitadores como el glutamato podría ser responsable del incremento en los potenciales excitadores postsinápticos (PEPS) observado en rebanadas de hipocampo expuestas a medios de baja osmolaridad (Huang et al., 1997). Por otra, la reducción en el espacio extracelular debido al hinchamiento de las células, podría generar fenómenos de transmisión electrotónica y actividad epileptogénica (Andrew et al., 1989; Roper et al., 1992). Se ha descrito por ejemplo que el hinchamiento de las dendritas favorece la propagación electrotónica originando fenómenos de depresión propagada de la excitabilidad neuronal (SD) (Baraban et al., 1997; Chebabo et al., 1995). Igualmente en condiciones hiposmóticas se favorece la actividad epileptogénica debido a la disminución en el espacio extracelular, lo que genera la sincronización de los disparos. También se ha visto que un incremento en el volumen celular puede favorecer interacciones efápticas durante la transmisión sináptica. (Andrew et al., 1989; Ballyk et al., 1991; Dudek et al., 1990; Rosen y Andrew., 1990). De manera general, se ha observado que disminuciones en la osmolaridad del medio aumentan la transmisión sináptica y la excitabilidad neuronal, mientras que aumentos en la osmolaridad tiene el efecto opuesto (Somjen, 2000). Para una mayor profundidad respecto al tema, ver artículo de revisión 4.

Si la liberación de aminoácidos neurotransmisores estuviese dada por un evento de exocitosis, ésta no contribuiría al DRV en condiciones hiposmóticas, por lo que su participación en la adaptación prolongada en condiciones de hiponatremia queda a

discusión. Es importante hacer hincapié en que los aminoácidos dentro de compartimentos vesiculares, no participan en la osmolaridad interna del citosol, por lo que su liberación vesicular no modificaría de alguna forma el gradiente osmótico de entrada de agua y no acarrearía ningún flujo neto de la misma dentro del DRV. Sin embargo, no se descarta el papel que puedan tener los aminoácidos en el control del volumen vesicular.
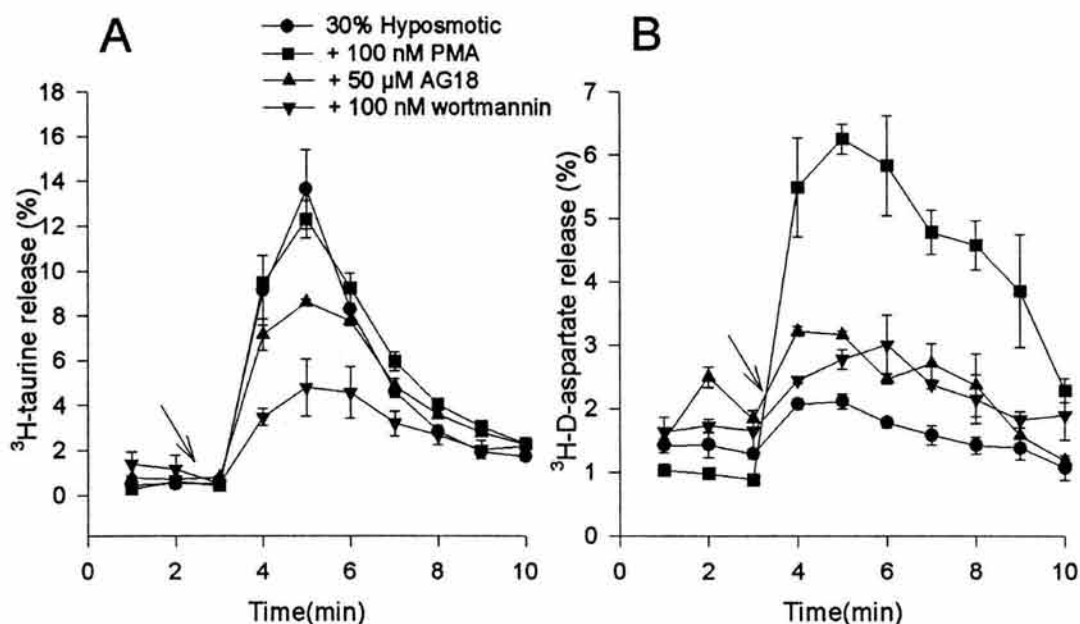
Figura 9. Vías de señalización involucradas en la liberación de taurina y glutamato activada por hiposmolaridad en astrocitos de corteza de rata. Los astrocitos de corteza sembrados a confluencia en cajas petri de 35 mm, fueron preincubados durante 1hr con los trazadores radioactivos. Posteriormente, la perfusión se realiza según lo descrito en (Morales-Mulia, et al., 2001). La flecha indica el cambio de medio isosmótico a hiposmótico. En **A**. Se observa el efecto del inhibidor general de cinasas de tirosina AG18 y del inhibidor de PI3K, wortmanina, sobre la liberación de taurina, la cual se reduce de manera importante en presencia de estos agentes. Estos agentes no modifican la liberación de glutamato por hiposmolaridad **B**., sin embargo, esta a su vez se incrementa por la activación de PKC por PMA, similar a lo observado en las rebanadas de hipocampo. Los datos se expresan como % de liberación y son promedios ± ES de n = 4. Controles (●) con el mismo tratamiento con el vehículo. El tratamiento con los fármacos es el mismo que el utilizado para las rebanadas de hipocampo.

Este fenómeno de dos vías o mecanismos de liberación de aminoácidos, parece presentarse principalmente en tejido nervioso. En astrocitos corticales se observa el mismo fenómeno, siendo la liberación de taurina modulada por cinasas de tirosina y PI3K, mientras que la de glutamato se regula por la actividad de PKC (Figura 9, datos no publicados). Existe evidencia que prueba la presencia de proteínas asociadas al complejo

de fusión vesicular en astrocitos, así como la presencia de mecanismos de liberación de glutamato independientes de la participación del transportador y sensible a neurotoxinas que bloquean la exocitosis en neuronas (Volknandt, 2002), por lo que no se descarta que un proceso de exocitosis medie la liberación de glutamato por hiposmolaridad en este tipo celular. En nuestros resultados, observamos que en fibroblastos en cultivo, la liberación de glutamato y taurina presenta las mismas características descritas para una vía difusional (VSOAC) (ver tercera sección de la tesis), lo que corrobora la hipótesis de que este fenómeno sólo se presenta en preparaciones de tejido o de células nerviosas. Esto también se ha observado en otros estudios (Sanchez-Olea et al., 1995, Shennan and McNeillie, 1995; Song, et al., 1998a; 1998b;).

TERCERA PARTE

PARTICIPACION DE RECEPTORES CON ACTIVIDAD DE CINASA DE TIROSINA EN LA CASCADA DE OSMOTRANSDUCCION QUE MODULA LA LIBERACION DE TAURINA POR HIPOSMOLARIDAD

ANTECEDENTES

Sensores de volumen.

La regulación del volumen debe involucrar mecanismos que le permitan detectar o sensar cambios en el volumen ya sean transitorios o prolongados, para después transducirlos en la activación de distintos mecanismos de translocación de osmolitos. Conforme se ha ido avanzando más en el estudio de estos candidatos y su participación en el control del volumen celular, el concepto de que no sólo un osmosensor, sino más bien un sistema sensorial complejo participan en la detección y transducción del cambio en el volumen ha ido ganando más fuerza. A continuación se describen brevemente algunos de los posibles sistemas de sensor de volumen propuestos a la fecha.

El agrupamiento de macromoléculas intracelulares es un término utilizado para describir el fenómeno, en el cual la concentración total de macromoléculas en el citosol es lo suficientemente alta que una proporción significativa del volumen está significativamente ocupado. Este fenómeno altera las tasas de reacción bioquímica alterando tanto las tasas de difusión molecular como la actividad termodinámica. Cambios en el agrupamiento de macromoléculas pueden funcionar como sensor de volumen y regular a su vez mecanismos involucrados en el control del volumen celular, debido por ejemplo, a la asociación reversible de proteínas de transporte iónico con proteínas reguladoras en el citosol. Existe evidencia experimental que demuestra que el agrupamiento macromolecular determina el umbral de activación de los mecanismos involucrados en la regulación del volumen celular en eritrocitos. Se postula que el umbral de regulación o activación no está determinado por el volumen celular, sino por la concentración proteica total. En éstas células, la dilución del citosol inducida por hinchamiento disminuye la actividad de una cinasa cuya actividad inhibe la actividad del cotransporte K^+/Cl^-, por lo que al incrementar la actividad de este transportador durante el

hinchamiento se permite el DRV (Colclasure y Parker, 1991; Minton, et al., 1992; Parker, et al., 1991). Aunque el agrupamiento intracelular de macromoléculas puede ser un mecanismo de detección de cambios del volumen celular, parece que no es la única señal involucrada.

Los cambios en el volumen celular, alteran las concentraciones intracelulares de iones y por consiguiente afectan la fuerza iónica total del citoplasma. A su vez, estos cambios en las concentraciones iónicas intracelulares alteran las interacciones específicas entre los lípidos de membrana y las proteínas, por consiguiente alterando su actividad. Estudios recientes demuestran que una disminución en la fuerza iónica subsecuente al hinchamiento celular funciona como señal primaria de la activación de I_{Clvol} y de la LTAH, independientemente del gradiente de sales a través de la membrana plasmática, de la concentración intracelular de las especies aniónicas o catiónicas y de la concentración intracelular total de electrolitos (Cannon, et al., 1998; Cardin, et al., 1999; Emma, et al., 1997; Motais, et al., 1991; Nilius, et al., 1998; Voets, et al., 1999). Estos estudios sugieren ya sea una directa modulación de la fuerza iónica sobre las vías de salida de osmolitos (de forma directa o mediante vías de señalización intermediarias), o un cambio en el umbral de volumen de activación de estas vías. Un estudio reciente demuestra que la actividad de cinasas de tirosina se modula por cambios en la fuerza iónica intracelular, las cuales a su vez modulan la LTAH (Wittels, et al., 2000). Sin embargo, esta teoría no explica la activación de las vías involucradas en la salida de osmolitos, en condiciones de hinchamiento isosmótico. La participación de estos fenómenos en el transporte de osmolitos en organismos unicelulares ha sido ampliamente estudiada (Poolman, et al., 2002).

El hinchamiento celular causa estiramiento membranal en distintos tipos celulares. Okada describió que la tensión membranal solo comenzaría a aumentar después que la célula alcanzara un volumen de 4.8 veces el original y que el hinchamiento celular es a expensas de la pérdida de invaginaciones en la membrana y no de un incremento en la tensión membranal (Okada, 1997). Sin embargo, esto sólo aplica a células con un alto reservorio membranal en invaginaciones como serían células epiteliales en las cuales se realizó este estudio. Para el caso de otros tipos celulares, como las neuronas, cuya membrana contiene pocas invaginaciones, el hinchamiento celular, podría incrementar substancialmente la tensión membranal. El hinchamiento celular, por lo tanto, podría

inducir transiciones conformacionales en los transportadores iónicos sensibles a volumen, en respuesta a estrés mecánico o compresión de la membrana lipídica, modulando su función, esto debido a su arquitectura molecular. La activación de I_{Clvol}, por presión intracelular o por sustancias que alteran la forma celular como la clorpromazina y el dipiridamol, sugiere que los cambios en la tensión membranal podrían ser el sensor para la activación de los mecanismos de control del volumen celular (Fan, et al., 1999; Hagiwara, et al., 1992; Lewis, et al., 1993; Nilius, et al., 1994;). Los cambios en el estiramiento de la membrana pueden activar distintas cascadas de señalización, como aquellas reguladas por la reorganización en el citoesqueleto, o por la liberación sensible a hinchamiento de segundos mensajeros como ATP o ácidos grasos (Kirber, et al., 1992). En este sentido se ha observado que la reorganización estructural de la bipaca lipídica por estiramiento membranal regula la actividad de la PLA_2 (Lehtonen y Kinnunen, 1995), la cual se ha observado que puede estar involucrada en la modulación de la salida de osmolitos por hiposmolaridad.

La estructura de la membrana plasmática no es homogénea, y en muchos tipos celulares contiene una gran variedad de dominios especializados denominados caveolas. Las caveolas son invaginaciones de la membrana plasmática con un diámetro de alrededor de 50nm. Estas están organizadas en cuatro isoformas de proteínas especializadas llamadas caveolinas las cuales contienen un alto contenido en colesterol, además de esfingolípidos. Se ha reportado que la caveola aglutina distintos tipos de proteínas de señalización incluyendo: receptores acoplados a proteínas G, proteínas G de la familia de Ras y p21Rho, cinasas de tirosina y receptores a factores de crecimiento (Anderson, 1998). Se ha sugerido que en respuesta a hiposmolaridad, el desdoblamiento de estas invaginaciones podría inducir la activación de distintas vías de señalización. En algunos tipos celulares se ha demostrado que la activación de I_{Clvol} es dependiente de la expresión de la proteína caveolina 1, la cual es una proteína de andamiaje en la caveola (Trouet, et al., 1999; 2001).

Los cambios en el volumen provocan perturbaciones en la morfología celular y en consecuencia en el citoesqueleto. El hecho de que los filamentos de actina están normalmente concentrados en la región submembranal cortical los hace posibles candidatos como sensores de volumen. Los segundos mensajeros que se han visto involucrados en la activación de las vías de transporte de osmolitos tienen interacciones

funcionales con el citoesqueleto e incluso los canales y transportadores involucrados pueden también estar asociados al citoesqueleto (Janmey, 1998). Esto sugiere que el citoesqueleto podría estar involucrado en la detección y transducción del cambio en volumen, e inclusive como un sistema de memoria del estado no-deformado de la célula o del volumen celular original. En muchos tipos celulares, el hinchamiento celular conlleva al remodelamiento del citoesqueleto. En algunos tipos celulares, el DRV se inhibe en presencia de citocalasinas, las cuales inhiben la formación de fibras de estrés. Existe evidencia experimental que indica que el citoesqueleto está involucrado en la regulación del DRV y de I_{Clvol} (Cornet, et al., 1988; 1993; Downey, et al., 1995; Foskett y Spring, 1985; Levitan, et al., 1995; Mills, et al., 1999; Pedersen, et al., 1999; Schwiebert, et al., 1994; Ziyadeh, et al., 1992). Sin embargo este fenómeno no es común a todos los tipos celulares estudiados. Para una mejor revisión respecto al papel del citoesqueleto en el control del volumen celular y en la regulación de mecanismos de transporte de osmolitos, referirse a Cantiello, 1997; Henson, 1999; Papakonstanti, et al., 2000; Pedersen, et al., 2001.

Las células se adhieren a la matriz extracelular (ECM) por medio de las integrinas. Aunque muchos grupos de investigación han propuesto a las integrinas como posibles sensores de cambios en volumen celular en condiciones hiposmóticas, a la fecha solo estudios en células hepáticas han demostrado la participación de las integrinas en la respuesta celular al estrés hiposmótico (Haussinger, et al., 2003; Vom Dahl, et al., 2003). Sin embargo, esta teoría no explicaría el porqué, aún en células en suspensión donde no se origina la activación de procesos de adhesión, varios tipos celulares presentan DRV inclusive más eficiente que al estar adheridos a una superficie. Se ha sugerido que los cambios en la tensión membranal originados por hinchamiento pueden inducir la activación de las integrinas en ausencia de señales de adhesión o de unión a ECM. La activación por estiramiento o por hiposmolaridad de p125FAK (Hamasaki, et al., 1995), la cinasa de tirosina directamente ligada a la activación o dimerización de las integrinas (Schaller, 2001; Schlaepfer, et al., 1999), y de p21Rho, reportada en distintos tipos celulares, es una evidencia indirecta de la participación de estas proteínas, así como de las integrinas en la cascada de osmotransducción en respuesta a hinchamiento celular.

Receptores transmembranales como mecanotransductores. Una hipótesis.

La habilidad de las células para interactuar y adaptarse al medioambiente es uno de los procesos fundamentales en la biología celular. Esta capacidad de responder está dada principalmente a través de la expresión en la superficie celular de un gran repertorio de receptores específicos que son sensibles a la composición del medio ambiente. Los receptores membranales transducen las señales extracelulares a través de la membrana hacia la activación de vías de señalización intracelular. Moléculas de señalización extracelular como los factores de crecimiento EGF (factor de crecimiento epidermal), PDGF (factor de crecimiento derivado de plaquetas), IGF (factor de crecimiento tipo insulina), etc., se unen a receptores con actividad intrínseca de tirosina cinasa (RTK). Estos receptores poseen una o dos regiones intracelulares de cinasa de tirosina. Al unir su ligando, los RTK se dimerizan y posteriormente se autofosforilan (transfosforilación) en residuos específicos. Los residuos de tirosina fosforilados resultantes funcionan como sitios de unión altamente selectivos para proteínas con dominios tipo SH2 (dominios homólogos a Src 2), que transducen la señal alterando su actividad enzimática o reclutando otras proteínas. Dentro de estas proteínas podemos encontrar proteínas activadoras de la actividad de GTPasa-Ras (GAP); PLCγ que liberan DAG e IP_3 liberando Ca^{++} intracelular y activando a PKC; proteínas adaptadoras como Shc y Grb2 (proteína homóloga a colágeno y src; proteína de unión a factores de crecimiento 2) que a su vez, unen a Sos y Ras activando la vía de las MAP cinasas. La subunidad p85 de la PI3K, también es reclutada por estos dominios SH2 a receptores a factores de crecimiento, o por la unión a proteínas adaptadoras como IRS (Hubbard y Hill, 2000; Fiorini, et al., 2001; Schenk y Snaar-Jagalska, 1999; Uings y Farrow, 2000).

Recientemente se ha observado que en respuesta a condiciones de estrés mecánico se pueden inducir la fosforilación de distintos receptores membranales incluyendo receptores a factores de crecimiento. Se ha postulado que el estrés mecánico por si solo puede alterar directamente la superficie de la célula y por ende la conformación estructural de los RTK, iniciando la activación de vías de señalización de manera ligando independiente. Esto debido posiblemente a un incremento en la elongación y movilidad membranal que como resultado expone los dominios de cinasa del receptor permitiendo así la autofosforilación (Chen, et al., 1999; Hu, et al., 1999; Li y Xu, 2000). A la fecha no

existe evidencia que sugiera este fenómeno para el caso de condiciones de hinchamiento hiposmótico.

Un ejemplo clásico de RTK es el receptor al factor de crecimiento epidermal o EGFR. La familia del EGFR esta compuesto por cuatro miembros que son: EGFR (ErbB1), HER2 (ErbB2/neu), HER3 (erbB3) y HER4 (erbB4). Al ser activados, los miembros de la familia del EGFR, forman complejos de homo o heterodímeros, que inician un sinnúmero de cascadas de señalización, muchas de las cuales atenúan o regulan de forma negativa la actividad del mismo (Wells, 1999). El EGFR o ErbB1, es un miembro prototípico de los RTK y es el subtipo más ampliamente distribuido en muchos tipos celulares (Bogdan y Klambt, 2001). El EGFR puede ser activado de manera ligando independiente en respuesta a distintas condiciones de estrés como son hiperosmolaridad, luz ultravioleta y radiación γ, varios oxidantes como el peroxido de hidrógeno (H_2O_2), permanganato, iones metálicos pesados y agentes alquilantes de sulfhidrilos (King, et al., 1989, Zwuick, et al., 1999). Aparentemente este efecto está mediado principalmente por interferencia con la acción de fosfatasas de tirosina de manera directa o por agentes secundarios. Esto por la inhibición reversible de la desfosforilación por oxidación de un grupo SH- catalítico crucial en su función o por alquilación irreversible de un residuo esencial en las fosfatasas de tirosina (Knebel, et al., 1996; Weiss, et al., 1997). A su vez, el EGFR puede ser transactivado (de forma ligando independiente) por receptores acoplados a proteínas G y por señales de adhesión mediadas por integrinas (Moro, et al., 1998; ver figura 14). Estos datos sugieren que los RTK, o receptores a factores de crecimiento, podrían funcionar como posibles sensores del cambio en volumen en condiciones anisosmóticas. La participación de RTK en la cascada de osmotransducción activada por hiposmolaridad no ha sido esclarecida aún.

OBJETIVOS PARTICULARES

El objetivo de esta sección es estudiar el papel de receptores transmembranales en la modulación de la salida de osmolitos por hiposmolaridad. En particular se estudiará el papel de los RTKs en la cascada de osmotransducción, activada por hinchamiento hiposmótico, involucrada en la liberación osmosensible de taurina, y su posible intervención como sensor de volumen. Como ya se mencionó el EGFR es un miembro representativo de la superfamilia de RTKs que se expresa en muchos tipos celulares.

Para este estudio se escogió en particular al EGFR por ser un miembro representativo de la familia de los RTKs que se expresa en muchos tipos celulares y debido a que se activa de manera ligando independiente en respuesta a muchos estímulos, como son señales de adhesión y condiciones de estrés. Para este trabajo, se utilizó una línea celular inmortalizada de fibroblastos Swiss 3T3, debido a que expresan este receptor y a que presentan un solo componente de liberación de aminoácidos a diferencia de las preparaciones nerviosas.

RESULTADOS

Los resultados de esta sección se anexan a continuación en el formato de los artículos correspondientes ya publicados, los cuales contienen, una breve introducción la tema, la metodología utilizada, los datos y la discusión la cual se amplia en la siguiente sección.

CELL AND MOLECULAR PHYSIOLOGY

Rodrigo Franco · Ruth Lezama · Benito Ordaz ·
Herminia Pasantes-Morales

Epidermal growth factor receptor is activated by hyposmolarity and is an early signal modulating osmolyte efflux pathways in Swiss 3T3 fibroblasts

Abstract Exposure of cultured Swiss 3T3 fibroblasts to 35% hyposmotic solution activated epidermal growth factor receptor (EGFR) phosphorylation to a greater extent than the ligand, EGF. Concanavalin A (Con A) and wheat-germ agglutinin (WGA) had the same effect. EGFR phosphorylation seems to be involved in the transduction signalling for hyposmotically induced taurine release, as suggested by the latter's reduction when EGFR phosphorylation was blocked by 50 μM AG213 or AG112 and, conversely, its potentiation by EGF (200 ng/ml). The relationship between hyposmotically induced taurine efflux and reduced osmolarity showed saturable kinetics, following a sigmoidal function. EGF shifted the relationship to the left, implying an increase in sensitivity to hyposmolarity. EGF increased taurine efflux only marginally under isosmotic conditions. EGF and agglutinins also potentiated the hyposmotically induced release of ^{86}Rb but, in contrast to taurine, the efflux was unaffected by EGFR inhibition. EGF and agglutinins markedly increased ^{86}Rb release under isosmotic conditions. The EGF-evoked isosmotic ^{86}Rb release, together with the hyposmotic efflux, accounted fully for the observed potentiation by EGF, raising the possibility of an overlapping of these two effects, rather than a true potentiation. A link between EGFR, phosphatidylinositide-3-kinase (PI3K) and hyposmotically induced taurine (but not ^{86}Rb) release is suggested by the increase in PI3K activity elicited by hyposmolarity, which was fully prevented by EGFR inhibition, and by a marked reduction of hyposmotically induced taurine (but not ^{86}Rb) release, by wortmannin. The present findings, together with results showing EGF activation of osmosensitive Cl⁻ fluxes implicate EGFR as an important modulator of osmolyte efflux pathways.

Introduction

Cell exposure to hyposmotic conditions results in cell swelling, followed by an active mechanism of volume recovery, accomplished by the extrusion of intracellular osmotically active solutes and obligated water. K^+ and Cl^-, as well as organic molecules play this role as osmolytes [15]. Osmolytes are translocated through different pathways: K^+ and Cl^- are released by independent ion channels while an anion channel-like molecular identity mediates organic osmolyte efflux [15]. Although the volume-sensitive K^+ and Cl^-/organic osmolyte channels have been studied in detail, many elements in the signalling cascade transducing the volume change into channel activation are as yet unknown, in particular the key element, the volume or osmolarity sensor [11]. In the present study on Swiss 3T3 fibroblasts, we explored the possibility of activation of the epidermal growth factor receptor (EGFR) as an early membrane signal involved in the operation of the corrective osmolyte fluxes. Previous reports have shown potentiation of the volume-sensitive Cl^- currents in mouse mammary C127 cells following EGFR over-expression [1] and of ^{125}I and ^{86}Rb fluxes in the intestinal cell line 407 [25].

There is now much experimental evidence for the role of transmembrane receptors with intrinsic protein tyrosine kinase activity (TKR) in sensing changes in the cell environment. TKR activation, either by specific ligands or by a number of other stimuli, triggers numerous downstream signalling pathways involved in a variety of cell responses [12, 35]. Tyrosine phosphorylation of TKR can be activated in the absence of ligands by a number of external conditions. Ligand-independent TKR activation is elicited by radiation, oxidants, heavy metal ions and alkylating agents [6]. A change in external osmolarity might be sensed by these receptors, as suggested by EGFR activation by hyperosmolarity [4, 22]. The ability of

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EGFR or other TKR to sense decreases in external osmolarity has not been examined. In the present study in Swiss 3T3 fibroblasts, we report the activation of EGFR by hyposmolarity and determined the influence of this activation on the efflux of taurine and K^+ efflux (traced by ^{86}Rb) in hyposmotic and isosmotic conditions. The activation state of EGFR influences taurine efflux in hyposmotic, but not in isosmotic conditions, while the opposite was the case for K^+. We selected EGFR as a representative TKR since it is one of the most widely distributed among these receptors and is expressed in most cells. EGFR activation mechanisms are well known, and pharmacological tools are available to manipulate its activity.

Materials and methods

Reagents

Tyrphostins (AG18, AG112, AG213 and AG1478), lavendustin A, herbimycin A, concanavalin A (Con A), wheat germ agglutinin (WGA) and EGF were from Calbiochem-Novabiochem (San Diego, Calif., USA). [^3H]-taurine and ^{86}Rb were from New England Nuclear (Boston, Mass., USA). All salts for solution preparation were from Merck (Darmstadt, Germany). The antibodies used, anti-phospho-EGF (Tyr 845) anti EGF, anti-phospho-AKT, anti-AKT and cell lysis buffer were from Cell Signaling Technology (Beverly, Mass., USA). Anti-phosphotyrosine (Py20) and secondary antibody goat anti-rabbit IgG tagged with fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRP) conjugated were from Zymed (San Francisco, Calif., USA). Fluorescent mounting medium was from Dako Corporation (Carpinteria Calif., USA). Complete proteinase inhibitor cocktail tablets were obtained from Roche Diagnostics (Mannheim, Germany). The enhanced chemiluminescence (ECL) Western detection system was from Amersham Pharmacia Biotech (Bucks., England). Wortmannin, formaldehyde, protein A-agarose and Ponceau S and other reagents were from Sigma (St. Louis, Mo., USA).

Cell culture

Stock cultures of Swiss 3T3 fibroblasts were maintained in Eagle basal medium (Gibco), supplemented with 10% fetal bovine serum, in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. For experimental purposes, Swiss 3T3 fibroblasts were plated in 100-mm dishes at a density of 1×10^6 cells/dish for Western blot immunoassays, in 35-mm dishes at 2.5×10^4 cells/dish for radiolabelled taurine fluxes and on rounded cover-slips at 1×10^4 cells/dish for immunofluorescence preparations. Cells were cultured in Eagle basal medium containing 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin, and used after 2–3 days, when the cells were confluent or subconfluent (immunofluorescence) and after 24 h serum starvation. The culture dishes were incubated at 37°C in an humidified 5% CO_2/95% air atmosphere.

Solutions and drugs

Isosmotic medium contained (in mM): 135 NaCl, 5 KCl, 1.17 $MgSO_4$, 1 $CaCl_2$, 10 glucose and 10 HEPES, pH 7.4, with an osmolarity of 300 mOsm. Hyposmotic solutions (35% reduction, H-35%: 195 mOsm) were prepared by reducing the concentration of NaCl. Medium osmolarities were verified in a freezing-point osmometer (Osmette A, Precision Systems, Natick, Mass., USA). In all experiments, EGF (200 ng/ml) was present only in the hyposmotic medium. For drug treatment, preincubation time is indicated in each figure. Agents were present throughout the experiment. Control cells were always treated in parallel with the vehicle used to prepare solutions containing the tested drugs (DMSO 0.1%, acetic acid 10 µM plus 0.1% BSA for EGF).

^3H-Taurine and ^{86}Rb release experiments

Swiss 3T3 fibroblasts were preloaded with ^3H-taurine (0.5 µCi/ml) or ^{86}Rb (0.25 µCi/ml) for 1 h in isosmotic medium. After incubation, cells were washed for 13 min with isosmotic medium and superfused at 1 ml/min for 5 min, after which a stable efflux baseline was attained. Then, the isosmotic medium was replaced by the 35% hyposmotic medium and samples collected for a further 8 min. At the end of the experiments, cells were lysed with 0.4 N NaOH and the radioactivity remaining in the cells and in the collected samples determined in a liquid scintillation counter. The radioactivity released per minute was expressed as a percentage of the total incorporated during loading.

Immunofluorescence and confocal microscopy

Cells were grown on glass cover-slips in complete medium, rinsed in PBS, and then fixed with 3.7% formaldehyde at room temperature for 20 min. Cells were permeabilized by 3 min treatment with 0.5% Triton X-100 and then blocked with 0.5% BSA in PBS for 30 min. After rinsing with PBS, cells were incubated overnight with the first antibody diluted 1:25 in PBS/BSA. After incubation, cells were rinsed with PBS and the secondary antibody goat anti-rabbit IgG tagged with FITC was added for 60 min at room temperature (1:50 dilution with PBS/BSA). After rinsing with PBS, the cover-slips were mounted on glass slides containing 50 µl of DAKO fluorescent mounting medium for observation in a confocal laser microscope (BioRad, Hercules, Calif., USA). Optical sections were collected at 0.5-µm intervals. The fluorescence intensity was analysed using the program Laser Sharp (BioRad). Five fields, containing 10–15 cells each, were analysed for at least three independent experiments. Laser output power and photomultiplier settings were kept at similar levels throughout all experiments.

Immunoprecipitation and Western blot analysis

Cells grown on Petri dishes were submitted to the experimental conditions, then lysed with ice-cold cell lysis buffer containing protease inhibitors and detached gently with a rubber policeman. The whole lysate was sonicated (three 30-s cycles) and centrifuged (10,000 rpm, 10 min, 4°C). Protein concentration in the supernatant was determined using the Bradford assay. Immunoprecipitation was carried out by overnight incubation (4°C) of lysates (0.5–1 mg protein) with the appropriate antibodies (1:50 dilution) plus protein A-agarose beads (1.2 mg). Beads were washed 3 times with lysis buffer and boiled in SDS/β-mercaptoethanol buffer. Immunoprecipitates were fractionated by 12% SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes. Protein loading after the immunoprecipitation was checked routinely by staining membranes with Ponceau S. Blots were blocked with 5% skim milk in TRIS-buffered saline (TBS) and then probed with the indicated antibodies (1:500 dilution) overnight at 4°C. Blots were washed 4 times (5 min each) with TBS and incubated for 1 h in a 1:2,000 dilution of peroxidase-conjugated anti-IgG, washed with TBS and developed with ECL reagent. The immunoblot reactive bands were quantified by fluorography scanning the blots from at least five independent experiments of the same type. Integration of the areas under the curves was performed by the gel analysis software (Sigma gel v. 1.0).

Results

### Hyposmolarity and agglutinins activate EGFR in fibroblasts

The effect of hyposmolarity on EGFR phosphorylation was assessed by confocal immunofluorescence and immunobloting. Phosphorylation by the ligand EGF (200 ng/ml) and by hyposmolarity was examined comparatively. After 3 min exposure to the 35% hyposmotic stimulus (H-35%), marked activation of EGFR was observed, which was significantly higher than the activation by EGF in isosmotic medium (Fig. 1A, B). The hyposmolarity-evoked activation of EFGR was potentiated by EGF, but the effects were not additive. Similar results were observed in immunoblots, although the magnitude of the differences observed was, in general, lower than that found by immunofluorescence (Fig. 2A, B). The increased EGFR activity induced by hyposmolarity was reduced in the presence of AG213, a tyrphostin with a fairly specific action blocking EGFR phosphorylation (Figs. 1, 2).

Several lectins are known to mimic typical signal transduction induced by EGF, including the EGFR tyrosine kinase activity. In particular Con A and WGA induce receptor dimerization and subsequent phosphorylation [34]. Such EGFR phosphorylation under isosmotic conditions was found in Swiss 3T3 fibroblasts treated with these lectins, as shown in Fig. 3. Both agents were more potent than either the ligand EGF or hyposmolarity, increasing the basal phosphorylation three- to fivefold (Fig. 3A, B).

Hyposmolarity-activated taurine release from Swiss 3T3 fibroblasts is sensitive to tyrosine kinase blockers

Tyrphostins are a group of synthetic blockers used widely to reduce tyrosine kinase phosphorylation of TKR. This reaction is particularly sensitive to tyrphostins, while it seems less affected by other blockers such as herbimycin and lavendustin [16]. The effect of tyrphostins was then examined on hyposmotic taurine release. Figure 4 shows the time course of ^{3}H-taurine release from cells exposed to a 35% hyposmotic solution. Shortly after the stimulus, taurine efflux increased from 0.3% to 4.6% (of total radioactivity accumulated), was maximal after 3 min and declined slowly thereafter towards basal levels (Fig. 4A). At the end of the experiment, 8 min after the stimulus, the amount of ^{3}H-taurine released corresponded to 24% of the total label accumulated (Fig. 4B). The general tyrosine kinase blocker tyrphostin AG18 exhibited a strong inhibitory action on the hyposmotic taurine release. The blocker reduced taurine efflux of the peak release fractions
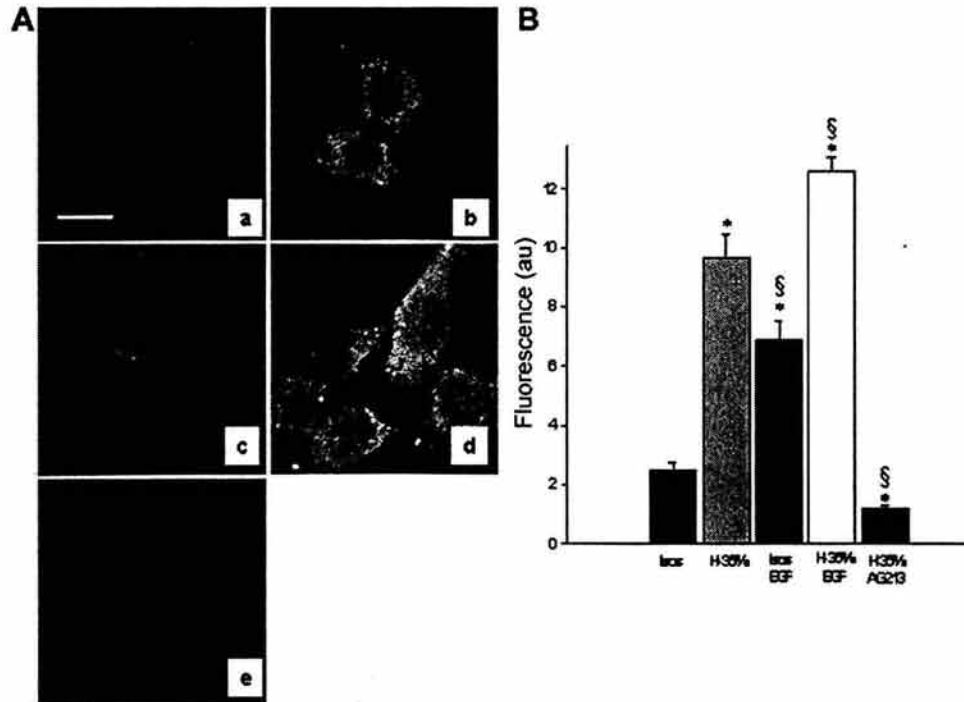
Fig. 1A, B Hyposmolarity-induced epidermal growth factor receptor (EGFR) phosphorylation in Swiss 3T3 fibroblast cell line cultures. Serum-starved cells (24 h) were exposed for 3 min to the conditions indicated, fixed, incubated overnight with phospho-EGFR (Tyr 845) antibody, incubated with a secondary fluorescent antibody and visualized by confocal microscopy in sections collected at 0.5-μm intervals. The images shown are from the second section in the series. **A** Isosmotic medium (*a*); 35% hyposmotic (H35%) medium (*b*); isosmotic medium plus EGF (200 ng/ml) (*c*); 35% hyposmotic medium plus EGF (*d*) and 35% hyposmotic medium plus 50 μM AG213 (*e*). *Bar* 10 μm. **B** Quantitative summary of fluorescence intensity. Means±SE from analysis of five fields, containing 10–15 cells each, from at least three independent experiments. *$P<0.001$ vs. isosmotic; $^§P<0.001$ vs. H35%
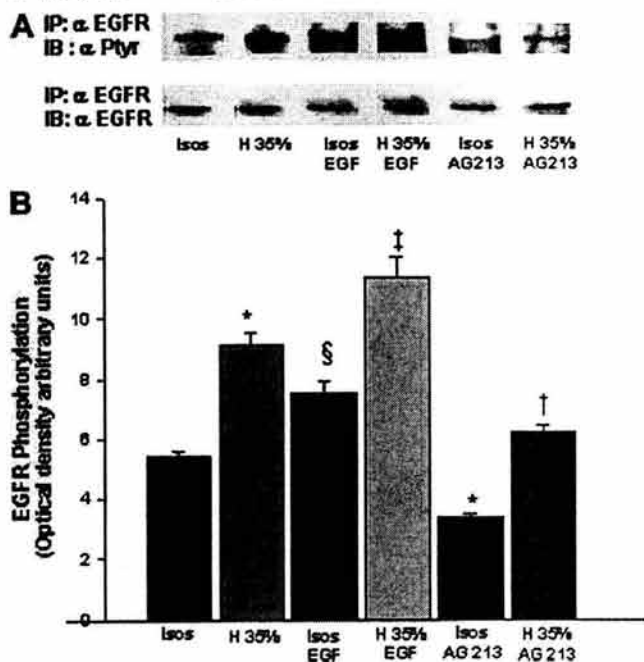
Fig. 2A, B Hyposmolarity-induced EGFR phosphorylation assayed by immunoblotting. Experimental conditions as in Fig. 1 and indicated in the corresponding lanes. **A** *Upper panel*: after treatment, cell lysates were immunoprecipitated overnight with anti-EGFR antibody (*IP*) and analysed by Western blotting with anti-phosphotyrosine PY20 (*IB*). *Lower panel*: blots reprobed with EGFR antibody after stripping to detect protein levels. **B** Quantitative summary of results in **A** by densitometric scanning, with *bars* representing the percentage increase of EGFR phosphorylation over that in isosmotic medium under the indicated conditions. Means ±SE, n=4–6 experiments. *P<0.001 vs. isosmotic; §P<0.002, ‡P<0.001; †P<0.01 vs. H35%

(fractions 2–5 after the stimulus) by about 63%, and the total release decreased by more than 50%. Genistein had a marginal effect reducing taurine release by only 9%. Herbimycin and lavendustin did not affect this release (Fig. 4B).

Hyposmotic taurine release is modulated by EGFR activity

AG18 is a general blocker of tyrosine kinase reactions, while other tyrphostins, such as AG213 and AG112, are more specifically directed to block EGFR phosphorylation. Figure 5A shows the effect of these agents: reduction of peak hyposmotic taurine release by 44% and 25% (fractions 2–5 after the stimulus) and total release by 30% and 25% respectively. Another EGFR blocker, AG1478 reduced taurine release similarly (not shown). These results implicate EGFR activation in the transduction signalling for hyposmotic taurine efflux. Moreover, activation of EGFR by the ligand EGF (200 ng/ml) potentiated taurine efflux. Figure 5B shows that hyposmotic taurine efflux is a saturable process following sigmoidal function. The increase of taurine efflux by EGF shifts this function leftwards, indicative of an increase in sensitivity to hyposmolarity (Fig. 5A, B). Under isosmotic conditions, EGF elicited only a small increase in taurine efflux (Fig. 5B, inset).

Fig. 3A, B EGFR phosphorylation by lectins concanavalin A (*Con A*) or wheat-germ agglutinin (*WGA*). **A** Cells were incubated for 10 min in isosmotic medium (*a*) or in medium containing 500 ng/ml Con A (*d*) or WGA (*e*). Images of cells exposed to H35% medium (*b*) or isosmotic medium plus EGF (*c*) are included for comparison. Preparation of samples for immunofluorescence visualization and quantification were as in Fig. 1. **B** Quantitative summary of results from **A** with *bars* representing fluorescence arbitrary units. *P<0.001 vs. isosmotic; §P<0.001 vs. H35% and isosmotic plus EGF
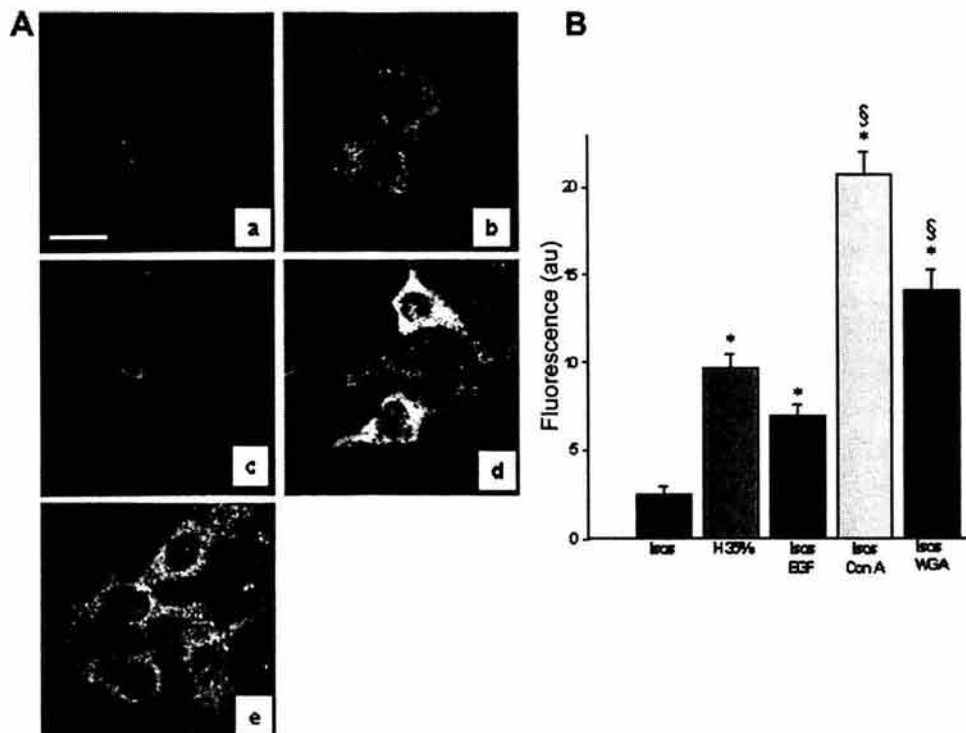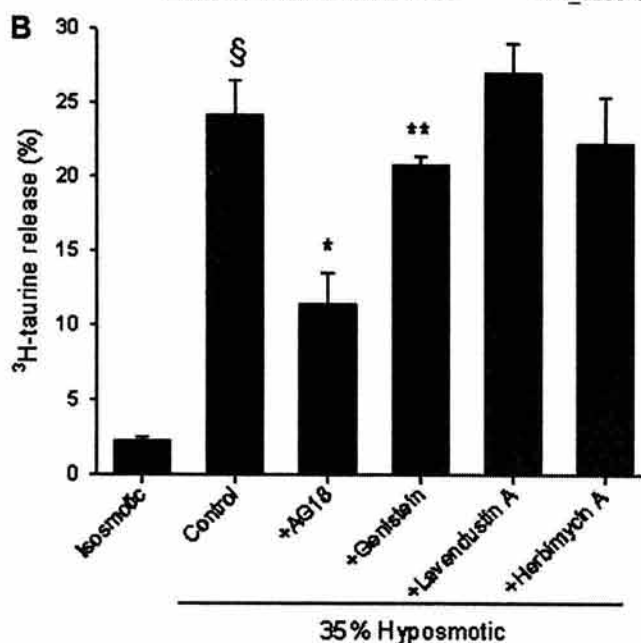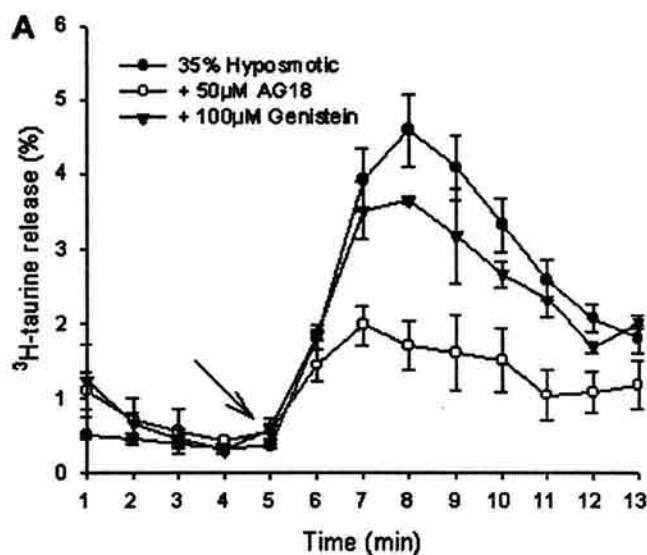
Fig. 4A, B Effect of general tyrosine kinase inhibitors on hyposmolarity-induced taurine release from Swiss 3T3 fibroblasts. A Cells preloaded with ^{3}H-taurine were superfused with isosmotic medium for 5 min and then (*arrow*) for 8 min with 35% hyposmotic medium (•) or hyposmotic medium plus 50 µM tyrphostin AG18 (○) or 100 µM genistein (▼). Cells were preincubated for 30 min with AG18 or 1 h with genistein before the experiment. The *points* represent the radioactivity released at each time, expressed as a percentage of the total radioactivity accumulated during loading. B Summary of the effect of AG18, genistein, lavendustin or herbimycin on ^{3}H-taurine released during 8 min superfusion with 35% hyposmotic medium. Tyrphostin AG18 and genistein conditions were as in A. Cells exposed to lavendustin A (10 µM) or herbimycin A (1 µM) were preincubated with these substances for 1 h. The blockers were present in all solutions throughout the experiment. Means±SE; $n=6$–8; $^§P<0.001$ vs. isosmotic; $*P<0.005$, $**P<0.01$ vs. 35% hyposmotic
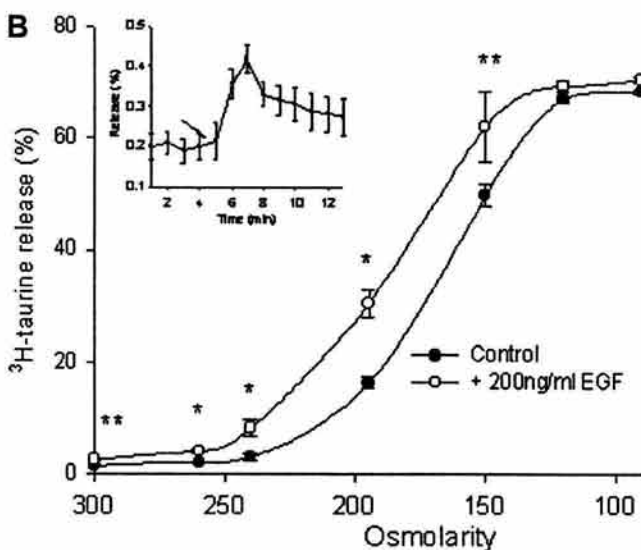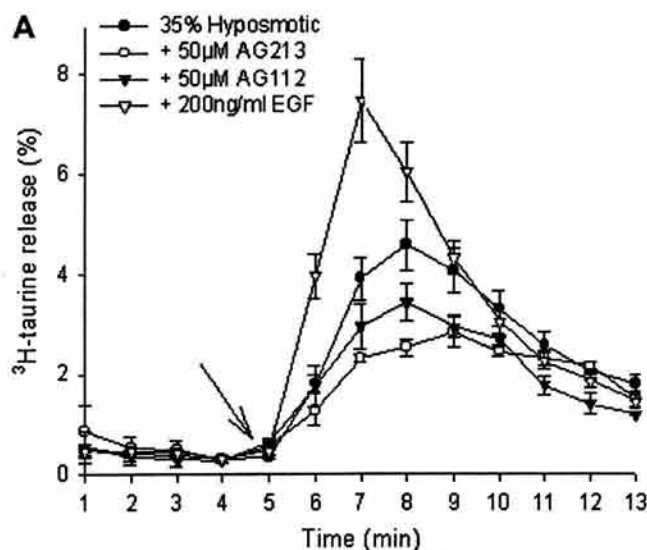
Fig. 5A, B Effect of activation or inhibition of EGFR on hyposmolarity-induced taurine release from Swiss 3T3 cultures. A Assay as in Fig. 4A. Control, 35% hyposmotic medium (•); hyposmotic medium plus 50 µM AG213 (○) or AG112 (▼) or EGF (200 ng/ml) (▽). Cells were preincubated with the blockers for 30 min; the latter were then present in all solutions throughout the experiment. Results are expressed as in Fig. 4A and are means±SE ($n=6$–8). B Stimulatory effect of EGF on ^{3}H-taurine efflux in response to hyposmolarity (270—90 mOsm), or in isosmotic medium (*inset*). The points represent taurine released in fractions 2–5 after the hyposmotic stimulus, with (○) or without (•) the addition of 200 ng/ml EGF. *Inset*: the effect of EGF on ^{3}H-taurine release in isosmotic medium. Means±SE; $n=6$–8; $*P<0.005$, $**P<0.01$ vs. medium without EGF

102

Hyposmotic taurine release is potentiated by lectins

Con A and the WGA (500 ng/ml) enhanced hyposmotic taurine efflux with a much higher potency than EGF. Con A and WGA increased taurine release by 146% and 138% (Fig. 6A, B). Con A (WGA was not tested) elicited a marginal increase in taurine efflux under isosmotic conditions (Fig. 6C). The taurine efflux potentiation by Con A was markedly reduced by AG213, stressing further the influence of EGFR in Con A-induced potentiation of taurine efflux under hyposmotic conditions (Fig. 6A).

EGFR activation increases isosmotic ^{86}Rb efflux but has no influence on the hyposmotic ^{86}Rb release

Reduction in osmolarity (35%) increased ^{86}Rb release over the basal efflux, with a maximum 2 min after the stimulus (Fig. 7A). Addition of EGF to the hyposmotic medium accelerated the activation of ^{86}Rb efflux and markedly increased the amount released in all fractions (Fig. 7A). This suggests a modulation of ^{86}Rb efflux by EGFR, as observed for the taurine efflux pathway. However, at variance with the effect on taurine, hyposmotic ^{86}Rb release was not reduced by the EGF blockers AG213 and AG1478 (Fig. 7B). Also, in contrast to the modest increase of taurine efflux by EGF under isosmotic medium, ^{86}Rb efflux was increased markedly (Fig. 7C). The insensitivity of hyposmotic ^{86}Rb release to EGFR blockers together with its marked increase under isosmotic

conditions raised the question of whether the observed EGF potentiation of hyposmotic ^{86}Rb release results from the overlapping of the EGF-activated isosmotic ^{86}Rb efflux. That this seems to be the case is suggested by Fig. 7D, which shows that the EGF-stimulated ^{86}Rb efflux in isosmotic medium (a) plus that elicited by hyposmolarity (b), account fully for the EGF potentiation (a+b). Effects similar to those evoked by EGF were found with Con A, i.e. a marked potentiation of hyposmotic ^{86}Rb release (higher than that by EGF) and a strong increase in isosmotic release (Fig. 8A, B).

EGF potentiation of hyposmotic taurine efflux but not of ^{86}Rb efflux involves PI3K activation

Activation of PI3K (particularly class-1 PI3K) via TKR is well documented [2, 10]. Moreover, hyposmolarity activates PI3K, and this kinase seems to play an important role in hyposmotic taurine efflux in a variety of cell types, as shown by its marked reduction by wortmannin [9,19]. The involvement of PI3K in the EGF-mediated taurine efflux potentiation is suggested by the results illustrated in Fig. 9. First, the hyposmolarity-evoked PI3K activation was prevented by AG213 (Fig. 9A) and, second, wortmannin reduced both hyposmotic taurine release and its potentiation by EGF (Fig. 9B, C). Wortmannin did not influence ^{86}Rb release (results not shown).

Fig. 6A–C Potentiation of the hyposmolarity-activated ^{3}H-taurine efflux by Con A and WGA. **A** Assay was as in Fig. 4A. *Symbols*: 35% hyposmotic medium (●); hyposmotic medium plus Con A (○) or WGA (▼) (500 ng/ml); Con A plus 50 μM AG213 (▽). The agglutinins were added to the hyposmotic medium. **B** Effect of Con A or WGA on ^{3}H-taurine released in fractions 2–5 after the stimulus. Means±SE, $n=6-8$; *$P<0.005$ vs. 35% hyposmotic. **C** Effect of Con A (*arrow*) on ^{3}H-taurine efflux in isosmotic conditions. Results in A and C are expressed as in Fig. 4A and are means±SE, $n=4$
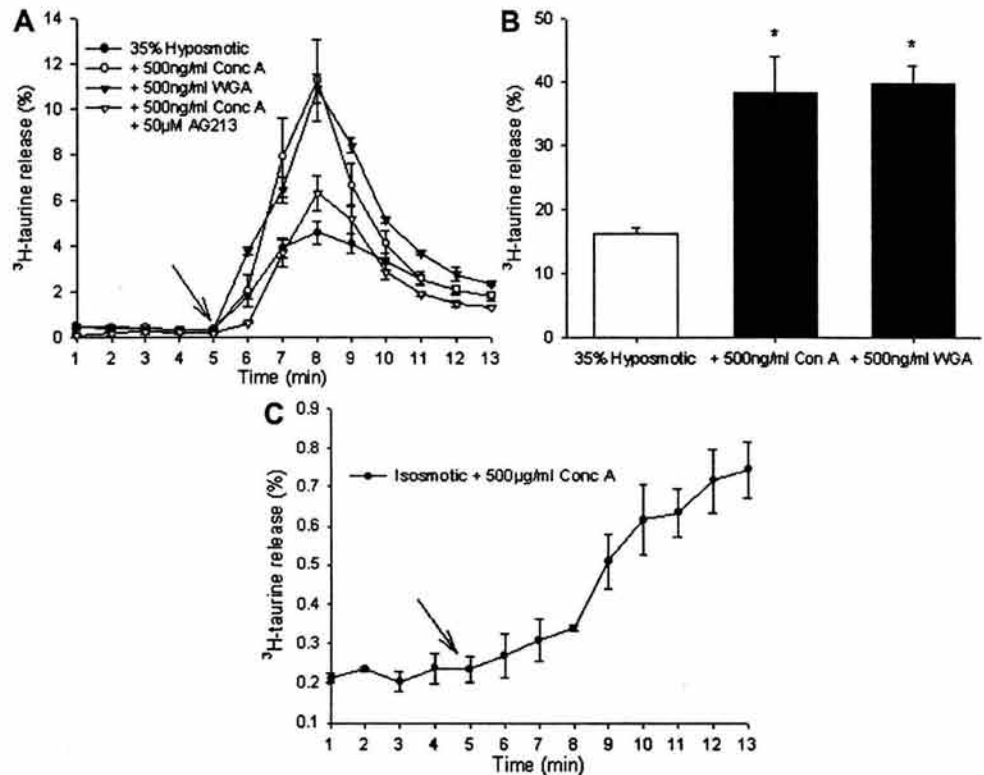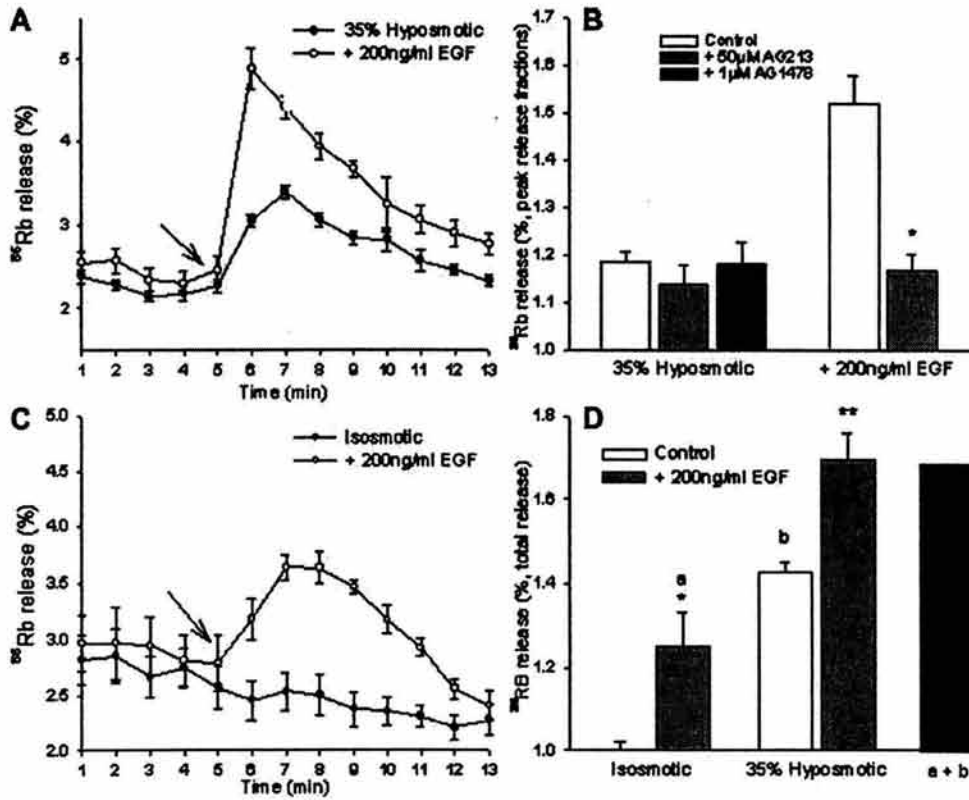
Fig. 7A–D Effects of EGF and EGFR blockers ^{86}Rb release from Swiss 3T3 fibroblasts under isosmotic or hyposmotic conditions. Cells were preloaded with ^{86}Rb and superfused with isosmotic or 35% hyposmotic medium as in Fig. 4A. **A** ^{86}Rb release in 35% hyposmotic medium (●) and in hyposmotic medium containing EGF (200 ng/ml) (○). **B** Effect of the EGFR blockers AG213 (50 µM) or AG1478 (1 µM) on ^{86}Rb release in 35% hyposmotic medium. *Bars* represent the release into fractions 2–5 in hyposmotic medium minus the corresponding fractions in isosmotic medium (normalized to 1.0) under the indicated conditions. **C** ^{86}Rb release in isosmotic medium (●) or in isosmotic medium plus EGF (○). **D** The *bars* represent the ^{86}Rb release during 8 min (total release) in isosmotic medium (normalized to 1.0) or in hyposmotic medium. The effect of addition of EGF in the two conditions is shown in the *shaded bars*. The *black bar* represents the release in *a* (hyposmotic) plus *b* (isosmotic plus EGF). Means±SE, n=6–8; *P<0.005 vs. absence of EGF (isosmotic); **P<0.005 vs. absence of EGF (hyposmotic)

Fig. 8A, B Effect of Con A and WGA on Rb^{86} efflux in cultured Swiss 3T3 fibroblasts. **A** Cells were preloaded with ^{86}Rb and superfused as in Fig. 4A, with isosmotic and 35% hyposmotic medium (arrow) (●) or with 35% hyposmotic medium with Con A (○). **B** ^{86}Rb release in isosmotic medium (●) or in isosmotic medium plus Con A (*arrow*) (○). Means±SE, n=6–8

A

B

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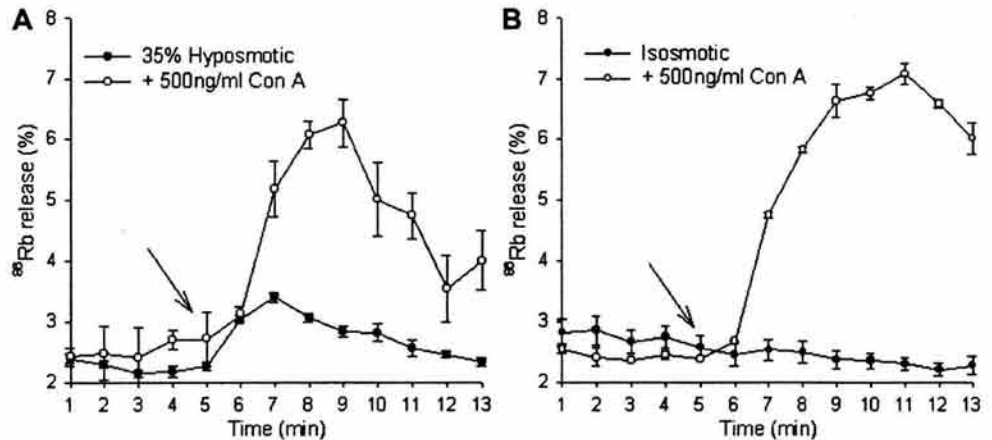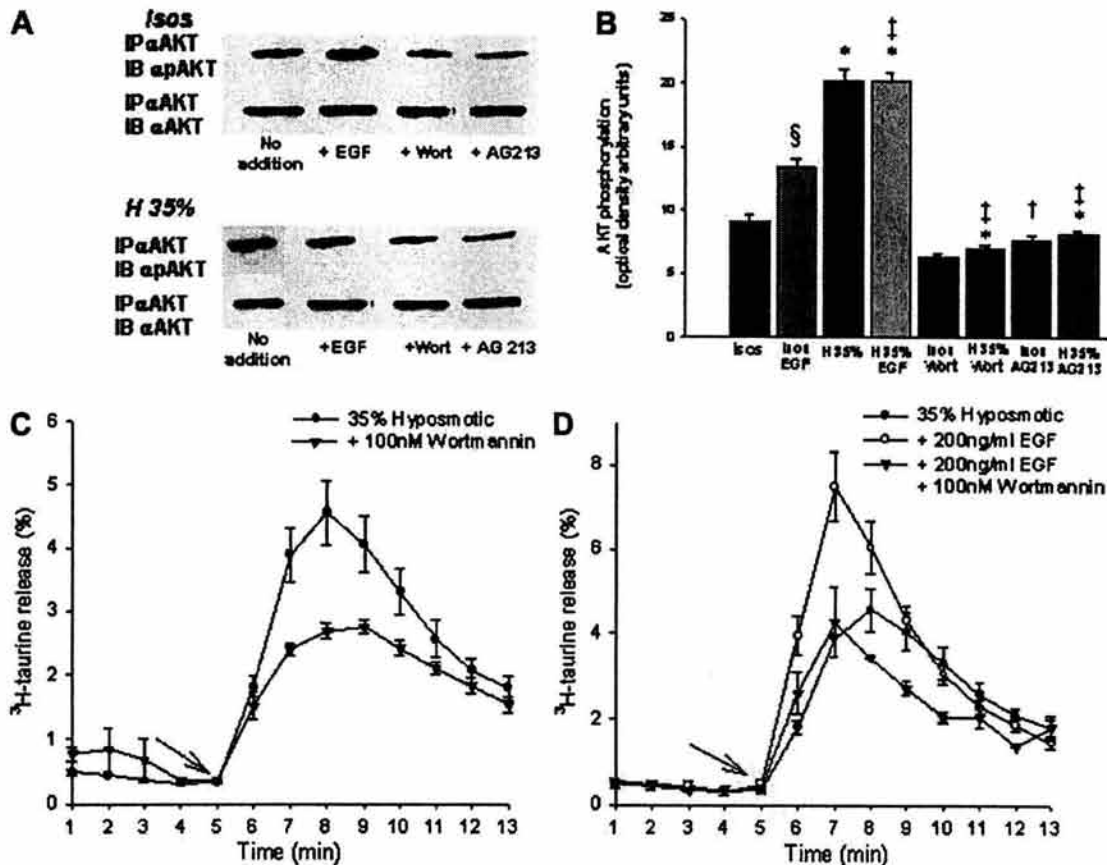
Fig. 9A–D Phosphatidylinositide-3-kinase (PI3K) activation by hyposmolarity and the effect of EGFR blockade. **A** Activation of PI3K by EGF in isosmotic medium and the effect of wortmannin and AG213. *Upper panel*: cell lysates prepared after treatment were immunoprecipitated overnight with anti-AKT antibody (*IP*) and analysed by Western blotting with anti-pAKT (*IB*). Blots were reprobed with AKT antibody after stripping to detect protein levels. *Lower panel*: PI3K activity in hyposmotic (H35%) conditions, and effects of EGF, wortmannin and AG213. **B** Quantitative summary of results in A, with *bars* representing the percentage increase of AKT phosphorylation by hyposmotic medium over that in isosmotic medium, under the indicated conditions. Means±SE, *n*=4–6 experiments. *$P<0.001$, §$P<0.005$, †$P>0.05$ vs. isosmotic; ‡$P<0.001$ vs. 35% hyposmotic. **C, D** Effect of wortmannin (▼) on the hyposmotic (●) and the EGF-potentiated efflux of ³H-taurine (○). The superfusion, release and results expression as in Fig. 4A. Cells exposed to wortmannin (100 nM) were preincubated with it for 1 h and wortmannin was present in all solutions throughout the experiment. Means±SE, *n*=6–8

Discussion

The present study in Swiss 3T3 fibroblasts showed the activation of EGFR by hyposmolarity, a finding that to our knowledge has not been reported so far. EGFR activation by hyposmolarity was higher than that elicited by a saturating EGF concentration and the hyposmotic effect was potentiated by the ligand. These results suggest either an increase in exposed EGFR in the hyposmotic condition, or a delay in the internalization process occurring subsequent to phosphorylation [32]. Another important finding of our study is the implication of EGFR as an element in the transduction signalling for the pathway operation releasing taurine after the hyposmotic stimulus. This is supported by the decrease of hyposmotic taurine efflux by EGFR blockade and by its potentiation by EGFR activation in the presence of EGF or agglutinins. The pattern of EGF influence on the seemingly saturable process of hyposmotic taurine release is suggestive of an increase by EGF of the sensitivity of taurine efflux to

hyposmolarity. Previous studies in intestinal cell lines 407 and C127 have shown potentiation by EGF of the hyposmotic efflux of Cl⁻ (traced by ¹²⁵I) and modulation of the osmosensitive Cl⁻ current by EGFR over-expression [1, 25]. A common hyposmotic release pathway for Cl⁻ and organic osmolyte has been proposed on the basis of their similar pharmacological profiles [13], and this pathway may be that modulated by EGFR activation. Similar to the effect of EGF on Cl⁻ efflux in intestinal 407 cells [25], our results showed that EGFR activation by EGF or Con A elicited only a small increase in taurine efflux under isosmotic conditions. These results suggest that although EGFR activation may be able to initiate taurine efflux by itself, other elements may be required concurrently with hyposmolarity to activate the taurine efflux pathway fully. A decrease in ionic strength may be one such element, as suggested by studies on taurine efflux in astrocytes and on the volume-sensitive Cl⁻ current in endothelial cells [3, 7, 29]. A study on cultured astrocytes [17] has shown, at variance with our results, a

decrease in hyposmotic taurine release by pretreatment of cells with EGF. The discrepancy with the present study may be due either to the longer exposure of astrocytes to EGF, which may lead to EGFR internalization [32], or to the cell culture conditions, particularly the presence or absence of fetal serum in the cultures.

The connecting steps linking EGFR and the taurine/Cl⁻ pathway are unknown. Activation of EGFR triggers multiple and diverse downstream signalling pathways involving a variety of elements, notably several tyrosine kinases and enzymes related to phospholipid metabolism [10, 20]. Among these later, PI3K may have an important role linking EGFR activation and hyposmotic taurine release. A well-known EGFR downstream effect is the recruitment and activation of PI3K (class 1), either directly or via ras- or src-mediated pathways [12]. The involvement of PI3K in hyposmotic taurine release has been shown by studies showing an increase in PI3K activity by hyposmolarity, and a potent effect of wortmannin reducing the hyposmolarity-elicited efflux of taurine [9, 19]. Wortmannin also decreases the efficacy of cell volume regulation [8, 24]. In support of an EGFR-PI3K-taurine release connection are our present results, showing prevention of the hyposmolarity-evoked increase in PI3K activity by blockade of EGFR phosphorylation, and the reduction by wortmannin of both hyposmotic taurine release and its potentiation by EGF. Wortmannin inhibition of about 50% was found for both taurine release and EGF potentiation, suggesting the involvement of additional PI3K-independent mechanisms, under the two conditions. The influence on taurine release of other elements in the downstream signalling of activated EGFR, such as the MAP kinases ERK1/ERK2 and p38 remains to be examined in fibroblasts. A link between ERK1/ERK2 and taurine/Cl osmosensitive translocation has been found in some cells [5, 23] but not in others [9, 19, 28].

EGFR phosphorylation also enhanced ⁸⁶Rb release in hyposmotic conditions but, in contrast to taurine, activation of EGFR either by the ligand EGF or by Con A resulted in a large increase in basal ⁸⁶Rb efflux. This raised the question of whether the observed EGF potentiation of ⁸⁶Rb in hyposmotic conditions, results from the overlapping of that enhanced in isosmotic medium, which may occur through a different pathway. This possibility is suggested by the insensitivity of the hyposmotic release to EGFR blockers, as well as by the fact that the EGF- and Con A-activated ⁸⁶Rb efflux in isosmotic medium plus the hyposmotic release fully account for the EGF/Con A potentiation. Growth factors have variety of effects on K⁺ channels, ranging from increasing expression in chronic experiments or decreasing the K⁺ currents in acute experiments. Most evidence relates to Ca²⁺-activated K⁺ channels, which are, in general, sensitive to growth factors [18, 21, 33]. The voltage-dependent channels are affected differently, being either activated or inhibited by growth factors depending on the subtype of channel. The ⁸⁶Rb efflux under isosmotic conditions enhanced by EGF may occur through one such channel, which remains to be identified. In any event, and whatever the mechanism

involved, EGFR activation by hyposmolarity contributes to K⁺ extrusion, and together with its effects enhancing taurine and Cl⁻ efflux, makes it an important modulator of the efficacy of cell volume regulation.

The present study focussed on EGFR, and our conclusions are based on the effect of agents believed to act as fairly specific blockers of EGFR (16). However, we cannot exclude the possibility of effects of these agents on other TKR that may also be sensitive to hyposmolarity, and exert similar effects on osmolyte translocation. In fact, this is suggested by the more potent effect stimulation of hyposmotic taurine release by Con A and WGA, which, besides EGFR, may also activate other TKR. Also, the more potent inhibition of taurine efflux by AG18, a general blocker of tyrosine kinases, than the EGFR blockers may support this notion. Further studies on the effect of hyposmolarity on different TKR and on their influence on the osmolyte efflux pathways are necessary to elucidate this question, and to establish a more general involvement of such receptors as early signals in the osmotransduction chain. The mechanisms or factors leading to EGFR activation by hyposmolarity are so far unknown. Factors that may be part of this sensing mechanism may include integrins and other adhesion molecules such as p125FAK, stress-sensitive proteins such as p38, or reactive oxygen species-generating systems, all of them known to be activated by hyposmotic swelling [14, 19, 26, 27, 30, 31].

References

1. Abdullaev IF, Sabirov RZ, Okada Y (2003) Upregulation of swelling-activated Cl⁻ channel sensitivity to cell volume by activation of EGF receptors in murine mammary cells. J Physiol (Lond) 549:749–758
2. Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296:1655–1657
3. Cardin V, Pena-Segura C, Pasantes-Morales H (1999) Activation and inactivation of taurine efflux in hyposmotic and isosmotic swelling in cortical astrocytes: role of ionic strength and cell volume decrease. J Neurosci Res 56:659–667
4. Cheng H, Kartenbeck J, Kabsch K, Mao X, Marques M, Alonso A (2002) Stress kinase p38 mediates EGFR transactivation by hyperosmolar concentrations of sorbitol. J Cell Physiol 192:234–243
5. Crepel V, Panenka W, Kelly ME, MacVicar BA (1998) Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. J Neurosci 18:1196–1206
6. Dent P, Yacoub A, Contessa J, Caron R, Amorino G, Valerie K, Hagan MP, Grant S, Schmidt-Ullrich R (2003) Stress and radiation-induced activation of multiple intracellular signaling pathways. Radiat Res 159:283–300
7. Emma F, McManus M, Strange K (1997) Intracellular electrolytes regulate the volume set point of the organic osmolyte/anion channel VSOAC. Am J Physiol 272:C1766–C1775

8. Feranchak AP, Roman RM, Schwiebert EM, Fitz JG (1998) Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. J Biol Chem 273:14906–14911

9. Franco R, Torres-Marquez ME, Pasantes-Morales H (2001) Evidence for two mechanisms of amino acid osmolyte release from hippocampal slices. Pflugers Arch 442:791–800

10. Grant S, Qiao L, Dent P (2002) Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. Front Biosci 7:d376–d389

11. Jakab M, Furst J, Gschwentner M, Botta G, Garavaglia ML, Bazzini C, Rodighiero S, Meyer G, Eichmueller S, Woll E, Chwatal S, Ritter M, Paulmichl M (2002) Mechanisms sensing and modulating signals arising from cell swelling. Cell Physiol Biochem 12:235–258

12. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW (2003) Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 284:31–53

13. Kirk K, Strange K (1998) Functional properties and physiological roles of organic solute channels. Annu Rev Physiol 60:719–739

14. Lambert IH (2003) Reactive oxygen species regulate swelling-induced taurine efflux in NIH3T3 mouse fibroblasts. J Membr Biol 192:19–32

15. Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev 78:247–306

16. Levitzki A, Gazit A (1995) Tyrosine kinase inhibition: an approach to drug development. Science 267:1782–1788

17. Mongin AA, Reddi JM, Charniga C, Kimelberg HK (1999) [^{3}H]taurine and D-[^{3}H]aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. Am J Physiol 276: C1226–C1230

18. Neylon CB (2002) Potassium channels and vascular proliferation. Vascul Pharmacol 38:35–41

19. Ochoa De La Paz LD, Lezama R, Torres-Marquez ME, Pasantes-Morales H (2002) Tyrosine kinases and amino acid efflux under hyposmotic and ischaemic conditions in the chicken retina. Pflugers Arch 445:87–96

20. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A (2001) The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer 8:11–31

21. Rane SG (1999) Ion channels as physiological effectors for growth factor receptor and Ras/ERK signaling pathways. Adv Second Messenger Phosphoprotein Res 33:107–127

22. Rosette C, Karin M (1996) Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. Science 274:1194–1197

23. Shen MR, Chou CY, Browning JA, Wilkins RJ, Ellory JC (2001) Human cervical cancer cells use Ca^{2+} signalling, protein tyrosine phosphorylation and MAP kinase in regulatory volume decrease. J Physiol (Lond) 537:347–362

24. Shi C, Barnes S, Coca-Prados M, Kelly ME (2002) Protein tyrosine kinase and protein phosphatase signaling pathways regulate volume-sensitive chloride currents in a nonpigmented ciliary epithelial cell line. Invest Ophthalmol Vis Sci 43:1525–1532

25. Tilly BC, Van den Berghe N, Tertoolen LG, Edixhoven MJ, De Jonge HR (1993) Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J Biol Chem 268:19919–19922

26. Tilly BC, Edixhoven MJ, Tertoolen LG, Morii N, Saitoh Y, Narumiya S, De Jonge HR (1996) Activation of the osmo-sensitive chloride conductance involves P21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. Mol Biol Cell 7:1419–1427

27. Tilly BC, Gaestel M, Engel K, Edixhoven MJ, de Jonge HR (1996) Hypo-osmotic cell swelling activates the p38 MAP kinase signalling cascade. FEBS Lett 395:133–136

28. Van der Wijk T, De Jonge HR, Tilly BC (1999) Osmotic cell swelling-induced ATP release mediates the activation of extracellular signal-regulated protein kinase (Erk)-1/2 but not the activation of osmo-sensitive anion channels. Biochem J 343:579–586

29. Voets T, Droogmans G, Raskin G, Eggermont J, Nilius B (1999) Reduced intracellular ionic strength as the initial trigger for activation of endothelial volume-regulated anion channels. Proc Natl Acad Sci USA 96:5298–5303

30. Vom Dahl S, Schliess F, Graf D, Haussinger D (2001) Role of p38(MAPK) in cell volume regulation of perfused rat liver. Cell Physiol Biochem 11:285–294

31. Vom Dahl S, Schliess F, Reissmann R, Gorg B, Weiergraber O, Kacalkova M, Dombrowski F, Haussinger D (2003) Involvement of integrins in osmosensing and signalling towards autophagic proteolysis in rat liver. J Biol Chem 278:27088–27095

32. Wiley HS (2003) Trafficking of the ErbB receptors and its influence on signaling. Exp Cell Res 284:78–88

33. Wonderlin WF, Strobl JS (1996) Potassium channels, proliferation and G1 progression. J Membr Biol 154:91–107

34. Zeng FY, Benguria A, Kafert S, Andre S, Gabius HJ, Villalobo A (1995) Differential response of the epidermal growth factor receptor tyrosine kinase activity to several plant and mammalian lectins. Mol Cell Biochem 142:117–124

35. Zwick E, Hackel PO, Prenzel N, Ullrich A (1999) The EGF receptor as central transducer of heterologous signalling systems. Trends Pharmacol Sci 20:408–412

Datos no incluidos en el artículo
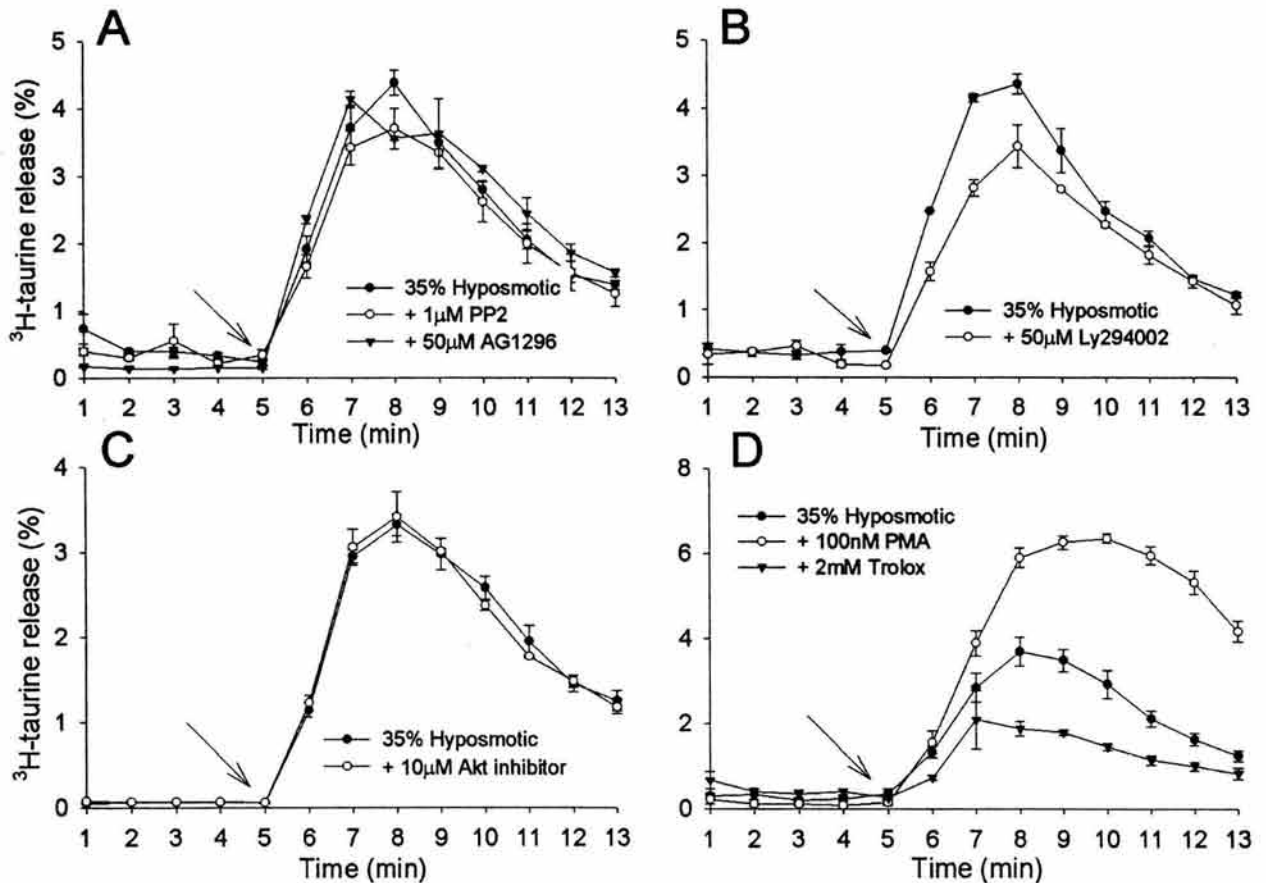
Figura 10. Efecto de distintos inhibidores de cinasas y del antioxidante Trolox, sobre la LTAH en fibroblastos Swiss 3T3. Los fibroblastos se sembraron en cajas petri 35 mm al 80% de confluencia. Las células se preincuban 1hr con ^{3}H-taurina en medio isosmótico, posteriormente se lavan durante 13 min con cambios de medio cada min. A partir de este momento, la perfusión se realiza con cambios de medio cada min y las muestras se recolectan en viales de centelleo. Se perfunde una basal de 5 min, y después se cambia el medio a una solución 35% hiposmótica y se perfunden las células durante 8 min de estímulo recolectándose las muestras cada min. La flecha indica el cambio de medio isosmótico a hiposmótico. **A.** Efecto del inhibidor específico de la cinasa de tirosina del PDGFR, tirfostina AG1296 (50μM), y del inhibidor de cinasas de tirosina tipo src, PP2 (1μM), sobre la LTAH en fibroblastos Swiss 3T3. **B.** Efecto del LY294002 (50μM), inhibidor de la PI3K, sobre la LTAH en fibroblastos. **C.** Efecto del inhibor de Akt, 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (10μM), sobre la LTAH. Los fármacos fueron preincubados 1h antes y estuvieron presentes durante todo el experimento. **D.** Efecto del antioxidante trolox (2mM) y del PMA (100nM) sobre la LTAH en fibroblastos Swiss 3T3. EL PMA y el trolox se preincubaron 30 min y estuvieron presentes durante todo el experimento. Los datos se expresan en % de liberación y son promedios ± ES de n = 4 experimentos (artículo 5 para más detalles sobre la metodología)

Como un acercamiento para estudiar la posible participación de otros RTKs en la cascada de osmotransducción involucrada en la liberación de taurina activada por hiposmolaridad, se estudió el efecto del inhibidor del receptor al PDGF sobre la LTAH en

fibroblastos Swiss 3T3, el cual se ha reportado se expresa también en este tipo celular, e inclusive comparte algunas vías de señalización (Lipson, et al., 1998). Como se observa en la figura 10A, la LTAH no se ve afectada en presencia de AG1296, inhibidor específico de la cinasa de tirosina acoplada al PDGFR (receptor al PDGF). Este dato es importante porque indica que la LTAH no es sensible a todo tipo de tirfostinas, sino sólo a aquellas que inhiben al EGFR, y habla de la especificidad de la cascada de osmotransducción.

Como se reportó en el artículo 5, la LTAH se modula por la actividad de la PI3K, sin embargo, esto lo corroboramos mediante el empleo de otro inhibidor específico de esta enzima, el LY294992, lo que descarta el posible efecto inespecífico de la wortmannina sobre la LTAH (Figura 10B). Por otra parte, aunque la LTAH depende de la actividad de la PI3K, la vía de señalización no involucra a Akt, cinasa que media gran parte de la vía de señalización activada por PI3K, ya que en presencia del inhibidor de Akt, 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, la LTAH no se ve afectada (Figura 10C)

El receptor a EGFR también puede ser activado por la generación de especies reactivas de oxígeno (ROS). Este mecanismo puede actuar de manera directa sobre el receptor o mediante la inhibición de fosfatasas de tirosina, permitiendo ya sea la activación del EGFR o un aumento en la señal. Recientemente se ha reportado que la hiposmolaridad (Lambert, et al., 2003; 2004; Ortenblad, et al., 2003) puede inducir la generación de ROS mediante la activación ya sea de la NADPH oxidasa o de la PLA_2. En este sentido, como un acercamiento a estudiar la participación de ROS en la activación del EGFR que modula la LTAH en fibroblastos, se probó el efecto del antoxidante trolox. En la Figura 10D, se observa que en presencia de 2mM de trolox la LTAH se inhibe de manera importante, lo que sugiere que la generación de ROS podría estar involucrada en este fenómeno. Por otra parte Lambert (2004) propone que la activación de la NADPH oxidasa por hiposmolaridad se potencia en presencia de esteres de forbol como el PMA debido a la fosforilación de la subunidad $p22^{phox}$. En nuestra preparación también observamos que el PMA induce una potenciación de la LTAH (Figura 10D). Estos resultados sugieren la posible participación de ROS en la regulación y/o activación de la LTAH, probablemente debido a la activación de la NADPH-oxidasa.

En la Figura 11, se observa el efecto de la adición del EGF en condiciones hiposmóticas en los cambios en volumen y el DRV subsecuentes al estrés hiposmótico. La adición del EGF previene el incremento máximo en volumen observado en los fibroblastos expuestos a un medio 35% hiposmótico. Esto refleja que el aumento en la liberación de osmolitos activada por hiposmolaridad en presencia del factor de crecimiento previene el incremento en volumen mayor observado en ausencia del ligando.
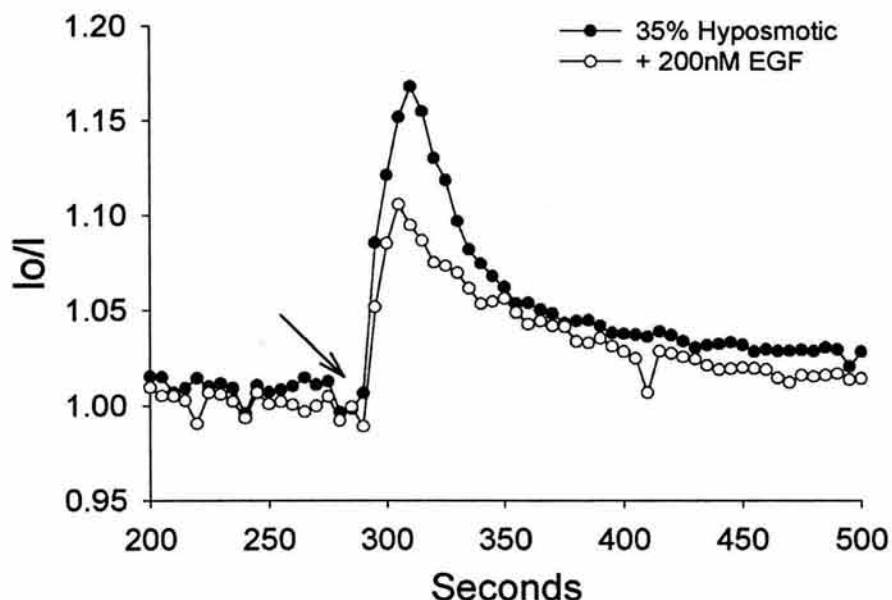
Figura 11. Cambios en el volumen celular en fibroblastos Swiss 3T3 en respuesta a hiposmolaridad (35%) en presencia o ausencia de EGF. Los fibroblastos fueron sembrados en cubreobjetos rectangulares (10 mm x 50 mm) a un 90% de confluencia como se explica en 8.3.1. Los cambios en el volumen celular en células en cultivo fueron realizados, estimando los cambios en el volumen relativo, utilizando un sistema de dispersión de luz de ángulo largo como el utilizado por McManus, et al., 1996 y Pedersen, et al., 2002. Las células se sembraron en cubreobjetos rectangulares (10 mm x 50 mm) a un 90% de confluencia al momento de los experimentos. Los cubreobjetos se colocaron a 50 grados respecto a la incidencia de la luz de excitación en una cámara de perfusión dentro de una celda de cuarzo con medio isosmótico. La celda se coloca en un espectrómetro de luminiscencia Aminco-Bowman Series 2. La celda está acoplada a un sistema de perfusión como lo muestra la Figura 11.5. Los cambios súbitos de osmolaridad se realizan por dilución del medio isosmótico en la celda hasta alcanzar la osmolaridad deseada por adición de agua bidestilada La celda se excita a 585 nm con una lámpara de arco de Argon, y la emisión se detecta a la misma longitud de onda. Los datos se expresan como el inverso de la intensidad de la señal de emisión registrada, debido a que la intensidad de la luz se correlaciona de manera inversa con el volumen celular. Los cambios en volumen son calculados de acuerdo a la ecuación: Io/I_t donde Io: es la señal promedio en condiciones isosmóticas; y I_t: es la emisión al tiempo t (tiempo indicado). El EGF (200nM) se adicionó junto con el estímulo hiposmótico. La flecha indica el momento del estímulo. Experimento representativo de n = 4.

Como se mencionó en los antecedentes, el EGFR es susceptible de ser transactivado por distintos receptores membranales como integrinas y receptores acoplados a proteínas G (Zwuick, et al., 1999). Es por esto que decidimos estudiar el efecto de alguno de estos receptores sobre la LTAH. Un estudio realizado por Nilius y colaboradores (Manolopoulos, et al., 1997a; 1997b), muestra que la activación del receptor a trombina en células endoteliales potencia I_{Clvol}, y la LTAH, siendo capaz a su vez de activar la salida de taurina en condiciones isosmóticas. La trombina (cuyo receptor es un receptor acoplado a proteínas G heterotriméricas) se ha visto puede inducir la transactivación del EGFR en distintos tipos celulares incluyendo fibroblastos (Daub, et al., 1997; Eguchi, et al., 1998, Macfarlane, et al., 2001), aunque este efecto puede estar mediado por el incremento en Ca^{++} intracelular en respuesta a este ligando (Wang, et al., 1996, Miyakawa, et al., 1998), no existe evidencia directa sobre los mecanismos involucrados en la potenciación de la LTAH por este agente.
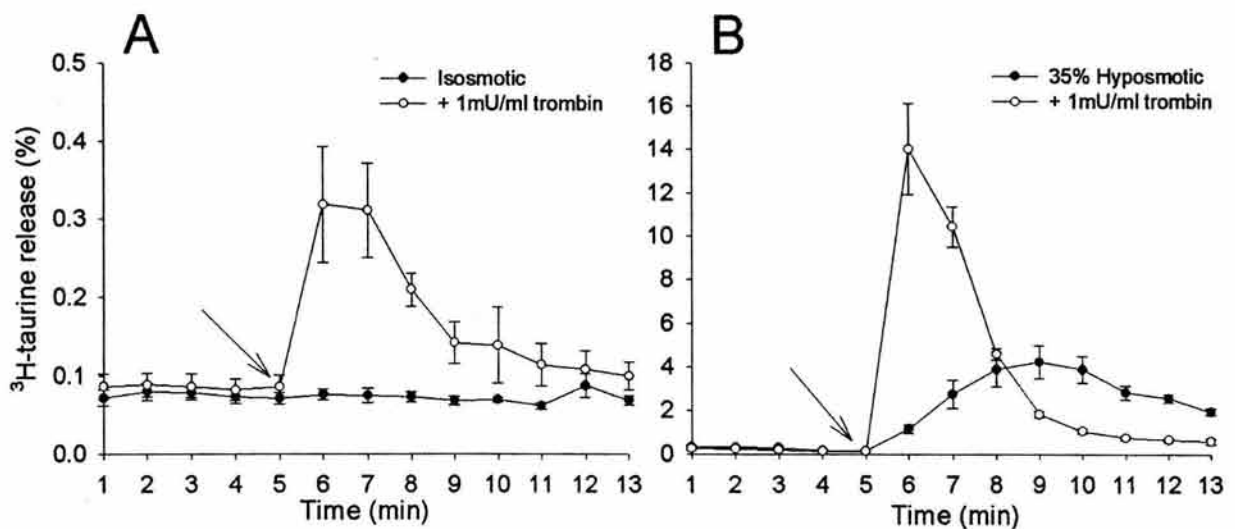
Figura 13. Efecto de la trombina (1mU/ml) sobre la liberación de taurina en fibroblastos Swiss 3T3. Los experimentos se realizaron como en la Figura 10. **A.** En condiciones isosmóticas (perfusión continua con medio isosmótico 300mOsm), la trombina es capaz de activar la liberación de taurina. La trombina se adicionó al medio isosmótico (flecha) y estuvo presente hasta el final del experimento. **B.** Efecto potenciador de la trombina sobre la LTAH. La trombina se adiciona con el estímulo hiposmótico, y está presente hasta el final del experimento. La flecha indica el cambio de medio isosmótico a hiposmótico. Los datos se expresan en % de liberación y son promedios ± ES de n = 4 experimentos (Ver 8.3.1 y 11.4 para más detalles de la metodología)

En la figura 13, se muestra cómo la trombina potencia la LTAH en mayor grado a la observada en presencia del EGF (artículo 5). De manera sorpresiva, la trombina indujo
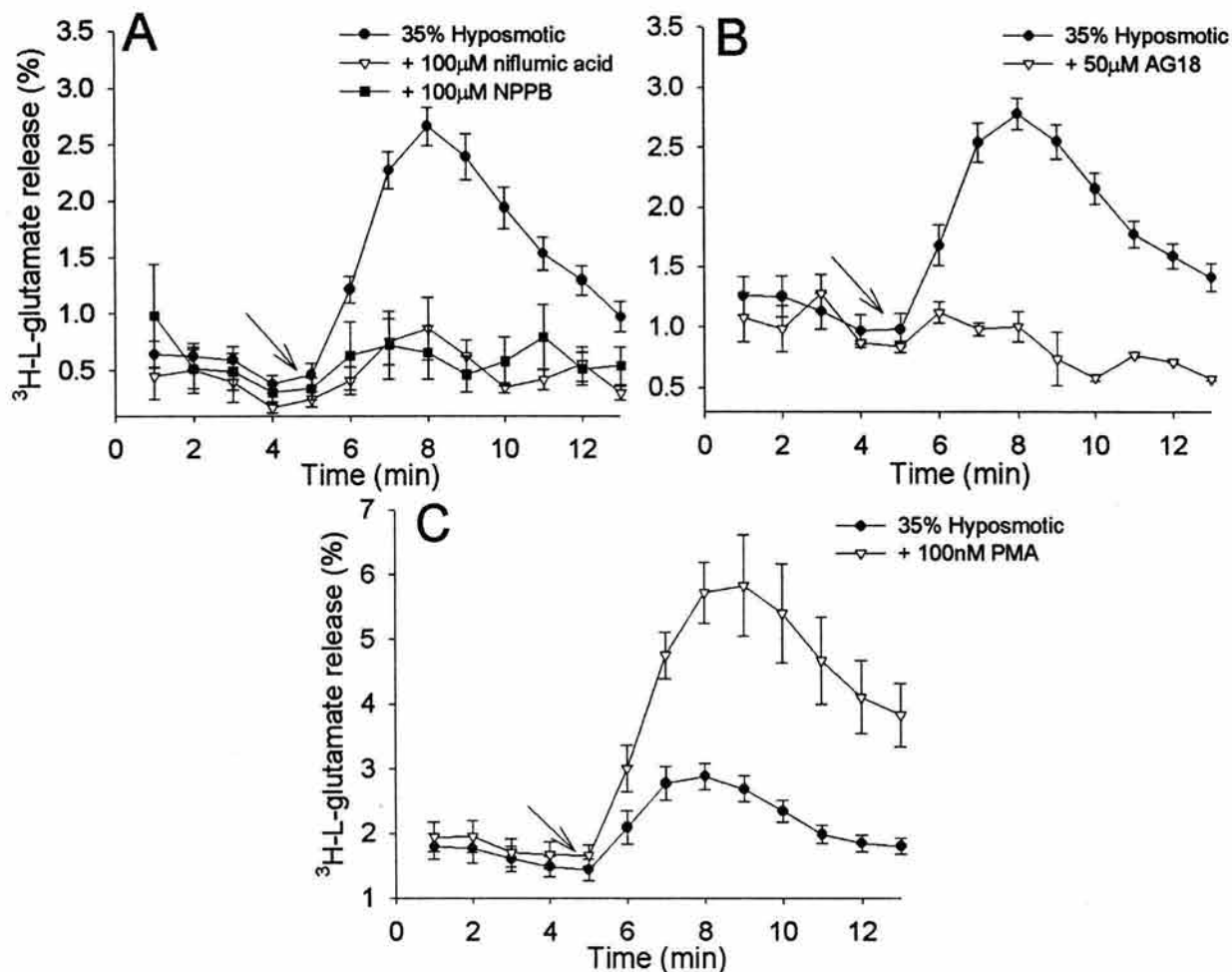
Figura 14. Caracterización de la liberación de glutamato por hiposmolaridad en fibroblastos Swiss 3T3. Los fibroblastos se sembraron en cajas petri 35 mm al 80% de confluencia. Las células se preincuban 1hr con ^{3}H-L-glutamato en medio isosmótico, posteriormente se lavan durante 13 min con cambios de medio cada min. La perfusión se realiza como en la figura 10. La flecha indica el cambio de medio isosmótico a hiposmótico. **A.** Efecto de bloqueadores de canales de Cl⁻, NPPB (100μM) y ácido niflúmico (100μM) sobre la liberación de glutamato. Los bloqueadores estuvieron presentes solo en el estímulo hiposmótico. **B.** Efecto del inhibidor general de cinasas de tirosina AG18 (50μM), sobre la liberación de glutamato por hiposmolaridad. La tirfostina AG18 se preincubo 30min y estuvo presente durante todo el experimento. **C.** Efecto del PMA (100nM) sobre la liberación de glutamato en fibroblastos Swiss 3T3. EL PMA se preincubo 30 min y estuvio presente durante todo el experimento. Los datos se expresan en % de liberación y son promedios ± ES de n = 4 experimentos.

la liberación de taurina en condiciones isosmóticas en mucho mayor grado que la inducida por EGF. Esto podría sugerir que la trombina además de activar al receptor EGF, induce la activación de otras vías de señalización que potencian aún más la LTAH, y que en condiciones isosmóticas pueden activar la vía de movilización de taurina de manera importante aún en ausencia de estímulo hiposmótico. Esta señal podría ser la formación de fibras de estrés que se forman por activación del receptor a trombina y que incluso

colocalizan con el EGFR. (Crouch, 1997; Crouch, et al., 2001). La formación de fibras de estrés en respuesta a hiposmolaridad también se ha observado en varios tipos celulares (Tilly, et al., 1996; Carton, et al., 2003).

Por último se investigó si la liberación de otros aminoácidos como el glutamato estaba siendo modulada de manera similar que la LTAH. En contraste con lo observado en preparaciones de células nerviosas, en los fibroblastos, la liberación del glutamato se inhibe por bloqueadores de canales de cloro e inhibidores de cinasas de tirosina. Por otra parte esta liberación también se potencia en presencia de PMA (Figura 14). Esto sugiere que la liberación de distintos aminoácidos en fibroblastos Swiss 3T3, y probablemente en general en células no excitables comparten la misma vía.

DISCUSION.

Participación de RTK como parte de la vía de osmotransducción que regula la liberación de osmolitos en condiciones hiposmóticas

Como se demostró en el artículo 5, la LTAH es dependiente de la activación del EGFR por hiposmolaridad. En células intestinales 407 (Tilly, et al., 1993) y en células mamarias C127 (Abdullaev, et al., 2003), se observó que la activación de EGFR potencia la activación de I_{Clvol}. En la línea celular C127, en fibroblastos de ratón (Bryan-Sisneros, et al., 2000) y en células endoteliales CPAE (Voets, et al., 1998), la activación de I_{Clvol} se inhibe en presencia de la tirfostina B46, un inhibidor de la cinasa de tirosina del EGFR. Estas observaciones, señalan la participación de este receptor como parte de la vía de señalización que activa la vía de LTAH y la de I_{Clvol}. Por otra parte, en células epiteliales del colon humano, el canal ClC-2 que se activa por hinchamiento celular, se modula por la activación del EGFR por TGF-α, otro ligando natural de este receptor (Bali, et al., 2001). En nuestros datos por primera vez se muestra que la hiposmolaridad es capaz de inducir la activación del EGFR, lo que puede sugerir que el receptor no es sólo parte de la vía de señalización, sino posiblemente parte del mecanismo de detección de cambios en volumen. Es importante señalar que la activación del EGFR no es el único evento necesario para la activación de la salida de osmolitos por hiposmolaridad como lo muestran nuestros datos y el trabajo de Tilly y colaboradores (1993). Probablemente la otra señal necesaria sea la formación de fibras de estrés como se muestra por los

resultados obtenidos con la trombina, o la formación de radicales libres que se generan por hiposmolaridad y que pueden amplificar la señal del EGFR (ver más adelante). En nuestra preparación la LTAH se potencia en presencia del EGF, lo que no se observa en el estudio de Mongin y colaboradores (1999b), en donde el EGF tiene un efecto inhibidor sobre la liberación de D-aspartato por hiposmolaridad. Esto puede explicarse debido a que este grupo preincubó al EGF (3 min) antes del estímulo hiposmótico, lo que pudo inducir la internalización del receptor, la cual se ha visto que sucede de manera inmediata como mecanismo de desensibilización del receptor (Wiley, 2003).

Como se mencionó en la introducción, el EGFR puede ser activado en ausencia de ligando por muchos estímulos extracelulares como son el estiramiento mecánico (Correa-Meyer, et al., 2002) y estrés osmótico (hiperosmolaridad) (Cheng, et al., 2002; King, et al., 1989; Reinehr, et al., 2003; Rodríguez, et al., 2002; Rosette y Karin, 1996). El hecho de que el EGFR pueda ser activado tanto por encogimiento (hiperosmolaridad) como por hinchamiento celular (hiposmolaridad), sustenta la participación del EGFR como sensor de osmolaridad o de volumen. Se ha visto que el EGFR puede regular la también la actividad de cotransportadores que participan en el IRV (Furukawa, et al., 1999; Jiang, et al., 2001; Yanaka, et al., 2002; Yang, et al., 2001; Xu, et al., 2001). No se descarta que otros RTK se activen por hiposmolaridad. Resultados recientes en el laboratorio muestran que el receptor al factor de crecimiento tipo insulina, el receptor tirosina cinasa tipo A (TRK-A) y el ErbB4 (otro subtipo de la familia del EGFR), se activan por hiposmolaridad (Lezama, et al., datos no publicados). Su implicación en la regulación de la salida de osmolitos no ha sido estudiada aún. Por otra parte en este estudio, al inhibir al receptor a PDGF, no se afectó la LTAH, similar a lo observado en la línea celular mamaria C127 (Abdullaev, et al., 2003) para el caso de la activación de I_{Clvol}. Sin embargo no se descarta que este receptor se active por hiposmolaridad, auque este fenómeno no estuviese ligado al de la salida de osmolitos por hiposmolaridad.

En cuanto a la activación de la liberación de K^+ activada por volumen (I_{Kvol}). Nuestros resultados y el trabajo de Tilly y colaboradores (1993) muestran que el EGFR no modula la activación I_{Kvol}, y que la potenciación observada en presencia de EGF se debe a la activación de una vía de salida de K^+ distinta, probablemente canales de K^+ activados por Ca^{++}, los cuales ha reportado se modulan por activación del EGFR en los tipos celulares estudiados. Sin embargo no se descarta la participación de canales

dependientes de voltaje tipo Kv, los cuales se activan también por receptores a factores de crecimiento (Bowlby, et al., 1997; Huang y Rane, 1994; Lovisolo, et al., 1992; Pandiella, et al., 1989; Peppelenbosch, et al., 1991; Rane, 1999; Wang, et al., 1997; Wonderlin y Strobl, 1996; Xu, et al., 1999).

¿Cómo es que la hiposmolaridad o el hinchamiento celular inducen la activación del EGFR? La activación del EGFR por hiposmolaridad puede ser de forma directa o indirecta. De manera indirecta, indicaría que el EGFR es un sensor de volumen (mecanosensor) o de osmolaridad. Como ya se mencionó, el estrés mecánico por si solo puede alterar directamente la conformación estructural de los RTK, iniciando la activación de vías de señalización de manera ligando independiente. Esto debido posiblemente a un incremento en la exposición de los dominios de cinasa del receptor permitiendo así su autofosforilación. A la fecha no existe evidencia que sustente este hecho para el caso de la activación del EGFR por hiposmolaridad
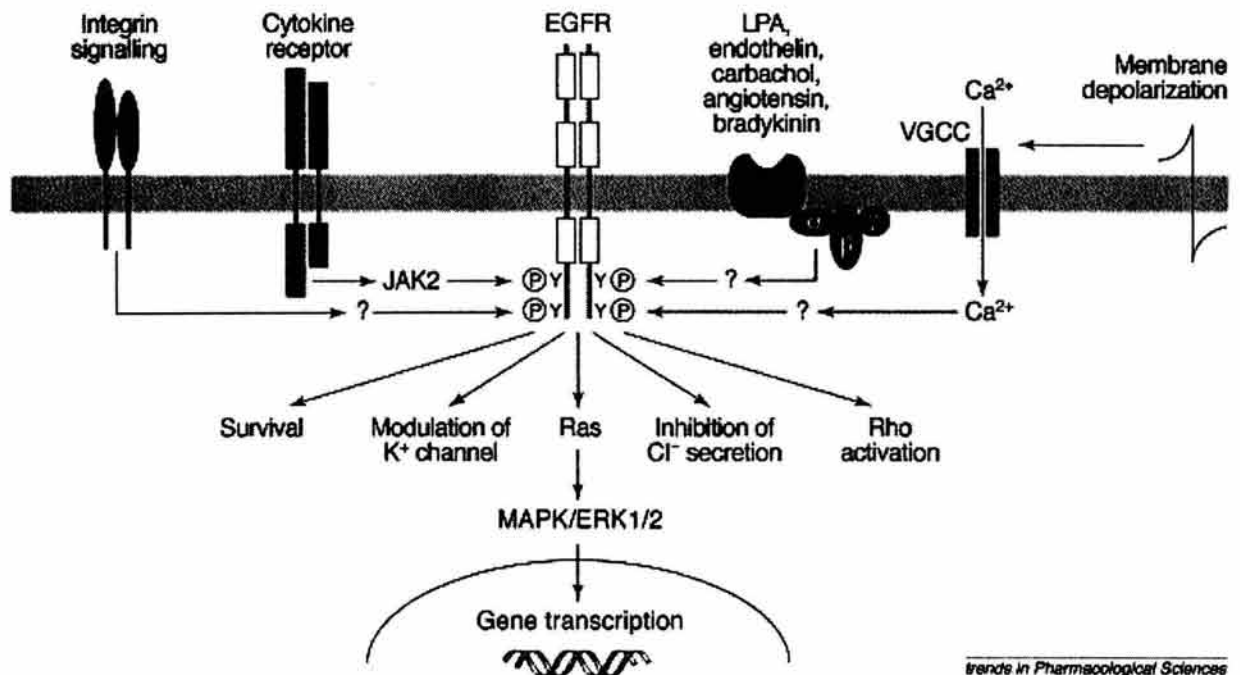
Figura 14. Transactivación del receptor a EGFR por otros sistemas de señalización. (Tomado de Zwuick, et al., 1999)

En condiciones hiposmóticas, la activación del EGFR podría ser debida a la participación de otros elementos de señalización cascada arriba del receptor, lo que implicaría a otra molécula como el sensor de volumen implicado en el fenómeno. En

células alveolares, la activación del EGFR por estrés mecánico es dependiente de proteínas G sensibles a toxina pertusis y a la activación de ERK1/2. En condiciones de estrés osmótico, se ha observado que la activación del EGFR depende de la activación a su vez de p38 (Cheng, et al., 2002). La activación de ERK1/2 y p38 y su papel en la activación del EGFR por hiposmolaridad y en la LTAH en este tipo celular, queda por resolver. La activación del EGFR en distintas condiciones de estrés depende de la inhibición de fosfatasas de tirosina que regulan de forma negativa la actividad de este receptor (Herrlich y Bohmer, 2000). Esta inhibición probablemente este dada por la generación de radicales libres (Huang, et al., 1996; Peus, et al., 1999), los cuales pueden inhibir la actividad catalítica de las fosfatasas de tirosina (Denu, et al. 1998; Hecht y Zick, 1992; Knebel, et al., 1996; Sullivan, et al., 1994), o activar directamente al EGFR (Meves, et al., 2001; Oeckler, et al., 2003; Thannickal y Fanburg, 2000). La formación de radicales libres también sirven como sistema de amplificación y prolongación de la cascada de señalización activada por el EGFR mediante la inhibición de las fosfatasas de tirosina, las cuales terminarían con la señalización (Droge, 2001). Recientemente se demostró la generación de radicales libres en condiciones hiposmóticas en distintos tipos celulares, por la activación de la NADPH-oxidasa o de PLA_2, los cuales a su vez regulan la LTAH (Lambert, et al., 2003; 2004; Ortenbald, et al., 2003). Es probable que estos radicales libres estén mediando la activación del EGFR por hiposmolaridad, o al menos amplificando la señal, lo que también se sugiere por los resultados obtenidos en presencia del antioxidante trolox y PMA (debido a que la PKC modula la translocación de la NADPH-oxidasa a la membrana, ver Figura 10D). Por su parte, el EGFR es capaz de inducir la generación de radicales libres probablemente por la activación de la PLA_2 y de la vía de las lipoxigenasas; si este fuera el caso en nuestro modelo, podría significar un sistema de amplificación de la señal (Thannickal y Fanburg, 2000).

La comunicación cruzada entre sistemas de señalización heteróloga es esencial en la integración de una amplia variedad de estímulos extracelulares dentro de un número limitado de vías de señalización (ver Figura 14.). Se ha reportado que el EGFR interactúa con las integrinas. Particularmente, la cinasa de adhesión focal (p125FAK) se ha observado que une las vías de señalización del EGFR y las integrinas (Miyamoto, et al., 1996; Schneller, et al., 1997; Sieg, et al., 2000; Prenzel, et al., 2001). Datos recientes señalan que las integrinas pueden inducir la activación del dominio de cinasa del EGFR en ausencia de ligando (Moro, et al., 1998), el mecanismo involucrado podría implicar la

generación de radicales libres por señales de adhesión (Droge, 2001). Otros fenómenos que podrían estar involucrados en la activación o en la regulación de la actividad del receptor en condiciones hiposmóticas, podrían ser la alta localización del EGFR en las caveolas, que muchos de los pasos críticos en la vía de señalización del EGFR ocurren en esos dominios de membrana especializado (Lehto, 2001), y que las caveolas parecen formar parte importante en la vía de osmotransducción que activa a I_{Clvol} (Trouet, et al., 1999; 2001). Otra observación importante es que la hiposmolaridad disminuye la tasa de endocitosis membranal lo cual podría estar disminuyendo la internalización del EGFR permitiendo que este se mantuviera activo en la membrana plasmática donde el hinchamiento celular es detectado (Wiley, 2003).
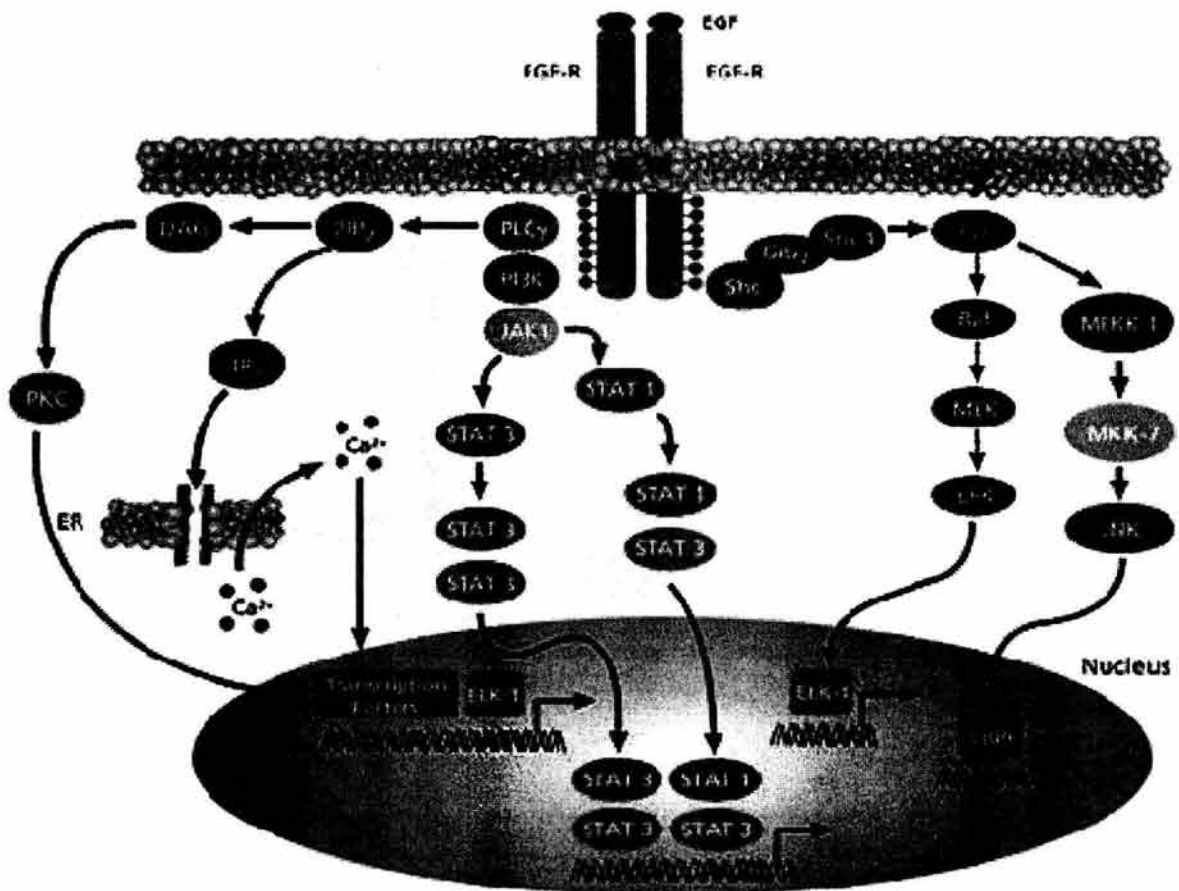
Figura 15. Vías de señalización activadas a partir de la activación del receptor al factor de crecimiento epidermal (EGFR)

¿Cómo es que la activación del EGFR modula o activa la LTAH? La activación del EGFR conlleva a su vez a la activación de otras proteínas de señalización como son MAP-cinasas, reclutamiento de proteínas de andamiaje, cinasas tipo Src, PLC y a la PI3K

de la familia Ia (ver Figura 15). La activación de la PI3K por activación del EGFR depende de la asociación de la subunidad p85 con los residuos autofosforilados en tirosina del receptor, vía el reconocimiento por el dominio SH2 de la subunidad reguladora. Con nuestros datos demostramos que la activación del EGFR por hiposmolaridad activa a la PI3K, la cual modula la activación de la LTAH (ver la segunda sección de esta tesis para una discusión sobre este punto y los posibles mecanismos involucrados en el fenómeno). Esto contrasta con lo observado en células C127 (Abdullaev, et al., 2003), en las que la activación de I_{Clvol} por el EGFR no se modula por PI3K. Esto sustentaría la participación de dos distintas vías de movilización para I_{Clvol} y para la LTAH, reguladas a su vez por dos vías de señalización distintas que divergen a partir de la activación del EGFR. Tilly y colaboradores (Van der Wijk, et al., 1998), reportaron que la potenciación de I_{Clvol} por EGF es dependiente de Ca^{++} intracelular. Por otra parte, en células T84 de colon (Bali, et al., 2001), la modulación del canal sensible a volumen ClC-2 por el EGFR esta mediada por la activación de PI3K y PKC. Esto no indica por supuesto que ClC-2 sea la identidad molecular de I_{Clvol}. El sustrato mayor para el reclutamiento de PI3K no es el EGFR en sí, sino el ErbB3, lo que podría sugerir que la hiposmolaridad induce la formación de heterodímeros ErbB1 (EGFR)/ErbB3, lo cual tendría implicaciones muy importantes a nivel de especificidad de la señal (Jorissen, et al., 2003).

CONCLUSIONES

El control del volumen celular es un proceso involucrado en la homeostasis la celular. Los cambios en el volumen celular están implicados tanto en el progreso de distintos procesos fisiológicos como en la regulación de vías metabólicas y cascadas de señalización. Asimismo, los cambios en el volumen celular están asociados a patologías tales como trauma, isquemia e hiponatremia. En este trabajo de tesis se estudiaron distintos aspectos del control del volumen celular en condiciones hiposmóticas, con el objetivo de comprender los mecanismos involucrados en la regulación del volumen en condiciones de hinchamiento celular. Este trabajo, originó resultados que representan un aporte importante al conocimiento de este fenómeno.

Aunque la discusión de los trabajos incluidos en esta tesis se desarrolla principalmente en cada sección, es importante remarcar ciertos puntos y aportaciones como resultado de este trabajo.

El control del volumen celular en condiciones hiposmóticas depende de la magnitud y tasa de cambio en la osmolaridad del medio (artículos 1 y 2). Algunos tipos celulares como las neuronas y células renales, así como preparaciones integras de tejido nervioso son capaces de mantener su volumen constante dentro de amplios márgenes de hiposmolaridad, debido a un ajuste constante del contenido intracelular de osmolitos (regulación isovolumétrica). Por otra parte en tipos celulares como los astrocitos y cardiomiocitos, los cambios graduales de osmolaridad permiten una mayor eficiencia en el control del volumen, previniendo el hinchamiento máximo observado en condiciones de cambios súbitos de osmolaridad del medio. Este fenómeno se debe a una mayor movilización de osmolitos orgánicos, en este caso particular, aminoácidos como la taurina juegan un papel importante. No se descarta la participación de otros osmolitos orgánicos en este fenómeno. Este paradigma representa un acercamiento mas real a condiciones patológicas como la hiponatremia crónica en la cual, la reducción en la osmolaridad del medio es gradual y no se observa edema cerebral.

En la segunda sección (artículos 3 y 4), se observó que la liberación de aminoácidos en preparaciones de células nerviosas presenta distintas vías de liberación. La taurina, cuya movilización no modifica la excitabilidad, ni el metabolismo celular, se

moviliza a través de una vía difusional denominada canal aniónico y de osmolitos sensible a volumen (VSOAC), la cual se modula por la actividad de cinasas de tirosina y de PI3K. Por el contrario la liberación de aminoácidos neurotransmisores como el GABA y el glutamato presenta una cinética de activación e inactivación más rápida con respecto a la de la taurina, y se modula por la actividad de PKC y por cambios en la integridad del citoesqueleto. Estos datos sugieren la posible participación de un proceso de exocitosis como mecanismo de liberación de estos aminoácidos. Se ha reportado, que la hiposmolaridad induce la liberación la liberación vesicular de distintas hormonas. Los mecanismos involucrados no han sido esclarecidos a un, pero pueden involucrar desde cambios en los niveles de Ca2+ intracelular, despolarización de membranal. Y tensión membranal. Sin embargo, se necesita mayor evidencia experimental para corroborar esto.

Por último, la tercera parte de este trabajo (artículo 5) estudia la participación de RTKs como posibles miembros de la cascada de señalización que modula la LTAH y su posible participación como sensores de volumen. El hinchamiento hiposmótico activa al EGFR de manera ligando independiente. Esta activación del EGFR activa a la PI3K, que a su vez modula la LTAH. Por lo tanto el EGFR es miembro de la cascada de osmotransducción que modula y/o activa la LTAH, y es probable que a su vez module a I_{Clvol} (conductancia de Cl$^-$ activada por volumen). Es probable que otras cinasas de tirosina, incluyendo otros RTK estén participando en la regulación, no sólo de la salida de osmolitos subsecuente a hinchamiento hiposmótico, sino como mecanismos de osmotransducción que regulan la respuesta celular al hinchamiento. El EGFR no regula la liberación de K$^+$ por hiposmolaridad, aunque su activación puede potenciar la salida de K$^+$ mediante la activación de otro tipo de canales de K$^+$. Si el EGFR es miembro del mecanismo de detección del cambio en volumen, este necesita de otra señal que converja en la activación de la LTAH. Esta señal podría involucrar la generación de ROS, cambios en la concentración de Ca2+ intracelular y otras proteínas de señalización como fosfolipasas. La participación de ROS se sustenta por el efecto de antioxidantes sobre la LTAH en distintas preparaciones.

En conjunto estos resultados señalan el hecho de que el control del volumen celular en condiciones hiposmóticas, esta determinado por diversos fenómenos o mecanismos como son:

a) La magnitud y rapidez del cambio en la osmolaridad del medio o del cambio en el volumen celular generado, lo que define la eficiencia y capacidad de regulación del mismo.

b) La activación de vías de salida de osmolitos orgánicos, como los aminoácidos, que permiten contrarrestar el cambio en volumen generado. Estas vías pueden ser distintas dependiendo el tipo de osmolito estudiado. Para el caso de preparaciones de tejido nervioso, la liberación de aminoácidos neurotransmisores esta dada por una vía distinta a la de osmolitos como la taurina.

c) La activación de vías de señalización que modulan y/o activan la salida de osmolitos, las cuales varían según el osmolito estudiado. Estas vías pueden involucrar cinasas de tirosina, PI3K, PKC y citoesqueleto según sea el caso

d) La presencia de mecanismos de detección del cambio en volumen que como se mencionó en el trabajo, puede involucrar la conjunción de distintos fenómenos dentro de un sistema sensorial complejo, Dentro de este mecanismo, la participación de RTKs puede ser un evento importante en el mecanismo de detección y transducción del cambio en volumen.

APENDICE I

ARTICULOS DE REVISION PUBLICADOS COMO PARTE DEL PROYECTO DE DOCTORADO.

ARTICULO 1

Isovolumic regulation in nervous tissue: a novel mechanism of cell volume regulation.

ARTICULO 2

Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: contribution and mechanisms.

ARTICULO 3

Mechanisms counteracting swelling in brain cells during hyponatremia.

ARTICULO 4

Osmosensitive release of neurotransmitter amino acids: relevance and mechanisms.

ARTICULO 5

Influence of protein tyrosine kinases on cell volume change-induced taurine release.

ISOVOLUMIC REGULATION IN NERVOUS TISSUE

A Novel Mechanism of Cell Volume Regulation

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INTRODUCTION

Cell volume regulation is a property present in most animal cell lineages that allows them to recover their original volume after events of swelling or shrinkage. Such events can be caused by changes in external osmolarity or to osmotic gradients generated during normal cell functioning[4,6]. The mechanism of cell volume regulation involves transmembrane fluxes of osmotically active solutes in the necessary direction to counteract the net gain or lose of intracellular water[9]. The process through which cells recover their normal volume after swelling is named Regulatory Volume Decrease (RVD). This consists of the efflux of inorganic osmolytes, such as K^+ and Cl^-, as well as organic compounds such as free amino acids, methyl amines and polyalcohols. These movements create a new osmotic gradient that leads to water efflux and volume recovery.

The simplest and more often experimental paradigm used in RVD studies consists of sudden exposures of cells/tissues to mild-to-acute hyposmotic media (~20 to 50% hyposmotic) and recording different cell parameters (cell volume, osmolyte release, membrane potential, conductances, etc.) These conditions, however, do not occur under normal, physiological circumstances, where changes in external or internal osmolarity are gradual, as the different homeostatic systems are challenged and activated[12]. Even in pathological situations, such as water intoxication or hyponatremia, the activation and eventual surpassing of the encephalic mechanisms of water and electrolyte control lead to *progressive* osmotic changes in the extracellular brain milieu[3].

123

Gradual and continuous changes in external osmolarity (in contrast to the sudden changes usually used) were introduced by Lohr and Grantham[8] to examine volume regulation properties in the S_2 proximal tubules. It was observed that regardless of the progressive reduction in the osmolarity medium *cells do not swell*, provided that the rate of change does not exceed – 3.0 mOsmol/min. The adaptive cell response occurred, however, as cells swell immediately after reintroduction of isosmotic medium[8]. The response was described as *Isovolumic Regulation* due to the lack of change in cell
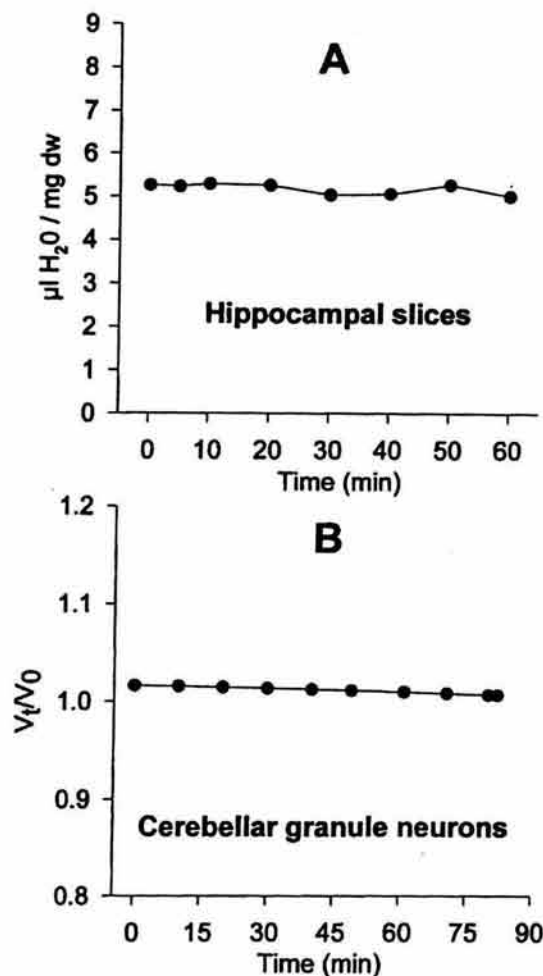
Figure 1. Cell volume changes of hippocampal slices and cerebellar granule neurones exposed to a continuous hyposmotic gradient (300 mOsmol/l $\Rightarrow$ 150 mOsmol/l). A gradient-generating system was constructed as described by Van Driessche *et al.*[13] The rate of change in osmolarity was adjusted at -2.5 or –1.8 mOsmol/min for the experiments with hippocampal slices or cell cultures, respectively. A and B: At time 0 in figures, slices or cerebellar granule neurones were superfused with the osmotic gradient and cell volume was determined at different times. A. Volume changes in hippocampal slices were indirectly estimated by quantification of tissue water content corrected by the interstitial space measured by ^{14}C-inulin distribution. B. Relative cell volume in cerebellar granule neurones was quantified fluorometrically, using calcein-AM as fluorescent dye[1]. Data are means $\pm$ SE of 6 (A) or 3 (B) individual experiments.
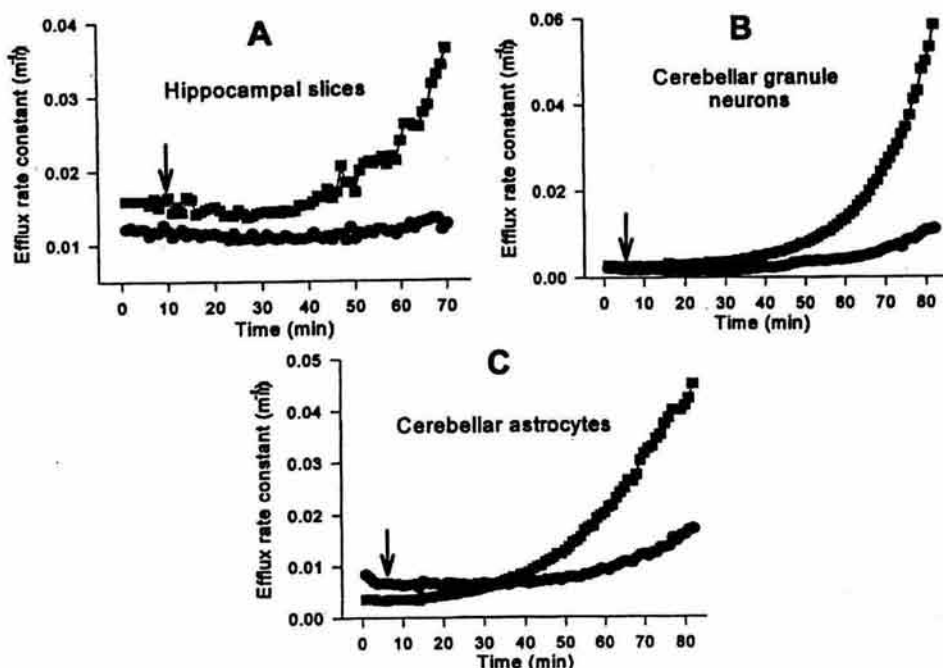
Figure 2. Amino acid efflux from hippocampal slices, cerebellar granule neurones and cerebellar astrocytes elicited by a continuous hyposmotic gradient. Preloaded tissues were initially superfused with isosmotic medium (6-10 min) and then (at arrow) exposed to the osmotic gradient (300 $\Rightarrow$ 150 mOsmol/l). (■): ^{3}H-taurine; (●): ^{3}H-D-aspartate in all figures. Data are expressed as efflux rate constants and are means ± SE of 6 (A), 8 (B) or 2 (C) individual experiments.

Table 1. Kinetic constants of ^{3}H-taurine and ^{3}H-D-aspartate fluxes from cerebellar granule neurones and hippocampal slices elicited by a continuous hyposmotic gradient

Cerebellar Granule Neurones	k_1 (x10^{-5})	k_2 (x10^{-5})	k_3 (x10^{-5})
External osmolarity range (mOsmol/l)	300 → 249	248 → 203	202 → 156
^{3}H-D-Aspartate	N.A.	10.37 ± 0.53	37.17 ± 1.26
^{3}H-Taurine	5.29 ± 0.33	34.97 ± 1.59	213.38 ± 10.12
Hippocampal Slices	k_1 (x10^{-5})	k_2 (x10^{-5})	k_3 (x10^{-5})
External osmolarity range (mOsmol/l)	300 → 250	249 → 178	175 → 150
^{3}H-D-Aspartate	-2.322 ± 0.111	2.070 ± 0.219	7.138 ± 1.070
^{3}H-Taurine	-4.066 ± 0.542	8.968 ± 0.502	34.829 ± 3.733

The efflux of ^{3}H-taurine and ^{3}H-aspartate under IVR conditions were kinetically analysed adjusting the experimental data of Fig. 1 to lineal regressions in different segments of the curves, as indicated (fractions). Values are the slopes of the adjusted averaged points ± S.E. (n= 4-8) N.A.: Not adjusted

volume. In the present study, we described the occurrence of Isovolumic Regulation in different preparations of nervous tissue, where it is observed, the early activation of taurine and glutamic acid efflux, as well as the relatively late (or absence of) mobilisation of K^+.

RESULTS AND DISCUSSION

Although the experimental model of large and sudden decreases in osmolarity had rendered valuable information to elucidate some basic mechanisms of cell volume control, such changes probably never occur in brain under physiological conditions. This is also true during pathological situations such as chronic hyponatremia, water intoxication or the inappropriate secretion of vasopressin, where the osmolarity changes in the brain interstitial space occurs most likely in a gradual manner, as the osmotic challenge from plasma progressively surpasses the brain homeostatic resistance[3,12]. Thus, the experimental approach of the present work, decreasing gradual and slowly the external osmolarity, could reflect more accurately physiological variations. Under these conditions, Figure 1 shows the lack of change in cell volume when hippocampal slices (A) or cultivated neurones (B) are exposed to the osmotic gradient, regardless of the low, final external osmolarity (~150 mOsmol/l or 50% hyposmotic). This constancy in cell volume appears to result from an active process of volume control accomplished by the adjustment of osmolyte intracellular content and its named Isovolumic Regulation (IVR). This is supported by the swelling observed in cells previously exposed to gradual hyposmotic changes and suddenly returned to isosmotic medium[8].

Figure 2 shows the efflux of ^3H-taurine and ^3H-D-aspartate from hippocampal slices (A), cerebellar granule neurones (B) or cerebellar astrocytes (C) elicited by a continuous hyposmotic gradient (300 $\Rightarrow$ 150 mOsmol/l). In these preparations it is observed first, that efflux of both amino acids is activated early during IVR; second, the lower the external osmolarity is, the faster the amino acid efflux, showing no inactivation phase; third, the release of taurine is larger than that of D-aspartate. A similar higher efflux rate for taurine as compared with other osmolytes has been observed in rat brain *in vivo* upon microdialysis perfusion with hyposmotic solutions[2,11].

In the present study, the release of taurine and D-aspartate during IVR were resolved into three first-order velocity components by fitting linear regressions to different segments of the efflux curves. Table 1 shows the derived kinetic constants for hippocampal slices and cerebellar granule neurones, along with the corresponding external osmolarity ranges. The osmotic intervals for each of the release components of taurine and D- aspartate are remarkably similar, suggesting a common efflux pathway.   126
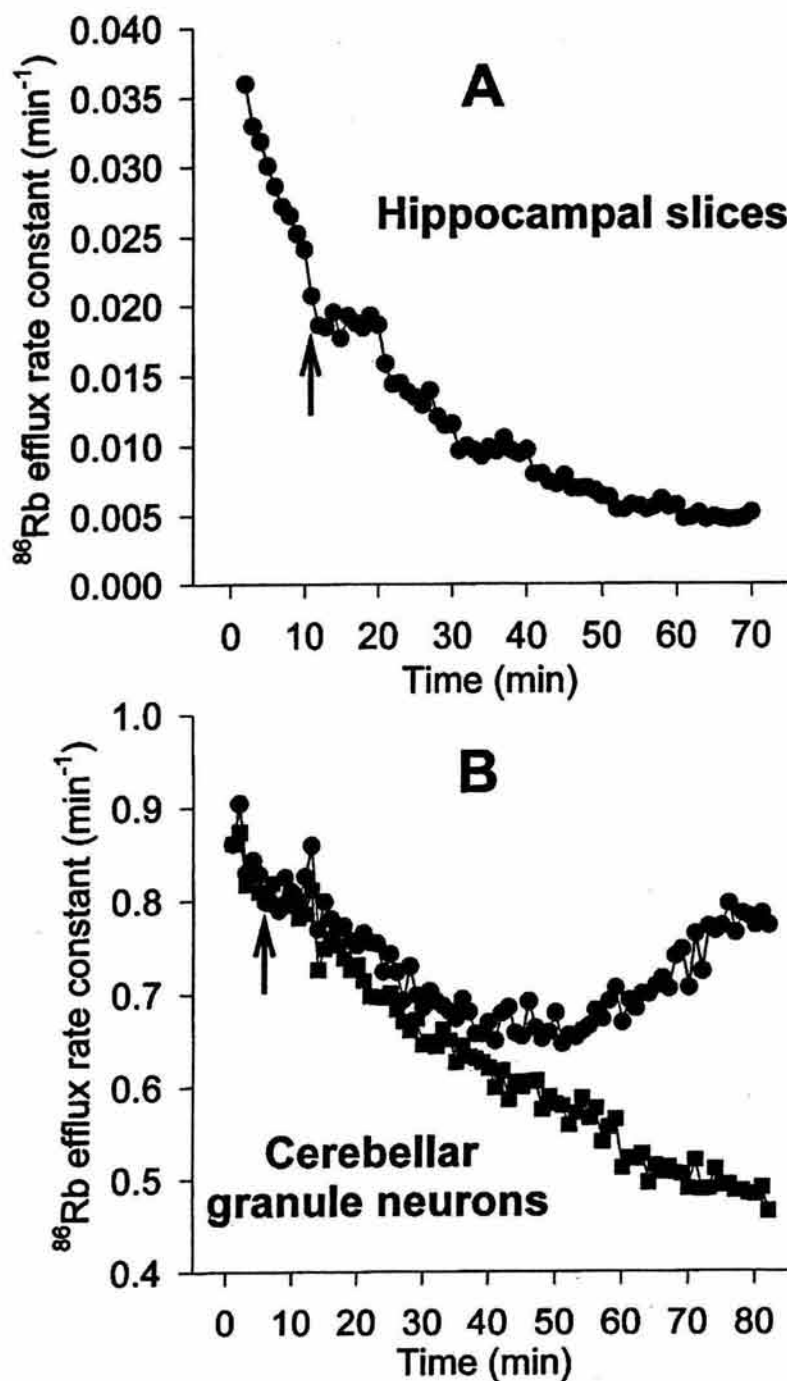
Figure 3. ^{86}Rb efflux from hippocampal slices and cerebellar granule neurones elicited by a continuous hyposmotic gradient. Preloaded tissues were initially superfused with isosmotic medium (6-10 min) and then (at arrow) exposed to the osmotic gradient (300 mOsmol/l $\Rightarrow$ 150 mOsmol/l). A and B (●): Hyposmotic gradient; B (■): Isosmotic medium. Data are expressed as efflux rate constants and are means ± SE of 6 (A) or 8 (B) individual experiments.

Moreover, such intervals are similar also between preparations, i.e. k_1 describe taurine and D-aspartate fluxes during the same osmotic range in hip-[127] pocampal slices and granule neurones. This suggests that the efflux mecha-

nisms present in both preparations have similar osmotic sensitivities. The magnitude of the rate release for each amino acid however, is notably different. In the hippocampal slices, k_1 for taurine is about half of that of D-aspartate (Table 1). When the amino acids efflux is described by k_2 and k_3, velocity of taurine efflux is 4 times higher than that of D-aspartate. Similar differences are observed in cerebellar granule neurones: k_2 and k_3 for taurine efflux are about 3.5 and 5.7 times higher than those of D-aspartate. These differences could be due to distinct permeability coefficients through the suggested common pathway, or/and to different availabilities of the intracellular pools. Brain glutamate is extremely active and deeply involved in synaptic transmission and thus, is sequestered into vesicles and other metabolic compartments. Taurine in contrast, is essentially an inert compound, not contributing to protein synthesis nor involved in any metabolic reaction, and is found essentially free in the cytosol[5]. All these results suggest a predominant role for taurine in volume regulation in brain.

Potassium is an important osmolyte due to its high intracellular content. An osmosensitive release of K^+ has been consistently described in cells showing regulatory volume decrease (RVD)[6,9]. In cerebellar astrocytes, it has been shown that K^+ efflux is the rate-limiting factor in an on-going RVD process[10]. In isovolumic conditions, an osmosensitive K^+ outflow has been clearly shown in renal cells. In the distal nephron cell line A6, K^+ efflux is activated with a threshold[13] of 210 mOsmol/l, while in renal proximal tubules K^+ content decreased after superfusion with an osmotic gradient[7]. Figure 3 shows the release of K^+ (traced with ^{86}Rb) during IVR, from hippocampal slices (A) and cerebellar granule neurones (B). In this last preparation, K^+ outflow initially follows a k_1 equal to $4.98 \pm 0.36 \times 10^{-5}$ until the external osmolarity has decreased 90 mOsmol ($\approx$ 30% hyposmotic). This value is in the same range than that measured in isosmotic conditions ($3.48 \pm 0.15 \times 10^{-5}$). When the external osmolarity has decreased $\approx$ 65 mOsmol, K^+ efflux is activated and its movement follows a second k, with a value of $39.97 \pm 1.04 \times 10^{-5}$ for the rest of the experiment. In contrast, ^{86}Rb outflow from hippocampal slices *does not change* during IVR, and its efflux runs parallel with that observed under isosmotic conditions. This is an unexpected result, since as previously mentioned, K^+ is a key osmolyte in essentially all cell types. The difference in K^+ efflux during RVD and IVR may be due to the involvement in each case, of different mechanisms of release. In renal proximal tubules, the Na^+-K^+ ATPase seems implicated in IVR[7] but not in RVD. Also, these two processes differ in A6 cells[13]. In addition, unlike in cells in culture, in the hippocampal slices which have an intact cytoarchitecture, buffering of extracellular K^+ by the efficient mechanisms known to exist in brain tissue, could mask an osmosensitive release occurring gradually as during IVR. Due to the key role played by K^+ in nervous excitability[28], its extracellular levels in brain have to be kept under strict control. Clearly,

studies on the occurrence and features of IVR in different cell types are essential for a better understanding of the physiological significance of this mechanism of volume regulation.

ACKNOWLEDGMENTS

REFERENCES

1. Altamirano, J., Brodwick, M.S. and Alvarez-Leefmans, F.J., 1998. Regulatory volume decrease and intracellular Ca^{2+} in murine neuroblastoma cells studied with fluorescent probes. *J. Gen Physiol.* 112: 145-160.
2. Estévez, A.Y., O'Regan, M.H., Song, D., Phillis, J.W., 1999. Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. *Neurochem Res* 24: 447-452.
3. Fraser, C.L., Arieff, A.I., 1997. Epidemiology, pathophysiology, and management of hyponatremic encephalopathy. *Am J Med* 102: 67-77.
4. Häussinger, D., 1996. The role of cellular hydration for the regulation of cell function. *Biochem J* 313: 697-710.
5. Huxtable, R.J., 1992. Physiological actions of taurine. *Physiol Rev* 72 : 101-163.
6. Lang, F., Busch, G.L., Ritter, M., Völki, H., Waldegger, S., Gulbins, E., Häussinger, D., 1998. Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* 78: 247-306.
7. Lohr, J.W., 1990. Isovolumetric regulation of renal proximal tubules in hypotonic medium. *Ren Physiol Biochem* 13: 233-240.
8. Lohr, J.W., Grantham, J.J., 1986. Isovolumetric regulation of isolated S_2 proximal tubules in anisotonic media. *J Clin Invest* 78: 1165-1172.
9. Pasantes-Morales, H., 1996. Volume regulation in brain cells: cellular and molecular mechanisms. *Metab Brain Dis* 11: 187-204.
10. Pasantes-Morales, H., Murray, R.A., Lilja, L., Morán, J., 1994. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. *Neurochem Res* 18: 445-452.
11. Solis, J.M., Herranz, A.S., Herras, O., Lerma, J., Del Río, R.M., 1988. Does taurine act as an osmoregulatory substance in the rat brain? *Neurosci Lett* 91: 53-58.
12. Trachtman, H., 1991. Cell volume regulation: a review of cerebral adaptive mechanisms and implications for clinical treatment of osmolal disturbances II. *Pediatric Nephrology* 5: 743-750.
13. Van Driessche, W., de Smet, P., Li, J., Allen, S., Zizi, M., Mountian, I., 1997. Isovolumetric regulation in a distal nephron cell line (A6). *Am J Physiol* 272: C1890-C1898.

Amino Acid Osmolytes in Regulatory Volume Decrease and Isovolumetric Regulation in Brain Cells: Contribution and Mechanisms

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Abstract

Brain adaptation to hyposmolarity is accomplished by loss of both electrolytes and organic osmolytes, including amino acids, polyalcohols and methylamines. In brain in vivo, the organic osmolytes account for about 35% of the total solute loss. This review focus on the role of amino acids in cell volume regulation, in conditions of sudden hyposmosis, when cells respond by active regulatory volume decrease (RVD) or after gradual exposure to hyposmotic solutions, a condition where cell volume remains unchanged, named isovolumetric regulation (IVR). The amino acid efflux pathway during RVD is passive and is similar in many respects to the volume-activated anion pathway. The molecular identity of this pathway is still unknown, but the anion exchanger and the phospholemman are good candidates in certain cells. The activation trigger of the osmosensitive amino acid pathway is unclear, but intracellular ionic strength seems to be critically involved. Tyrosine protein kinases markedly influence amino acid efflux during RVD and may play an important role in the transduction signaling cascades for osmosensitive amino acid fluxes. During IVR, amino acids, particularly taurine are promptly released with an efflux threshold markedly lower than that of K^+, emphasizing their contribution (possibly as well as of other organic osmolytes) vs inorganic ions, in the osmolarity range corresponding to physiopathological conditions. Amino acid efflux also occurs in response to isosmotic swelling as that associated with ischemia or trauma. Characterization of the pathway involved in this type of swelling is hampered by the fact that most osmolyte amino acids are also neuroactive amino acids and may be released in response to stimuli concurrent with swelling, such as depolarization or intracellular Ca^{++} elevation.

Introduction

Cell volume perturbation is a challenge for homeostasis in all animal organs, but has particularly dramatic consequences in brain. The limits to expansion imposed by the rigid skull give narrow margins for the

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Fig. 1. Contribution of different types of osmolytes to volume adjustment in brain in vivo during chronic hyponatremia. Recalculated from: Lien et al, 1991; Verbalis and Gullans, 1991, 1993; Lien, 1995; Sterns et al, 1993. Glycero-phosphoryl choline (GPC), Phosphoetanolamine (PEA), N-acetylaspartate (NAA), Creatine (Cr), Phosphocreatine (PCr), Myo-inositol (Myo-I) and Amino acids (AA).
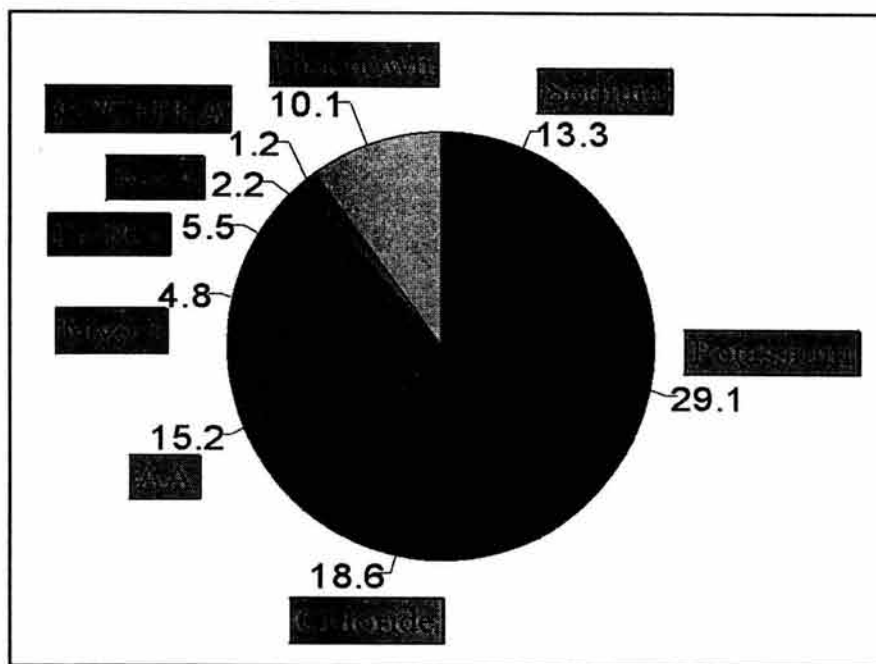
buffering of intracranial volume changes, leading to compression of small vessels generating episodes of anoxia and ischemia. As pressure increases, herniation leads to respiratory and cardiac arrest. Besides these extreme effects, brain cell swelling may also lead to hyperexcitability [1]. Early studies in chronic hyponatremia, showed that brain does not behave as a perfect osmometer, and the initial swelling is followed by progressive water loss until almost complete normalization, despite the persistence of hyponatremia. The observed electrolyte decrease was not sufficient to compensate for the loss of water and evidence was then obtained pointing to a significant contribution of organic osmolytes, including the most abundant amino acids, as well as of N-acetylaspartate, myo-inositol, creatine, phosphocreatine, phosphoetanolamine and glycero-phosphoryl choline [2, 3] (Fig. 1). In rodents, taurine is the most important organic osmolyte, because it is highly concentrated and shows the largest reduction during hyponatremia. In other species with lower brain taurine content, compounds such as N-acetylaspartate, may have an important role [4]. The estimation of organic osmolyte change in all these studies does not discriminate neither the regional variation within the brain nor possible differences in the cell type. *In vitro* studies in tissue slices as well as in cells in culture exposed to hyposmotic media represent a convenient model to address these questions.

Amino acids and regulatory volume decrease (RVD) during hyposmosis

Activation and inactivation of corrective fluxes

In cultured astrocytes and neurons, hyposmosis leads to rapid swelling followed by typical RVD. The efflux pattern of amino acids closely parallels the time course of the change in cell volume [5] in contrast to Cl⁻ and K⁺ fluxes which are faster (Cl⁻) or slower (K⁺) than the change in cell volume. Osmosensitive efflux of amino acids has also been reported in hippocampal and cortical slices [6, 7] and in vivo during continuous superfusion of cerebral cortex [8] or by microdialysis [9]. In all these preparations, taurine is the most sensitive to the osmotic perturbation, with the lowest release threshold and the largest amount released. Interestingly, in the neuroblastoma cell line CHP-100, glutamate is not responsive to hyposmosis [10].

Pasantes-Morales/Franco/Torres-Marquez/Hernández-Fonseca/Ortega

131

The amino acid efflux pathway

A leak pathway rather than an energy-dependent cotransporter, was first suggested as the mechanism for osmosensitive taurine release in Ehrlich ascites cells [11] and this was confirmed in brain cells, in which taurine translocation is passive, directed only by the concentration gradient. [12]. Unexpectedly, taurine efflux was found sensitive to Cl channel blockers [5], and this led to propose an anion channel-like molecule as the transport pathway for Cl and organic osmolytes [13]. The Cl channel involved in RVD is a volume-sensitive outwardly rectifying Cl channel (VSCC) of broad spectrum, permeable to most monovalent anions, and to some extent to large anions and to organic anions [14-17]. The properties of VSSC have been recently reviewed in detail [14, 15]. In brain cells, this channel has been characterized in the C6 glioma cell line [13], in cerebellar granule neurons [17] and in the N2A neuroblastoma [18]. Evidence in support of this VSCC as the common pathway for organic osmolytes is rather indirect, based essentially on the similar pharmacological profile of swelling-activated Cl currents and the swelling-induced osmolyte release [5, 13]. Currents carried through VSCC by glutamate, taurine and aspartate in the anion form, have been observed in MDCK, glioma and IMCD cells [19, 20]. Although these experiments do not prove the transport of neutral amino acids, they at least demonstrate that the size of the pore is sufficiently large for the passage of amino acids. Against this common pathway are findings of cell lines exhibiting Cl channel but not taurine fluxes and vice versa [16]. Also, different actions of blockers (arachidonic acid and DIDS) suggest different pathways [21, 22]. Swelling-induced taurine release without chloride channel activity in oocytes expressing anion channels and transporters also strongly favors the idea of separate pathways for taurine and Cl [23]. If this is the case, it should be a remarkable similarity between the molecular species permeating the two types of osmolytes, or a close interconnection between the fluxes of Cl and organic osmolytes.

Osmosensitive taurine fluxes appear to be carried by the anion exchanger in fish but not in mice erythrocytes [24, 25]. The protein domains responsible for the differences between mouse and trout anion exchangers have been identified, thus opening the possibility to select the anion exchanger forms in different cell types by the presence of these protein domains [26]. The phospholemman another candidate for taurine transport is a member of a superfamily of proteins with single trans-membrane domains exhibiting markedly high permeability to taurine, a feature possibly due to the presence of binding sites for cations and anions within the pore. Phospholemman is present in cultured astrocytes and neurons. Overexpression in HEK cells increases RVD, osmosensitive Cl currents and taurine fluxes [27, 28].

Activation and transduction signalling cascades

The trigger to activate the osmosensitive Cl/amino acid pathways and the identification of transduction signalling cascades are still unresolved. Available information refers mainly to the volume-activated Cl currents, with scarce confirmation about similarities or dissimilarities with the amino acid efflux pathways. Hyposmotic swelling leads to changes in the concentration of second messengers, such as Ca^{2+}, cAMP, IP3 and arachidonic acid [29]. It is still undefined whether these signalling systems are part of the mechanisms directly activating the osmolyte corrective fluxes or occur in connection with the set of other cell responses generated by hyposmosis. Cell swelling and RVD are complex phenomena involving cell reactions to stress, reorganization of the cytoskeleton, and adhesion or retraction mechanisms, among others. All of them activate their own signals, which may or may not be implicated in the activation of corrective osmolyte fluxes.

Calcium and calmodulin
Hyposmotic swelling is associated with an increase in $[Ca^{2+}]i$ levels, which occurs also in brain cells. However, with few exceptions, Cl currents and osmosensitive taurine fluxes are essentially Ca^{2+}-independent [30], although a minimal amount of cell Ca^{2+} (>50 nM), referred as permissive Ca^{2+}, appears necessary for the activation of Cl/taurine osmosensitive fluxes [31]. It might thus be that the swelling-induced $[Ca^{2+}]i$ increase is an epiphenomenon, unrelated to the corrective fluxes of osmolytes. Taurine fluxes are blocked by pimozide and trifluoperazine [6] but their effects appear unrelated to calmodulin systems since the inhibition persists in cells where taurine efflux is Ca^{2+}-independent or even in those where swelling does not elicit any Ca^{2+} increase.

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Protein kinases (PK)
PKC, PKA and cAMP. The osmosensitive amino acid fluxes appear largely PKC independent, as shown by the failure of PKC blockers or to maneuvers directed

to activate or down regulate the enzyme [32]. Taurine fluxes are PKC-independent in rat supraoptic nucleus and in cultured cerebellar granule neurons [33, 34]. Swelling- and stress-induced changes in cAMP levels have been reported in some but not in all cell types [15]. cAMP potentiates osmosensitive taurine efflux in C6 cells and in brain cortex [32, 35], but not in cerebellar granule neurons nor in supraoptic nucleus slices [33, 34]. Information about modulation of VSCC by protein kinases is reviewed in detail in [14, 15].

Protein tyrosine kinases

A role for protein tyrosine kinases (PTK) in the volume-related signalling is suggested by the numerous PTK which phosphorylate by swelling: p125[FAK], p38, JNK, p56[lck], p72[syk] and ERK1/ERK2 [36-40]. Further support comes from the potent inhibitory effect of PTK blockers on Cl- and taurine fluxes and the corresponding potentiation by the tyrosine phosphatase blocker o-vanadate [34, 36]. The specific kinases involved in amino acid release and the precise step of reaction have not been identified. Swelling activation of PTK does not necessarily imply a link with osmolyte fluxes, as occurs for ERK1/ERK2, for which prevention of the hyposmosis-induced phosphorylation has no effect on taurine fluxes or Cl- currents [34, 36]. The same lack of correlation is found for the stress-activated protein kinase p38 [41]. This dissociation suggests the involvement of some PTK in phenomena coincident with swelling, but not necessarily in the activation of osmosensitive fluxes. However, such correlation may be cell specific, as shown for ERKs phosphorylation and the osmosensitive Cl- current in cortical astrocytes [37]. Swelling-induced activation of p56lck in lymphocytes is required for VSCC functioning as p56lck deficiency by genetic knockout, leads to defective VSCC and RVD, a condition reversed by retransfection of the protein [39]. Swelling-induced tyrosine phosphorylation of band 3 (anion exchanger) in skate erythrocytes is also linked to p72[syk] and p56[lyn] [42]. This is the first report showing direct tyrosine phosphorylation of the osmolyte translocation pathway.

Tyrosine kinases and cytoskeleton

Cell swelling and RVD require a substantial reorganization of the cytoskeleton, to cope with the changes in cell volume and cell adhesion, but it is unclear whether these changes are directly involved in activation of osmolyte fluxes. A connection may be established through p21Rho, which is closely involved in the reor-

ganization of the actin cytoskeleton and also modulates osmosensitive Cl- currents [43, 44]. Downstream Rho, two possibilities have been explored, one of them suggesting a link with p125[FAK] and PI3 kinase, and another one proposing Rho kinase as the downstream target [43, 44]. Manipulation of these pathways has clear effects on VSCC, but less is known about their influence in amino acid fluxes. A cytoskeleton connection with taurine is suggested by decreased hyposmotic taurine efflux in astrocytes from vimentin/GFAP-deficient mice as compared to cells from the wild type mice [45].

PI3 kinase

Hyposmosis activates PI3K in some cells, and blockade by wortmannin, LY294002 or antibodies to the 110-catalytic subunit impairs cell volume recovery, VSCC activation, and the osmosensitive I[125] and taurine fluxes [34, 43, 46]. In cerebellar granule neurons, wortmannin but not LY294002, decreases the osmosensitive taurine efflux [34]. This difference may be due to permeability restrictions to LY294002 or to different sensitivity of PI3K isoforms. However, results based only on effects of wortmannin should be taken with caution, further considering that wortmannin may also affect phospholipase A and the myosin light chain, two proteins which also appear involved in osmolyte fluxes [47].

Phosholipases (PL)

Implication of PLAs in osmolyte fluxes came from the early work by Hoffman, Lambert and coworkers [48] in Ehrlich ascites cells, showing an effect of leukotrienes LTC4 and LTD4 accelerating RVD and enhancing taurine efflux under isotonic conditions. This may not be a general mechanism of osmolyte activation since LTD4 does not affect the osmosensitive Cl- currents in many other cell types [14,15]. However, PLA2 may still modulate the taurine/Cl efflux pathways as shown by a study in neuroblastoma CP100 cells, where swelling increases arachidonic acid release, which, if prevented by AACOCF3, inhibits taurine and Cl- fluxes [49]. At variance with these results in isolated cells, in rat brain cortex in vivo, amino acid fluxes are essentially unaffected by the PLA2 blockers pBPB, DEDA and AACOCF3 [32].

The connection between all these enzymes remains to be established. PI3K is a key intermediate in signaling cascades, interacting notably with the Rho GTP-binding proteins, which as discussed above, appear to criti-

Pasantes-Morales/Franco/Torres-Marquez/Hernández-Fonseca/Ortega

cally influence VSCC. An association between PI3K, Rho GTPases and phospholipases has been shown in a variety of pathways, some of them regulating the dynamics of the cortical and cytoplasmic actin cytoskeleton. A possibility that cannot be ruled out is that PLA2 is acting at the very early steps of the signalling cascades acting as a volume sensor, since there is some evidence in support of PLA2 as a mechanosensor [50].

The influence of ionic strength

The importance of intracellular ionic strength as a regulatory signal for activation of taurine fluxes in trout erythrocytes was first proposed by Motais et al. [51]. Consistent with this proposal are findings in C6 glioma and in CHO cells showing that, as intracellular ionic strength increases, larger volume changes are progressively required to activate taurine efflux [52]. In cortical astrocytes, taurine efflux is notably higher when swelling decreases ionic strength as in hyposmotic- or urea-induced swelling, as compared with K-generated swelling occurring without a decrease in ionic strength [53]. In CPAE cells, a Cl⁻ current identical to that elicited by hyposmotic swelling is activated by reducing the ionic strength at constant osmolarity. All these results suggest an effect of intracellular ionic strength either shifting the volume set point [52] or directly acting as activation signal [54]. A recent study in skate red blood cells confirms the above results, but in addition, demonstrate the influence of ionic strength on the activity of some PTK directly involved in the activation of taurine fluxes [55].

Amino acids and isosmotic swelling

Brain cell edema in isosmotic conditions (also called cytotoxic edema) conveys more risks than hyposmotic swelling, since in cytotoxic swelling there is no clear evidence of efficient cell volume correction. Ischemic stroke, head trauma and hepatic encephalopathy, are pathological conditions associated with brain edema, leading to a critical clinical challenge. Swelling also occurs in excitotoxicity and seizures [56]. The mechanisms generating swelling may be somewhat different in each pathology, but in all cases, the influx of anions, Cl⁻ or/and bicarbonate-, is a consistent causal factor.

As mentioned above, in brain cells adaptive mechanisms during isosmotic swelling appear less efficient than

in hyposmotic swelling. This may have to do with a difficulty for ionic osmolytes to be released when ion accumulation is the condition generating swelling. In this case, the contribution of organic osmolytes may not be sufficient to regulate cell volume, although it is certainly important to attenuate the magnitude of swelling. Amino acid efflux during cytotoxic swelling has been observed in experimental models of ischemia, from *in vitro* chemical models or *in vivo*, by vein occlusion. Cell exposure to high K⁺ concentrations, as occurs in most situations leading to cytotoxic swelling, is often used to generate cytotoxic edema. Activation of the various excitatory receptor subtypes by glutamate, kainate and other agonists, are also experimental models simulating cytotoxic swelling. In all these cases, fluxes of taurine, glutamate, GABA and glycine, consistently increase. However, it should be mentioned that these amino acids are all neuroactive compounds, and some of them are important synaptic transmitters. Therefore, when evaluating the effect of cytotoxic edema on amino acid release, it is necessary to discriminate between a pure response to swelling and that related to other signals, as depolarization or Ca²⁺ entry, concurrent with ischemia, epileptic activity or excitotoxicity. Conversely, increase of extracellular K⁺ or glutamate concentration known to occur in ischemia or epileptic episodes, and currently attributed to neuronal hyperexcitability, may rather be a response to swelling. This point has been addressed recently in studies about the mechanism of glutamate efflux in ischemia. Two possibilities have been considered. One explains the increase in extracellular glutamate concentration as a result of swelling-activated corrective efflux [56, 57]. The other one implicates a reverse operation of the energy-dependent glutamate transporters, due to intracellular Na⁺ accumulation resulting from the energy failure, and thus, unrelated to swelling. Both possibilities have experimental support, but the option involving an impaired transport mechanism is more favored at present [58]. An effect of swelling on glutamate transporters has been described, which may link the two options [56]. The same situation applies for other amino acids such as aspartate, GABA, taurine and glutamine, which are also released in ischemic conditions, and are also transported by energy-dependent carriers [59-61]. Depolarization concurrent with ischemia may also trigger amino acid release. Differences may exist, though, in the relative sensitivity of amino acids to swelling or depolarization, which may be useful to estimate their role

as osmolytes in cytotoxic edema. In any event, the release of inhibitory amino acids such as taurine, GABA or glycine, which in contrast to glutamate, do not generate *per se* a secondary volume increase, nor excitotoxicity, may contribute to attenuate swelling and additionally, to counteract the hyperexcitability generated by K^+ and glutamate.

Studies in ischemic models of vein occlusion, have shown a blockade of amino acid fluxes by anion channel inhibitors, as in hyposmotic swelling, suggesting similar translocation pathways in the two conditions [61,62]. However, it is worthy to emphasize that these agents affect most Cl^- channel types and may affect Cl^- influx which is an essential causal element of swelling. Therefore, a reduced efflux of amino acids may be due, not to an effect on the efflux pathway, but a consequence of less swelling by Cl^- influx reduction. Cytotoxic swelling associated with hyperamonemia or with head trauma also involves an increased efflux of amino acids, including excitotoxic amino acids. The mechanism of this release is still unclear, but at least for glutamate and taurine, it seems not directly related to a swelling-induced efflux [63]. Lactacidosis is a prominent sequel in ischemic and traumatic brain tissue resulting in glial cell swelling. The swelling mechanism involves activation of coupled Na^+/H^+ and Cl^-/HCO_3^- antiporters, resulting in intracellular accumulation of NaCl and water. There is no evidence for compensatory mechanisms to regulate swelling under these conditions. Also there is as yet only scarce information about activation of osmolyte fluxes associated with this model of swelling. A microdialysis study on NH_4^+ -induced acidosis reports an increase in extracellular N-acetyl-aspartate, an amino acid present in large amounts in neurons, which may have an important role as osmolyte [4].

There is scarce information about possible signalling cascades for activation of mechanisms of cell volume control in isosmotic swelling. The work by Phillis, O'Regan and their collaborators [60] in a model of ischemic rat brain cortex, have shown the influence of PKC based on the stimulatory effect of phorbol esters and the inhibition by chelerythrine on glutamate and aspartate fluxes increased during ischemia. PKA seems not involved in this process. It also documents the importance of phospholipases, PLC and particularly of PLA2, in this mechanism of ischemia-induced amino acid efflux [60]. They also suggest an influence of PTK, since the tyrosine kinase inhibitor genistein, attenuates neurotransmitter release from the ischemic rat cerebral cortex [64]. Recently these authors have made a comparison between the features of amino acid release during ischemia and during hyposmotic swelling in the rat brain. They found important differences, particularly regarding the role of PLA2, which being critical for activation of amino acid release in ischemia, seems to play a minor role in the hyposmotic-stimulated release of amino acids in the same preparation [32]. Also, while PKC modulates ischemia-induced amino acid release, it has no major influence on the hyposmolarity-associated release [32]. These comparative studies are crucial to identify the signalling elements associated with isosmotic swelling within the complex set of responses evoked by ischemia.

Identification of the transduction cascades in isosmotic swelling may be further complicated by the fact that essentially all conditions generating this type of swelling represent severe stressful situations, resulting in activation of numerous signalling elements associated with stress [65]. Some of them such as MAPK and PI3K, are also activated during hyposmotic swelling. The associated events of depolarization and excitotoxicity specific to brain tissue, also activate numerous signalling cascades [66]. All this makes it very difficult to discriminate among the spectrum of responses, those solely attributable to swelling. An experimental maneuver to circumvent this problem, which could be approached in preparations *in vitro*, is to elicit the ischemic or any other situation of cytotoxic swelling, but reducing the external concentration of Cl^-, which would largely attenuate swelling without decreasing the depolarization or other stimuli. Then, a parallel analysis of the signalling cascades activated in both conditions, may help to identify those associated with swelling, and from them, those related to the corrective fluxes of osmolytes.

It has been consistently observed that cytotoxic edema *in vivo* is more prominent in astrocytes than in neurons, being so far unclear whether this difference is due to a selective localization of the swelling-generating mechanisms in astrocytes, or to the presence in neurons of more efficient mechanisms of cell volume control. In this respect, a most interesting observation is the transfer of taurine and glutamate from neurons to astrocytes during experimental ischemia [67]. By this mechanism, neurons are spared and protected from the deleterious effects of swelling.

Pasantes-Morales/Franco/Torres-Marquez/Hernández-Fonseca/Ortega

136

Isovolumetric regulation: role of amino acids

Although the experimental model of sudden and marked decreases in osmolarity has rendered valuable information to elucidate the basic mechanisms of cell volume control, such changes probably never occur in brain under physiological conditions. Even during pathological situations such as chronic hyponatremia, water intoxication or the inappropriate handling of antidiuretic hormone, the osmolarity changes in the interstitial space in brain occur most likely in a gradual manner as the osmotic challenge from plasma progressively surpasses the brain homeostatic resistance. A paradigm closer to the situation in vivo, was that assayed first by Lohr and Grantham [68] in renal proximal tubules, in which cells were exposed to small and gradual decreases in osmolarity. Under these conditions, cell volume remains stable over a broad range of osmolarities, even when the osmolarity drops up to 50%. This constancy in cell volume was named "isovolumetric regulation" (IVR). This term has implicit the idea of an active mechanism of cell adjustment, based on the shrinkage in cells returned to an isosmotic medium, which due to the loss of intracellular osmolytes is now hyperosmotic with respect to the intracellular medium [68]. After this early report, IVR has been observed in the renal cell line A6, in the glioma cells C6 [69, 70], in cerebellar granule neurons (unpublished), in cardiomyocytes [71] and in a more integrated preparation, the hippocampal slice [72]. In contrast, partial IVR is found in cardiomyocytes and no IVR is observed in trout erythrocytes [71, 73] . The mechanisms subserving IVR have not been explored in detail. In A6 cells, increased K^+ efflux is observed at 30% reductions in external osmolarity [68]. K^+ efflux with a similar threshold was found in cultured cerebellar granule neurons (unpublished results) and in cardiomyocytes [71]. K^+ efflux during IVR is decreased by Ba^{2+}, but is insensitive to 4AP, TEA and charybdotoxin (unpublished results). The Cl^- pathway activated during IVR seems to have marked differences with the VSCC, as IVR is impaired when Cl^- is replaced by other anions which permeate through VSCC [69]. This may suggest the involvement of electroneutral cotransporters, known to be more selective for anions than VSCC. In hippocampal slices, IVR occurred without any measurable release of K^+ [72]. This is an unexpected finding which may be attributable to the large K^+ buffering capacity of astrocytes. We have addressed the role of amino acids in IVR in hippocampal slices and found increased fluxes of taurine, GABA and glutamate (Fig. 2). The efflux of taurine shows the lowest threshold, and the highest efflux rate with 4-10-fold differences at essentially all osmolarities. This may reflect a higher permeation through the efflux pathway or/and more availability of the taurine pools to be released in response to the change in cell volume. This may be related to features of taurine such as its metabolic inercy and its mainly cytoplasmic location, while GABA, glycine and glutamate, which have a prominent role as synaptic transmitters or are part of numerous metabolic cascades, may be sequestered in compartments which restrict their availability for osmosensitive release. Taurine efflux during IVR has been shown in cardiac myocytes and in trout erythrocytes, with reductions of 10-17% in taurine cell content [71, 73].

About 30% in average, of the amino acid content in cells or slices is released during IVR. This is clearly insufficient to compensate for the change in external osmolarity when K^+ fluxes have not yet been activated. Therefore, other factors should be considered to explain the maintenance of cell volume under these conditions. One or several of the following possibilities are plausible: 1) swelling is overall restricted when the osmolarity change is small and gradual, 2) other organic osmolytes, such as creatine, myo-inositol, sorbitol, N-acetyl aspartate, phosphocreatine and phosphoethanol amine, are also contributing to counteract the external osmolarity, and altogether compensate for the initial phase of hyposmotic stress, 3) a Cl^- efflux activates, accompanied by cations other than K^+, 4) rapid metabolic changes such as synthesis of macromolecules, i.e. glycogen, may contribute to reduce the intracellular osmolyte pool necessary to reach the osmotic equilibrium [74]. In more integrated preparations, such as the hippocampal slice, it may happen that swelling occurs in some but not in all cells, and therefore, the decrease in amino acids and other osmolytes is required to compensate the change in cell volume only in a minor population of cells. Also, a redistribution of osmolyte amino acids between different types of cells i.e. neurons and astrocytes may occur, as observed in mice cerebellum where taurine is translocated from Purkinje cells to astrocytes in response to hyponatremia [75]. In this situation, even though amino acids contribute importantly to regulate cell volume in specific types of cells, this may not result in a large net efflux. Finally, it should be no-

Fig. 2. Amino acid release from hippocampal slices exposed to gradual and progessive reductions in external osmolarity. Slices preloaded with [³H]-taurine (●), D-[³H]aspartate (■), or [³H]GABA (▲), were superfused 10 min with isosmotic medium. At the time pointed by the arrow the external osmolarity was continuosly decreased at a rate of −2.5 mOsm/min until the medium osmolarity reached 150 mOsm (50% hyposmotic). Data are expresed as efflux rate constant (min⁻¹) and are means ± SE (n = 8-10).
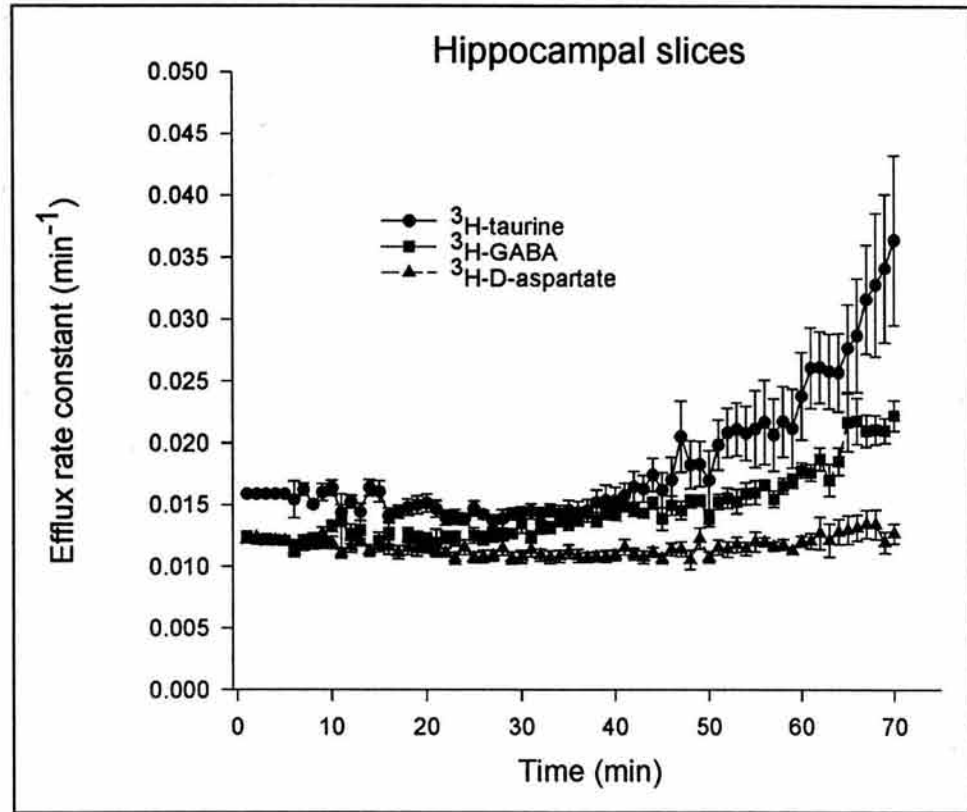
ticed that in pathological situations, such as chronic hyponatremia, when the external osmolarity decrease is small but persists during several hours, or even days, the decrease in amino acids and other osmolytes during this period is substantial, being almost 90% in the case of taurine [2]. This has been reproduced in vitro by Olson [76] in cultured astrocytes, which showed no change in cell volume after 24 h of hyposmolarity, coincident with an almost total depletion of the taurine pool, and no significant changes in the concentration of glutamate and K^+. This points to the role played by taurine as an osmolyte of choice for cell volume control in physiopathological conditions.

The similarities or differences, which may exist between the amino acid osmolyte pathway activated during IVR and RVD, have not been explored in detail. There is also no information about transduction signalling cascades leading to activation of this mechanism of cell volume control. In this respect, it is worthy to mention that IVR may be a better system than RVD for the study of signalling cascades primarily associated with osmotransduction. In the absence of the dramatic changes in cell volume occurring during RVD, changes associated with cytoskeleton reorganization, adhesion and even stress, would be reduced and consequently, the remaining set of signals expressed during IVR may be more easily adscribed to specific aspects of cell volume regulation.

Acknowledgements

Pasantes-Morales/Franco/Torres-Marquez/Hernández-Fonseca/Ortega

138

References

1　Chebabo SR, Hester MA, Jing J, Aitken PG, Somjen GG: Interstitial space, electrical resistance and ion concentrations during hypotonia of rat hippocampal slices. J Physiol 1995;487:679-689.

2　Verbalis JG, Gullans SR: Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res 1991;567:274-282.

3　Sterns RH, Baer J, Ebersol S, Thomas D, Lohr JW, Kamm DE: Organic osmolytes in acute hyponatremia. Am J Physiol 1993;264:F833-F836.

4　Gotoh M, Davies SE, Obrenovich TP: Brain tissue acidosis effects on the extracellular concentration of N-acetylaspartate. J Neurochem 1997;69:655-661.

5　Pasantes-Morales H: Volume regulation in brain cells: cellular and molecular mechanisms. Met Brain Dis 1996;11:187-204.

6　Law RO: Taurine efflux and the regulation of cell volume in incubated slices of rat cerebral cortex. Biochim Biophys Acta 1994;1221:21-28.

7　Haugstad T S, Langmoen IA: Release of brain amino acids during hyposmolar stress and energy deprivation. J Neurosurg Anesthesiol 1996;8:159-168.

8　Estevez AY, O'Regan MH, Song D, Phillis JW: Effects of anion channel blockers on hyposmolarity induced amino acid release from the in vivo rat cerebral cortex. Neurochem Res 1999;24:447-452.

9　Solís JM, Herranz AS, Herraz O, Lerma J, Del Rio RM: Does taurine act as an osmoregulatory substance in the rat brain? Neurosci Lett 1988;91:53-58.

10　Basavappa S, Huang CC, Mangel AW, Lebedev DV, Knauf PA, Ellory JC: Swelling-activated amino acid efflux in human neuroblastoma cell line CHP-100. J Neurophysiol 1996;76:764-769.

11　Hoffmann EK, Lambert IH, Simonsen LO: Mechanisms in volume regulation in Ehrlich ascites tumor cells. Renal Physiol Biochem 1988;11:221-247.

12　Sánchez-Olea R, Morán J, Schousboe A, Pasantes-Morales H: Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. Neurosci Lett 1991;130:233-236.

13　Strange K, Jackson PS: Swelling-activated organic osmolyte efflux: a new role for anion channel. Kidney Int 1995;48:994-1003.

14　Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G: Properties of volume-regulated anion channels in mammal cells. Prog Biophys Mol Biol 1997;68:69-119.

15　Okada Y: Volume expansion-sensing outward-rectifier Cl- channel: fresh star to the molecular identity and volume sensor. Am J Physiol 1997;273:C755-C789.

16　Kirk K: Swelling-activated organic osmolyte channels. J Membr Biol 1997;158:1-6.

17　Patel AJ, Lauritzen I, Lazdunski M, Honoré E: Disruption of mitochondrial respiration inhibits volume-regulated anion channels and provoques neuronal cell swelling. J Neurosci 1998;18:3117-3123.

18　Carpaneto A, Accardi A, Pisciotta M, Gambale F: Chloride channel activated by hypotonicity in N2A neuroblastoma cell line. Brain Res 1999;124:193-199.

19　Roy G: Amino acid current through anion channel in cultured human glial cells. J Membr Biol 1995;147:35-44.

20　Boese SH, Wehner F, Kinne RK: Taurine permeation through swelling-activated anion conductance in rat IMCD cells in primary culture. Am J Physiol 1996;271:F498-F507.

21　Lambert IH, Hoffmann EK: Cell swelling activates separate taurine and chloride channels in Ehrlich mouse ascites tumor cells. J Membr Biol 1994;142:289-298.

22　Stutzin A, Torres R, Oporto M, Pacheco P, Eguiguren AL, Cid LP, Sepulveda FV: Separate taurine and chloride efflux pathways activated during regulatory volume decrese. Am J Physiol 1999;277:C392-C402.

23　Stegen C, Matskevich I, Wagner CA, Paulmilch M, Lang F, Broer S: Swelling-induced taurine release without chloride channel activity in Xenopus laevis oocytes expressing anion channels and transporters. Biochim Biophys Acta 2000;31:91-100.

24　Goldstein L, Brill SR: Volume activated taurine efflux from skate erythrocytes: possible band 3 involvement. Am J Physiol 1991;260:R1014-R1020.

25　Motais R, Fievet B, Borgese F, Garcia-Romeu F: Association of the band 3 protein with a volume-activated, anion and amino acid channel: a molecular approach. J Exp Biol 1997;200:361-367.

26　Fievet B, Gabillat N, Borgese F, Motais R: Expression of band 3 anion exchanger induces chloride current and taurine transport: structure-function analysis. EMBO J 1995;14:5158-5169.

27　Moorman JR, Jones L: Phospholemman: a cardiac taurine channel involved in regulation of cell volume. Adv Exp Med Biol 1998;442:219-228.

28　Morales-Mulia, M., Pasantes-Morales, H. and Morán, J. Volume-sensitive efflux of taurine in HEK293 cells overexpressing phospholemman. Biochim Biophys Acta 2000;1496:252-260.

29　Hoffmann EK, Dunham PB: Membrane mechanisms and intracellular signalling in cell volume regulation. Int Rev Cytol 1995;161:173-262.

30　Pasantes-Morales H, Morales-Mulia S: Influence of calcium on regulatory volume decrease: role of potassium channels. Nephron 2000, in press.

31　Szucs G, Heinke S, Droogmans G, Nilius B: Activation of the volume-sensitive chloride current in vascular endothelial cells requires a permissive intracellular Ca^{2+} concentration. Pflügers Arch 1996;431:467-469.

32　Estevez AY, O'Regan MH, Song D, Phillis JW: Hyposmotically induced amino acid release from the rat cerebral cortex: role of phospholipases and protein kinases. Brain Res 1999;844:1-9.

33　Deleuze C, Duvoid A, Moos FC, Hussy N: Tyrosine phosphorylation modulates the osmosensitivity of volume-dependent taurine efflux from glial cells in the rat supraoptic nucleus. J Physiol 2000;523:291-299.

34　Morales-Mulia S, Cardin V, Torres-Márquez ME, Crevenna A, Pasantes-Morales H: Influence of protein kinases on the osmosensitive release of taurine from cerebellar granule neurons. Neurochem Int 2000, in press.

35　Jackson PS, Strange K: Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. Am J Physiol 1993;265:C1489-C1500.

36　Tilly BC, van den Berghe N, Tertoolen LG, Edixhoven MJ, de Jonge HR: Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J Biol Chem 1993;268:19919-19922.

37　Crepel V, Panenka W, Kelly ME, MacVicar BA: Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. J Neurosci 1998;18:1196-11206.

38　Niisato N, Post M, Van Driessche W, Marunaka Y: Cell swelling activates stress-activated protein kinases, p38 MAP kinase and JNK, in renal epithelial A6 cells. Biochem Biophys Res Commun 1999;266:547-550.

39　Lepple-Wienhues A, Szabo I, Laun T, Kaba NK, Gulbins E, Lang F: The tyrosine kinase p56lck mediates activation of swelling-induced chloride channels in lymphocytes. J Cell Biol 1998;141:281-286.

40　Musch MW, Hubert EM, Goldstein L: Volume expansion stimulates p72(syk) and p56(lyn) in skate erythrocytes. J Biol Chem 1999;274:7923-7928.

41　Tilly BC, Gaestel M, Engel K, Edixhoven MJ, de Jonge HR: Hypo-osmotic cell swelling activates the p38 MAP kinase signalling cascade. FEBS Lett 1996;395:133-136.

42　Hubert EM, Musch MW, Goldstein L: Inhibition of volume-stimulated taurine efflux and tyrosine kinase activity in the skate red blood cell. Pflügers Arch 2000;440:132-139.

43 Tilly BC, Edixhoven MJ, Tertoolen LG, Morii N, Saitoh Y, Narumiya S, de Jonge HR: Activation of the osmo-sensitive chloride conductance involves P²¹rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. Mol Biol Cell 1996;7:1419-1427.

44 Nilius B, Voets T, Prenen J, Barth H, Aktories K, Kaibuchi K, Droogmans G, Eggermont J: Role of Rho and Rho kinase in the activation of volume-regulated anion channels in bovine endothelial cells. J Physiol 1999;516:67-74.

45 Ding M, Eliasson C, Betsholtz C, Hamberger A, Pekny M: Altered taurine release following hypotonic stress in astrocytes from mice deficient for GFAP and vimentin. Brain Res Mol Brain Res 1998;62:77-78.

46 Feranchak AP, Roman RM, Schwiebert EM, Fitz JG: Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. J Biol Chem 1998;273:14906-14911.

47 Nilius B, Prenen J, Walsh MP, Carton I, Bollen M, Droogmans G, Eggermont J: Myosin light chain phosphorylation-dependent modulation of volume-regulated anion channels in macrovascular endothelium. FEBS Lett 2000;466:346-350.

48 Lambert IH: Regulation of the taurine content in Ehrlich ascites tumour cells. Adv Exp Med Biol 1998;442:269-276.

49 Basavappa S, Pedersen SF, Jorgensen NK, Ellory JC, Hoffmann EK: Swelling-induced arachidonic acid release via the 85-kDa cPLA2 in human neuroblastoma cells. J Neurophysiol 1998;79:1441-1449.

50 Lehtonen JY, Kinnunen PK. Phospholipase A2 as a mechanosensor. Biophys J 1995;68:1888-1894.

51 Motais R, Guizouarn H, Garcia-Romeu F: Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems. Biochim Biophys Acta 1991;1075:169-180.

52 Cannon CL, Basavappa S, Strange K: Intracellular ionic strength regulates the volume sensitivity of a swelling-activated anion channel. Am J Physiol 1998;275:C416-C422.

53 Cardin V, Pena-Segura C, Pasantes-Morales H. Activation and inactivation of taurine efflux in hyposmotic and isosmotic swelling in cortical astrocytes: role of ionic strength and cell volume decrease. J Neurosci Res 1999;56:659-667.

54 Voets T, Droogmans G, Raskin G, Eggermont J, Nilius B: Reduced intracellular ionic strength as the initial trigger for activation of endothelial volume-regulated anion channels. Proc Natl Acad Sci 1999;96:5298-5303.

55 Wittels KA, Hubert EM, Musch MW, Goldstein L: Osmolyte channel regulation by ionic strength in skate RBC. Am J Physiol 2000;279:R69-R76.

56 Kimelberg HK:Current concepts of brain edema. Review of laboratory investigations. J Neurosurg 1995;83:1051-1059.

57 Seki Y, Feustel PJ, Keller RW Jr, Tranmer BI, Kimelberg HK: Inhibition of ischemia-induced glutamate release in rat striatum by dihydrokinate and an anion channel blocker. Stroke 1999;30:433-440.

58 Rossi DJ, Oshima T, Attwell D: Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 2000;403:316-321.

59 Phillis JW, Smith-Barbour M, Perkins LM, O'Regan MH: Characterization of glutamate, aspartate, and GABA release from ischemic rat cerebral cortex Brain Res Bull 1994;34:457-466.

60 Phillis JW, O'Regan MH: Mechanisms of glutamate and aspartate release in the ischemic rat cerebral cortex. Brain Res 1996;730:150-164.

61 Phillis JW, Song D, O'Regan MH: Inhibition by anion channel blockers of ischemia-evoked release of excitotoxic and other amino acids from rat cerebral cortex. Brain Res 1997;758:9-16.

62 Estevez AY, Song D, Phillis JW, O'Regan MH: Effects of the anion channel blocker DIDS on ouabain- and high K(+)-induced release of amino acids from the rat cerebral cortex. Brain Res Bull 2000;52:45-50.

63 Albrecht J: Roles of neuroactive amino acids in ammonia neurotoxicity. J Neurosci Res 1998;51:133-138.

64 Phillis JW, Song D, O'Regan MH: Inhibition of tyrosine phosphorylation attenuates amino acid neurotransmitter release from the ischemic/reperfused rat cerebral cortex. Neurosci Lett 1996;207:151-154.

65 Irving EA, Barone FC, Reith AD, Hadingham SJ, Parsons AA: Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat. Brain Res Mol Brain Res 2000;77:65-75.

66 Mielke K, Brecht S, Dorst A, Herdegen T: Activity and expression of JNK1, p38 and ERK kinases, c-Jun N-terminal phosphorylation, and c-jun promoter binding in the adult rat brain following kainate-induced seizures. Neurosci 1999;91:471-483.

67 Torp R, Andine P, Hagberg H, Karagulle T, Blackstad TW, Ottersen OP: Cellular and subcellular redistribution of glutamate-, glutamine- and taurine-like immunoreactivities during forebrain ischemia: a semiquantitative electron microscopic study in rat hippocampus. Neurosci 1991;41:433-447.

68 Lohr JW, Grantham JJ: Isovolumetric regulation of isolated S2 proximal tubules in anisotonic media. J Clin Invest 1986;78:1165-11672.

69 Van Driessche W, De Smet P, Li J, Allen S, Zizi M, Mountian I: Isovolumetric regulation in a distal nephron cell line (A6). Am J Physiol 1997;272:C1890-C1898.

70 Lohr JW, Yohe L: .Isovolumetric regulation of rat glial cells during development and correction of hypo-osmolality. Neurosci Lett 2000;286:5-8.

71 Souza MM, Boyle RT, Melvyn L. Different physiological mechanisms control isovolumetric regulation and regulatory volume decrease in chick embryo cardiomyocytes. Cell Biol Intern 2000;24:713-721.

72 Franco R, Quesada O, Pasantes-Morales H: Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J Neurosci Res 2000;61:701-711.

73 Godart H, Ellory JC, Motais R: Regulatory volume response of erythrocytes exposed to a gradual and slow decrease in medium osmolality. Pflügers Arch 1999;437:776-779.

74 Häussinger D: Regulation of cell function by level of hydration. Naturwissenschaften 1996;83:264-271.

75 Nagelhus EA, Lehmann A, Ottersen OP: Neuronal-glial exchange of taurine during hypo-osmotic stress: a combined immunocytochemical and biochemical analysis in rat cerebellar cortex. Neurosci 1993;54:615-631.

76 Olson JE: Osmolyte contents of cultured astrocytes grown in hypoosmotic medium. Biochim Biophys Acta 1999;1453:175-179.

140

REVIEW ARTICLE

Mechanisms Counteracting Swelling in Brain Cells During Hyponatremia

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Water gain in the brain consequent to hyponatremia is counteracted by mechanisms that initially include a compensatory displacement of liquid from the interstitial space to cerebrospinal fluid and systemic circulation and subsequently an active reduction in cell water accomplished by extrusion of intracellular osmolytes to reach osmotic equilibrium. Potassium (K^+), chloride (Cl^-), amino acids, polyalcohols, and methylamines all contribute to volume regulation, with a major contribution of ions at the early phase and of organic osmolytes at the late phase of the regulatory process. Experimental models *in vitro* show that osmolyte fluxes occur via leak pathways for organic osmolytes and separate channels for Cl^- and K^+. Osmotransduction signaling cascades for Cl^- and taurine efflux pathways involve tyrosine kinases and phosphoinositide kinases, while Ca^{2+} and serine-threonine kinases modulate K^+ pathways. In-depth knowledge of the cellular and molecular adaptive mechanisms of brain cells during hyponatremia contributes to a better understanding of the associated complications, including the risks of inappropriate correction of the hyponatremic condition. © 2002 IMSS. Published by Elsevier Science Inc.

Key Words: Volume regulation, Taurine, Hyposmolarity, Regulatory volume decrease.

Introduction

The ability to regulate cell volume is an ancient conserved trait present in essentially all species throughout evolution. Maintenance of constant cell volume is a homeostatic imperative in animal cells. Changes in cell water content by affecting the concentration of messenger molecules impair the complex signaling network crucial for cell functioning and intercellular communication. Although under physiologic conditions extracellular fluids have a highly controlled osmolarity, a variety of diseases is paralleled by alterations of systemic osmolarity. In addition, the intracellular volume constancy is continuously compromised by the generation of local and transient osmotic microgradients associated with uptake of nutrients, secretion, cytoskeletal remodeling, and transsynaptic ionic gradients.

Cell volume disturbances have particularly dramatic consequences in the brain. The limits to expansion imposed by

the rigid skull give narrow margins for buffering of intracranial volume changes. As expansion occurs, constraining of small vessels generates episodes of anoxia ischemia, infarct, excitotoxicity, and neuronal death. Under extreme conditions, caudal herniation of the brain parenchyma through the foramen magnum affects brain stem nuclei, resulting in death by respiratory and cardiac arrest (1).

Hyponatremia is the most common cause of hyposmotic swelling in brain cells. This condition results from an imbalance between intake and excretion of water and electrolytes derived from either an excess of water or a sodium (Na^+) deficit. Water excess may derive from excessive oral intake as in psychotic polydipsia, or more commonly from impaired renal elimination as a consequence of inappropriate secretion of antidiuretic hormone, glucocorticoid deficiency, hypothyroidism, use of thiazide diuretics, and renal or hepatic failure. A variety of diseases or conditions such as head trauma, brain tumor, and cerebrovascular accidents result in hyponatremia associated with the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) or the cerebral salt-wasting syndrome (CSWS). Both syndromes have marked similarities with regard to clinical context and presentation, with euvolemia in SIADH and hypovolemia in

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CSWS the most clear contrasting variables. CSWS is characterized by excessive renal sodium loss (eventually in relation to a natriuretic factor) resulting in volume depletion and hyponatremia (2). Na^+ loss also results from mineralocorticoid deficiency, nephrotic syndrome, osmotic diuresis, vomiting, or diarrhea. Hyponatremia may also be caused by rapid correction of uremia by excessive hemodialysis and by infusion of hypotonic solutions in the perioperative period. Hyponatremia is a common state in the elderly and during pregnancy (3–5). Fatal hyponatremia-induced cerebral edema has been recently associated with the use of the drug Ecstasy (6).

Adaptive Response of Brain Cells to Hyposmotic Conditions. Studies *In Vivo*

In the face of a decrease in external osmolarity, the brain does not exhibit the behavior predicted for a perfect osmometer. During chronic hyponatremia, only approximately 40% of the expected water gain occurs within the first hours; thereafter, total water content decreases progressively to nearly complete normalization (7). The first adaptive response is a compensatory displacement of liquid from the interstitial space to the cerebrospinal fluid; thereafter, the excess in cerebrospinal fluid enters the systemic circulation. The next adaptive brain reaction is the extrusion of intracellular solutes, mainly the inorganic ions potassium (K^+) and chloride (Cl^-) and a number of small organic molecules, prominently amino acids with osmotically obligated water. Some loss of Na^+ is also observed in whole brain studies, likely displaced from the extracellular space (8). Studies in animal models of chronic hyponatremia have shown the greatest loss of Na^+ and Cl^- during the first 3 h, while K^+ loss is slower, achieving significance only after this first time period (8). From early studies on this subject, it was evident that the decrease in electrolytes was not sufficient to compensate for the loss of water observed and that the involvement of other osmotically active solutes needed to be considered. These molecules, initially referred to as idiogenic osmolytes, were further identified as organic molecules such as amino acids, polyalcohols, and methylamines, which were found to contribute significantly to the adaptive brain response to hyponatremia (8). A decrease in the concentration of myo-inositol, phosphocreatine/creatine, glycerophosphoryl choline and of the most abundant amino acids (glutamate, glutamine, taurine, and glycine) has been consistently observed in chronic hyponatremia (9,10). The contribution of organic osmolytes and electrolytes to the total brain osmolarity change has been estimated as 23–29% and 62–70%, respectively (Table 1). While decreases of electrolytes reverse with time, decreases of organic osmolytes, particularly taurine, are sustained as long as hyponatremic conditions persist (9).

Taken together, these studies show that immediate response to hyponatremia in brain is in charge of K^+ and Cl^-

Table 1. Electrolytes and organic osmolyte content in rat brain during chronic hyponatremia

Osmolyte (nmol/kg DBW)[a]	Normonatremic	Hyponatremic (nmol/kg DBW)	Decrease
Electrolytes			
Sodium	279	250	29
Potassium	480	424	56
Chloride	152	118	34
All electrolytes	911	792	192
Organic osmolytes			
Glutamate	52.9	32.5	20.4
Glutamine	14.2	6.5	7.7
Taurine	13.8	2.1	11.7
GABA	1.7	0.9	0.8
Aspartate	2.2	1.7	0.5
N-acetylaspartate	7.5	5.9	1.6
Myo-inositol	16	5.3	10.7
Creatine	34.8	17.1	17.7
Phosphoethanolamine	1.2	0.8	0.4
GPC[b]	1.1	0.6	0.5
All organic osmolytes	145.4	73.4	72

[a]DBW: dry brain weight. Recalculated from (8,9). Data are from rats after 2–3 days of hyponatremia. [b]GPC: glycerophosphorylcholine.

efflux, and that sustained adaptation is carried out by organic osmolytes, particularly taurine. This has been confirmed in studies *in vitro* in astrocytes demonstrating how myo-inositol- and taurine swelling-activated efflux persists for several hours after the hyposmotic stimulus, in contrast to glutamate and K^+, which remained unchanged (11,12). These results highlight differences in handling the various osmolytes. Loss of K^+ and Cl^- is an emergency mechanism to counteract brain swelling rapidly, but it is potentially harmful on a long-term basis, in contrast to the relative innocuousness of most organic osmolytes. Taurine in particular may be a perfect osmolyte because it is metabolically inert and exhibits only weak synaptic interaction (13).

Estimation of osmolyte change in all these studies does not discriminate among regional variations within the brain or possible differences in cell type. Studies *in vitro*, in tissue slices as well as in homogeneous cultured cells exposed to media of reduced osmolarity (by decreasing NaCl concentration), represent a suitable initial approach to clarify these questions and to obtain insight into the mechanisms of brain adaptation to hyponatremia. In-depth knowledge of these mechanisms is important to determine the development of symptoms in patients with hyponatremia and is critical for avoiding risks of inadequate correction procedures.

Regulatory Volume Decrease. Cellular and Molecular Mechanisms

Studies in cells such as neurons as well as glial cells in culture have contributed enormously to our knowledge concerning the basic mechanisms of adaptive cell volume re-

covery after hyposmotic swelling. Cells are, in general, highly permeable to water; therefore, any difference in osmolarity across the membrane results in net water movements in the direction necessary to reach osmotic equilibrium. In the face of a decrease in external osmolarity, cells initially behave as nearly perfect osmometers and swell with a magnitude proportional to the osmolarity reduction. Immediately after, an active volume correction begins, based on the extrusion of intracellular solutes together with osmotically obligated water; this tends to reduce osmotic difference and normalize cell volume. This adaptive mechanism is known as regulatory volume decrease (RVD). The time necessary to fully activate RVD and regain cell volume is variable in the different cell types. In brain cells *in vitro*, RVD occurs rapidly, with a 70–80% recovery reached within a few minutes as a result of osmolyte activation (Figure 1A).

RVD is a complex chain of events requiring a sensor to detect transient changes in cell volume, a signaling cascade to transduce information on volume change into activation of pathways for osmolyte extrusion, and a memory of the original cell volume that sets the timing for inactivation of the regulatory process. During the past years, the majority of efforts have been directed toward identifying and characterizing the osmolyte efflux pathways; thus, it is only recently that interest have been aroused in understanding osmotransduction mechanisms. There is at present only scarce information concerning the nature of volume-sensing mechanisms.

RVD has been studied in detail in astrocytes and neurons from primary cultures (14,15), in neuroblastoma (16), glioma cells lines (17), and in snail neurons (18). RVD has also been found in freshly isolated cells from hippocampus (19). The situation is unclear in more integrated preparations because in some of these preparations RVD has been undetectable (20); nevertheless, the results *in vivo* previously described clearly indicate the occurrence of compensatory mechanisms, although variations in ability to regulate volume may occur within brain regions.

Pathways for Osmolyte Fluxes Activated During RVD. The osmolytes responsible for RVD are essentially the same in most cell types including brain cells and are grouped into two broad categories: the most concentrated intracellular ions (K^+ and Cl^-) and small organic molecules, prominently amino acids, polyalcohols, sugars, and methylamines. In most cells examined to date, osmolyte fluxes occur essentially by diffusive pathways, i.e., K^+ and Cl^- efflux through separate channels with marginal participation of electroneutral cotransporters, and organic osmolytes through leak pathways with no contribution of energy-dependent carriers (21).

Osmosensitive Channels. Volume-sensitive K^+ and Cl^- fluxes in most cell types occur through separate channels that may possess some interdependence but that clearly exhibited a different selectivity. Cl^- channels activated by hyposmotic swelling are typically outward rectifiers with an intermediate unitary conductance of 40–78 pS, inactivating at potentials of +60 mV and above. These channels have been characterized in numerous cell types (22,23). In brain cells, the volume-sensitive Cl^- channel (VSCC) has been studied in astrocytes (24), C6 glioma cells (25), and cerebellar granule neurons (26). The VSCC has high selectivity of anions over cations but exhibits broad anion selectivity, being permeable to the majority of monovalent anions and even to large anions such as gluconate and methansulfonate. Activation of VSCC requires ATP but not its hydrolysis. Typical Cl^- channel blockers such as DIDS, SITS, 9-AC, and DPC inhibit VSCC with different potencies according to cell type. Other agents with inhibitory effects on the VSCC include NPPB, DDF, niflumic acid, and flufenamic acid (23,24). Notably, arachidonic acid and other polyunsaturated fatty acids are potent VSCC blockers (27,28). For details on the basic properties of VSCC, readers are referred to recent reviews on this topic (22,23).

The molecular species of VSCC are as yet unidentified. Approximately eight members of a family of voltage-gated Cl^- channels have been cloned and characterized. Some are activated by swelling (29), but none unequivocally corresponds to VSCC. Some evidence appears to support the ClC3 channel gene as encoding the channel protein responsible for the volume-sensitive Cl^- current (30), but recent evidence argues against this channel being indeed the VSCC (31). Some molecules with Cl^- permeability properties, namely Icln and the P-glycoprotein, are suggested to play a role in osmosensitive Cl transport either as a Cl^- pathway properly, a possibility recently questioned, or as regulating elements of the functional Cl^- channels (review in Reference 30). Not unlikely but at present undefined is the question of whether different types of VSCC and other anion-permeating molecules coincide in the same cell. An interesting avenue for future research may be the identification
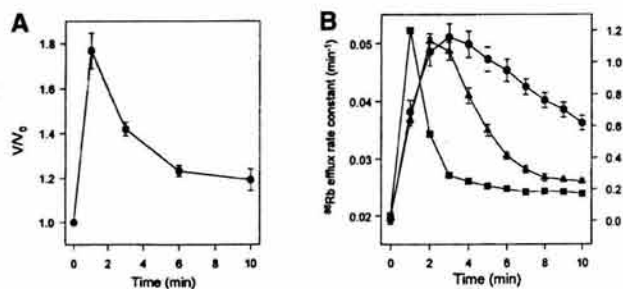
Figure 1. Regulatory volume decrease in cultured cerebellar granule neurons. A. Upon exposure to media of reduced osmolarity (50%), cerebellar granule neurons exhibit a rapid increase in cell volume followed by an active phase of volume regulation occurring despite the persistence of the hyposmotic medium. Volume recovery is approximately 60% within 15 min. B. Volume recovery is accomplished by the efflux of inorganic and organic osmolytes (▲) ^{3}H-taurine, (■) ^{125}I (as tracer for Cl^-), and (●) ^{86}Rb (as tracer for K^+). Results are expressed as efflux rate constants, as described in the work of Sánchez-Olea et al. (14,26).

of the factors and situations that determine the functioning of one or another of these different osmosensitive channels.

In contrast to the broad similarities found for VSCC in different types of cells, swelling activates at least two different types of K$^+$ channels. In some—mainly epithelial—cells, volume-sensitive K$^+$ channels (VSKC) are calcium (Ca^{2+})-dependent, large-conductance (100–200 pS) channels. In other cell types, VSCK channels are small channels with conductances of 20–30 pS, the majority Ca^{2+}-independent (review in Reference 32). While the first group of channels has been clearly identified as high conductance K$^+$ channels, the identity of the second group is still unclear. Some types of voltage-gated K$^+$ channels appear to permeate K$^+$ efflux during RVD in lymphocytes, and Kv channels are activated by hyposmolarity in hippocampal pyramidal neurons, although not all Kv subtypes are responsive (32).

Volume-Sensitive Pathways for Organic Osmolytes. A number of organic osmolytes are released during hyposmotic swelling, but the details of their efflux pathways are known for only a few. The best-characterized organic osmolytes are those for taurine and myo-inositol (33–35); additionally, there is some information on N-acetyl aspartate and ascorbate (36,37). In general, these are bidirectional leak pathways with net solute movement depending on concentration gradient direction. Remarkably, organic osmolyte pathways commonly exhibit a pharmacologic profile similar to that of the VSCC, suggestive of a common pathway with Cl$^-$ or of a close connection between the two pathways (21,35). Other amino acids also responsive to swelling are glycine, GABA, glutamate, and aspartate, which contribute to correction of osmotic disturbance (38). However, this may create additional risks of excitability imbalance, due to their prominent role as synaptic transmitters, to be discussed later. There is recent evidence on hyposmolarity-induced glutamate release insensitive to Cl$^-$ channel blockers, at clear variance with other organic osmolytes (39). This is suggestive of either different pathways or different stimuli and mechanisms for release of this particular amino acid.

Volume Sensor and Osmotransductive Signaling. How cells sense volume changes is the initial and critical step in the chain of reactions activated for volume correction, yet this has remained elusive to date. Among possible mechanisms considered to play this role are membrane receptors such as integrins or receptors with intrinsic tyrosine kinase activity, cytoskeleton rearrangements, dilution of cytosolic macromolecules, decrease in intracellular ionic strength, stretch-induced activation of adhesion molecules, activation of phospholipases, or changes in the concentration of signaling molecules such as Ca^{2+} or magnesium (Mg^{2+}) (Figure 2). Although to date none of these possesses sufficient supporting experimental evidence, this is at present a very active field of research (40). The question of volume sensing is also closely
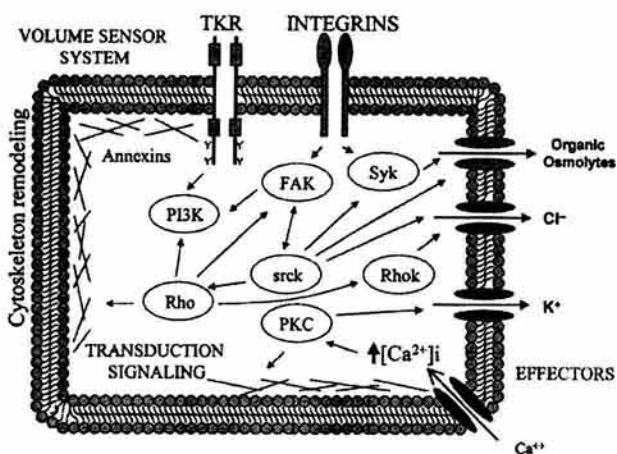
Figure 2. Hypothetical scheme of the elements of volume sensing, osmotransduction, and activation of osmolyte pathways. The volume sensor system includes membrane and submembrane elements such as integrins, membrane receptors with intrinsic tyrosine kinase activity (TKR), annexins, and cortical actin cytoskeleton remodeling. Osmotransduction signaling includes Ca^{2+} and a number of protein tyrosine kinases, including focal adhesion kinase (FAK), SYK, src-related kinases (srck), the tyrosine-kinase-activated kinase phosphoinositide-3 kinase (PI3K), and protein kinase C (PKC). The small GTPase Rho and its kinase RhoK may also act as osmo-signaling elements. Not all enzymes appear involved in the same cell type.

related to mechanisms of osmolyte flux inactivation. At present, this is an essentially unexplored aspect of RVD.

Calcium and protein kinases are among the most likely candidates to act as osmotransductory elements. One of the most constant features of hyposmotic swelling is an increase in cystolic Ca^{2+} (32) (Figure 2). Despite this, the main corrective osmolyte efflux pathways and consequently RVD are Ca^{2+}-independent in a large variety of cell types. This is the case for brain cells, in which VSCC, VSKC, and organic efflux pathways are largely Ca^{2+}-independent (32). The more commonly accepted interpretation of these results is that cytosolic Ca^{2+} increase is an epiphenomenon resulting from activation by swelling of Ca^{2+} influx pathways and/or of release mechanisms from intracellular stores (41), but that this increase is not part of the osmosignaling cascades. In cells such as epithelial cells, the magnitude of hyposmolarity-evoked cytosolic Ca^{2+} elevation is sufficient to activate Ca^{2+}-dependent large conductance K$^+$ channels, which once activated, predominantly contribute to RVD. As a consequence, RVD is Ca^{2+}-dependent in these cells (32).

Protein kinases of different types modulate some osmolyte pathways. In contrast to the constancy of the cytosolic Ca^{2+} elevation, the effect of protein kinases appears to be cell-specific. Protein kinase C (PKC) appears involved in the function of the VSCC but with different effects (activation or inhibition) according to cell type (22). Protein kinase A does not influence RVD in most cells but may be involved in the modulatory action of hormones and other factors on cell volume regulation. Protein tyrosine kinases (PTK) have re-

cently received special attention as elements of the osmotrans-duction cascades as a result of the potent effect of blockers of PTK reducing the osmosensitive efflux of Cl^- and tau-rine (42,43). This effect has been reported in cultured astro-cytes and neurons and in more integrated preparations such as the supraoptic nucleus and hippocampal slices (34,39). *In vivo*, PTK appear related to swelling-evoked amino acid re-lease in heart Langendorff preparations (44). Furthermore, in cultured cells inhibition of tyrosine phosphatases, which prolong the protein phosphorylation reaction, increases os-molyte fluxes (42) (Figure 2). A modulatory role of PTK on volume-sensitive K^+ channels has not been reported but Ca^{2+} and PKC are involved in VSKC in some cell types, as previously mentioned (Figure 2).

The site within the complex signaling network modu-lated by PTK has not been identified. A possible target is the phosphoinositide kinase PI3K, a tyrosine-kinase-acti-vated kinase, because inhibition of this enzyme has a marked influence in reducing the volume-corrective fluxes of Cl^- and taurine (42,43). PI3K is a key element in signal-ing cascades with links to tyrosine-kinase membrane recep-tors and the integrin-FAK pathway. PI3K also relates to small GTP-ases of the Rho family that in turn modulate the dynamics of the cytoskeleton (45) (Figure 2).

A role for phospholipases in osmotransduction, in partic-ular the cPLA2 form, is suggested by reports in neuroblas-toma and in Ehrlich ascites cells, showing a strong correla-tion between arachidonic acid release and volume-sensitive taurine efflux, which are blunted by blockers of this specific enzyme (46).
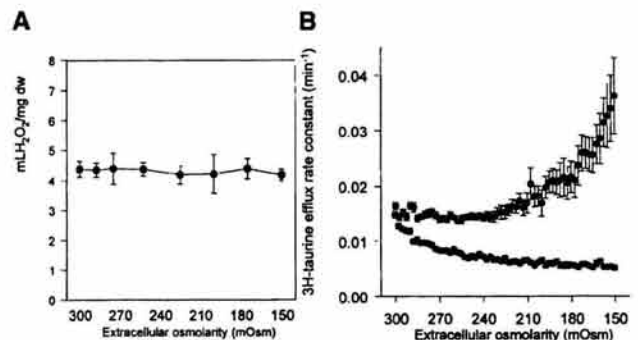
Isovolumetric Regulation

The experimental model of abrupt and large reduction in exter-nal osmolarity has contributed importantly to our knowledge concerning the mechanisms by which cells face osmotic chal-lenges. However, this model does not closely reproduce the conditions *in vivo*. Even in acute hyponatremia, changes in ex-ternal osmolarity never exceed −16% and onset of hyposmo-larity appears gradually. A better paradigm for approaching these conditions was developed by Lohr and Grantham (47) in renal tubule cells exposed to small and gradual changes in os-molarity. Under these conditions, cells do not swell even if ex-ternal osmolarity is drastically decreased. This constancy in cell volume is not due to swelling being restricted, but is rather due to rapid and efficient correction of the continuous change in water content. This volume adjustment, similar to models of abrupt hyposmotic shock, appears to be accomplished by ac-tive extrusion of intracellular osmolytes (48). Molecules in-volved and mechanisms in operation are not known in detail, as studies on isovolumetric regulation (IVR) are still scarce. Oc-currence of IVR has been found in only two types of renal cells (47,49), in hippocampal slices (50) (Figure 3), and with lower efficiency in C6 glioma cells (51) and in cardiomyocytes (52).

In A6 cells and in myocytes, IVR stimulates K^+ release, but only at delayed phase of IVR, with an efflux threshold at −30% hyposmotic external osmolarity. In contrast, amino ac-ids, particularly taurine, appear involved at earlier phases of volume regulation, showing efflux thresholds at approximately −10 to 12% hyposmolarity (50) (Figure 3). There is no infor-mation on osmotransduction factors or signaling messengers involved in this model of volume regulation, and possible simi-larities or dissimilarities of this mechanism of volume control in different cell types have not been explored to date. This is an interesting avenue of research because as previously men-tioned, this experimental model approaches physiologic and pathologic situations generating changes in cell volume in brain.

Hyponatremia and Hyperexcitability

A serious clinical consequence of acute and severe hy-ponatremia is the generation of epileptiform activity and in-creased susceptibility to seizures (53). In studies in hippo-campal slices, osmolarity reduction causes an increase in amplitude of evoked field potentials and of excitatory postsynaptic potentials, which is inversely related to osmo-larity (54). Hyposmolarity does not affect cell properties such as resting membrane potential, cell input resistance, and action potential threshold and duration (55). The man-ner in which osmolarity alters synaptic transmission is not fully understood. It may result from either hyperfunction of excitatory synapses and/or from non-synaptic mechanisms derived from reductions in the size of the extracellular space. The increase in excitatory synaptic activity may be the consequence of the well-documented, swelling-acti-vated glutamate release (39,55). On the other hand, narrow-ing of the extracellular space due to cell swelling may also be a source of hyperexcitability, either by enhancing ephap-tic interactions and/or because increase of extracellular K^+ concentration and reduced diffusion of neurotransmitters

Figure 3. Isovolumetric regulation in hippocampal slices. A. Slice water con-tent remains unchanged when slices are exposed to small and gradual changes in osmolarity (1.8 mosm/min). B. Constancy in cell volume is an active pro-cess accomplished by the efflux of osmolytes, as illustrated for taurine efflux. Details of experiments are described according to Franco et al. (49).
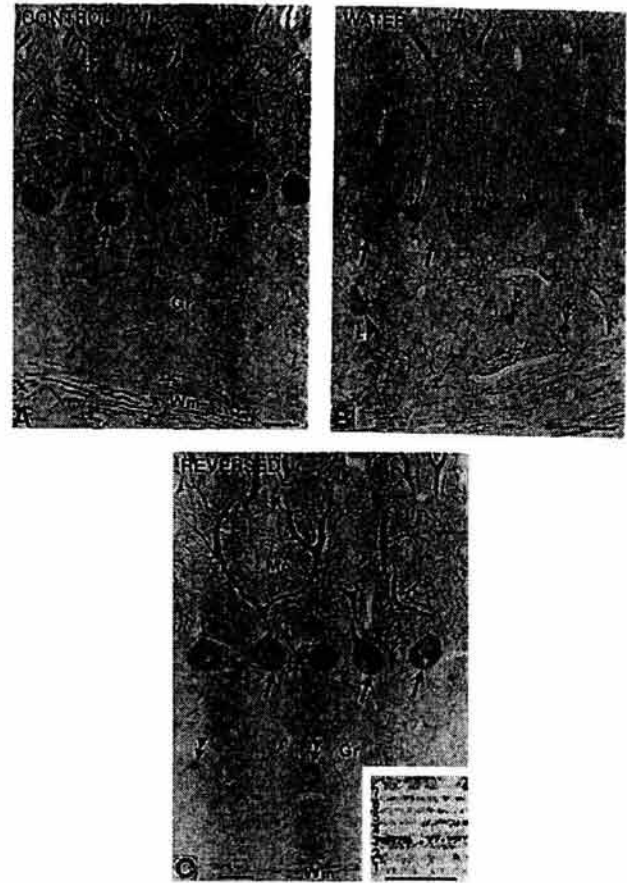
prolong the synaptic function (53,56). These possibilities, which do not exclude each other, may all contribute to generate hyposmotic-associated hyperexcitability. In support of this interpretation, the Cl⁻ channel blocker furosemide prevents extracellular space reduction and blocks epileptiform activity in a variety of *in vitro* models (57). Furosemide prevents the kainic acid-induced, synchronized burst discharges in hippocampal slices (57).

Differences in Brain Cell Swelling in Hyponatremia

Cultured brain cells exposed to hyposmolarity reductions exhibit an immediate and general increase in cell swelling. However, this homogeneity has not been observed in more integrated preparations, in which the response of individual neurons to lowering osmolarity varies greatly. In pyramidal cells freshly isolated from CA1 region of the hippocampus, at least three different populations of cells could be identified according to their response to decreasing osmolarity. One group of cells swells immediately, while other groups exhibit a delayed response or are resistant to swelling (19). Differences are also observed between hippocampal regions in which CA1 and the dentate gyrus swell more than CA3 (58). Within the same region, as in CA1, the stratum radiatum and the stratum oriens containing the apical and basal dendrites, respectively, are notably more responsive to hyposmotic swelling than the stratum pyramidale, formed by the cell somata (58). The reason for these differences is unclear to date. Lack of swelling in some cells or regions may result from i) an intrinsic mechanism preventing water entry such as reduced expression of aquaporins, ii) activation of highly efficient processes of volume adjustment, or iii) temporary redistribution of osmolytes to nearby cell compartments. In this respect, an elegant *in vivo* study by Nagelhus and co-workers (59) in cerebellum of water-loaded rats shows an immediate redistribution of taurine-cell content in Purkinje cells to nearby glial elements in response to the hyposmotic condition. As a result, astrocytes swell while neurons are spared (Figure 4).

Figure 4. Redistribution of taurine-like immunoreactivity in rat cerebellar cortex following water loading. Details of the experiment are as in Reference 58. Photomicrographs of pairs of neighboring semi-thin sections treated with taurine antiserum are as described in Reference 58. Animals were treated with isotonic saline (A) or water (B) 4 h prior to killing, or with water followed by hypertonic saline (C). The figure shows the highly concentrated taurine in Purkinje cells (large arrows), which upon water load is transferred to the adjacent glial elements (arrows). The original distribution is restored after hyposmolarity correction. Double arrowheads represent different types of cells labeled with taurine. This is part of Figure 2 from the work by Nagelhus et al. (58), reproduced with permission.

Risks of Rapid Correction of Hyponatremia

Acute, severe hyponatremia (Na⁺ serum concentration <115 mM) produces clear symptoms of neurologic damage due to brain edema, with the consequent increase in intracranial pressure. The commonly used procedure to correct hyponatremia is administration of hypertonic saline solutions. Saline/acetate solutions have also proved highly efficient to correct mild hyponatremia (60). In cases of SIADH, fluid restriction is usually the first line of treatment. It is important to mention in this respect that a clear distinction should be made between a diagnosis of SIADH and of CSWS, which as mentioned previously may have similar symptoms. While fluid restriction is appropriate to correct hyponatremia associated with SIADH, it is detrimental to patients with CSWS

and can even be lethal (2,61). An increase in plasma Na⁺ concentration of approximately 10–15 mmol/L is normally sufficient to prevent permanent brain damage in severe, acute hyponatremia. It is currently accepted that the rate of correction of acute hyponatremia should be no more than 0.5 mmol/L/h and should be interrupted when serum Na⁺ levels have increased to 125–130 mmol/L (62). These precautions are necessary because overly excessive and rapid corrective procedures may result in brain injury, likely due to the adaptive mechanisms developed to counteract hyponatremia described in this review. As a consequence of these adjustments, solute intracellular pools change their concentration to attain an osmotic balance with the external modified condition, i.e., the osmolarity of the cytosol is in equi-

librium with an external hyposmotic environment. When the increase in plasma tonicity that accompanies correction of chronic hyponatremia restores the normal isosmotic condition, this condition is now sensed as hyperosmotic by brain cells, which consequently dehydrate until new adaptive mechanisms are activated. The main risk of this situation is a neurologic sequel of demyelinating lesions in the brain, a pathology known as osmotic demyelination syndrome (8). This pathologic entity is characterized by a symmetric focus prominently in the basis pontis, but extrapontine demyelinating lesions have also been found in the basal ganglia, internal capsule, lateral geniculate body, and cortex (63). The salient clinical features of the syndrome include motor abnormalities progressing to flaccid quadriplegia, occasional respiratory paralysis, mental state disturbances, lethargy, and coma. Why demyelination develops is not well understood. Recent studies have focused on a disruption of the brain blood barrier as the gating factor of the degenerative process (64,65). The current hypothesis is that disruption of the tight junctions of the blood brain barrier as a consequence of brain dehydration might expose oligodendrocytes to substances normally excluded from the brain, such as complement, which could be the precipitous factor of demyelination. Situations predisposing to the development of the demyelination syndrome associate with preexisting conditions such as alcoholism and malnutrition (8). Particular care should be taken in correcting hyponatremia in patients with an associated clinical condition of hypoxia. Studies in animals as well as in humans (66–68) have shown that hypoxia combined with hyponatremia produces a major increase in brain edema, injury, and mortality. This is possibly a consequence of inefficiency of the compensatory mechanisms of cell volume regulation due to intracellular Na^+ increase and subsequent Cl^- influx occurring in the hypoxic condition as a result of the energetic failure (69).

Conclusions

Knowledge concerning the cellular and molecular mechanisms subserving brain adaptation to hyponatremia has notably progressed in the last decade. All of this information has contributed to understanding the risk of the pathologic consequences of an inappropriate correction of the hyponatremic patient and to guide the clinicians toward a rational, optimal therapeutic approach.

Acknowledgments

References

1. Kimelberg HK. Current concepts of brain edema. Review of laboratory investigations. J Neurosurg 1995;83:1051–1059.
2. Harrigan MR. Cerebral salt wasting syndrome. Crit Care Clin 2001;17:125–138.
3. Verbalis JG. Adaptation to acute and chronic hyponatremia: implications for symptomatology, diagnosis, and therapy. Semin Nephrol 1998;18:3–19.
4. Fall PJ. Hyponatremia and hypernatremia. A systematic approach to causes and their correction. Postgrad Med 2000;107:75–85.
5. Law RO. Effects of pregnancy on the contents of water, taurine, and total amino nitrogen in rat cerebral cortex. J Neurochem 1989;53:300–302.
6. Holmes SB, Banerjee AK, Alexander WD. Hyponatraemia and seizures after ecstasy use. Postgrad Med J 1999;75:32–33.
7. Verbalis JG, Drutarosky MD. Adaptation to chronic hypoosmolality in rats. Kidney Int 1988;34:351–360.
8. Berl T. Treating hyponatremia: damned if we do and damned if we don't. Kidney Int 1990;37:1006–1018.
9. Verbalis JG, Gullans SR. Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res 1991;567:274–282.
10. Sterns RH, Baer J, Ebersol S, Thomas D, Lohr JW, Kamm DE. Organic osmolytes in acute hyponatremia. Am J Physiol 1993;264:F833–F836.
11. Olson JE. Osmolyte contents of cultured astrocytes grown in hypoosmotic medium. Biochim Biophys Acta 1999;1453:175–179.
12. Isaacks RE, Bender AS, Kim CY, Shi YF, Norenberg MD. Effect of osmolality and anion channel inhibitors on myo-inositol efflux in cultured astrocytes. J Neurosci Res 1999;57:866–871.
13. Huxtable RJ. Physiological actions of taurine. Physiol Rev 1992;72:101–163.
14. Pasantes-Morales H, Maar TE, Morán J. Cell volume regulation in cultured cerebellar granule neurons. J Neurosci Res 1993;34:219–224.
15. Sánchez-Olea R, Peña C, Morán J, Pasantes-Morales H. Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl^- transport in cultured astrocytes. Neurosci Lett 1993;156:141–144.
16. Basavappa S, Huang CC, Mangel AW, Lebedev DV, Knauf PA, Ellory JC. Swelling-activated amino acid efflux in the human neuroblastoma cell line CHP-100. J Neurophysiol 1996;76:764–769.
17. Strange K, Morrison R. Volume regulation during recovery from chronic hypertonicity in brain glial cells. Am J Physiol 1992;263:C412–C419.
18. Alvarez-Leefmans FJ, Gamino SM, Reuss L. Cell volume changes upon sodium pump inhibition in *Helix aspersa* neurons. J Physiol 1992;458:603–619.
19. Aitken PG, Borgdorff AJ, Juta AJ, Kiehart DP, Somjen GG, Wadman WJ. Volume changes induced by osmotic stress in freshly isolated rat hippocampal neurons. Pflugers Arch 1998;436:991–998.
20. Andrew RD, Lobinowich ME, Osehobo EP. Evidence against volume regulation by cortical brain cells during acute osmotic stress. Exp Neurol 1997;143:300–312.
21. Pasantes-Morales H. Volume regulation in brain cells: cellular and molecular mechanisms. Metab Brain Dis 1996;11:187–204.
22. Okada Y. Volume expansion-sensing outward-rectifier Cl^- channel: fresh start to the molecular identity and volume sensor. Am J Physiol 1997;273:C755–C789.
23. Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G. Properties of volume-regulated anion channels in mammalian cells. Prog Biophys Mol Biol 1997;68:69–119.
24. Jalonen T. Single-channel characteristics of the large-conductance anion channel in rat cortical astrocytes in primary culture. Glia 1993;9:227–237.
25. Jackson PS, Strange K. Characterization of the voltage-dependent properties of a volume-sensitive anion conductance. J Gen Physiol 1995;10:661–676.
26. Patel AJ, Lauritzen I, Lazdunski M, Honore E. Disruption of mitochondrial respiration inhibits volume-regulated anion channels and provokes neuronal cell swelling. J Neurosci 1998;18:3117–3123.
27. Sánchez-Olea R, Morales-Mulía M, Morán J, Pasantes-Morales H. Inhibition by polyunsaturated fatty acids of cell volume regulation and osmolyte fluxes in astrocytes. Am J Physiol 1995;269:C96–C102.

28. Sakai H, Kakinoki B, Diener M, Takeguchi N. Endogenous arachidonic acid inhibits hypotonically-activated Cl⁻ channels in isolated rat hepatocytes. Jpn J Physiol 1996;46:311–318.

29. Jentsch TJ, Friedrich T, Schriever A, Yamada H. The CLC chloride channel family. Pflugers Arch 1999;437:783–795.

30. Duan D, Winter C, Cowley S, Hume JR, Horowitz B. Molecular identification of a volume-regulated chloride channel. Nature 1997;390:417–420.

31. Weylandt KH, Valverde MA, Nobles M, Raguz S, Amey JS, Díaz M, Nastrucci C, Higgins CF, Sardini A. Human ClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. J Biol Chem 2001;276:17461–17467.

32. Pasantes-Morales H, Morales Mulía S. Influence of calcium on regulatory volume decrease: role of potassium channels. Nephron 2000;86:414–427.

33. Pasantes-Morales H, Schousboe A. Role of taurine in osmoregulation: mechanisms and functional implications. Amino Acids 1997;12:281–293.

34. Hussy N, Deleuze C, Desarmenien MG, Moos FC. Osmotic regulation of neuronal activity: a new role for taurine and glial cells in a hypothalamic neuroendocrine structure. Prog Neurobiol 2000;62:113–134.

35. Kirk K. Swelling-activated organic osmolyte channels. J Membr Biol 1997;158:1–6.

36. Davies SE, Gotoh M, Richards DA, Obrenovitch TP. Hyposmolarity induces an increase of extracellular N-acetylaspartate concentration in the rat striatum. Neurochem Res 1998;23:1021–1025.

37. Siushansian R, Dixon SJ, Wilson JX. Osmotic swelling stimulates ascorbate efflux from cerebral astrocytes. J Neurochem 1996;66:1227–1233.

38. Pasantes-Morales H, Alavez S, Sánchez Olea R, Morán J. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem Res 1993;18:445–452.

39. Franco R, Torres-Márquez ME, Pasantes-Morales H. Evidence of two mechanisms for amino acid osmolyte release from hippocampal slices. Pflugers Arch 2001;442:791–800.

40. Hoffmann EK. Intracellular signalling involved in volume regulatory decrease. Cell Physiol Biochem 2000;10:273–278.

41. McCarty NA, O'Neil RG. Calcium signaling in cell volume regulation. Physiol Rev 1992;72:1037–1061.

42. Pasantes-Morales H, Cardin V, Tuz K. Signaling events during swelling and regulatory volume decrease. Neurochem Res 2000;25:1301–1314.

43. van der Wijk T, Tomassen SF, de Jonge HR, Tilly BC. Signalling mechanisms involved in volume regulation of intestinal epithelial cells. Cell Physiol Biochem 2000;10:289–296.

44. Wymann MP, Pirola L. Structure and function of phosphoinositide-3-kinases. Biochim Biophys Acta 1998;436:127–150.

45. Toker A. Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mol Pharmacol 2000;57:652–658.

46. Hoffmann EK. Intracellular signalling involved in volume regulatory decrease. Cell Physiol Biochem 2000;10:273–288.

47. Lohr JW, Grantham JJ. Isovolumetric regulation of isolated S2 proximal tubules in anisotonic media. J Clin Invest 1986;78:1165–1672.

48. Pasantes-Morales H, Franco R, Torres-Márquez ME, Hernández-Fonseca K, Ortega A. Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: contribution and mechanisms. Cell Physiol Biochem 2000;10:361–370.

49. Van Driessche W, De Smet P, Li J, Allen S, Zizi M, Mountian I. Isovolumetric regulation in a distal nephron cell line (A6). Am J Physiol 1997;272:C1890–C1898.

50. Franco R, Quesada O, Pasantes-Morales H. Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J Neurosci Res 2000;61:701–711.

51. Tuz K, Ordaz B, Vaca L, Quesada O, Pasantes-Morales H. Isovolumetric regulation mechanisms in cultured cerebellar granule neurons. J Neurochem 2001;79:143–151.

52. Lohr JW, Yohe L. Isovolumetric regulation of rat glial cells during development and correction of hyposmolality. Neurosci Lett 2000;286:5–8.

53. Souza MM, Boyle RT, Lieberman M. Different physiological mechanisms control isovolumetric regulation and regulatory volume decrease in chick embryo cardiomyocytes. Cell Biol Int 2000;24:713–721.

54. Andrew RD. Seizure and acute osmotic change: clinical and neurophysiological aspects. J Neurol Sci 1991;101:7–18.

55. Schwartzkroin PA, Baraban SC, Hochman DW. Osmolarity, ionic flux, and changes in brain excitability. Epilepsy Res 1998;32:275–287.

56. Chebabo SR, Hester MA, Aitken PG, Somjen GG. Hypotonic exposure enhances synaptic transmission and triggers spreading depression in rat hippocampal tissue slices. Brain Res 1995;695:203–216.

57. Hochman DW, Baraban SC, Owens JW, Schwartzkroin PA. Dissociation of synchronization and excitability in furosemide blockade of epileptiform activity. Science 1995;270:99–102.

58. Johnson LJ, Hanley DF, Thakor NV. Optical light scatter imaging of cellular and sub-cellular morphology changes in stressed rat hippocampal slices. J Neurosci Methods 2000;98:21–31.

59. Nagelhus EA, Lehmann A, Ottersen OP. Neuronal-glial exchange of taurine during hypo-osmotic stress: a combined immunocytochemical and biochemical analysis in rat cerebellar cortex. Neuroscience 1993;54:615–631.

60. Suárez JI, Quareshi AI, Parekh PD, Razumovsky A, Tamargo RJ, Bhardwaj A, Ulatowski JA. Administration of hypertonic (3%) sodium chloride/acetate in hyponatremic patients with symptomatic vasospasm following subarachnoid hemorrhage. J Neurosurg Anesthesiol 1999;11:178–184.

61. Zafonte RD, Mann NR. Cerebral salt wasting syndrome in brain injury patients: a potential cause of hyponatremia. Arch Phys Med Rehabil 1997;78:540–542.

62. Gross P, Reiman D, Henschkowski J, Damian M. Treatment of severe hyponatremia: conventional and novel aspects. J Am Soc Nephrol 2001;17:S10–S14.

63. Brown WD. Osmotic demyelination disorders: central pontine and extrapontine myelinolysis. Curr Opin Neurol 2000;13:691–697.

64. Baker EA, Tian Y, Adler S, Verbalis JG. Blood-brain barrier disruption and complement activation in the brain following rapid correction of chronic hyponatremia. Exp Neurol 2000;165:221–230.

65. Adler S, Martínez J, Williams DS, Verbalis JG. Positive association between blood brain barrier disruption and osmotically-induced demyelination. Mult Scler 2000;6:24–31.

66. Knochel JP. Hypoxia is the cause of brain damage in hyponatremia. JAMA 1999;281:2342–2346.

67. Vexler Z, Ayus JC, Roberts TPL, Fraser CL, Kuchaczyk J, Arieff AI. Hypoxic and ischemic hypoxia exacerbate brain injury associated with metabolic encephalopathy in laboratory animals. J Clin Invest 1994;93:256–264.

68. Ayus JC, Arieff AI. Chronic hyponatremic encephalopathy in postmenopausal women: association of therapies with morbidity and mortality. JAMA 1999;28:2299–2304.

69. Rosenberg GA. Ischemic brain edema. Prog Cardiovasc Dis 1999;42:209–216.

Osmosensitive Release of Neurotransmitter Amino Acids: Relevance and Mechanisms*

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Hyposmolarity activates amino acid efflux as part of the corrective volume process in a variety of cells. This review discusses the mechanism of amino acid release in brain cells preparations. Results present evidence of substantial differences between the efflux of taurine and that of GABA and glutamate, which besides a possible role as osmolytes, have a main function as synaptic transmitters. The differences found concern the efflux time course, the sensitivity to Cl^- channel blockers, the modulation by tyrosine kinases, the influence of PKC and the effect of cytoskeleton disruptive agents. While taurine efflux features fit well with the mechanisms so far described in most cell types, the efflux of GABA and glutamate does not. Alternate mechanisms for the release of these two amino acids are discussed, including a PKC-modulated, actin-dependent exocytosis.

KEY WORDS: Hyposmolarity; tyrosine kinases; hyperexcitability; PKC.

INTRODUCTION

Most cells respond to decreases in external osmolarity by rapid swelling, followed by a corrective process leading to cell volume recovery, usually referred as regulatory volume decrease (RVD) (1). This is an active process accomplished by the extrusion of intracellular osmolytes, occurring essentially through leak pathways. The main intracellular ions K^+ and Cl^- are important osmolytes, but also a number of organic molecules, polyols, organic amines and particularly amino acids, are significantly involved in RVD (Fig. 1). In fact, the contribution of organic osmolytes in brain during chronic hyponatremia, is crucial for maintain-

ing the brain water content within limits compatible with survival (2) (Fig. 1).

A simple approach to consider a molecule as potential osmolyte is to examine its response to hyposmotic swelling. A large number of reports in the last years document the osmosensitive efflux of amino acids in many cell types, including brain cells, glial cells as well as neurons (3–5). Amino acid release has been also observed in more integrated preparations such as brain slices (6–8), supraoptic nucleus (9) and in the brain in vivo using paradigms of microdialysis or superfusion (10,11). Amino acids released upon hyposmosis are preferentially taurine, GABA, glycine and glutamate. N-acetyl aspartate also seems to be released by hyposmolarity, particularly from neurons (12). The increased efflux of amino acids consistently observed in all these preparations has its counterpart in the decrease in tissue levels (10). In chronic hyponatremic mice, taurine concentrations are reduced to only 15% of its initial value, while glutamate levels are reduced to 60%. Studies about the mechanism of this release have been carried out preferentially on
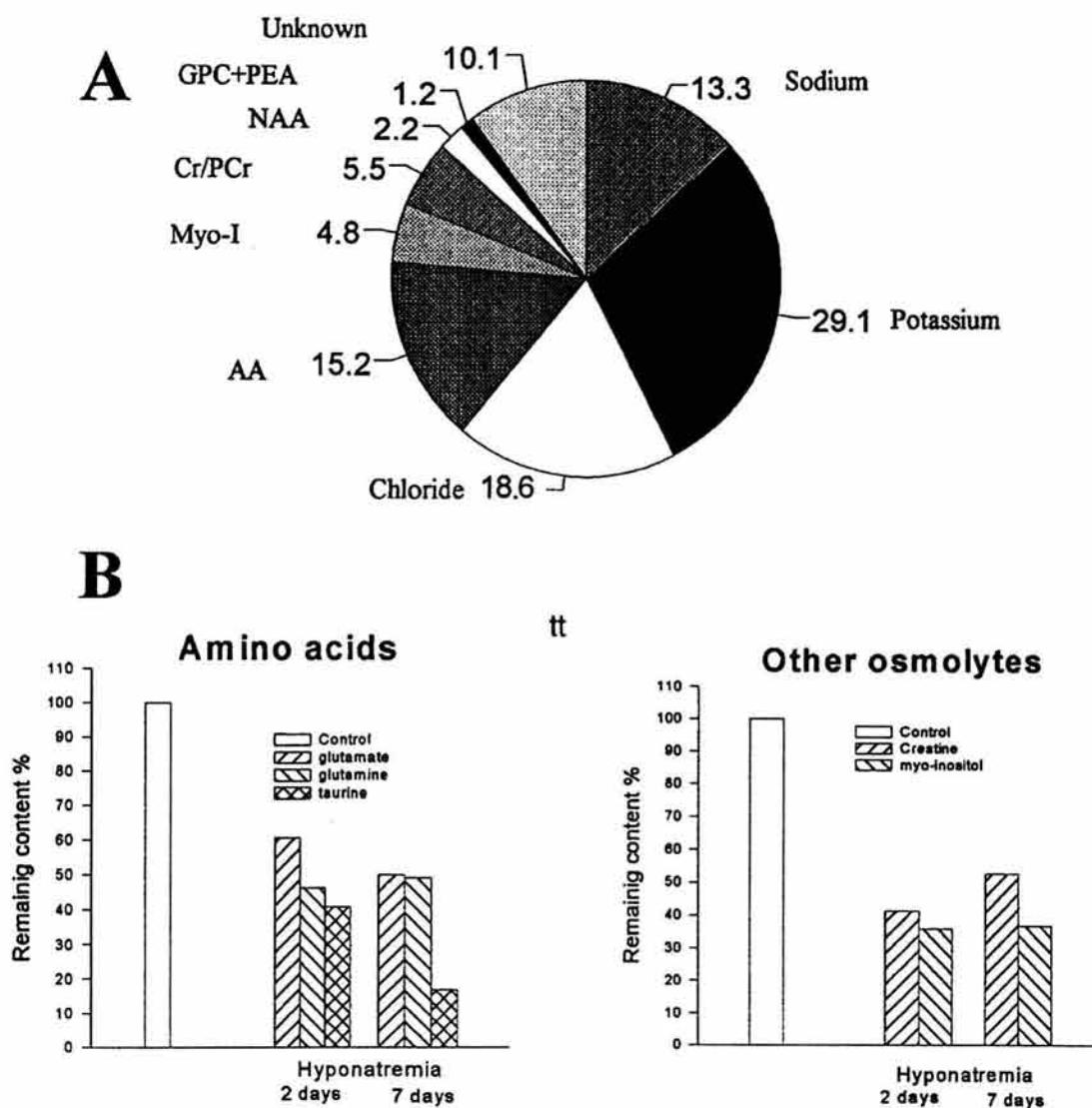
Fig. 1. Inorganic and organic osmolytes involved in the control of brain water content during chronic hyponatremia in mice. A: Relative contribution of the various osmolytes to cell volume correction. B: Decrease in the brain content of amino acids, creatine and myo-inositol after 2–7 days of chronic hyponatremia in mice. Empty bar: normal brain content of osmolytes. Recalculated from (2). GPC: glycerophosphorylcholine, PEA: phosphatidyl ethanolamine, NAA: N-acetyl aspartate, Cr/PCr: creatine/phosphocreatine; Myo-I: myo-inositol, AA: amino acids.

taurine, because of its particular features such as metabolic inercy, high concentrations, and prompt release upon the hyposmotic stimulus (13). For these reasons, taurine has been considered as representative of amino acid osmolytes, and it has been currently assumed that results obtained for taurine are valid for other amino acids as well. In the present work, we present evidence about substantial differences between the mechanisms of hyposmolarity-activated release of taurine and those GABA and glutamate, which not only suggest differences in the pathways but likely also in the functional meaning of this release.

The Efflux Pathway

Studies in cultured astrocytes and neurons document the efflux of taurine, GABA, glutamate and glycine in response to hyposmotic stimulus (3). Taurine efflux occurs through a leak pathway, with essentially

no contribution of the energy-dependent carrier (4,5). The diffusive nature of the taurine permeation pathway is suggestive of a channel-like molecule as the mechanism for efflux. Since osmosensitive taurine release in most cell types, including cultured astrocytes and neurons, is sensitive to Cl^- channel blockers (14), an anion channel-like molecule has been proposed as the common pathway for the corrective fluxes for Cl^- and amino acid during RVD. In fact, it is known that the volume-activated Cl^- channel exhibits a broad range of permeability, which includes all monovalent anions and even large molecules such as benzoate (15). Taurine, glutamate and aspartate, all permeate through this channel when are present in anionic form (15), suggesting that the size of the pore is large enough to allow the passage of these amino acids. Still, the question remains about how amino acids, which are found in the cell mostly as zwitterions, may translocate through an anion channel.

It has been often assumed that other amino acids released by hyposmolarity permeate by a similar pathway as taurine, but this has not been studied in detail. In a study in hippocampal slices directed to address to this question, we found remarkable differences between the efflux of taurine and the release of GABA and glutamate (16). The first main difference is the time course, as illustrated in Fig. 2A. While the efflux of taurine slowly activates and essentially does not inactivate, those of glutamate and GABA are characterized by rapid activation and inactivation. The sensitivity to Cl^- channel blockers further reveals these differences. In most cell types, the osmosensitive efflux of taurine is markedly decreased by agents such as NPPB (5-Nitro-[3-phenylpropylamino]benzoic acid), DDF (dideoxyforskolin), niflumic acid and DIDS (4,4'-diisothiocyanato-stilbene-2-2' disulfonic acid), which also effectively block the volume-activated Cl^- channel (1,17). A report in cultured astrocytes shows inhibition by DIDS of glutamate efflux but none of the other blockers was examined (18). The referred study in hippocampal slices revealed the insensitivity of the release of GABA and glutamate to NPPB and niflumic acid, which are potent blockers of taurine efflux also in this preparation (16) (Fig. 2B). Only DIDS, at high concentrations, was an efficient blocker of the efflux of GABA and glutamate.

Release of taurine, glutamate and GABA in response to hyposmolarity, has been also observed in vivo using microdialysis or superfusion of the cortex surface (10,11). Here again, the release of aspartate and glutamate was insensitive to Cl^- channel blockers (11). Altogether, these findings showing major differ-

ences in the mechanism of amino acid release, are suggestive of different pathways, which in the case of GABA and glutamate would also considerably differ from the volume-sensitive Cl^- channel.

Transduction Signalling

Volume regulation is a complex process which initiates by detection of the change in cell volume, followed by the trigger of signalling cascades, ultimately leading to activation of effectors, i.e. the various translocation pathways in charge of the osmolyte extrusion necessary for volume correction. At a certain
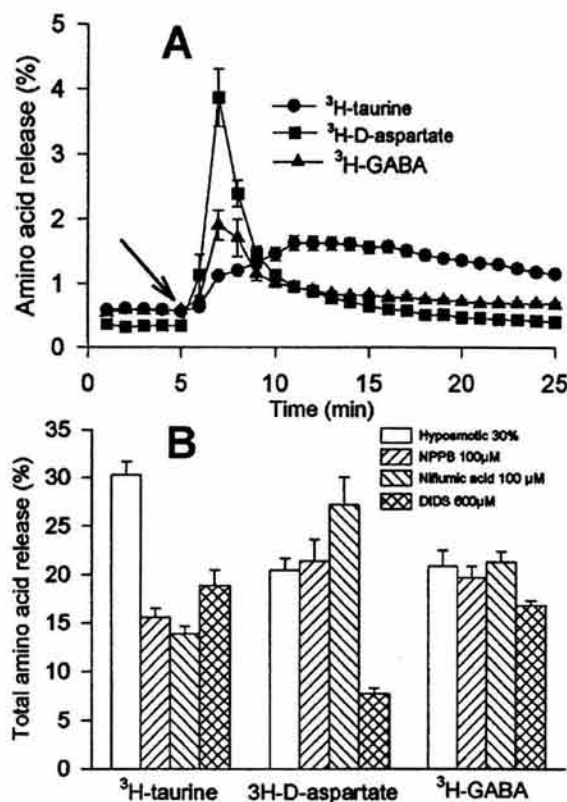
Fig. 2. Time course and pharmacological profile of hyposmosis-induced amino acid efflux from rat hippocampal slices. A: Efflux time course. Slices were loaded with [^{3}H]taurine, [^{3}H]GABA, and [^{3}H]D-aspartate (as tracer for glutamate), washed and superfused with warmed isosmotic medium (1 ml/min) and after reaching stable basal efflux (arrow), superfusion continued with 30% hyposmotic medium, made by reducing NaCl. B: Effect of Cl^- channel blockers. Slices were preincubated 15 min in isosmotic medium with 100 μM NPPB, 100 μm niflumic acid or 600 μM DIDS. Blockers were present in all superfusion solutions. Bars represent the amino acid released during 20 min of exposure to hyposmotic medium minus the release in isosmotic medium during the same time period. Data are arranged from Franco et al. (16).

point, the cell should "remember" its initial value and inactivate the efflux pathways.

Most work about the mechanisms of RVD has focussed on the characterization of the osmolyte efflux pathways, and it is only recently that other aspects of the volume adjustment process have been examined. As the above mentioned, the first signal in the chain is necessarily the detection of the change in cell volume, and nevertheless, this volume sensor mechanism is essentially unknown in all cell types. As for the transduction signalling, some recent evidence relates second messengers such as Ca^{2+}, such protein phosphorylation and phospholipases as elements of the transduction cascade (19–22). Here again, marked differences have been found between a typical organic osmolyte such as taurine, and amino acids such as GABA and glutamate.

Calcium. A change in cytosolic calcium ($Ca^{2+}i$) could be a key transduction messenger, since with few exceptions, hyposmotic swelling always results in an increase in $Ca^{2+}i$ levels (20). In spite of this general response, RVD and osmolyte fluxes are either Ca^{2+}-dependent or Ca^{2+}-independent in the different cell types. Activation of the volume-sensitive Cl^- channel is essentially Ca^{2+}-independent in most cells. (rev. in 20). Similarly, taurine efflux is insensitive to changes in $Ca^{2+}i$ in most cell types, including cultured neurons and astrocytes (23,24). Taurine release from brain slices is also essentially Ca^{2+}-independent (16). The influence of Ca^{2+} on the efflux of glutamate and GABA has not been examined in detail. In hippocampal slices, it seems independent of external and internal Ca^{2+}, although cytosolic Ca^{2+} cannot be fully depleted (16). In contrast, to taurine and Cl^- fluxes, the volume-activated K^+ fluxes are Ca^{2+}-dependent in numerous cell types, but not in many others (20). It is worthy to note that most cells showing these Ca^{2+} requirement for swelling-activated K^+ channels, are epithelial cells and that the Ca^{2+}-activated BK-type channels appear those involved in K^+ permeation during swelling. Obviously, in these cells, RVD is also Ca^{2+}-dependent, while it is not in cells where all the permeation pathways activated by swelling do not require Ca^{2+} (19).

Protein Phosphorylation. Recent evidence points to a key role of protein kinases as part of the signalling cascades to activate osmolyte fluxes (22,25). Tyrosine kinases appear involved in the mechanisms of osmosensitive release of Cl^- and taurine, as shown by the inhibitory effect of tyrosine kinase blockers such as tyrphostins, genistein, herbymicin and lavendustin (19,26,27). Accordingly, blockers of tyrosine phosphatases, such as *ortho*-vanadate, which prolong the tryosine phosphorylation reactions, lead to marked potentiation of taurine and Cl^- fluxes in various cell types, including cultured neurons and astrocytes (19,28). These results further emphasize the close similarities between Cl^- and taurine translocation pathways. The specific tyrosine kinases involved in osmolyte fluxes are not well identified. This is further complicated by the known activation of a number of tyrosine kinases by hyposmolarity and swelling, which however, appear unrelated to the operation of the osmolyte efflux pathways. This is the case of the MAP kinases ERK1/ERK2 and p38, which are indeed activated during swelling and RVD but have no influence on taurine and Cl^- release in various cell types, including neurons (28,29), and in hippocampal slices (Fig. 3) (16). (An exception are cortical astrocytes in which ERK1/ERK2 appear connected with the volume-activated Cl^- channel) (30). This is not unexpected, since the hyposmotic shock and the subsequent swelling are complex phenomena, involving numerous processes such as cell adhesion and retraction, as well as dramatic changes in the cytoskeleton organization. All these adaptations represent also stressful situations for the cell and some of these kinases are stress-activated enzymes. Therefore, it is necessary to discriminate among the plethora of signals activated by hyposmosis and swelling, those being strictly connected with the activation of corrective osmolyte pathways leading to RVD.

Studies so far have identified the tyrosine-kinase activated kinase PI3K, as one directly related to taurine efflux during RVD. PI3K is activated by hyposmolarity, and this reaction is blocked by wortmannin and LY294002 (16,28,31). These agents, particularly wortmannin, markedly inhibit taurine (and Cl^-) fluxes (16,28,31). The magnitude and the time course of taurine efflux reduction by PI3K blockers are rather similar to those due to general blockers of tyrosine kinases, suggesting this enzyme as a main target of tyrosine kinase activity. A noteworthy effect of the Cl^- channel and tyrosine kinase blockers observed in hippocampal slices is the persistence of a small peak of taurine, evident only when the blockers have blunted most of the efflux. This component, which accounts for about 17%, shows rapid activation and inactivation and seems similar in all respects, to the D-aspartate efflux (Fig. 4).

In contrast to these important influences of tyrosine- and tyrosine-activated kinases on taurine fluxes, D-aspartate efflux is resistant to these agents, as reported in cultured astrocytes and hippocampal slices (16,32) (Fig. 4). This remarkable difference is a clear indication of different signalling pathways for trigger

A

B
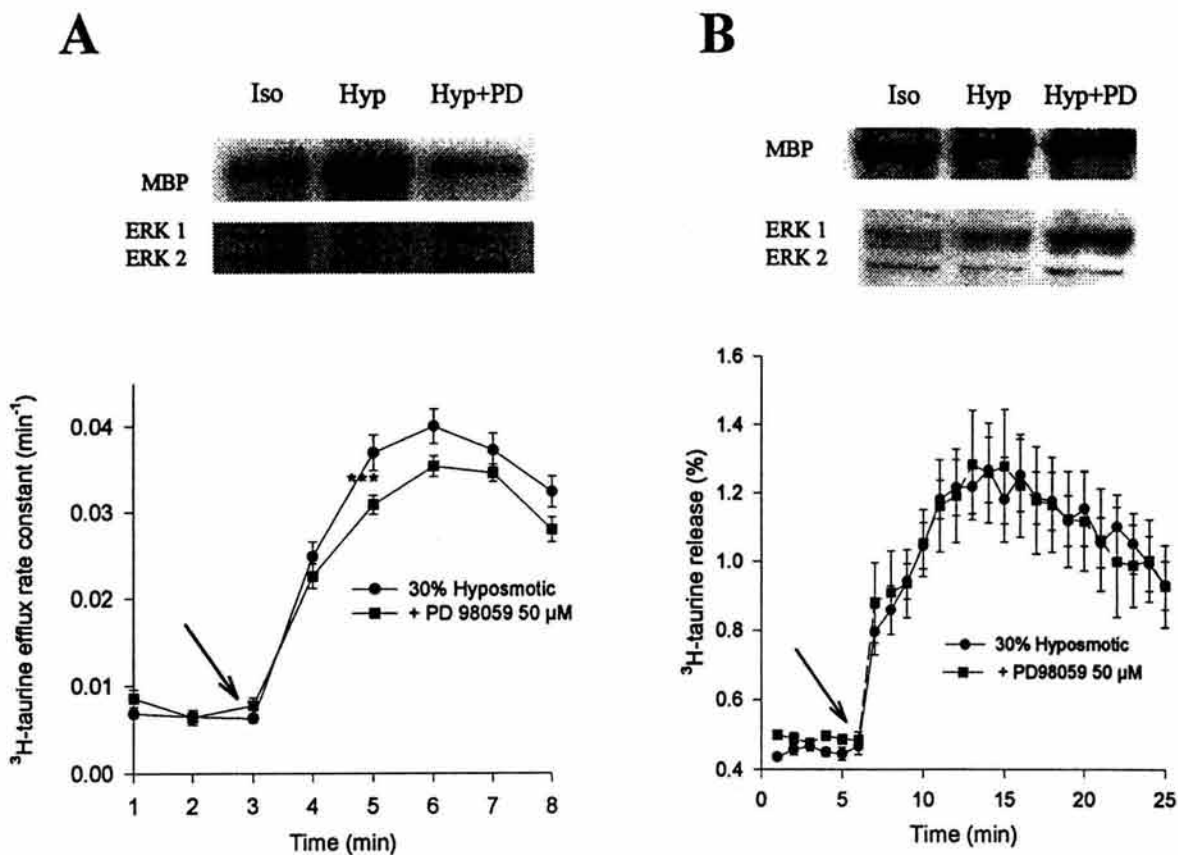
Fig. 3. Activation of ERK1/2 by hyposmosis in cerebellar granule neurons and in hippocampal slices, and lack of connection of this activity with the osmosensitive taurine efflux. Cultured cerebellar granule neurons (A) or hippocampal slices (B) were exposed to media: isosmotic (Iso), 30% hyposmotic (Hyp) or 30% hyposmotic + 50 µM PD98058 (Hyp + PD). Upper panel: ERK1/ERK2 activity in the different conditions. ERK1/ERK2 assay was performed as described in Morales-Mulia et al. (26). Briefly, aliquots of the cell lysate were immunoprecipitated with a polyclonal antibody to ERK1/ERK2. Immune complexes were collected by protein A sepharose and incubated with myelin basic protein (MBP) as substrate and [^{32}P]ATP. The activity of ERK1/ERK2 was monitored via autoradiography after SDS/PAGE electrophoresis. Lower panel: Lack of effect of the ERK1/2 activation blocker PD98058 on the hyposmolarity-induced release of taurine, despite the reduction in ERK activity. A. reproduced from Morales-Mulia et al. (26) and B. from Franco et al. (16).

of taurine and glutamate release. Being glutamate a neurotransmitter, its release may respond to other stimuli associated with events concurrent with swelling or/and volume regulation, such as swelling-associated depolarization, known to occur in various cell types, including astrocytes. However, this response is likely due to rapid Cl^- extrusion through the volume-activated pathway, and should be consequently prevented by niflumic acid or NPPB. Thus, the insensitivity of GABA/glutamate release to these blockers is against the notion of depolarization as the trigger for glutamate release. The Na^+ and Cl^- independence of GABA and glutamate release as well as the insensitivity of this efflux to carrier blockers observed in hippocampal slices (16) seem to exclude the reverse operation of the energy-dependent transporter. The efflux is also independent of

external Ca^{2+}, and insensitive to internal Ca^{2+} stores depletion, thus making unlikely the involvement of the typically Ca^{2+}-dependent vesicular release. However, this possibility cannot be definitely ruled out in the light of some recent evidence next discussed.

Calcium-Independent Exocytosis?

The hyposmotic stimulus trigger active phenomena of exocytosis in various cell types, which are either Ca^{2+}-dependent or -independent (33). This raises the possibility of exocytosis as the mechanism of GABA and glutamate release associated with swelling, although the vesicular release of these amino acids is typically Ca^{2+}-dependent. However, recent evidence
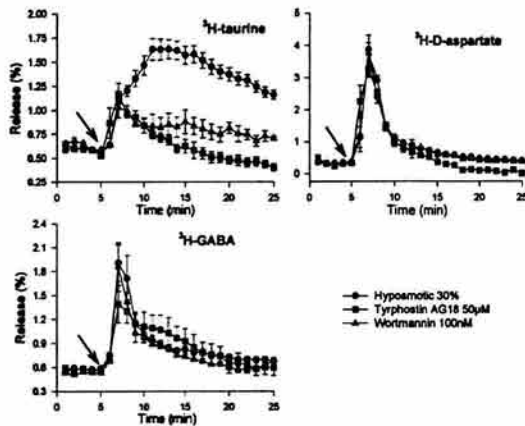
Fig. 4. Effects of tyrosine kinase blockers on hyposmolarity-elicited release of taurine, GABA and D-aspartate. Loading and superfusion of slices as in Fig. 2. Tyrphostine AG18 (50 μM), a blocker of tyrosine kinases, markedly reduced [³H]taurine release without significantly affecting the efflux of [³H]GABA and [³H]D-aspartate. The same different effects are observed in the presence of wortmannin (100 nM), a blocker of the tyrosine kinase-activated kinase PI3K. Both blockers revealed a resistant component of taurine efflux which, similar to those of GABA and glutamate appears not modulated by tyrosine kinases. Data arranged from Franco et al. (16).

points to occurrence of exocytotic release without a previous Ca²⁺ signal (34,35). The focus of this argument is that the submembrane actin filaments are organized as a mesh preventing exocytosis of the pool of synaptic vesicles trapped in this network. The disassembly dynamics occurs via Ca²⁺ and associated proteins and seems modulated by PKC, likely via an effect increasing vesicular release sensitivity to Ca²⁺. Thus it can be especulated whether hyposmolarity in association with PKC could activates exocytosis at basal Ca²⁺ levels, or even at residual Ca²⁺ levels in Ca²⁺-depleted preparations. In support of this hypothesis are results in hippocampal slices (16) showing that the osmosensitive release of glutamate is modulated by PKC (reduced by the blocker chelerythrine and enhanced by phorbol-12-myristate-13 acetate) and potentiated by the cytoskeleton disruption elicited by cytochalasin E. Noteworthy, these maneuvers do not affect taurine release, further emphasizing the differences in the mechanisms for release between these two amino acids.

These findings, although intriguing, have to be considered as preliminary, since they have so far been observed in only a few preparations. Such kind of studies should be extended to other brain preparations, including neurons of various types and different brain regions, and more importantly, to non nervous cell

types. It is not unlikely that in cells where these amino acids do not play a role as synaptic transmitters, their response to hyposmotic swelling is restricted to an osmolyte type and the mechanisms of release is in all alike to that of taurine.

In any event, the observations here presented explain a number of effects of osmolarity described in hippocampal slices, such as the reversible hyposmolarity-dependent enhancement of excitatory postsynaptic potentials, and to a lesser extent, also the inhibitory postsynaptic currents (36). This response of brain tissue to hyposmolarity, likely mediated by the mechanisms here described, may also explain the the increased seizure susceptibility observed during hyponatremia (37).

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REFERENCES

1. Pasantes-Morales, H. 1996. Volume regulation in brain cells: cellular and molecular mechanisms. Metab. Brain. Dis. 11:187–204.
2. Verbalis, J. G. and Gullans, S. R. 1991. Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res. 567:274–282.
3. Pasantes-Morales, H., Alavez, S., Sanchez Olea, R., and Moran, J. 1993. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem. Res. 18:445–452.
4. Pasantes-Morales, H. and Schousboe, A. 1988. Volume regulation in astrocytes: a role for taurine as osmoeffector, J. Neurosci. Res. 20:505–509.
5. Schousboe, A., Sanchez-Olea, R., Moran, J., and Pasantes-Morales, H. 1991. Hyposmolarity-induced taurine release in cerebellar granule cells is associated with diffusion and not with high-affinity transport. J. Neurosci. Res. 30:661–665.
6. Fránco, R., Quesada, O., and Pasantes-Morales, H. 2000. Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J. Neurosci. Res. 61:701–711.
7. Law, R. O. 1996. Volume regulation and the efflux of amino acids from cells in incubated slices of rat cerebral cortex. I. Characteristics of transport mechanisms. Biochim. Biophys. Acta. 1314:34–42.
8. Law, R. O. 1996. Volume regulation and the efflux of amino acids from cells in incubated slices of rat cerebral cortex. II. Dependence on Ca2+ ions. Biochim. Biophys. Acta. 1314:43–48.
9. Deleuze, C., Duvoid, A., and Hussy, N. 1998. Properties and glial origin of osmotic-dependent release of taurine from the rat supraoptic nucleus. J. Physiol. 507:463–471.
10. Solis, J. M., Herranz, A. S., Herreras, O., Lerma, J., and Martin del Rio, R. 1988. Does taurine act as an osmoregulatory substance in the rat brain?. Neurosci. Lett. 91:53–58.
11. Estevez, A. Y., O'Regan, M. H., Song, D., and Phillis, J. W. 1999. Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. Neurochem. Res. 24:447–452.

12. Taylor, D. L., Davies, S. E., Obrenovitch, T. P., Doheny, M. H., Patsalos, P. N., Clark, J. B., and Symon, L. 1995. Investigation into the role of N-acetylaspartate in cerebral osmoregulation. J. Neurochem. 65:275–281.
13. Pasantes-Morales, H. and Schousboe, A. 1997. Role of taurine in osmoregulation: mechanisms and functional implications. Amino Acids, 12:281–292.
14. Sanchez-Olea, R., Pena, C., Moran, J., and Pasantes-Morales, H. 1993. Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl-transport in cultured astrocytes. Neurosci. Lett. 156:141–144.
15. Roy, G. 1995. Amino acid current through anion channels in cultured human glial cells. J. Membr. Biol. 147:35–44.
16. Franco, R., Torres-Marquez, M. E., and Pasantes-Morales, H. 2001. Evidence of two mechanisms for amino acid osmolyte release from hippocampal slices. Pflügers. Arch. (in press).
17. Okada, Y. 1997. Volume-expansion-sensing, outward-rectifier Cl⁻ channel: fresh start to the molecular identity and volume sensor. Amer. J. Physiol. 273:C755–C789.
18. Rutledge, E. M., Aschner, M., and Kimelberg, H. K. 1998. Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. Am. J. Physiol. 274:C1511–C1520.
19. Tilly, B. C., Van den Berghe, N., Tertoolen, L. G., Edixhoven, M. J., and de Jonge, H. R. 1993. Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J. Biol. Chem. 268:19919–19922.
20. Pasantes-Morales, H. and Morales Mulia, S. 2000. Influence of calcium on regulatory volume decrease: role of potassium channels. Nephron 86:414–427.
21. Hoffmann, E. K. 2000. Intracellular signalling involved in volume regulatory decrease. Cell. Physiol. Biochem. 10:273–288.
22. Van der Wijk, T., Tomassen, S. F., de Jonge, H. R., and Tilly, B. C. 2000. Signalling mechanisms involved in volume regulation of intestinal epithelial cells. Cell. Physiol. Biochem. 10:289–296.
23. O'Connor, E. R. and Kimelberg, H. K. 1993. Role of calcium in astrocyte volume regulation and in the release of ions and amino acids. J. Neurosci. 13:2638–2650.
24. Moran, J., Morales-Mulia, S., Hernandez-Cruz, A., and Pasantes-Morales, H. 1997. Regulatory volume decrease and associated osmolyte fluxes in cerebellar granuleneurons are calcium independent. J. Neurosci. Res. 47:144–154.
25. Pasantes-Morales, H., Cardin, V., and Tuz, K. 2000. Signaling events during swelling and regulatory volume decrease. Neurochem. Res. 25:1301–1314.
26. Hubert, E. M., Musch, M. W., and Goldstein, L. 2000. Inhibition of volume-stimulated taurine efflux and tyrosine kinase activity in the skate red blood cell. Pflugers. Arch. 440:132–139.
27. Deleuze, C., Duvoid, A., Moos, F. C., and Hussy, N. 2000. Tyrosine phosphorylation modulates the osmosensitivity of volume-dependent taurine efflux from glial cells in the supraoptic nucleus. J. Physiol. 1:291–299.
28. Morales-Mulia, S., Cardin, V., Torres-Marquez, M. E., Crevenna, A., and Pasantes-Morales, H. 2001. Influence of protein kinases on the osmosensitive release of taurine from cerebellar granule neurons. Neurochem. Int. 38:153–161.
29. Tilly, B. C., Gaestel, M., Engel, K., Edixhoven, M. J., and de Jonge, H. R. 1996. Hypo-osmotic cell swelling activates the p38 MAP kinase signalling cascade. FEBS Lett. 395:133–136.
30. Crepel, V., Panenka, W., Kelly, M. E., and MacVicar, B. A. 1998. Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. J. Neurosci. 18:1196–1206.
31. Tilly, B. C., Edixhoven, M. J., Tertoolen, L. G., Moril, N., Saitoh, Y., Narumiya, S., and de Jonge, H. R. 1996. Activation of the osmo-sensitive chloride conductance involves P21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. Mol. Biol. Cell. 7:1419–1427.
32. Mongin, A. A., Reddi, J. M., Charniga, C., and Kimelberg, H. K. 1999. [3H]taurine and D-[3H]aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. Am. J. Physiol. 276:C1226–C1230.
33. Strbak, V., Greer, M. A. 2000. Regulation of hormone secretion by acute cell volume changes: Ca(2+)-independent hormone secretion. Cell. Physiol. Biochem. 10:393–402.
34. Knight, D. E., Von Grafenstein, H., and Athayde, C. M. 1989. Calcium-dependent and calcium-independent exocytosis. Trends Neurosci. 12:451–458.
35. Hille, B., Billiard, J., Babcock, D. F., Nguyen, T., and Koh, D. S. 1999. Stimulation of exocytosis without a calcium signal. J. Physiol. 520:23–31.
36. Andrew, R. D. 1991. Seizure and acute osmotic change: clinical and neurophysiological aspects. J. Neurol. Sci. 101:7–18.
37. Arieff, A. I. 1986. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgeryin healthy women. N. Engl. J. Med. 314:1529–1535.

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Influence of protein tyrosine kinases on cell volume change-induced taurine release

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Taurine efflux occurs in association with cell swelling in both hyposmotic and isosmotic conditions and during cell shrinkage in apoptotic death. Release occurs through a leak pathway, is largely Ca^{2+}-independent and is sensitive to Cl^- channel blockers. Taurine efflux elicited by hyposmolarity is reduced or suppressed by tyrosine kinase blockers and increased by tyrosine phosphatase inhibitors. The specific kinases involved are still unknown and may be different in the various cell types. Non-receptor and scr-related protein kinases have been identified in some cells as elements that directly phosphorylate the taurine efflux pathway. Possible tyrosine kinase targets are the phosphinositide kinase (PI3K), which if inhibited, prevents the osmosensitive taurine efflux in brain cells, or the small GTP-binding proteins associated with remodeling of the cytoskeleton. The similar effects of tyrosine kinase modulators on volume-activated taurine fluxes and Cl^- currents are suggestive of either a shared translocation pathway or a common step in the signaling network. The effects of tyrosine kinases on taurine efflux activated in isosmotic swelling and in the release associated with apoptosis are essentially unexplored.

Introduction

Taurine is released from diverse brain tissue preparations in response to a variety of stimuli, one of the most conspicuous being the reduction in external osmolarity.[1] Taurine release is activated by cell membrane depolarization and in conditions of ischemia, hypoxia, hyperammonemia and subsequent to generation of oxygen free radicals and membrane lipid peroxidation.[2-5] Another stimulus consistently increasing taurine release from brain preparations is the activation of excitatory amino acid receptors by agonists such as glutamate, kainate, NMDA and quinolinic acid. Nitric oxide has been reported to increase taurine efflux from cultured neurons and brain slices, an effect that could be either direct or through modulation of NMDA receptors.[2] It is so far unclear whether the release of taurine in all these different conditions is triggered by a common factor, or whether in each case the release operates through different avenues. A possibility is that taurine release is

primarily a cell response to swelling. The bases to consider this possibility are, first, that all these conditions involve a certain degree of swelling and second, that swelling consistently evokes taurine efflux. Taurine is particularly suited for this osmolyte role, due to its metabolic inactivity and its high concentration as a free solute in the cytosol.[6]

Taurine efflux is evoked by hyposmotic and isosmotic cell swelling

Swelling of brain cells may occur in hyposmotic or isosmotic conditions. The first condition is the consequence of hyponatremia of various origins.[7] Isosmotic cell swelling, also referred as cytotoxic edema, occurs in association with ischemia, epilepsies, hepatic encephalopathy, hyperammonemia, head trauma and hypoglycemia.[8-11] Swelling results from changes in ion distribution by dysfunction of ion transport mechanisms or by disruption of the membrane barrier subsequent to attack by free radical and lipoperoxidation, leading to ionic imbalance and overload, with the consequent water accumulation.[8-11] In all cases, brain cell edema represents a situation of high risk, since the restriction to tissue expansion imposed by the rigid cranium, commonly leads to compression of small vessels generating episodes of ischemia, excitotoxicity and neuronal death

Experimental paradigms *in vitro* and *in vivo* have been designed to simulate the conditions leading to brain cell edema *in vivo*. The most commonly used model for hyponatremia is to expose cells or tissue to a hyposmotic medium made by decreasing the concentration of NaCl. Under these conditions, cells initially swell and then undergo an active process of volume recovery accomplished by the extrusion of intracellular osmolytes. Hyposmolarity elicits taurine efflux in essentially all cell types, including brain cells and also in brain tissue preparations.[1,12,13] *In vivo*, taurine efflux from brain superfused with hyposmotic medium has been also consistently observed, and the increase of taurine in the extracellular space mirrors the decrease in intracellular levels.[14,15,16] The magnitude of taurine efflux is proportional to the reduction in osmolarity, its time course roughly parallels that of the volume recovery and blockade of taurine efflux or taurine deficiency impairs cell volume regulation.[1] In models of chronic hyponatremia *in vivo*, a continuous and progressive decrease in brain taurine levels is observed, to an extent that the intracellular pool is essentially depleted.[17] These results underlie the importance of taurine in the maintenance of normal brain water content, preventing the sequels of injury due to cell edema.

There is general agreement that osmosensitive taurine efflux occurs through a leak pathway, with no contribution of the Na^+-dependent carriers.[18] In most cell types taurine efflux is sensitive to Cl^- channel blockers, such as 5-nitro-(3-phenyl-propylamino) benzoic acid (NPPB), tamoxifen, and to the anion exchanger blockers niflumic acid, 4,4'-diisothiocyanatostil loene-2, 2'-discultonic acid (DIDS) and pyridoxal phosphate.[19-21] These features have raised the proposal of a common pathway (a Cl^- channel-like molecule), for the osmosensitive transport of Cl^-, taurine, and other organic osmolytes.[19] This common pathway hypothesis is not yet conclusive[21] and awaits the molecular identity of the volume-sensitive Cl^- channel as well as more insight into the molecular nature of the taurine pathway itself.

While cell volume regulation after swelling evoked by hyposmolarity has been extensively investigated, it is still unclear whether such regulation occurs in cytotoxic edema and to what extent organic osmolytes may participate in the volume correction, or at least contribute to reduce the magnitude of swelling. Taurine release occurs consistently under conditions of cytotoxic edema[2] and its release is sensitive to Cl^- channel blockers, suggesting a similarity with the mechanism of release in hyposmotic conditions.[22-24] However, it should be emphasized that even when cell swelling occurs by different mechanisms in each pathology, in most of them Cl^- influx is a causal factor. Therefore, the inhibitory action of Cl^- channel blockers may be exerted on the Cl^- influx pathways, thus preventing swelling and consequently, the primary cause of taurine release.

Tyrosine kinases are involved in the hyposmolarity-evoked release of taurine

Most studies aiming to understand the influence of tyrosine kinase-activated pathways during volume correction after hyposmotic swelling have been focussed on the volume-activated Cl^- channel,[25] while similar investigations for taurine efflux are still scarce. As mentioned before, the one pathway hypothesis for Cl^- and taurine is not yet conclusive and therefore, results about signaling mechanisms modulating volume-activated Cl^- channels should not be directly extrapolated to the osmosensitive taurine efflux. Similarly, transduction steps leading to activation of taurine efflux should not be naturally assumed to be the same for other organic osmolytes, nor even for other amino acids. This may be particularly true for tyrosine kinases as will be later discussed.

Evidence in support of the involvement of tyrosine kinases in the osmosensitive taurine efflux relies on the effect of several tyrosine kinase blockers reducing the efflux of taurine, while correspondingly, agents which inhibit tyrosine kinase phosphatases and prolong tyrosine phosphorylation, potentiate taurine release.

The general tyrosine kinase blockers tyrphostins, inhibit taurine release evoked by swelling in cultured cerebellar granule neurons and astrocytes,[26,27] in the supraoptic nucleus[28] and in hippocampal slices[29] (Table 1). Ortho-vanadate potentiates taurine efflux in cerebellar granule neurons and astrocytes and in the supraoptic nucleus whereas it has no effect on taurine efflux in hippocampal slices. These differences, also found in other cells (see below) may be due either to the involvement of tyrosine phosphatases with different sensitivities to the blocker or to differences in the permeability of ortho-vanadate in the various preparations.

Tyrosine kinase blockers also reduce the hyposmolarity-elicited taurine efflux in non brain cells (Table 2). In skate blood erythrocytes, tyrphostins reduce hyposmolarity-induced taurine release[30] and in HeLa cells, genistein decreases and ortho-vanadate potentiates taurine efflux. Also genistein reduces taurine efflux in trout erythrocytes, but in the latter cells, the release is either unaffected or reduced by ortho-vanadate.[32] In a Langendorff heart preparation, taurine efflux is reduced by genistein and lavendustin A.[33] Remarkably, this is the only study *in vivo*, addressing the influence of tyrosine kinases on the mechanism of taurine release in hyposmotic conditions.

While taurine efflux stimulated by hyposmolarity is clearly affected by tyrosine kinase blockers, other amino acids (GABA and glutamate) which are also released from brain cells by the hyposmotic stimulus, are insensitive to tyrosine kinase blockers, suggesting a lack of influence of these kinases.[29] A similar insensitivity to Cl^- channel blockers, in contrast to taurine, suggests a different pathway for the osmosensitive release of these amino acids.[29]

Table 1

Effect of tyrosine kinase blockers in hyposmotic-induced taurine efflux in brain preparations

Cell type	Agent	[µM]	Effect	Reference
Cerebellar granule neurons	Tyrphostin AG18	50	Inhibition 90%	(26)
	Genistein	50	Inhibition 30%	
	PD98059	25	Unaffected	
Cerebellar astrocytes	Tyrphostin A23	0.32–320	Inhibition 90%	(27)
	Tyrphostin A51	0.1–100	Inhibition 50–55%	
Supraoptic nucleus	Tyrphostin B44	50	Inhibition 27–32%	(28)
	Genistein	50	Inhibition	
	PD98059	50	Unaffected	
Hippocampal slices	Tyrphostin AG18	50	Inhibition 61%	(29)
	Tyrphostin AG112	100	Inhibition 29%	
	Tyrphostin AG879	25	Inhibition 78%	
	Genistein	100	Unaffected	
	Lavendustin A	1	Unaffected	
	Herbimycin A	10	Unaffected	
	PD98059	25	Unaffected	
	SB202190	25	Unaffected	

Table 2

Effect of tyrosine kinase blockers in hyposmotic-induced taurine efflux in non-brain preparations

Cell type	Agent	[µM]	Effect	Reference
Skate red blood cells	Tyrphostin 46	25–200	Inhibition	(30)
	Tyrphostin A23	25–200	Inhibition 50%	
	Genistein	25–200	Stimulation <20%	
	PD98059	25–200	Stimulation <20%	
Trout red blood cells	Genistein	45–100	Inhibition	(32)
Jurkat T-cells	Herbimycin A	10	Unaffected	(53)
HeLa cells	Genistein	100	Inhibition 60%*	(31)
Langendorff preparation	Genistein	1	Inhibition	(33)
	Lavendustin A	0.5	Inhibition	

* Recalculated from (31).

In most cell types, the specific kinases involved in the osmosensitive taurine efflux have not yet been identified

The consistent effect of tyrosine kinase blockers reducing taurine efflux in hyposmotic conditions, point to these enzymes as part of the osmotransduction signaling, connecting the change in cell volume and the activation of the taurine efflux pathway. So far, though, in most cells the specific kinases involved and the sites of their action remain to be identified. The most consistent work on the involvement of tyrosine kinases in hyposmolarity-activated taurine release has been carried out by Goldstein and coworkers, in a series of studies in the skate nucleated erythrocytes. The hyposmolarity-induced release of taurine in these cells appears to occur through the band 3 anion exchange protein.[34] Taurine release is reduced by tyrphostin A23, although it is insensitive to other tyrphostins and to genistein.[30] Hyposmosis leads to band 3 phosphorylation by the tyrosine kinases p76syk and p56lyn[30] and p72syk directly phosphorylates the cytoplasmic domain of the band 3 protein with a time course parallel to that of the swelling-activated taurine efflux.[30] Accordingly, the p72syk inhibitor piceatannol blocks, at about the same concentration, both taurine efflux and p72syk-dependent band 3 phosphorylation.[30] Moreover, the phosphatase blocker pervanadate similarly potentiates taurine efflux and the activity of p72syk phosphorylation.[30] In this same preparation, various experimental paradigms were used to produce cell swelling with differences in the intracellular ionic strength, as this parameter seems to regulate the set point for taurine efflux activation by cell swelling.[35,36] The effect of intracellular strength reduction on the activity of the two tyrosine kinases involved in the band 3 phosphorylation and taurine efflux was examined, and results are suggestive

of some sensitivity of p72syk and p56lyn to the change in intracellular ionic strength.[37] In this respect, in the supraoptic nucleus, the magnitude of the effect of tyrosine kinase and phosphatase blockers is dependent on the external osmolarity, suggesting that tyrosine kinases modulate the sensitivity to hyposmolarity rather than the efflux pathway activation.[28]

The src-related tyrosine kinase p56lck is found to be a key signaling element for osmosensitive Cl⁻ currents in lymphocytes, as shown by its activation upon swelling, suppression of osmosensitive currents in p56lck-deficient cells by genetic knockout and its restitution after retransfection of the kinase.[38] The involvement of this kinase on taurine efflux has not yet been examined.

Apart from these studies in blood cells, the tyrosine kinases involved in taurine efflux, and in that of other osmolytes as well, have not been identified. This is further complicated because tyrosine kinases are steps in the signaling cascades of numerous cell responses, many of which are activated by processes occurring in association with swelling and volume regulation, such as cell adhesion and retraction, reorganization of the cytoskeleton and gating of stretch-activated channels. Besides, swelling is a stressful condition for the cells, also activating signals associated with stress responses. Therefore, even the identification of the kinases activated by hyposmolarity (Figure 1) may not be sufficient to ascribe them to the activation or operation of osmolyte extrusion pathways, including those for taurine. Examples of

this situation are the phosphorylation by hyposmotic swelling of the mitogen-activated protein (MAP) kinases extracellular signal-related kinase-1/2 (ERK1/ERK2) observed in various cell types.[25,39] This reaction mediates the swelling-induced transcription of early expression genes,[40] the volume-sensitive channels in astrocytes[41] and taurocholate excretion in hepatocytes;[39] but in most cell types and brain preparations such as cerebellar granule neurons, hippocampal slices, supraoptic nucleus, intestinal 407 cells and skate erythrocytes, even when MAP kinases are activated by hyposmolarity, preventing this reaction has no effect on osmolyte fluxes, including Cl– and taurine.[25,26,28,29,42]

An attractive hypothesis recently considered is that the integrin receptor could play a role in the sensing of cell volume changes by detecting membrane expansion or unfolding of membrane invaginations.[43] Integrin activation, possibly by receptor clustering, could initiate a signaling cascade via focal adhesion kinase (FAK), which is known to be activated by hyposmolarity. FAK, in turn, connects with a variety of signaling proteins such as src-related protein kinases, PI3K and small GTP-binding proteins associated with remodeling of the cytoskeleton. All these proteins have been found to be activated by hyposmolarity in different cells and many of them are related to taurine (or Cl⁻) corrective fluxes. Besides the already discussed link of taurine release with src-related kinases and other non receptor kinases in blood cells, other elements of this signaling cascade
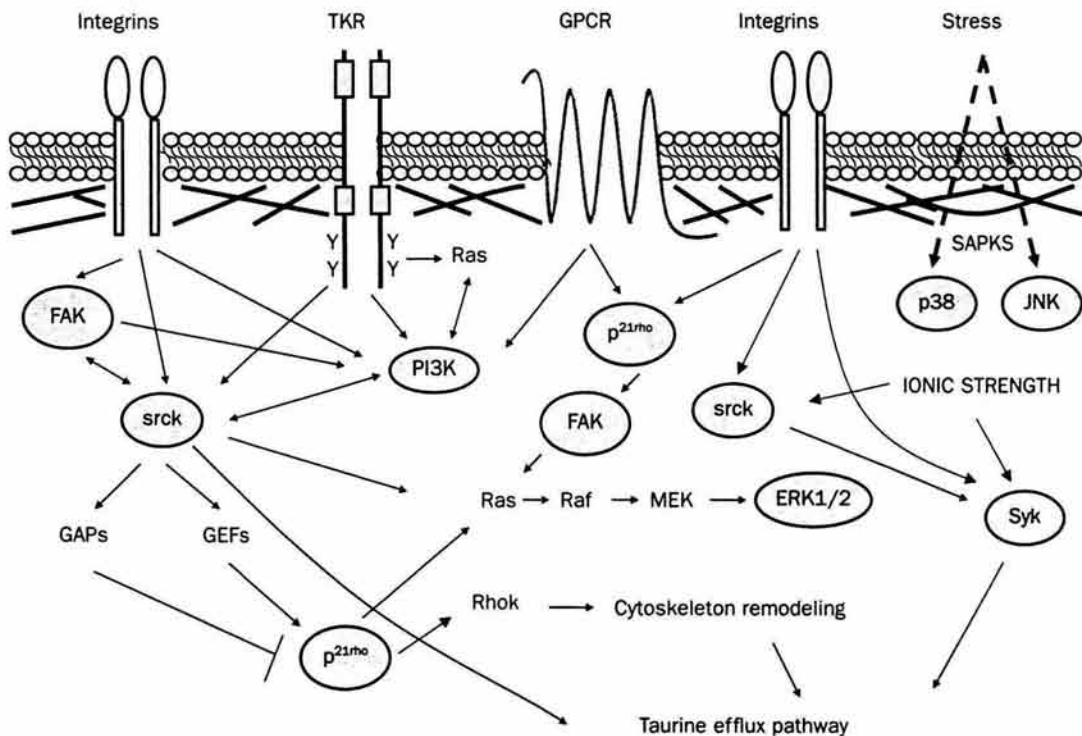
Figure 1
Interactions between some membrane associated receptors and signaling cascades involving tyrosine kinases and tyrosine kinase-activated kinases. Enzymes in shaded circles are those activated by hyposmolarity. Receptors may be activated by adhesion reactions or by clustering due to membrane cytoskeleton reorganization as a consequence of swelling. TKR: tyrosine kinase receptors, GPCR: G protein coupled receptors, FAK: focal adhesion kinase, PI3K: phoshoinositide 3-kinase, SAPKS: stress-activated kinases, ERK1/2: extracellular regulated kinases 1 and 2, MEK: MAPK/ERK-kinase, GEEs: guanine exchange factors, GAPs: GTPase activating proteins, srck: src related kinases, Rhok: Rho kinase
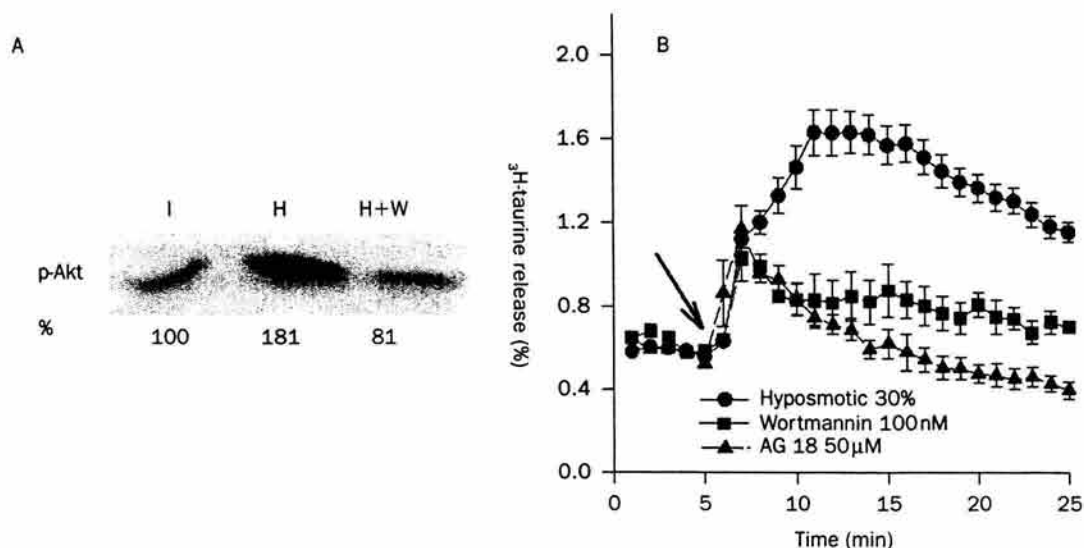
Figure 2
Effect of tyrosine kinases and PI3K inhibition on the osmosensitive taurine efflux hippocampal slices. (A) Hyposmotic (30%) medium activates PI3K, sensitive to wortmannin. I: isosmotic, H: 30%, hyposmotic, H+W: 30% hyposmotic + 100 nM wortmannin. Representative results of three independent experiments are shown. PI3K was quantified by detection of the phosphorylated form of Akt phosphorylated at Ser-473. Lower panel shows the % of activity respectively to the isosmotic condition. (B) Inhibitory effect of 50 µM tyrphostin AG18 (s) and 100 nM wortmannin (n) on taurine efflux elicited by 30% hyposmotic medium (l) (arrow) from hippocampal slices preloaded with 3H-taurine. Data represent the radioactivity released per min expressed as percentage of the total incorporated and are means ± SE (*n* = 6). Details of the experimental conditions as in Ref. 29.

involved in osmolyte fluxes include the small GTPase p21Rho,[44,45] and particularly the phosphoinositide kinase PI3K. This enzyme, which is a key element in signaling networks connecting with integrins, FAK, src-related kinases and tyrosine kinase membrane receptors, appears to have a relevant role in the mechanisms of taurine release. In cerebellar granule neurons, astrocytes and hippocampal slices, hyposmolarity activates PI3K, and this reaction is blocked by wortmannin (Figure 2A).[26,29] This agent is also a potent blocker of hyposmotic taurine release in these preparations (Figure 2B), suggesting a direct link of PI3K and the activation or/and operation of the taurine efflux pathway. Wortmannin also blocks anion fluxes activated by hyposmolarity in various cell types.[44,47,48] The similar effect of tyrphostins and wortmannin on taurine efflux illustrated in Figure 2 is suggestive of PI3K as a main target of tyrosine kinase reactions in osmotransduction.

Tyrosine kinase receptors such as integrin, associated with many of those signaling elements may also have an influence on osmolyte release, as suggested by a study on myoblasts, in which taurine efflux is activated by thrombin and hyposmolarity, with similar characteristics.[46] The idea of integrin or other membrane receptors acting as a very early and basic mechanism of the signaling osmotransduction cascade, might also explain the many common effects of tyrosine kinases on Cl⁻ and taurine fluxes, and possibly on other organic osmolytes as well.

Are tyrosine kinases involved in the mechanism of taurine release in isosmotic conditions?

As previously mentioned, taurine efflux is elicited in isosmotic conditions by a variety of stimuli, most of them having in common some extent of cell swelling due to intracellular ion redistribution. The mechanisms of taurine release in these conditions are still poorly defined and may or may not be similar to those activated by hyposmotic swelling. The influence of tyrosine kinases on taurine release is essentially unknown. Only two reports document the inhibitory effect of genistein and lavendustin on taurine release evoked by ischemia-reperfusion in brain and heart.[49,50] It should be noticed that, similar to hyposmolarity, or even more, ischemia evokes a large number of reactions and cell responses, many of them involving tyrosine kinase phosphorylations, thus making it difficult to identify among those activated by the ischemic condition, which are directly related to the mechanism of taurine release.

Taurine release and apoptosis

Cell shrinkage is one of the hallmarks of apoptosis, though the mechanisms leading to this remarkable reduction in cell volume are still poorly understood. Moderate increases in external osmolarity inhibit apoptosis, suggesting an active role of cell volume as part of the signals leading to cell death.[51] Increasing external

osmolarity may prevent the loss of osmotic solutes, and cell shrinkage, which may be a determinant in the initiation of the apoptotic events. Taurine appears to be one of those osmolytes. In Jurkat T lymphocytes, apoptosis induced by stimulation of the CD95 receptor, evokes a release of taurine.[52] An apoptosis-associated taurine release has been also found in cerebellar granule neurons.[53] At variance with the osmosensitive release of taurine, the apoptosis-related taurine efflux is insensitive to Cl⁻ channel blockers.[52,53] The influence of tyrosine kinases is essentially unknown, with only one report in Jurkat cells showing that src-related kinases are not involved, since neither herbimycin A, vanadate nor p56lck deficiency influenced taurine release.[54]

Conclusions

The release of taurine associated with cell volume regulation is well established in conditions of hyposmolarity and tyrosine kinases likely play a critical role in the mechanisms of this release. The present knowledge, however, is still fragmentary, with studies carried out in different cell types or tissue preparations, different blockers and different experimental approaches, which makes it difficult to identify the specific tyrosine kinases involved in each cell type. As for the release of taurine in isosmotic conditions, the influence of tyrosine kinases is essentially unexplored, as is that of the apoptosis-associated taurine efflux. This is an interesting avenue for future research, which may give important clues about the similarities and dissimilarities of taurine release evoked by different stimuli.

Acknowledgements

References

1. Pasantes-Morales H, Schousboe A. Role of taurine in osmoregulation: mechanisms and functional implications. Amino Acids 1997; 12: 281–292.
2. Oja SS, Saransaari P. Modulation of taurine release by glutamate receptors and nitric oxide. Prog Neurobiol 2000; 62: 407–425.
3. Albrecht J, Bender AS, Norenberg MD. Ammonia stimulates the release of taurine from cultured astrocytes. Brain Res 1994; 660: 288–292.
4. Menéndez N, Solis JM, Herreras O, Herranz AS, Martin del Rio R. Extracellular taurine release in rat hippocampus evoked by specific glutamate receptor activation is related to the excitatory potency of glutamate agonists. Neurosci Lett 1989; 102: 64–69.
5. O'Regan MH, Song D, VanderHeide SJ, Phillis JW. Free radicals and the ischemia-evoked extracellular accumulation of amino acids in rat cerebral cortex. Neurochem Res 1997; 22: 273–280.
6. Huxtable RJ. Physiological actions of taurine. Physiol Rev 1992; 72: 101–163.
7. Kimelberg HK. Current concepts of brain edema. Review of laboratory investigations. J Neurosurg 1995; 83: 1051–1059.
8. Chan PH, Yurko M, Fishman RA. Phospholipid degradation and cellular edema induced by free radicals in brain cortical slices. J Neurochem 1982; 38: 525–531.
9. Kempski O, Staub F, Schneider GH, Weigt H, Baethmann A. Swelling of C6 glioma cells and astrocytes from glutamate, high K⁺ concentrations or acidosis. Prog Brain Res 1992; 94: 69–75.
10. Kreisman NR, Soliman S, Gozal D. Regional differences in hypoxic depolarization and swelling in hippocampal slices. J Neurophysiol 2000; 83: 1031–1038.
11. Rosenberg GA. Ischemic brain edema. Prog Cardiovasc Dis 1999; 42: 209–216.
12. Law RO. Taurine efflux and the regulation of cell volume in incubated slices of rat cerebral cortex. Biochim Biophys Acta 1994; 1221: 21–28.
13. Franco R, Quesada O, Pasantes-Morales H. Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J Neurosci Res 2000; 61: 701–711.
14. Estevez AY, O'Regan MH, Song D, Phillis JW. Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. Neurochem Res 1999; 24: 447–452.
15. Lehmann A. Effects of microdialysis-perfusion with anisoosmotic media on extracellular amino acids in the rat hippocampus and skeletal muscle. J Neurochem 1989; 53: 525–535.
16. Solis JM, Herranz AS, Herreras O, Lerma J, Martin del Rio R. Does taurine act as an osmoregulatory substance in the rat brain? Neurosci Lett 1988; 91: 53–58.
17. Verbalis JG, Gullans SR. Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res 1991; 567: 274–282.
18. Sanchez-Olea R, Moran J, Schousboe A, Pasantes-Morales H. Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. Neurosci Lett 1991; 130: 233–236.
19. Strange K, Emma F, Jackson PS. Cellular and molecular physiology of volume-sensitive anion channels. Am J Physiol. 1996; 270: C711–730.
20. Pasantes-Morales H. Volume regulation in brain cells: cellular and molecular mechanisms. Metab Brain Dis 1996; 11: 187–204.
21. Kirk K. Swelling-activated organic osmolyte channels. J Membr Biol 1997; 158: 1–16.
22. Estevez AY, Song D, Phillis JW, O'Regan MH. Effects of the anion channel blocker DIDS on ouabain- and high K(+)-induced release of amino acids from the rat cerebral cortex. Brain Res Bull 2000; 52: 45–50.
23. Phillis JW, Song D, O'Regan MH. Tamoxifen, a chloride channel blocker, reduces glutamate and aspartate release from the ischemic cerebral cortex. Brain Res 1998; 780: 352–355.
24. Phillis JW, Song D, O'Regan MH. Inhibition by anion channel blockers of ischemia-evoked release of excitotoxic and other amino acids from rat cerebral cortex. Brain Res 1997; 758(1–2): 9–16.
25. van der Wijk T, Tomassen SF, de Jonge HR, Tilly BC. Signalling mechanisms involved in volume regulation of intestinal epithelial cells. Cell Physiol Biochem 2000; 10: 289–296.
26. Morales-Mulia S, Cardin V, Torres-Marquez ME, Crevenna A, Pasantes-Morales H. Influence of protein kinases on the osmosensitive release of taurine from cerebellar granule neurons. Neurochem Int 2001; 38: 153–161.
27. Mongin AA, Reddi JM, Charniga C, Kimelberg HK. [3H]taurine and D-[3H]aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. Am J Physiol 1999; 276: C1226–C1230.

28. Deleuze C, Duvoid A, Moos FC, Hussy N. Tyrosine phosphorylation modulates the osmosensitivity of volume-dependent taurine efflux from glial cells in the rat supraoptic nucleus. J Physiol 2000; 523: 291–299.

29. Franco R, Torres-Marquez ME, Pasantes-Morales H. Evidence of two mechanisms of amino acid osmolyte release from hippocampal slices. Pflugers Arch 2001; 442: 791–800.

30. Hubert EM, Musch MW, Goldstein L. Inhibition of volume-stimulated taurine efflux and tyrosine kinase activity in the skate red blood cell. Pflugers Arch 2000; 440: 132–139.

31. Lambert IH, Falktoft B. Lysophosphatidylcholine induces taurine release from HeLa cells. J Membr Biol 2000; 176: 175–185.

32. Kiessling K, Ellory JC, Cossins AR. The relationship between hypotonically-induced taurine and K fluxes in trout red blood cells. Pflugers Arch 2000; 440: 467–475.

33. Song D, O'Regan MH, Phillis JW. Protein kinase inhibitors attenuate cardiac swelling-induced amino acid release in the rat. J Pharm Pharmacol 1998; 50: 1280–1286.

34. Goldstein L, Brill SR. Volume-activated taurine efflux from skate erythrocytes: possible band 3 involvement. Am J Physiol 1991; 260: R1014–R1020.

35. Motais R, Guizouarn H, Garcia-Romeu F. Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems. Biochim Biophys Acta. 1991; 1075: 169–180.

36. Cannon CL, Basavappa S, Strange K. Intracellular ionic strength regulates the volume sensitivity of a swelling-activated anion channel. Am J Physiol 1998; 275: C416–C422.

37. Wittels KA, Hubert EM, Musch MW, Goldstein L. Osmolyte channel regulation by ionic strength in skate RBC. Am J Physiol Regul Integr Comp Physiol 2000; 279: R69–R76.

38. Lepple-Wienhues A, Szabo I, Wieland U, Heil L, Gulbins E, Lang F. Tyrosine kinases open lymphocyte chloride channels. Cell Physiol Biochem 2000; 10: 307–312.

39. Haussinger D, Schliess F. Osmotic induction of signaling cascades: role in regulation of cell function. Biochem Biophys Res Commun 1999; 255: 551–555.

40. Sadoshima J, Qiu Z, Morgan JP, Izumo S. Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. EMBO J 1996; 15: 5535–5546.

41. Crepel V, Panenka W, Kelly ME, MacVicar BA. Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. J Neurosci 1998; 18: 1196–1206.

42. Tilly BC, Gaestel M, Engel K, Edixhoven MJ, de Jonge HR. Hypo-osmotic cell swelling activates the p38 MAP kinase signalling cascade. FEBS Lett 1996; 395: 133–136.

43. Pedersen SF, Hoffmann EK, Mills JW. The cytoskeleton and cell volume regulation. Comp Biochem Physiol (in press).

44. Tilly BC, Edixhoven MJ, Tertoolen LG, Morii N, Saitoh Y, Narumiya S, de Jonge HR. Activation of the osmo-sensitive chloride conductance involves P21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. Mol Biol Cell 1996; 7: 1419–1427.

45. Nilius B, Voets T, Prenen J, Barth H, Aktories K, Kaibuchi K, Droogmans G, Eggermont J. Role of Rho and Rho kinase in the activation of volume-regulated anion channels in bovine endothelial cells. J Physiol. 1999; 516: 67–74.

46. Manolopoulos VG, Droogmans G, Nilius B. Hypotonicity and thrombin activate taurine efflux in BC3H1 and C2C12 myoblasts that is down regulated during differentiation. Biochem Biophys Res Commun 1997; 232: 74–79.

47. Feranchak AP, Roman RM, Doctor RB, Salter KD, Toker A, Fitz JG. The lipid products of phosphoinositide 3-kinase contribute to regulation of cholangiocyte ATP and chloride transport. J Biol Chem 1999; 274: 30979–30986.

48. Feranchak AP, Roman RM, Schwiebert EM, Fitz JG. Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. J Biol Chem 1998; 273: 14906–14911.

49. Song D, O'Regan MH, Phillis JW. Mechanisms of amino acid release from the isolated anoxic/reperfused rat heart. Eur J Pharmacol 1998; 351: 313–322.

50. Phillis JW, Song D, O'Regan MH. Inhibition of tyrosine phosphorylation attenuates amino acid neurotransmitter release from the ischemic/reperfused rat cerebral cortex. Neurosci Lett 1996; 207: 151–154.

51. Bortner CD, Cidlowski JA. Absence of volume regulatory mechanisms contributes to the rapid activation of apoptosis in thymocytes. Am J Physiol 1996; 271: C950–C961.

52. Lang F, Madlung J, Uhlemann AC, Risler T, Gulbins E. Cellular taurine release triggered by stimulation of the Fas(CD95) receptor in Jurkat lymphocytes. Pflugers Arch 1998; 436: 377–383.

53. Moran J, Hernandez-Pech X, Merchant-Larios H, Pasantes-Morales H. Release of taurine in apoptotic cerebellar granule neurons in culture. Pflugers Arch 2000; 439: 271–277.

54. Lang F, Madlung J, Siemen D, Ellory C, Lepple-Wienhues A, Gulbins E. The involvement of caspases in the CD95(Fas/Apo-1)- but not swelling-induced cellular taurine release from Jurkat T-lymphocytes. Pflugers Arch 2000; 440: 93–99.

APENDICE II

ABREVIATURAS

Abreviación	Significado
DRV	Decremento regulador del volumen.
RIV	Regulación Isovolumetrica
LTAH	Liberación de taurina activada por hiposmolaridad
I_{Clvol}	Conductancia de cloro activada por volumen
VSOAC	Canal de osmolitos y/o aniones sensible a volumen
PI3K	Fosatidil inositol-3 cinasa
RTKs	Receptores con actividad intrínseca de cinasa de tirosina

REFERENCES

- Abdullaev I. F., Sabirov E. Z., Okada Y. 2003. Upregulation of swelling-activated Cl- channel sensitivity to cell volume by activation of EGF receptors in murine mammary cells. J. Physiol (Lond) 349: 749-758.
- Al-Obeidi F. A., Wu J. J., Lam K. S. 1998. Protein tyrosine kinases: structure, substrate specificity, and drug discovery. Biopolymers. 47: 197-223.
- Al-Salman J. 2002. Hyponatremia. West. J. Med. 176: 173-6.
- Alvarez-Leefmans F., Reus L. 1996. Operación orquestada de los sistemas de transporte a través de la membrana plasmática: regulación y mantenimiento del volumen celular, en: Biofísica y Fisología Celular, Latorre R., López-Barneo J., Bezanilla F., Llinás R., Eds., Universidad de Sevilla, 177-214
- Ameder T., Robert A., Coles J. A. 1997. Potassium homeostasis and glial energy metabolism. Glia. 21: 41-55.
- Anderson R. G. W. 1998. The caveolae membrane system. Annu. Rev. Biochem. 67: 199-225.
- Andrew R. D., Fagan M., Ballyk A., Rosen A. S. 1989. Seizure susceptibility and the osmotic state. Brain Res. 498: 175-180.
- Aschner M., Mutkus L., Allen J. W. 2001. Aspartate and glutamate transport in acutely and chronically ethanol exposed neonatal rat primary astrocyte cultures. Neurotoxicology. 22: 601-605.
- Ashrafian H, Davey P. 2001. A review of the causes of central pontine myelinosis: yet another apoptotic illness. Eur. J. Neurol. 8: 103-109
- Badaut J., Lasbennes F., Magistretti P. J., Regli L. 2002. Aquaporins in brain: distribution, physiology, and pathophysiology. J. Cereb. Blood Flow Metab. 22: 367-378.
- Bajnath R. B., de Jonge H. R., Borgdorff A. J., Zuiderwijk M., Groot J. A. 1997. Characterization of swelling-induced ion transport in HT-29Cl.19A cells. Role of inorganic and organic osmolytes during regulatory volume decrease. Pflugers Arch. 433: 276-286.
- Bali M., Lipecka J., Edelman A., Fritsch J. 2001. Regulation fo ClC-2 chloride channels in T84 cells by TGF-α. Am. J. Physiol. Cell. Physiol. 280: C1588-C1598.
- Ballantyne J. S., Mary E. C. 1994, en Cellular and Molecular Physiology of Cell Volume Regulation, Strange K., Eds., CRC Press, Boca Ranton, 111-122
- Ballanyi K., Grafe P., 1988. Cell Volume Regulation in the Nervous System. Ren. Physiol. Biochem. 3-5: 142-157.
- Ballyk A. B., Quackenbush J. S., Andrew D. R. 1991. Osmotic effects on the CA1 neuronal population in hippocampal slices with special reference to glucose. J. Neurophysiol. 65: 1055-1066.
- Baraban S. C., Bellingham M. C., Berger A. J., Schwartzkroin P. A. 1997. Osmolarity modulates K⁺ channel function on rat hippocampal interneurons but not CA1 pyramidal neurons. J. Physiol. 498: 679-689.
- Basarsky T. A., Feighan D., MacVicar B. A. 1999. Glutamate release through volume-activated channels during spreading depression. J. Neurosci. 19: 6439-6445.
- Basavappa S., Huang C. C., Mangel A. W., Lebedev D. V., Knauf P. A., Ellory J. C. 1996. Swelling-activated amino acid efflux in the human neuroblastoma cell line CHP-100. J. Neurophysiol. 76: 764-769.
- Basavappa S., Pedersen S. F., Jorgensen N. K., Ellory J. C., Hoffmann E. K.1998. Swelling-induced arachidonic acid release vía the 85-kDa cPLA₂ in human neuroblastoma cells. J. Neurophysiol. 79: 1441-1449.
- Baslow M. H. 1999. The existence of molecular water pumps in the nervous system: a review of the evidence.Neurochem. Int. 34: 77-90.
- Bedford, J. J., Leader J. P. 1993. Response of tissues of the rat to anisosmolality in vivo. American Journal of Physiology. 264: R1164-R1169.
- Boese S. H., Glanville M., Gray M. A., Simmons N. M. 2000. The swelling-activated anion conductance in the mouse renal inner medullary collecting duct cell line mIMCD-K2. J. Membr. Biol. 177: 51-64.
- Bogdan S., Klambt C. 2001. Epidermal growth factor receptor signaling. Curr. Biol. 11: R292-R295.
- Bothwell J. H., Rae C., Dixon R. M., Styles P., Bhakoo K. K. 2001. Hypo-osmotic swelling-activated release of organic osmolytes in brain slices: implications for brain oedema in vivo. J. Neurochem. 77: 1632-1640.
- Bouron A. 2001. Modulation of spontaneous quantal release of neurotransmitters in the hippocampus. Prog. Neurobiol. 63: 613-635.
- Bowlby M. R., Fadool D. A., Holmes T. C., Levitan I. B. 1997. Modulation of the Kv1.3 potassium channel by receptor tyrosine kinases. J. Gen. Physiol. 110: 601-610.
- Browe D. M., Baumgarten C. M. 2003. Stretch of {beta}1 integrin activates an outwardly rectifying chloride current via FAK and src in rabbit ventricular myocytes. J. Gen. Physiol. 122: 689-702.
- Bryan-Sisneros A., Sabanov V., Thoroed S. M. D. 2000. Dual role of ATP in supporting volume-regulated chloride channels in mouse fibroblasts. Biochim. Biophys. Acta. 1468: 63-72.
- Bursell J. D., Kirk J., Hall S. T., Gero A. M., Kirk K. 1996. Volume-regulatory amino acid release from the protozoan parasite Crithidia luciliae. J. Membr. Biol. 154: 131-141.
- Cannon C. L., Basavappa S., Strange K. 1998. Ionic strength regulates the volume sensitivity of a swelling-activated anion channel. Am. J. Physiol. 275: C416-C422.
- Cantiello H. F. 1997. Role of actin filament organization in cell volume and ion channel regulation. J. Exp. Zool. 279: 425-435
- Cantley L. C. 2002. The phsophoinositide 3.kinase pathway. Science 296: 1655-1657.
- Cantrell D. A. 2003. Phosphoinositide 3-kinase signalling pathways. J. Cell Sci. 144: 1439-1445.
- Cardin V., Peña-Segura C., Pasantes-Morales H. 1999. Activation and inactivation of taurine efflux in hyposmotic and isosmotic swelling in cortical astrocytes: role o ionic strength and cell volume decrease. J. Neurosci. Res. 56 : 659-667
- Cardin V., Lezama R., Torres-Marquez M. E., Pasantes-Morales H. 2003. Potentiation of the osmosensitive taurine release and cell volume regulation by cytosolic Ca2+ rise in cultured cerebellar astrocytes. Glia. 44: 119-128.
- Carton I., Hermans D., Eggermont J. 2003. Hypotonicity induces membrane protrusions and actin remodeling vía activation of small GTPases Rac and Cdc42 in Rat-1 fibroblasts. Am. J. Physiol. 285: C935-944
- Cassimeris L., McNeill H., Zigmond S. H. 1990. Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. J. Cell Biol. 110: 1067-1075.
- Chamberlain M. E., Strange K. 1989. Anisosmotic cell volume regulation: a comparative view. Am. J. Physiol. 257: C159-C173.
- Chebabo S. R., Hester M. A., Jing J., Aitken P. G., Somjen G. G., 1995. Hypotonic exposure enhances synaptic transmission and triggers spreading depression in rat hippocampal tissue slices. Brain Res. 695: 203-216.
- Chen K. D., Li Y. S., Kim M., Li S., Yuan S., Chen S., Shyy J. Y. 1999. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins and shc. J. Biol. Chem. 274: 18393-18400.
- Cheng H., Kartenbeck J., Kabsch K., Mao X, Marques M., Alonso A. 2002. Stress kinase p38 mediates EGFR transactivation by hyperosmolar concentrations of sorbitol. J. Cell. Physiol. 192: 234-243.
- Chou C. Y., Shen M. R., Hsu K. S., Huang H. Y., Lin H. C. 1998. Involvement of PKC-alpha in regulatory volume decrease responses and activation of volume sensitive chloride channels in human cervical cancer HT-3 cells. J. Physiol. 512: 435-448.

- Colclasure G. C., Parker J. C. 1991. Cytosolic protein ceoncentration is the primary volume signal in dog red cells. J. Gen. Physiol. 881-892.
- Cornet M., Delpire E., Giles R. 1988. Relations between cell volume control, microfilaments and microtubules networks in T2 and PC12 cultured cells. J. Physiol. (Paris) 83: 43-49.
- Cornet M., Lambert I. H., Hoffmann E. K. 1993. Relation between cytoskeleton, hypo-osmotic treatment and volume regulation in Ehlrich ascites tumor cells. J. Membr. Biol. 131: 55-66.
- Correa-Meyer E., Pesce L., Guerrero C., Sznajder J. I. 2002. Cyclic stretch activates ERK1/2 vía G proteins and EGFR in alveolar epithelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 282: L883-L891.
- Cousin M. A., Hurst H., Nicholls D. G. 1997. Presynaptic calcium channels and field-evoked transmitter exocitosis form cultured cerebellar granule cells. Neuroscience. 81: 151-161.
- Crepel V., Panenka W., Kelly M. E., MacVicar B. A. 1998. Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. J. Neurosci. 18: 1196-1206.
- Crouch M. F. 1997. Regulation of thrombin-induced stress fibre formation in Swiss 3T3 cells by the 70kDa S6 kinase. Biochem. Biophys. Res. Commun. 233: 193-199.
- Crouch M. F., Davy D. A., Willard F. S., Berven L. A. 2001. Activation of endogenous thrombin receptors causes clustering and sensitization of epidermal growth factor receptors of Swiss 3T3 cells without transactivation. J. Cell Biol. 152: 263-273.
- Daub H., Wallasch C., Lankenau A., Herrlich A., Ullrich A. 1997. Signal characteristics of G protein-transactivated EGF receptor. EMBO J. 16: 101-113.
- de La Paz L. D., Lezama R., Torres-Marquez M. E., Pasantes-Morales H. 2002. Tyrosine kinases and amino acid efflux under hyposmotic and ischaemic conditions in the chicken retina. Pflugers Arch. 445: 87-96.
- Deleuze C., Duvoid A., Moos F. C., Hussy N. 2000. Tyrosine phosphorylation modulates the osmosensitivity of volume-dependent taurine efflux from glial cells in the rat supraoptic nucleus. J. Physiol. 523: 291-299.
- Denu J. M., Tanner K. G. 1998. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. Biochemistry. 37: 5633-5642.
- Djali S., Dawson L. A. 2001. Characterization of endogenous amino acid efflux from hippocampal slices during chemically-induced ischemia. Neurochem. Res. 26: 135-143.
- Djordjevic S., Driscoll P. 2001. Structural insight into substrate specificity and regulatory mechanisms of phosphoinositide 3-kinases. Trends Biochem. Sci. 27: 426-432.
- Doroshenko P. 1998. Pervanadate inhibits volume-sensitive chloride current in bovine chromaffin cells. Pflugers Arch. 435: 303-309.
- Doussau F., Augustine G. J. 2000. The actin cytoskeleton and neurotransmitter release: an overview. Biochimie. 82: 353-363.
- Dove S. K., Cooke F. T., Douglas M. R., Sayers L. G., Parker P. J., Mitchell R. H. 1997. Osmotic stress activates phosphatidylinositol-3,5-biphosphate synthesis. Nature. 390: 187-192.
- Downey G. P., Grinstein S., Sue A. Q. A., Czaban B., Chan C. K. 1995. Volume regulation in leukocytes: requirement for an intact cytoskeleton. J. Cell. Physiol. 163: 96-104.
- Dresbach T., Qualmann B., Kessels M. M., Garner C. C., Gundelfinger E. D. 2001. The presynaptic cytomatrix of brain synapses. Cell. Mol. Life Sci. 58: 94-116.
- Droge W. 2001. Free radicals in the physiological control of cell function. Physiol. Rev. 82: 47-95.
- Dudek F. E., Obenaus A., Tasker J. G. 1990. Osmolarity-induced changes in extracellular volume alter epileptiform bursts independent of chemical synapses in the rat: importance of non-synaptic mechanisms in hippocampal epiletogenesis. Neurosc. Lett. 120: 267-270.
- Eggermont J., Trouet D., Carton I, Nilius B. 2001. Cellular function and control of volume-regulated anion channels. Cell. Biochem. Biophys. 35: 1-12.
- Eguchi S., Numaguchi K., Iwasaki H., Matsumoto T., Yamakawa T., Utsunomiya H., Motley E. D., Kawakatsu H., Owada K. M., Hirata Y., Marumo F., Inagami T. 1998. Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. J. Biol. Chem. 273: 8890-8896.
- El-Zarruk A. A., Van den Berg H. W. 1999. The anti-proliferative effects of tyrosine kinase inhibitors towards tamoxifen-sensitive and tamoxifen-resistant human breast cancer cell lines in relation to the expresión of epidermal growth factor receptors (EGF-R) and the inhibition of EGF-R tyrosine kinase. Cancer Lett. 142: 185-193.
- Emma F., McManus M., Strange K. 1997. Intracellular electrolytes regulate the volume set point of the organic osmolyte/anion channel VSOAC. Am. J. Pysiol. Cell Physiol. 272: C1766-C1775.
- Estevez A. Y., O'Regan M. H., Song D., Phillis J. W., 1999a. Effects of anion channel blockers on hyposmotically induced amino acid release from the in vivo rat cerebral cortex. Neurochem. Res. 24: 447-452.
- Estevez A. Y., O'Regan M. H., Song D., Phillis J. W., 1999b. Hyposmotically induced aminoacid release from the rat cerebral cortez:role of phospholipases and protein kinases. Brain Res. 844: 1-9.
- Estevez A. Y., Song D., Phillis J. W., O'Regan M. H. 2000. Effects of the anion channel blocker DIDS on ouabain- and high K(+)-induced release of amino acids from the rat cerebral cortex. Brain Res. Bull. 52: 45-50.
- Fall P. J 2000. Hyponatremia and hypernatremia: A systemic approach to causes and their correction. Post. Med. 107: 1-10.
- Fan S. F., Christ G. J., Melman A., Brink P. R., 1999. A stretch-activated Cl⁻ channel in human corpus cavernosal myocytes. Int. J. Impot. Res. 11: 1-7.
- Fang K. S., Ionides S., Oster G. Nuccitelli R., Isseroff R. R. 1999. Epidermal frowth factor receptor relocalization and kinase activity are necessary for directional migration of keratinocytes in DC electric fields. J. Cell. Sci. 112: 1967-78.
- Feranchak A. P., Roman R. M., Schwiebert E. M., Fitz J. G. 1998. Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. J. Biol. Chem. 273: 14906-14911.
- Feranchak A. P., Roman R. M., Doctor R. B., Salter K. D., Toker A., Fitz J. G. 1999. The lipid products of phosphoinositide 3-kinase contribuye to regulation of cholangiocyte ATP and chloride transport. J. Biol. Chem. 274: 30979-30986
- Feranchak A. P., Berl T., Capasso J., Wojtaszek P. A., Han J., Fitz J. G. 2001. p38 MAP kinase modulates liver cell volume through inhibition of membrane Na+ permeability. J. Clin. Invest. 108: 1495-1504.
- Finkenzeller G., Newsome W., Lang F., Haussinger D. 1994. Increase of c-jun mRNA upon hypo-osmotic cell swelling of rat hepatoma cells. FEBS Lett. 340: 163-166.
- Fiorini M., Alimandi M., Fiorentino L., Sala G., Segatto O. 2001. Negative regulation of receptor tyrosine kinase signals. 490: 132-141.
- Fleck M.W., Barrionuevo G., Palmer A.M. 2001. Synaptosomal and vesicular accumulation of L-glutamate, L-aspartate and D-aspartate. Neurochem. Int. 39: 217-223.
- Foskett J. K., Spring K. R. 1985. Involvement of calcium and cytoskeleton in gallbladder epithelial cell volume regulation. Am. J. Physiol. 248: C27-C36.
- Franco R. 2000. Regulación isovolúmica en el hipocampo de rata. Tesis de Licenciatura de Biología. Facultad de Ciencias. Universidad Nacional Autónoma de México. Julio 2000.
- Fruman D. A., Meyers R. E., Cantley L. C. 1998. Phosphinositide kinases. Annu. Rev. Biochem. 67: 481: 507.
- Fry D. W. 2003. Mechanism of action of erbB tyrosine kinase inhibitors. Exp. Cell. Res. 284: 131-139.

- Furst J., Gschwentner M., Ritter M., Botta G., Jakab M., Mayer M., Garavaglia L., Bazzini C., Rodiguiero S., Meyer G., Eichmuller S., Woll E., Paulmichl M. 2003. Molecular and functional aspects of anionic channels activated during regulatory volume decrease in mammalian cells. Pflugers Arch. 444: 1-25.
- Furukawa O., Matsui H., Suzuki N., Okabe S. 1999. Epidermal growth factor protects rat epithelial cells against acid-induced damage through the activation of Na+/H+ exchangers. J. Pharmacol. Exp. Ther. 288: 620-626.
- Galietta L. J., Falzoni S., Di Virgilio F., Romeo G., Zegarra-Moran O. 1997. Characterization of volume-sensitive taurine- and Cl(-)-permeable channels. Am. J. Physiol. 273: C57-C66.
- Gasnier B. 2000. The loading of neurotansmitters into synaptic vesicles. Biochimie. 82: 327-337
- Gazit A., Osherov N., Posner I., Yaish P., Poradosu E., Gilon C., Levitzki A. 1991. Tyrphostins. Heterocyclic and alpha-substituted benzylidenemalononitrile tyrphostins as potent inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. J. Med. Chem. 34: 1896-1907.
- Girault J. A., Barbeito L., Spampinato U., Gozlan H., Glowniski J., Besson M. J. 1986. In vivo release of endogenous amino acids from the rat striatum: further evidence for a role of glutamate and aspartate in corticostriatal neurotransmission. J. Neurochem. 47: 98-106
- Gnatenco C., Han J., Snyder A. K., Kim D. 2002 Functional expression of TREK-2 K+ channel in cultured rat brain astrocytes. Brain Res. 931: 56-67.
- Godart H., Ellory J. C., Motais R., 1999. Regulatory volume response of erythrocytes exposed to a graudal and slow decrease in medium osmolality. Pflügers Arch. 437: 776-779.
- Gosling M., Smith J. W., Poyner D. R. 1995. Characterization of a volume-sensitive chloride current in rat osteoblast-like (ROS 17/2.8) cells. J.Physiol (Lond) 485: 671-682.
- Grant A. C., Thomson J., Zammit V. A., Shennan D. B. 2000. Volume-sensitive amino acid efflux from a pancreatic beta-cell line. Mol. Cell. Endocrinol. 162: 203-210.
- Gross P. 2001. Correction of Hyponatremia. Sem Nephrol. 21: 269-272.
- Gullans S. R., Verbalis J. G. 1993. Control of brain volume during hyperosmolar and hyposmolar conditions. Ann. Rev. Med. 44: 289-301.
- Haas M., Wang H., Tian J., Xie Z. 2002. Src-mediated inter-receptor cross-talk between the Na+/K+-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases. J. Biol. Chem. 277: 18694-18702.
- Hagiwara N., Masuda H., Shoda M., Irisawa H. 1992. Stretch-activated anion currents of rabbit cardiac myocytes. J. Physiol. (Lond) 456: 285-302.
- Hallows K. R., Knauf P. A. 1994. , en Cellular and Molecular Physiology of Cell Volume Regulation, Strange K., Eds., CRC Press, Boca Ranton, 3-30 pp.
- Hamasaki K., Mimura T., Furuya H., Morino N., Yamazaki T., Komuro I., Yaski Y., Nijima, Y. 1995. Stretching mesangial cells stimulates tyrosine phosphorylation of focal adhesion kinase pp125[FAK]. Biochem. Biophys. Res. Commun. 212: 544-549.
- Haugstad, T. Karlsen H. E., Krajtci P., Due-Tonnessen B., Laisen M., Sandberg C., Sand O., Brandtaeg P., Langmoen I. A. 1997. Efflux of gamma-aminobutyric acid caused by changes in ion concentrations and cell swelling simulating the effect of cerebral ischaemia. Acta Neurochir. Wien. 139: 453-63.
- Haussinger D., Schliess F. 1999. Osmotic induction of signaling cascades: role in regulation of cell function. Biochem. Biophys. Res. Com. Haussinger D. 1996. The role of cellular hydratation for the regulation of cell function. Biochem. J. 313: 697-710.
- 255: 551-555.
- Haussinger D., Schliess F., Dombrowski F., Vom Dahl S. 1999b. Involvement of p38MAPK in the regulation of proteolysis by liver cell hydration. Gastroenterology. 116: 921-935.
- Haussinger D., Kurz A. K., Wettstein M., Graf D., Vom Dahl S., Schliess F. 2003. Involvement of integrins and Src in tauroursodeoxycholate-induced and swelling-induced choleresis. Gastroenterology. 124: 1476-1487.
- Haynes J. K., Goldstein L. 1993. Volume-regulatory amino acid transport in erythrocytes of the little skate, Raja erinacea. Am. J. Physiol. 265: R173-R179.
- Hecht D., Zick Y. 1992. Selective inhibition of protein tyrosine phsophatase activities by H_2O_2 and vanadote in vitro. Biochem. Biophys. Res. Commun. 188: 773-779.
- Henson J. H. 1999. Relationships between the actin vytoskeleton and cell volume regulation. Micros. Res. Tech. 47. 155-162.
- Herrlich P., Bohmer F. D. 2000. Redox regulation of signal transduction in mammalian cells. Biochem. Pharmacol. 59: 35-41.
- Hoffmann E. K. 2000. Intracellular signalling involved in volume regulatory decrease. Cell. Physiol. Biochem. 10: 273:288.
- Hu Y., Zou Y., Dietrich H., Wick G., Xu Q. 1999. Inhibition of neointima hyperplasia of mouse vein grafts by locally applied suramin. Circulation. 100 : 861-868.
- Huang C. C., Chang C. B., Liu J. Y., Basavappa S, Lim P. H. 2001. Effects of calcium, calmodulin, protein kinase C and protein tyrosine kinases on volume-activated taurine efflux in human erythroleukemia cells. J. Cell. Physiol. 189: 316-322.
- Huang R., P., Wu J., X., Fan Y., Adamson E. D. 1996. UV activates growth factor receptors via reactive oxygen intermediates. J. Cell. Biol. 133: 211-220.
- Huang R., Bossut D. R., Somjen G. G. 1997. Enhancement of whole cell synaptic currents by low osmolarity and by low [NaCl] in rat hippocampal slices. J. Neurophysiol. 77: 2349-2359.
- Huang Y., Rane S. G. 1994. Potassium channel induction by the Ras/Raf signal transduction cascade. J. Biol. Chem. 269: 31183-31189.
- Hubbard S. R., Till J. H. 2000. Protein tyrosine kinase structure and function. Annu. Rev. Biochem. 69: 373-398.
- Hubert E. M., Musch M. W., Goldstein L. 2000. Inhibition of volume-stimulated taurine efiflux and tyrosine kinase activity in the skate red blood cell. Pflugers Arch. 440: 132-139
- Hubner S., Couvillon A. D., Kas J. A., Bankaitis V. A., Vegners R., Carpenter C. L., Janmey P. A. 1998. Enhancement of phosphoinositide 3-kinase (PI 3-kinase) activity by membrane curvature and inositol-phospholipid-binding peptides. Eur. J. Biochem. 258: 846-853.
- Iannazzo L. 2001. Involvement of B-50 (GAP-43) phosphorylation in the modulation of transmitter release by protein kinase C. Clin. Exp. Pharmacol. Physiol. 28: 901-904.
- Jakab M., Furst J., Gschwentner M., Botta G., Garavaglia M. L., Bazzini C., Rodighiero S., Meyer G., Eichmueller S., Woll E., Chwatal S., Ritter M., Paulmichl M. 2002. Mechanisms sensing and modulating signals arising from cell swelling. Cell. Physiol. Biochem. 12: 235-258.
- Jaken S., Parker P. J. 2000. Protein kinase C binding partners. Bioessays. 22: 245-254.
- Jakobsson, E., 1980. Interactions of cell volume, membrane potencial, and membrane transport parameters. Am. J. Physiol. 238: C196-C203.
- Janmey P. A. 1994. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Annu Rev. Physiol. 56: 169-191.
- Jiang G., Klein J. D., O'Neill W. C. 2001. Growth factors stimulate the Na-K-2Cl cotransporter NKCC1 through a novel Cl(-)-dependent mechanism. Am. J. Physiol. Cell. Physiol. 281: C1948-C1953.
- Jorissen R. N., Walker F., Pouliot N., Garrett T. P. J., Ward C. W., Burgess A. W. 2003. Epidermal growth factor receptor: mechanisms of activation and signaling. Exp. Cell Res. 284: 31-53

- Junankar P. R., Kirk K. Organic osmolyte channels: a comparative view. Cell. Physiol. Biochem. 10: 355-360
- Katso R., Okkenhaug K., Ahmadi K., White S., Timms J., Waterfield M. D. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, immunity, homeostasis and cancer. Annu. Rev. Cell. Dev. Biol. 17: 615-675.
- Keenan C., Kelleher D. 1998. Protein kinase C and the cytoskeleton. Cell Signal. 10: 225-232.
- Kenneth R. H. P. 1994. Principles of Cell Volume Regulation, en Cellular and Molecular Physiology of Cell Volume Regulation, Strange
- Kim R. D., Roth T. P., Darling C. E., Ricciardi R., Schaffer B. K., Chari R. S. 2001. Hypo-osmotic stress stimulates growth in HepG2 cells via protein kinase B-dependent activation of activator protein-1. J. Gastrointest. Surg. 5: 546-555.
- Kimelberg H. K., Goderie S. K., Higman S., Pang S., Waniewski R. A. 1990. Swelling induced release of glutamate, aspartate and taurine from astrocyte cultures. J. Neurosci. 10: 1583-1591.
- Kimelberg H. K. 1995. Current concepts of brain edema. Review of laboratory investigations. J. Neurosurg. 83: 1051-1059.
- Kimelberg HK, Rutledge E, Goderie S, Charniga C. 1995b. Astrocytic swelling due to hypotonic or high K+ medium causes inhibition of glutamate and aspartate uptake and increases their release. J. Cereb. Blood. Flow. Metab. 15: 409-416.
- Kimelberg H. K. 2000. Cell volume in the CNS: Regulation and implications for nervous system function and pathology. The Neuroscientist. 6: 14-25
- King C. R., Borrello I., Porter L., Comoglio P., Schlessinger J. 1989. Ligand-independent tyrosine phosphorylation of EGF receptor and the erbB-2/neu proto-oncogene product is induced by hyperosmotic shock. Oncogene. 4: 13-18.
- Kirber M. T., Ordway R. W., Clapp L. H., Walsh Jr. J. V., Singer J. J. 1992. Both membrane stretch and fatty acids directly activate large conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells. FEBS Lett. 297: 24-28.
- Kirk K., 1997. Topical review: Swelling-activated Organic Osmolyte Channels. J. Mem. Biol. 158: 1 – 16.
- Kirk K., Strange K. 1998. Functional properties and physiological roles of organic solute channels. Annu. Rev. Physiol. 60: 719-739.
- Klacknik J. M., Cuenod M., Gahwiler B. H., Jiang Z. P., Do K. 1992. Release of endogenous amino acids, including homocysteic acid and cysteine sulphinic acid, from rat hippocampal slices evoked by electrical stimulation of Schaffer collateral-commisural fibres. Neuroscience. 49: 557-570.
- Knauf P. A., Anion transport in erythrocytes, en Physuikigy of Membrane Disorders, 2nd ed., Androceoli, T. E., Hoffman, J. F., Fanestil, D. D., Schultz, S. G., Eds., Plenum Press, New York, 1986, 191.
- Knebel A., Rahmsdorf H. J., Ullrich A., Herrlich P. 1996. Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. EMBO J. 15: 5314-5325.
- Knoublauch C., Montrose M. H., Murer H. 1989. Regulatory volume decrease in cultured renal cells, Am. J. Physiol., 256: C252-C257.
- Kontro P, Oja SS. 1983. Binding of taurine to brain synaptic membranes. Adv Biochem Psychopharmacol. 37:23-34.
- Kontro P., Oja S. S. 1985. Properties of sodium-independent taurine binding to brain synaptic membranes. Prog. Clin. Biol. Res. 179:249-59.
- Kontro P., Oja S. S. 1987. Co-operativity in sodium-independent taurine binding to brain membranes in the mouse. Neuroscience. 23:567-70.
- Krause U., Rider M. H., Hue L. 1996. Protein kinase signaling pathway triggered by cell swelling and involved in the activation of glycogen synthase and acetyl-CoA carboxylase in isolated rat hepatocytes. J. Biol. Chem. 271: 16668-16673.
- Kugler J. P., Hustead T. 2000. Hyponatremia and Hypernatremia. Am. Fam. Physician. 61: 1051-1059.
- Kultz D., Burg M. 1998. Evolution of osmotic stress signaling via MAP kinase cascades. J. Exp. Biol. 201: 3015-3021.
- Lambert I. H. 2003. Reactive oxygen species regulate swelling –induced taurine efflux in NIH3T3 mouse fibroblasts. J. Membr. Biol. 192: 19-32.
- Lambert I. H. 2004. Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. Neurochem. Res. 29: 27-63.
- Ortenblad N., Young J. F., Oksbjerg N., Nielsen J. H., Lambert I. H. 2003. Reactive oxygen species are important mediators of taurine release from skeletal muscle cells. Am. J. Physiol. Cell. Physiol. 284: C1362-C1373.
- Laming P. R. 2000. Potassium signalling in the brain: its role in behaviour. Neurochem. Int. 36: 271-290.
- Lampl C., Yazdi K. 2002. Central Pontine Myelinolysis. Eur. Neurol. 47: 3-10.
- Lang F., Busch G. L., Ritter M., Völki H., Waldegger S., Gulbins E., Häussingeret D. 1998. Functional Significance of Cell Volume Regulatory Mechanisms. Physiol. Rev. 78: 247-305.
- Lang F., Busch G. L., Volkl H. 1998b. The diversity of volume regulatory mechanisms. Cell. Physiol. Biochem. 8: 1-45.
- Law R. O., 1994 Effects of extracelular bicarbonate ions and pH on volume-regulatory taurine efflux from rat cerebral cortical slices in vitro: evidence for separate neutral and anionic transport mechanisms. Biochym. Biophys. Acta 1224: 377-383.
- Law R. O., 1996a. Volume regulation and the efflux of amino acids from cells in incubated slices of rate cerebral cortex. I. Characteristics of transport mechanisms. Biochym. Biophys. Acta 1314: 34-42
- Law R. O., 1996b. Volume regulation and the efflux of amino acids from cells in incubated slices of rate cerebral cortex. II. Dependence on Ca^{2+} ions. Biochym. Biophys. Acta 1314: 43-48.
- Law R. O. 1999a. Amino acid efflux and cell volume regulation in cerebrocortical minislices prepared from chronically hyponatraemic and hypernatraemic rats. Neurochem. Int. 35: 423-430.
- Law R. O. 1999b. The role of taurine in the regulation of brain cell volume in chronically hyponatraemic rats. Neurochem. Int. 33: 467-472.
- Leeple-Wienhues A., Szabo I., Laun T., Kaba n. K., Gulbins e., Lang F. 1998. The tyrosine kinase p56lck mediates activation of swelling-induced chloride channels in lymphocytes. J. Cell. Biol. 141: 281-286.
- Leeple-Wienhues A., Szabo I., Wieland U., Heil L., Gulbins e., Lang F. 2000. Tyrosine kinases open lymphocyte chloride channels. Cell. Physiol. Biochem. 10: 5-6.
- Leevers S. J., Vanhaesebroeck B., Waterfield M. D. 1999. Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr. Opin. Biol. 11: 219-225.
- Lehto V. P. 2001. EGF receptor: which way to go? FEBS Lett. 491: 1-3.
- Lehtonen J. Y. A., Kinnunen P. K. 1995. Phospholipas A_2 as a mechanosensor. Biophys. J. 68: 1888-1894.
- Leschinger A., Stabel J., Igelmud P., Heinemann U. 1993. Pharmacological and electrographic properties of epileptiform activity induced by elevated K^+ and lowered Ca^{2+} and Mg^{2+} concentration in rat hippocampal slices. Exp. Brain Res. 96(2): 230-240.
- Levitan I. Almonte C. Mollard P. Garber S. S. 1995. Modulation of a volume-regulated chloride current by F-actin. J. Membr. Biol. 147: 283-294.
- Levitzki A. 1990. Tyrphostins: Potential antiproliferative agents and novel molecular tools. Biochem. Pharmacol. 40: 913-918.
- Levitzki A. 1992. Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. FASEB J. 6: 3275-3282.
- Levitzki A., Gazit A. 1995. Tyrosine kinase inhibition: An approach to drug development. Science. 267: 1782-1788.
- Levitzki A., Bohmer F. D. 1998. Altered efficacy and selectivity of tyrosine kinase inhibitors of the activated status of protein tyrosine kinases. Anti-Can- Res. 13: 731-734.
- Lewis R. S., Ross P. E., Cahalan M. D. 1993. Chloride channels activated by osmotic stress in T lymphocytes. J. Gen. Physiol. 101:801-826.
- Li C., Xu Q. 2000. Mechanical stress-initiated signal transductions in vascular smooth muscle cells. Cell. Signal. 12: 435-445.

- Lien, Y. H., Shapiro, J. I., Chan, L. 1991. Study of brain electrolytes and organic osmolytes during correction of chronic hyponatremia. J. Clin. Invest. 88: 303-309.
- Lipson K., E. Pang L., Huber J., Chen H., Tsai J. M., Hirth P., Gazit A., Levitzki A., Mcmahon G. 1998. Inhibition of platelet-derived growth factor and epidermal growth factor receptor signaling events after treatment of cells with specific synthetic inhibitors of tyrosine kinase phsophrylation. J. Pharmacol. Exp Ther. 285: 844-852.
- Liu X., Zhang M. I. N., Peterson L. B., O'Neil R. G. 2203. Osmomechanical stress selectively regulates translocation of protein kinase C isoforms. FEBS Let. 538: 101-106
- Lohr J.W., Grantham J. J., 1986. Isovolumetric Regulation of Isolated S2 Proximal Tubules in Anisotonic Media. J. Clin. Invest. 78: 1165-1172.
- Lohr J. W., Sullivan L. P., Cragoe E. J., Grantham J. J. 1989. Volume regulation determinants in isolated proximal tubules in hypertonic medium. American Journal of Physiology. 256: F622-F631
- Lohr J. W., 1990. Isovolumetric regulation of renal proximal tubules in hypotonic medium. Ren. Physiol. Biochem. 13:233-240.
- Lohr J. W., Yohe L. 2000. Isovolumetric regulation of rat glial cells during development and correction of hypo-osmolality. Neurosci. Lett. 286: 5-8.
- Loo D. D., Wright E. M., Zeuthen T. 2002. Water pumps. J. Physiol. 542: 53-60.
- Lopez-Colome A. M. 1981. Taurine receptors in CNS membranes: binding studies. Adv. Exp. Med. Biol. 139:293-310.
- Lopez-Colome A. M., Pasantes-Morales H. 1981. Taurine binding to membranes from rat brain regions. J. Neurosci. Res. 6: 475-85.
- Lovisolo D., Bonelli G., Baccino F. M., Peres A., Alonzo F., Munaron L. 1992.Two currents activated by epidermal growth factor in EGFR-T17 fibroblasts. Biochim. Biophys. Acta. 1104: 73-82.
- Lunn J. A., Wong H., Rozengurt E., Walsh J. H. 2000. Requirement of cortical actin organization for bombesin, endothelin, and EGF receptor internalization. Am. J. Physiol. Cell. Physiol. 279: C2019-C2027.
- Lux H. D., Heinemann U., Dietzel I. 1986. Ionic changes and alteratios in the size of the extracellular space during epileptic activity. Adv. Neurol. 44: 619-39.
- Macfarlane S. R., Seatter M. J., Kanke T., Hunter G. D., Plevin R. 2001. Proteinase-activated receptors. Pharmacol. Rev. 53: 245-282.
- Macknight, A. D. C. 1988. Principles of cell volume regulation. Renal Physiol.Biochem. 3: 114-141.
- MacVicar B. A., Feighan D., Brown A., Ransom B. 2002. Intrinsic optical signals in the rat optic nerve: role for K(+) uptake via NKCC1 and swelling of astrocytes. Glia. 37: 114-123.
- Majewski H., Iannazzo L. 1998. Protein kinase C: a physiological mediator of enhanced transmitter output. Prog. Neurobiol. 55: 463-475.
- McMahon H. T., Foran P., Colly J. O., Verhage M., Wiegant V. M., Nicholls D. G. 1992. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate and met-enkephalin release from synaptosomes: clues to the locus of action. J. Biol. Chem. 267: 21338-21343.
- McManus M., Fischbarg J., Sun A., Hebert S., Strange K. 1993. Laser light-scattering system for studying cell volume regulation and membrane transport processes. Am. J. Physiol. 265: C562-C570.
- Meijer A. J. 2003. Amino acids as regulators and components of nonproteinogenic pathways. J. Nutr. 133: 2057S-2062S.
- Melton J. E., Patlack C. S., Pettigrew K. D., Cserr H. F., 1987. Volume regulatory loss of Na, Cl, K from rat brain during acute hyponatremia. Am J. Physiol 252: F661-F669.
- Meves A., Stock S. N., Beyerle A., Pittelkow M. R., Peus D. 2001. H(2)O(2) mediates oxidative stress-induced epidermal growth factor receptor phosphorylation. Toxicol. Lett. 122: 205-214.
- Mills J. W., Schwiebert E. M., Stanton B. A. 1999. Evidence for the role of actin filaments in regulating cell swelling. J. Exp. Zool. 268: 111-120.
- Mills J. W., Falsig Pedersen S., Walmod P. S., Hoffmann E. K. 2000. Effect of cytochalasins on F-actin and morphology of Ehrlich ascites tumor cells. Exp. Cell Res. 261: 209-219.
- Minton A. P. 2001. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. J Biol. Chem. 276: 10577-10580.
- Mitchell C. H., Zhang J. J., Wang L., Jacob T. J. C. 1997. Volume-sensitive chloride current in pigmented ciliary epithelial cells: role of phospholipases. Am. J. Physiol. 272: C212-C222.
- Miyakawa T., Kojima M., UI M. 1998. Differential routes of Ca^{2+} influx in Swiss 3T3 fibroblasts in response to receptor stimulation. Biochem. J. 329: 107-114.
- Miyamoto S., Teramoto H., Gutkind J. S., Yamada K. M. 1996 Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J. Cell. Biol. 135: 1633-16342.
- Miyata S., Matsushima O., Hatton G. I. 1997. Taurine in rat posterior pituitary: localization in astrocytes and selective release by hypoosmotic stimulation. J. Comp. Neurol. 381: 513-523.
- Mongin A. A., Cai Z., Kimelberg H. K. 1999. Volume-dependent taurine release from cultured astrocytes requires permissive [Ca2+]I and calmodulin. Am. J. Physiol. Cell. Physiol. 277: C823-C832.
- Mongin A. A., Reddi J. M., Charniga C., Kimelberg H. K. 1999b. [3H]taurine and D-[3H]aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. Am. J. Physiol. 276: C1226-C1230.
- Mongin AA, Orlov SN. Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology. 2001 Dec;8(2):77-88.
- Morales-Mulia S., Cardin V., Torres-Marquez M. E., Crevenna A., Pasantes-Morales H. 2001. Influence of protein kinases on the osmosensitive release of taurine from cerebellar granule neurons. Neurochem. Int. 38: 153-161.
- Moran J., Morales-Mulia M., Pasantes-Morales H. 2001. Reduction of phospholemman expresión decreases osmosensitive taurine efflux in astrocytes. Biochim. Biophys. Acta. 1538: 313-320
- Morgan J. M. 1984. Osmoregulation and water stress in higher plants. Ann. Rev. Plant Phy. 35: 299-319.
- Moro L, Moro L, Venturino M., Bozzo C., Silengo L., Altruda F., Beguinot L., Tarone G., Defilippi P. 1998. Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. EMBO J. 17: 6622-6632.
- Motais R., Guizouarn H., Garcia-Romeu F. 1991. Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems. Biochim. Biophys. Acta. 1075: 169-80.
- Mountain I., Van Driessche W. 1997. Isovolumetric regulation of C6 rat glioma cells in hyperosmotic media. American Journal of Physiology. 272: C318-C323.
- Musch M. W., Hubert E. M., Goldstein L. 1999. Volume expansion stimulates p72(syk) and p56(lyn) in skate erythrocytes. J. Biol. Chem. 274: 7923-7928.
- Nadler J V., Vaca K. W., White W. F., Lynch G. S., Cotman C. W. 1976. Aspartate and glutamate as possible transmitter of excitatory hippocampal afferents. Nature. 260: 538-540
- Nagelhus E. A., Lehmann A., Ottersen O. P. 1993. Neuronal exchange of taurine during hypo-osmotic stress: a combined immunocytochemical and biochemical analysis in rat cerebellar cortex. Neurosci. 54: 615-631.
- Naito S., Ueda T. 1985. Characterization of glutamate uptake into synaptic vesicles. J. Neurochem. 44: 99-109.
- Nicholls D. G. 1998. Presynaptic modulation of glutamate release. Prog. Brain Res. 116: 15-22.

- Nilius B., Oike M., Zahradnik I., Droogmans G. 1994. Activation of a Cl- current by hypotonic volume increase in human endothelial cells. J. Gen. Physiol. 103: 787-805.
- Nilius B., Eggermont J., Voets T., Buyse G., Manolopoulos V., Droogmans G. 1997. Properties of volume-regulated anion channels in mammalian cells. Prog. Biophys. Mol. Biol. 68: 69-119.
- Nilius B., Prenen J., Voets T., Eggermont J., Droogmans G. 1998. Activation of volume-regulated chloride currents by reduction of intracellular ionic strength in bovine endothelial cells. J. Physiol. (Lond) 506: 353-361.
- Nishizuka Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9: 484-496.
- O'Neill W. C. 1999. Physiological significance of volume-regulartory transporters. Am. J. Physiol. 276: C995-C1011.
- Oeckler R. A., Kaminski P. M., Wolin M. S. 2003. Stretch enhances contraction of bovine coronary arteries via an NAD(P)H oxidase-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein kinase cascade. Circ. Res. 92: 23-31.
- Oja S. S., Saransaari P. 1996. Kinetic analysis of taurine influx into cerebral cortical slices from adult and developing mice in different incubation conditions. Neurochem. Res. 2: 161-166.
- Okada Y. 1997. Volume expansion-sensing outward-rectifier Cl− channel: fresh start to the molecular identity and volume sensor.
- Okkenhaug K., Vanhaesebroeck B. 2001. New resposabilities for the PI3K regulatory subunit p85α. Sci STKE. 65: 1-5
- Osherov N., Gazit A., Gilon C., Levitzki A. 1993. Selective inhibition of the epidermal growth factor and HER2/neu receptors by tirphostins. J. Biol. Chem. 268: 11134-11142.
- Oster J. R., Singer I. 1999. Hyponatremia, hyposmolality, and hypotonicity: tables and fables. Arch. Intern. Med. 159: 333-336.
- Pandiella A., Magni M., Lovisolo D., Meldolesi J. 1989. The effect of epidermal growth factor on membrane potential. Rapid hyperpolarization followed by persistent fluctuations. J. Biol. Chem. 264: 12914-12921.
- Papadopoulos M. C., Krishna S., Verkman A. S. 2002. Aquaporin water channels and brain edema. Mt. Sinai. J. Med. 69: 242-248.
- Papakonstanti E. A., Vardaki E. A., Stournaras C. 2000. Actin cytoskeleton: a signaling sensor in cell volume regulation. Cell. Physiol and Biochem. 10: 257-264.
- Parker J. C., Colclasure G. C., McManus T. J. 1991. Coordinated regulation of shrinkage-induced Na+/H+ exchange and swelling induced [K-Cl] cotransport in dog red cells. Further evidence from activation kinetics and phosphatase inhibition. J. Gen. Physiol. 98: 869-880.
- Pasantes-Morales H., Alavez S., Sanchez-Olea R., Moran J. 1993. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem. Res. 18: 445-452.
- Pasantes-Morales H., Murray R. A., Sanchez-Olea R., Morán, J. 1994b. Regulatory volume decrease in cultured astrocytes II. Activated Permeability to amino acids and polyalcohols. Am. J. Physiol. 266: C172-C178.
- Pasantes-Morales, H. 1996. Volume Regulation in Brain Cells: Cellular and Molecular Mechanisms. Met. Brain Dis. 11: 187-203.
- Pasantes H., Massieu L., Arias C., Tapia R. 1998. Mecanismos celulares y moleculares de la neurodegeneración. Gac Med Mex. 134:685-703.
- Pasantes-Morales H., Ochoa de la Paz L. D., Sepulveda J., Quesada O. 1999. Amino acids as osmolytes in the retina. Neurochem. Res. 24:1339-1346.
- Pasantes-Morales H., Cardin V., Tuz K. 2000 Signaling events during swelling and regulatory volume decrease. Neurochem. Res. 25: 1301-1314.
- Pasantes-Morales H., Morales Mulia S. 2000b. Influence of calcium on regulatory volume decrease: role of potassium channels. Nephron. 86: 414-427.
- Paulsen R. E., Fonnum F. 1989. Role of glial cells for the basal and Ca2+-dependent K+-evoked release of transmitter amino acids investigated by microdialysis. J. Neurochem. 52: 1823-1829.
- Payrastre B., Missy K., Giuriato S., Bodin S., Plantavid M., Gratacp M. 2001. Phosphoinositides: key players in cell signaling in time and space.
- Pedersen S., Lambert I. H., Thoroed S. M., Hoffmann E. K. 2000. Hypotonic cell swelling induces translocation of the α isoform of cytosolic phospholipase A2 but not the γ isoform in Ehrlich ascites tumor cells. Eur. H. Biochem. 267: 5531-5539.
- Pedersen S. F., Hoffmann E. K., Mills J. W. 2001. The cytoskeleton and cell volume regulation. Com. Biochem Physiol P. A. 130: 385-399.
- Pedersen S. F., Mills J. W., Hoffmann E. K. 1999. Role of the F-actin cytoskeleton in the RVD and RVI processes in Ehrlich ascites tumor cells. Exp. Cell Res. 252: 63-74.
- Pedersen S. F., Beisner K. H., Hougaard C., Willumsen B. M., Lambert I. H., Hoffmann E. K. 2002. Rho family GTP binding proteins are involved in the regulatory volume decrease process in NIH3T3 mouse fibroblasts. J. Physiol. 541: 779-796.
- Peppelenbosch M. P., Tertoolen L. G., de Laat S. W. 1991. Epidermal growth factor-activated calcium and potassium channels. J. Biol. Chem. 266: 19938-19944.
- Peus D., Meyes A., Vasa R. A., Beyerle A., O'Brien T., Pittelkow M. R. 1999. H2O2 is required for UVB-induced EGF receptor and downstream signaling pathway activation. Free Radic. Biol. Med. 27: 1197-2124.
- Phillis J. W., Song D., O'Regan M. H. 1997. Inhibition by anion channel blockers of ischemia-evoked release of excitotoxic and other amino acids from rat cerebral cortex. Brain Res. 758: 9-16.
- Phillis J. W., Song D., O'Regan M. H. 1998. Tamoxifen, a chloride channel blocker, reduces glutamate and aspartate release from the ischemic cerebral cortex. Brain Res. 780: 352-355.
- Phillis J. W., Song D., O'Regan M. H. 1999. Effects of hyperosmolarity and ion substitutions on amino acid efflux from the ischemic rat cerebral cortex. Brain Res. 828: 1-11.
- Poolman B., Blount P., Folgering J. H. A., Friesen R. H. E., Moe P. C., Van der Heide T. 2002. How do membrana proteins sense water stress? Mol. Microbiol. 44: 889-902.
- Prenzel N., Fischer O. M., Streit S., Hart S., Ullrich A. 2001. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocrin. Rel. Can. 8: 11-31
- Rane S. G. 1999. Ion channels as physiological effectors for growth factor receptor and Ras/ERK signaling pathways. Adv. Second Messenger. Phosphoprotein Res. 33: 107-127.
- Rasmusson R. L., Davis D. G., Lieberman M. 1993. Amino acid loss during volume regulatory decrease in cultured chick heart cells. Am. J. Physiol. 264: C136-C145.
- Rausche G., Igelmud P., Heinemann U. 1990. Effects of changes in extracellular potassium, magnesium and calcium concentration on synaptic transmission in area CA! And the dentate gyrus of rat hippocampal slices. Pflugers Arch. 415: 588-93.
- Reinehr R., Schliess F., Haussinger D. 2003. Hyperosmolarity and CD95L trigger CD95/EGF receptor association and tyrosine phosphorylation of CD95 as prerequisites for CD95 membrane trafficking and DISC formation. FASEB J. 17: 731-733.
- Rizoli S. B., Rotstein O. D., Parodo J., Phillips J., Kapus A. 2000. Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling. Am. J. Physiol. 279: C-619-C633.
- Robson L., Hunter M. 1994. Role of cell volume and protein kinase C in regulation of a Cl− conductance in single proximale tubule cells of Rana temporaria. J. Physiol (Lond). 480: 1-17.

- Rodriguez I., Kaszkin M., Holloschi A., Kabsch K., Marques M. M., Mao X., Alonso A. 2002. Hyperosmotic stress induces phosphorylation of cytosolic phospholipase A(2) in HaCaT cells by an epidermal growth factor receptor-mediated process. Cell. Signal. 14: 839-848.
- Roman R. M., Bodily K. O., Wang Y., Raymond J. R., Fitz J. G. 1998. Activation of protein kinase Calpha couples cell volume to membrane Cl- permeabilitu in HTC hepatoma and Mz-ChA-1 cholangiocarcinoma cells. Hepatology. 28: 1073-1080
- Roper S. N., Obenaus A., Dudek F. E. 1992. Osmolarity and nonsynaptic epileptiform bursts in rat CA1 And dentate gyrus. Ann. Neurol. 31: 81-85.
- Rosen A. S., Andrew R. D. 1990. Osmotic effects upon excitability in rat neocortical slices. Neuroscience 38: 579-590.
- Rosette C., Karin M. 1996. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. Science. 274: 1194-1197.
- Rossi D. J., Oshima T., Attwell D. 2000. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature. 403: 316-321.
- Roy G. 1994. Channels for amino acids and metabolites activated by cell volume regulation. Jpn J. Physiol. 44 Suppl 2: S37-S42.
- Roy G., Banderali U. 1994. Channels for ions and amino acids in kidney cultured cells (MDCK) during volume regulation. J. Exp. Zool. 268: 121-126.
- Roy G. 1995. Amino acid current through anion channels in cultured human glial cells. J. Membr. Biol. 147: 35-44.
- Rutledge E. M., Kimelberg H. K. 1996. Release of [3H]-D-aspartate from primary astrocyte cultures in response to raised external potassium. J. Neurosci. 16: 7803-7811.
- Rutledge E. M., Aschner M., Kimelberg H. K. 1998. Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. Am. J. Physiol. 274: C1511-C1520.
- Sadoshima J., Qiu Z., Morgan J. P., Izumo S. 1996. Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. EMBO J. 15: 5535-5546.
- Sanchez Olea R., Pasantes-Morales H., Lazaro A., Cereijido M. 1991. Osmolarity-sensitive release of free amino acids from cultured kidney cells (MDCK). J. Membr. Biol. 121: 1-9.
- Sanchez-Olea R., Moran J., Schousboe A. Pasantes-Morales H. 1991. Hyposmolarity-activated fluxes of taurine in astrocytes are mediated bydiffusion. Neurosci. Lett. 130:233-236.
- Sanchez-Olea R., Pena C., Moran J., Pasantes-Morales H. 1993. Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl- transport in cultured astrocytes. Neurosci. Lett. 156: 141-144.
- Sanchez-Olea R., Fuller C., Benos D., Pasantes-Morales H. 1995. Volume-associated osmolyte fluxes in cell lines with or without the anion exchanger. Am. J. Physiol. 269: C1280-C1286.
- Sanchez-Olea R., Morales M., Garcia O., Pasantes-Morales H. 1996. Cl channel blockers inhibit the volume-activated efflux of Cl and taurine in cultured neurons. Am. J. Physiol. 270: C1703-C1708.
- Saransaari P., Oja S. S. 1992. Release of GABA and taurine from brain slices. Prog. Neurobiol. 38: 455-482.
- Saransaari P., Oja S. S. 1999. Mechanisms of D-aspartate release under ischemic conditions in mouse hippocampal slices. Neurochem. Res. 24: 1009-1016.
- Schaller M. D. 2001. Biochemial signals and biological responses elicited by the focal adhesion kinase. Biochim. Ciophys Acta. 1540: 1-21.
- Schenk P. W., Snaar-Jagalska B. E. 1999. Signal perception and transduction: role of protein kinases. Biochim. Biophys. Acta. 1449: 1-24.
- Schlaepfer D. D., Hauck C. R., Sieg D. J. 1999. Signaling through focal adhesion kinase. Prog. Biophys. Mol. Biol. 71: 435-478.
- Schneller et al., 1997
- Schwartzkroin P. A., Baraban S. C., Hochman D. W. 1998. Osmolarity, ionic flux, and changes in brain excitability. Epilepsy Res. 32: 275-285.
- Schwiebert E. M., Mills J. W., Stanton B. A. 1994. Actin-based cytoskeleton regulates a chloride channel and cell volume in a renal cortical collecting duct cell line. J. Biol. Chem. 269: 7081-7089.
- Seki Y., Feustel P. J., Keller R. W. Jr, Tranmer B. I., Kimelberg H. K. 1999. Inhibition of ischemia-induced glutamate release in rat striatum by dihydrokinate and an anion channel blocker. Stroke. 30: 433-440.
- Shen M. R., Chou C. Y., Browning J. A., Wilkins R. J., Ellory J. C. 2001. Human cervical cancer cells use Ca2+ signalling, protein tyrosine phosphorylation and MAP kinase in regulatory volume decrease. J. Physiol. 537: 347-362.
- Shennan D. B., McNeillie S. A. 1995. Volume-activated amino acid efflux from term human placental tissue: stimulation of efflux via a pathway sensitive to anion transport inhibitors. Placenta. 16: 297-308.
- Shi C., Barnes S., Coca-Prados M., Kelly M. E. 2002. Protein tyrosine kinase and protein phosphatase signaling pathways regulate volume-sensitive chloride currents in a nonpigmented ciliary epithelial cell line. Invest. Ophthalmol. Vis. Sci. 43: 1525-1532.
- Shuba Y. M., Prevarskaya N., Lemonnier L., Van Coppenolle F., Kostyuk P. G., Mauroy B., Skryma R. 2000. Volume-regulated chloride conductance in the LNCaP human prostate cancer cell line. Am. J. Physiol. 279: C1144-C1154.
- Sieg et al., 2000.
- Somjen G. G. 2000. Ion regulation in the Brain: Implications for Pathophysiology. The Neuroscientist. 8: 254-267.
- Song D., O'Regan M. H., Phillis J. W. 1998. Amino acid release during volume regulation by cardiac cells: cellular mechanisms. Eur. J. Pharmacol. 341: 273-280.
- Song D., O'Regan M. H., Phillis J. W. 1998. Protein kinase inhibitors attenuate cardiac swelling-induced amino acid release in the rat. J. Pharm. Pharmacol. 50: 1280-1286.
- Sorota S. 1995. Tyrosine protein kinase inhibitors prevent activation of cardiac swelling-induced chloride current. Pflugers Arch. 431: 178-185.
- Souza M. M., Boyle R. T., Lieberman M. 2000. Different physiological mechanisms control isovolumetric regulation and regulatory volume decrease in chick embryo cardiomyocytes. Cell. Biol. Int. 24: 713-721.
- Sterns R. H., Baer J., Ebersol S., Darbie T., Lohr J. W., Kamm D. E. 1993. Organic osmolytes in acute hyponatremia. Am. J. Physiol. 264: F833-F836.
- Strange, K. 1994. Cellular and Molecular Physiology of Cell Volume Regulation. CRC Press. Boca Ranton; Florida. 400 pag.
- Strange K., Jackson P. S. 1995. Swelling-activated organic osmolyte efflux: A new role for anion channels. Kidney. Int. 48: 994-1003
- Strbak V., Greer M. A. 2000. Regulation of hormone secretion by acute cell volume changes: Ca(2+)-independent hormone secretion. Cell Physiol. Biochem. 10: 393-402.
- Stringer J. L., Lothman E. W. 1988. Epileptiform discharges induced by altering extracellular potassium and calcium in the rat hippocampal slice. Exp. Neurol. 101: 147-157.
- Su G., Kintner D. B., Sun D. 2002. Contribution of Na(+)-K(+)-Cl(-) cotransporter to high-[K(+)](o)- induced swelling and EAA release in astrocytes. Am. J. Physiol. Cell Physiol. 282: C1136-C1146.
- Sundstrom E., Mo L. L. 2002. Mechanisms of glutamate release in the rat spinal cord slices during metabolic inhibition. J. Neurotrauma. 19: 257-266.

Sullivan S. G., Chiu D. T., Errasfa M., Wang J. M., Qi J. D., Stern A. 1994. Effects of H_2O_2 on protein tyrosine phosphates activity in HER14 cells. Free Radical Biol. Med. 16: 399-403.

Sykova E. 2001 Glial diffusion barriers during aging and pathological states. Prog. Brain. Res. 132: 339-363.

Szucs G., Heinke S., Droogmans, G., Nilius B. 1996. Activation of the volume-sensitive chloride current in vascular endothelial cells requires a permissive intracellular Ca^{2+} concentration. Pflugers Arch. 431: 467-469.

Szucs G., Heinke S., De Greef C., Raeymackers L., Eggermont J., Droogmans G., Nilius B. 1996b. The volume-activated chloride current in endothelial cells from bovine pulmonary artery is not modulated by phosphorylation. Pflugers Arch. 431: 5403548.

Takenawa T., Itoh T. 2001. Phosphoinositide, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochim. Biophys. Acta. 1533: 190-206.

Tancredi V., Avol M. 1987. Control of spontaneous epileptiform discharges by extracellular potassium: an "in vitro" study in the CA1 subfield of hippocampla slice. Exp. Brain. Res. 67: 363-372.

Thannickal V., Fanburg B. L. 2000. Reactive oxygen species in cell signaling. Am. J. Physiol. Lung. Cell. Mol. Physiol. 279: L1005-L1028

Thomson A. M. 2000. Facilitation, augmentation and potentiation at central synapses. Trends Neurosci. 23: 305-312.

Thoroed S. M., Lauritzen L., Lambert I H., Hansen H. S., Hoffmann E. K. 1997. Cell swelling activates phospholipase A_2 in Ehrlich ascites tumor cells. J. Membr. Biol. 160: 47-58.

Thoroed S. M., Bryan-Sisnetos P., 1999. Doroshenko P. Protein phosphotyrosine phosphatase inhibitors suppress regulatory volume decrease and the volume-sensitive Cl- conductance in mouse fibroblasts. Pflugers Arch. 438: 133-140.

Thurston J. H., Huhart R. D., Dirco J. A. 1980. Taurine: role in osmotic regulation in mammalian brain and possible clinical significance. Life Sci. 26: 1562-1568.

Thurston, J. H., Hauhart, R. E., Nelson, J. S., 1987. Adanpative decreases in amino acids (taurine in particular) creatine and electrolytes prevent cerebral edema in chronically hiponatremic mice: rapid correction (experimental model of central pontine myelinolysis) causes dehydration and shrinkage of brain. Met. Brain Des. 2: 223-241.

Thurston J. H., Hauhart R. E. 1992. Brain amino acids decrease in chronic hyponatremia and rapid correction causes brain dehydration: clinical significance. Life Sci. 40: 2539-2542.

Tilly B. C., Van den Berghe N., Tertoolen L. G., Edixhoven M. de Jonge H. R. 1993. Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J. Biol. Chem. 268: 19919-19922.

Tilly B. C., Edixhoven M. J., Tertoolen L. G., Morri N., Saitoh Y., Narumiya S., de Jonge H. R. 1996. Activation of the osmosensitive chloride conductance involves p21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. Mol. Biol. Cell. 7: 1419-1427

Tilly B. C., Gaestel M., Engel K., Edizhoven M. J., De Jonge H. R. 1996b. Hyposmotic cell swelling activates the p38 MAP kinase signaling cascade. FEBS Lett. 395: 133-136

Tinel H., Kinne-Saffran E., Kinne R. K. 2000. Calcium signalling during RVD of kidney cells. Cell. Physiol. Biochem. 10: 297-302.

Toker A. 1998. Signaling through protein kinase C. Front. Biosci. 3: D1134-D1147.

Toker A. 2000. Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mo. Pharm. 57: 652-658

Tosteson, D. C. Hoffman, J. F. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J. Gen. Physiol. 44: 169-175.

Trachtman H., del Pizzo R., Sturman A. 1990. Taurine and osmoregulation. III. Taurine deficiency protects against cerebral edema during acute hyponatremia. Pediatr. Res. 27:85-8.

Trachtman H., 1992. Cell volume: a review of cerebral adaptive mechanisms and implicatios for clinical treatment of osmolar disturbances: II. Ped. Neph. 6: 104-112.

Trouet D., Nilius B., Jacobs A., Remacle C., Droogmans G., Eggermont J. 1999. Caveolin-1 modulates the activity of the volume-regulated chloride channel. J. Physiol. (Lond) 520: 113-119.

Trouet D., Hermans D., Droogmans G., Nilius B., Eggermont J. 2001. Inhibition of volume-regulated anion channels by dominant-negative caveolin-1. Biochem. Biophys. Res. Commun. 284: 461-465.

Tuz K., Ordaz B., Vaca L., Quesada O., Pasantes-Morales H. 2001. Isovolumetric regulation mechanisms in cultured cerebellar granule neurons. J. Neurochem. 79: 143-151.

Tuz K., Pena-Segura C., Franco R., Pasantes-Morales H. 2004. Depolarization, exocytosis and amino acid release evoked by hyposmolarity from cortical synaptosomes. Eur. J. Neurosci. 19: 916-924

Uings I. J., Farrow S. N. Cell receptors and cell signaling. Mol. Pathol. 53: 295-299.

Valentijn K., Valentijn J. A., Jamieson J. D. 1999. Role of actin in regulated exocytosis and compensatory membrane retrieval: insights from an old acquaintance. Biochem. Biophys. Res. Commun. 266: 652-661.

van der Wijk T., Dorrestijn J., Narumiya S., Maassen J. A., de Jonge H. R., Tilly B. C. 1998. Osmotic swelling-induced activation of the extracellular-signal-regulated protein kinases Erk-1 and Erk-2 in intestine 407 cells involves the Ras/Raf-signalling pathway. Biochem. J. 331: 863-869.

Van Wijk T., Tomassen S. F., de Jonge H. R., Tilly B. C. 2000. Signalling mechanisms involved in volume regulation of intestinal epithelial cells. Cell. Physiol. Biochem. 10: 289-296.

Van Driessche W., de Smet P., Li J., Allen S., Zizi M., Mountain I. 1997. Isovolumetric regulation in a distal nephron cell line (A6). Am. J. Physiol. 272: C1890-C1898.

Vanhaesebroeck B., Leevers S. J., Ahmadi K., Timms J., Katso R., Driscoll P. C., Woscholski R., Parker P. J., Waterfield M. D. 2001. Synthesis and function of 3-phosphorylated inositol lipids. Annu. Rev. Biochem. 70: 535-602

Vaughan P. F., Walker J. H., Peers C. The regulation of neurotransmitter secretion by protein kinase C. Mol. Neurobiol. 18: 125-155.

Venero J. L., Vizuete M. L., Machado A., Cano J. 2001. Aquaporins in the central nervous system. Prog. Neurobiol. 63: 321-336.

Verbalis J. G., Drutarosky M. D. 1988. Adaptation to chronic hypoosmolality in rats. Kidney Int. 34: 351-360.

Verbalis J. G., Gullans S. R. 1990. Decreased brain concentrations of multiple organic osmolytes accompanies volume regulatory electrolyte losses during chronic hyponatremia. J. Am. Soc. Nephrol 1: 709-713.

Verbalis J. G., Gullans S. R. 1991. Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res. 567: 274-282.

Verbalis J. G., Gullans S. R. 1993. Rapid correction of hyponatremia produces differential effects on brain osmolyte and electrolyte reaccumulation in rats. Brain Res. 606: 19-27.

Verbalis J. G. 1998. Adaptation to acute and chronic hyponatremia: Implications for symptomatology, diagnosis and therapy. Sem. Nephrol. 18: 3-19

Verdon B., Winpenny J. P., Whitfield K. J., Argent B. E., Gray M. A. 1995. Volume-activated chloride currents in pancreatic duct cells. J. Membr. Biol. 145-87-98.

Voets T., Manolopoulos V., Eggermont J., Ellory C., Droogmans G. Nilius B. 1998. Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. J. Physiol. (Lond) 506: 341-352.

Voets T., Droogmans G., Raskin G., Eggermont J., Nilius B. 1999. Reduced intracellular ionic strength as the initial trigger for activation of endothelial volume-regulated anion channels. Proc. Natl. Acad. Sci. 96: 5298-5303.

Voisin D. L., Bourque C. W. Integration of sodium and osmosensory signals in vasopressin neurons. Trends Neurosci. 25: 199-205

- Volknandt W. 2002. Vesicular release mechanisms in astrocytic signalling. Neurochem. Int. 41: 301-306.
- Vom Dahl S., Schliess F., Graf D., Haussinger D. 2000. Role of p38(MAPK) in cell volume regulation of perfused rat liver. Cell. Physiol. Biochem. 11: 285-294.
- Vom Dahl S., Schliess F., Reissmann R., Gorg B., Weiergraber O., Kacalkova M., Dombrowski F., Haussinger D. 2003. Involvement of integrins in osmosensing and signaling towards autophagic proteolysis in rat liver. J. Biol. Chem. 278: 27088-27095
- Von Weikersthal S. F., Hickman M. E., Hladsky S. B., Barrand M. A. 1997. Hypotonicity-induced changes in anion permeabilitu of cultured rat brain endothelial cells. Biochim. Biophys. Acta. 1325: 99-107.
- Waldegger S., Steuer S., Risler T., Heidland A., Capasso G., Massry S., Lang F. 1998. Mechanisms and clinical significance of cell volume regulation. Nephrol. Dial. Transplant. 13: 867-874.
- Wang J. L. Kalyanaraman S., De Vivo M., Gautam N. 1996. Bombesin and thrombin affect discrete pools of intracellular calcium through different G-proteins. 320: 87-91.
- Wang L., Xu B., White R. E., Lu L. 1997. Growth factor-mediated K+ channel activity associated with human myeloblastic ML-1 cell proliferation. Am. J. Physiol. 273: C1657-C1665.
- Wei H., Mei Y. A., Sun J. T., Zhon H. Q., Zhang Z. H. 2003. Regulation of swelling-activated chloride channels in embryonic chick heart cells. Cell Res. 13: 21-28.
- Weiss F. U., Daub H., Ullrich A. 1997. Novel mechanisms of RTK signal generation. Curr. Opin. Gen. Dev. 7: 80-86.
- Wells A. 1999. EGF receptor. Int. J. Biochem. Cell. Biol. 31: 637-643.
- Widmann C., Gibson S., Jarpe M., J ohnson G. L. 1999. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to Human. Physiol. Rev. 79: 143-180.
- Wiley H. S. 2003. Trafficking of the ErbB receptors and its influence on signaling. Exp. Cel.. Res. 284: 78-88.
- Winter H.C., Ueda T. 1993. Glutamate uptake system in the presynaptic vesicle: glutamic acid analogs as inhibitors and alternate substrates. Neurochem. Res. 18: 79-85.
- Wittels K. A., Hubert E. M., Musch M. W., Goldstein L. 2000. Osmolyte channel regulation by ionic strength in skate RBC. Am. J. Physiol. 279: R69-R76.
- Wolfgang W. 2000. Role of astrocytes in the clearance of excess extracellular potassium. Neurochem. Int. 36: 291-300.
- Wonderlin W. F., Strobl J. S. 1996. Potassium channels, proliferation and G1 progression. J. Membr. Biol. 154: 91-107.
- Wymann M. P., Pirola L. 1998. Structure and function of phosphoinositide 3-kinase. Biochim. Biophys. Acta: 1436: 127-150
- Wymann M. P., Pirola L., Katanaev V. L., Bulgarelli-Leva G. 1999. Phosphoinositide 3-kinase signaling: no lipids. Biochem. Soc. Trans. 27: 629-634.
- Xu D., Wang L., Dai W., Lu L. 1999. A requirement for K+-channel activity in growth factor-mediated extracellular signal-regulated kinase activation in human myeloblastic leukemia ML-1 cells. Blood. 94: 139-145.
- Xu H., Collins J. F., Bai L., Kiela P. R., Lynch R. M., Ghishan F. K. 2001. Epidermal growth factor regulation of rat NHE2 gene expression. Am. J. Physiol. Cell. Physiol. 281: C504-C513.
- Yanaka A., Suzuki H., Shibahara T., Matsui H., Nakahara A., Tanaka N. 2002. EGF promotes gastric mucosal restitution by activating Na(+)/H(+) exchange of epithelial cells. Am J Physiol Gastrointest Liver Physiol. 282: G866-G876.
- Yancey H. P., 1994 Compatible and Counteracting Solutes , en Cellular and Molecular Physiology of Cell Volume Regulation, Strange K., Eds., CRC Press, Boca Ranton, 81 – 109
- Yang H., Wang Z., Miyamoto Y., Reinach P. S. 2001. Cell signaling pathways mediating epidermal growth factor stimulation of Na:K:2Cl cotransport activity in rabbit corneal epithelial cells. J. Membr. Biol. 183: 93-101.
- Yatsushiro S., Tamada H., Kozaki S., Kumon H., Michibata H., Yamamoto A., Moriyama Y. 1997. L-aspartate but not D-form is secreted through microvesicle-mediated exocitosis and is sequesteed through Na+-dependent transporter in rat pinealocytes. J. Neurochem. 69: 340-347.
- Zhang Z., Yang X. Y., Cohen D. M. 1998. Hypotonicity activates transcription through ERK-dependent and –independent pathways in renal cells. Am. J. Physiol. 275: C1104-C1112.
- Zhang Z., Bourque C. W. 2003. Osmometry in osmosensory neurons. Nat. Neurosci. 6: 1021-1022.
- Zhong J., Wang G. X., Hatton W. J., Yamboliev I. A., Walsh M. P., Hume J. R. 2002. Regulation of volume-sensitive outwardly rectifying anion channels in pulmonary arterial smooth muscle cells by PKC. Am. J. Physiol. 283: C1627-C1636.
- Zhou M., Peterson C .L., Lu Y .B., Nadler V. 1995. Release of glutamate and aspartate from CA1 synaptosomes: selective modulation of aspartate release by ionotropic glutamate receptors ligands. J. Neurochem. 64: 1556-1566.
- Ziyadeh F. N., Mills J. W., Kleinzeller A. 1992. Hypotonicity and cell volume regulation in shark rectal gland: role of organic osmolytes and F-actin. Am. J. Physiol. 262: F468-F479.
- Zwuick E., Hackel P. O., Prenzel N., Ullruch A. 1999. The EGF receptor as central transducer of heterologous signaling systems. Trends Pharmacol. Sci. 20: 408-412.