UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

“EFECTO DE LA HIPOSMOLARIDAD EN LA LIBERACIÓN DE NEUROTRANSMISORES. UN ESTUDIO EN TERMINALES NERVIOSAS AISLADAS”

T E S I S

QUE PARA OBTENER EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

KARINA LIZETH TUZ MOYA

DIRECTOR DE TESIS

DRA. HERMINIA PASANTES ORDOÑEZ

México, D.F. 2006

DEDICATORIAS

A Germán, las semillas del futuro, porque gracias a tí todo tiene sentido.

A Oscar, mi compañero de vida, con quien he crecido y compartido muchas satisfacciones y alegrías.

A mis padres y hermanos, a quienes agradezco haber crecido en el seno de una familia grande, leal y comprensiva, pero sobre todo por su amor.

A mi abuelita, quien me dio el mejor ejemplo.

A mi familia Juárez Mendoza por todo su cariño, apoyo y comprensión.

A mis amigas Rosa Alvarado, Miriam Bojorge, Claudia Peña y Karla Pulido.

AGRADECIMIENTOS

Agradezco de manera especial a la Dra. Herminia Pasantes, por todas las facilidades que se me otorgaron en su laboratorio para llevar a cabo este trabajo, por darme la oportunidad de formarme bajo su tutela, por sus enseñanzas, apoyo y compresión.

A los miembros del Comité Tutoral: Dra. Ana María López Colomé y Dr. Arturo Ortega Soto, por sus críticas y atinados comentarios durante la realización de la presente tesis.

A los miembros del jurado del examen: Dra. Elvira Galarraga, Dra. María Luisa Fanjul, Dra. Herminia Pasantes, Dra. Limei Zhang, Dr. Mauricio Díaz, Dr. Arturo Hernández y Dr. Arturo Ortega, por sus comentarios y revisión crítica de la presente tesis.

A la Biól. Claudia Peña Segura por su apoyo técnico.

A mis compañeros del laboratorio BL-301, quienes hacen posible el trabajo diario.

Al Dr. Julio Morán Andrade por las facilidades y amabilidad con la que fui tratada en su laboratorio.

Esta tesis se realizó en el Instituto de Fisiología Celular de la UNAM con los donativos 46465/A1 del CONACyT e IN206403/17 de la DGEP y con las becas para estudios de posgrado del CONACYT y de la DGEP.

ÍNDICE

RESUMEN

Las células animales tienen la capacidad de responder a cambios en la osmolaridad externa mediante mecanismos activos de adaptación consistentes en la movilización de osmolitos, principalmente iones y moléculas orgánicas, incluyendo aminoácidos, que contribuyen a restablecer el equilibrio osmótico. En células nerviosas, algunos de los aminoácidos que se movilizan en respuesta a la hiposmolaridad cumplen una doble función: como osmolitos y como neurotransmisores, por lo que su movilización en respuesta a una condición hiposmótica llevaría a una modificación en la excitabilidad nerviosa. Este trabajo tuvo como objetivo inicial investigar si la hiposmolaridad produce la liberación de aminoácidos en las terminales nerviosas aisladas (sinaptosomas), e identificar el mecanismo de esta liberación. Los resultados muestran que la hiposmolaridad induce la liberación de taurina, glutamato y GABA a través de distintos mecanismos con una diferente contribución para la liberación de cada aminoácido. Una aportación importante de nuestro trabajo fue el de encontrar que la hiposmolaridad induce la siguiente serie de eventos: despolarización dependiente de Na^+ y sensible a La^{3+} y Gd^{3+}, liberación de Ca^{2+} de la mitocondria a través del intercambiador Na^+/Ca^{2+} mitocondrial y exocitosis sensible a toxina tetánica (TeTX) y modulada por PKC. Este mecanismo es responsable del 44% de la liberación del glutamato, 30% del GABA y 20% de la taurina. La operación reversa de los transportadores contribuye también a la liberación hiposmótica de los aminoácidos. La vía difusional característica de los osmolitos que se caracteriza por ser sensible a bloqueadores de canales de Cl^- y requerir de la actividad de cinasas de tirosina, contribuye con el 55% a la liberación de taurina y 18% a la de GABA, pero no participa en la liberación de glutamato. Con base en los procesos concurrentes con la hiposmolaridad, nuestra predicción de si esta condición afectaría la liberación de otros neurotransmisores aunque no tengan una función como osmolitos fue confirmada experimentalmente. La norepinefrina se libera en condiciones hiposmóticas, por un mecanismo vesicular en su totalidad y que difiere de la de los aminoácidos por su dependencia de Ca^{2+} extracelular además del Ca^{2+} mitocondrial. La liberación hiposmótica de neurotransmisores, en particular de los excitadores, podría explicar el incremento en duración y amplitud de los potenciales excitadores postsinápticos en medios de baja osmolaridad y la susceptibilidad a las convulsiones asociada a la hiponatremia. En conjunto, estos resultados sugieren un efecto general de la hiposmolaridad sobre la función sináptica cuyas implicaciones habrán de conocerse en el futuro.

ABSTRACT

Animal cells respond to hyposmotic swelling by an active mechanism of cell volume recovery, accomplished by the extrusion of ions and organic molecules of low molecular weight, mainly amino acids. In brain cells, astrocytes as well as neurons, some amino acids released by hyposmolarity such as glutamate and GABA, have a dual role as osmolytes and neurotransmitters. If this release occurs at nerve endings, it may affect neuronal excitability. In the present study we found that hyposmolarity evokes a series of process which may directly affect the synaptic function: Na^+ dependent, La^{3+} and Gd^{3+} sensitive-depolarization, mitochondrial Ca^{2+}-release through the mitochondrial Na^+/Ca^{2+} exchanger and TeTX sensitive, PKC modulated-exocytosis. We also found that hyposmolarity elicits the release of taurine, glutamate and GABA from isolated nerve endings. The Ca^{2+}-dependent exocytosis, the reverse transporter operation and the difusional pathway characteristic of osmolytes are all activated during hyposmolarity and contribute differently to the amino acid release. Glutamate is preferentially released (44%) by the vesicular mechanism followed by the transporter reversal (37%), and is not mediated by the diffusion pathway. The opposite is found for taurine which is released preferentially (55%) by the diffusion pathway and with low contribution of the vesicular mechanism. The release pattern for GABA is intermediate. Since we found that hyposmolarity is a condition that promotes exocytosis, we may predict that other neurotransmitters should be also released in this condition, independently if they function or not as osmolytes. We found indeed a norepinephrine release evoked by hyposmolarity, which is completely vesicular and only differs from that of amino acids by its dependence on an external source of Ca^{2+} besides of that from mitochondrial origin. Altogether these results show a general influence of hyposmolarity on the synaptic function. In the case of the excitatory neurotransmitter, this hyposmotic-release could explain, at least in part, why hyposmolarity produces an increase in amplitude and duration of postsynaptic excitatory potentials and why during hyponatremia there is an increment of susceptibility to seizures.

INTRODUCCIÓN

La capacidad de regulación de volumen que tienen las células es una propiedad homeostática importante, así como una de las más antiguas, preservada a través de las ramas filogenéticas de la evolución biológica. Un evento crucial en la evolución de la vida ocurrió cuando altas concentraciones de sustancias no difusibles se rodearon de una bicapa lípidica semipermeable. Para llevar a cabo este paso evolutivo, tuvieron que haber mecanismos para contrarrestar la diferencia en la presión osmótica debido al equilibrio de las sustancias difusibles y el subsecuente establecimiento de la presión hidrostática en la célula (Baslow, 1999). Los organismos han desarrollado diversas estrategias para mantener el balance osmótico.

Las bacterias y plantas utilizan un mecanismo estático para contrarrestar la presión hidrostática ya que la pared celular rígida les permite, en cierto intervalo, ignorar la presión osmótica (Morgan, 1984). Los protozoarios presentan vacuolas contráctiles que les permiten contrarrestar la entrada de agua cuando el medio extracelular es hiposmótico, es decir, evaden la presión osmótica (Guarner, 1996).

Las membranas de las células animales son sumamente permeables al agua. Por ello, siempre que existan diferencias en la concentración de los solutos celulares con el exterior, es decir, un gradiente osmótico entre el interior y el exterior celular, se observarán movimientos netos de agua. Ante un gradiente de presión osmótica, el agua se moviliza del compartimiento de menor al de mayor presión osmótica; como consecuencia, el volumen celular aumenta en soluciones hiposmóticas y disminuye en soluciones hiperosmóticas. Las células animales en condiciones fisiológicas, se encuentran expuestas a un líquido intersticial isosmótico, cuya composición osmótica se encuentra finamente regulada debido a que los organismos presentan mecanismos de ingestión y excreción de agua y electrolitos que les permiten mantener la osmolaridad de los líquidos circulantes en un intervalo muy estrecho. En los mamíferos, se han desarrollado mecanismos renales. El riñón está dotado de nefronas, unidades funcionales que filtran la sangre y mantienen su composición y osmolaridad controladas, y de manera

indirecta, la del intersticio. A pesar de esta regulación, las células se pueden encontrar con cambios en la concentración de solutos extracelulares, como es el caso de las células epiteliales y renales. De manera local, también se pueden producir cambios en la concentración de solutos extracelulares, llamados microambientes osmóticos; como es el caso de las células nerviosas, las cuales se encuentran constantemente expuestas a un medio extracelular anisosmótico debido a la transmisión sináptica, durante la cual se producen variaciones temporales en la concentración de neurotransmisores y iones. Por otro lado, las células también sufren cambios en la concentración intracelular de solutos, debido a los procesos de síntesis y degradación de macromoléculas, ingestión de nutrientes, entre otros. Más aún, en condiciones de isosmolaridad, las células pueden enfrentar cambios en la presión osmótica debido a cambios en el coeficiente de reflexión dados por aumentos en la permeabilidad de la membrana a solutos cuyo gradiente electroquímico favorezca su influjo y produzcan hinchamiento, como el Na^+ durante un potencial de acción; o su eflujo y generen encogimiento, como el Cl^- en neuronas sensoriales activadas por GABA (Álvarez-Leefmans y Reuss, 1996). Un soluto con un coeficiente de reflexión alto o bajo disuelto en el medio isosmótico, también puede generar cambios en la presión osmótica, como es el caso de la sacarosa o la urea. La sacarosa genera encogimiento en condiciones isosmóticas debido a que su coeficiente de reflexión en muy alto, es decir, la membrana plasmática es virtualmente impermeable a la sacarosa. En el caso de la urea, esta produce hinchamiento celular debido a que su coeficiente de reflexión es muy bajo, es decir, la membrana plasmática es altamente permeable a ella (Álvarez-Leefmans y Reuss, 1996). Todas estas circunstancias fisiológicas producen alteraciones en la osmolaridad tanto del medio intra como extracelular lo que se traduce en un cambio en el volumen celular.

IMPORTANCIA DEL CONTROL DEL VOLUMEN CELULAR

El control del volumen es crítico para la supervivencia y funcionamiento de la célula, es necesario para mantener constante la concentración de solutos

intracelulares, algunos de los cuales participan en la transducción de señales o forman parte de la fuerza iónica (O'Neill, 1999). El control del volumen celular está relacionado a la proliferación, al crecimiento y división celular, a la migración, a los movimientos del citoesqueleto y a la inserción de proteínas en la membrana (Lang et al., 1998; Strbak y Greer, 2000).

La proliferación celular y la apoptosis son procesos fundamentales para mantener el número de células funcionalmente adecuado. Antes de iniciar la fase de mitosis, las células tienen que atravesar por las fases G1, S y G2 del ciclo celular. El tránsito por las diferentes fases se acompaña de un aumento en el volumen celular, que de no ser alcanzado impide la proliferación (Lang et al., 2000). Los cambios en la osmolaridad extracelular alteran la proliferación, ya que el medio hiperosmótico la inhibe y el medio hiposmótico la acelera (Lang et al., 1998). La apoptosis es el proceso de muerte celular que permite la remoción de células sin la liberación del contenido citosólico en el espacio extracelular y el inicio subsecuente de un proceso inflamatorio. Los puntos clave de este proceso son el encogimiento celular, la condensación del núcleo, la fragmentación del DNA, la pérdida de la función mitocondrial, la formación de vesículas apoptóticas y la activación de proteasas. El encogimiento coincide o precede a los eventos mencionados y para que se lleve a cabo es necesaria la liberación, acompañada de agua, de osmolitos intracelulares. En linfocitos Jurkat, la inducción de apoptosis a través de la estimulación del receptor CD95 provoca la activación de una corriente de Cl^- que también es sensible a hiposmolaridad (Lang et al., 2000). En estas células (Lang et al., 1998b) y en neuronas granulares de cerebelo (Morán et al., 2000) se libera taurina y otros osmolitos orgánicos durante la apoptosis. El volumen celular es importante también en la migración de leucocitos. Este proceso se activa por quimiotácticos como los formilpéptidos, que estimulan el cotransportador Na^+/H^+ y provocan hinchamiento. El bloqueo del cotransportador y la hiperosmolaridad externa inhiben la migración. Este proceso también se impide con la inhibición de canales de K^+ dependientes de Ca^{2+}, activados por las oscilaciones intracelulares de Ca^{2+}. Se postula entonces que en el extremo líder de la célula migrante es necesaria la polimerización de actina y la activación de

transportadores, mecanismos asociados a la regulación del volumen en condiciones hiperosmóticas; en el extremo posterior se necesita la despolimerización de actina, oscilaciones en la concentración intracelular de Ca^{2+} y activación de canales de K^+, eventos que se observan en condiciones hiposmóticas. Para esto se requiere que la célula distribuya de manera diferencial los canales iónicos en la membrana, como así ocurre (Lang et al., 1998).

El hígado, sujeto a irrigación sistémica y portal, está sometido a diferencias sustanciales en la concentración de aminoácidos, ácidos biliares y glucosa entre el ayuno y la ingestión de alimentos. El hinchamiento celular representa una señal anabólica, estimulando la síntesis de proteínas, la gluconeogénesis, la exocitosis y el flujo biliar. Por el contrario, una reducción en el volumen celular desencadena procesos catabólicos, como la proteólisis y la glucogenólisis. Las hormonas que regulan la función hepática tienen efectos sobre el volumen celular: insulina y fenilefrina provocan hinchamiento, mientras que el ATP, el AMPc y el glucagon reducen el volumen (Dunkelberg et al., 2001).

En los tejidos epiteliales, el transporte transcelular de iones, nutrientes y productos de desecho, se logra por la incorporación de estos a través de una de las membranas y la expulsión por la contraria. Esta constante entrada y salida de sustancias osmóticamente activas durante el transporte epitelial se traduce en cambios continuos en el volumen. En el intestino, la vejiga y los túbulos proximales del riñón, la incorporación luminal de sustratos, como glucosa o aminoácidos, está acoplada al gradiente de Na^+, lo que provoca hinchamiento celular y consecuente activación de canales de K^+ en la membrana basolateral como mecanismo de regulación. Este mecanismo no sólo ayuda a la regulación del volumen, sino que también mantiene el gradiente electroquímico regenerando el proceso (Lang et al., 1998).

REGULACIÓN CRÓNICA DEL VOLUMEN CELULAR

Las membranas plasmáticas de la mayoría de las células son impermeables a las proteínas, altamente permeables al agua, y en menor grado y de manera selectiva, a varios solutos de bajo peso molecular. Dado que el pH intracelular se

mantiene a niveles alcalinos (7.2-7.4) con respecto al punto isoeléctrico de las proteínas, éstas se comportan como aniones polivalentes. Esto trae como consecuencia que la presión coloidosmótica intracelular sea mayor que la del líquido extracelular y como la membrana es permeable al agua y a pequeños solutos tales como Na^+, K^+ y Cl^-, si los iones y el agua se distribuyesen tal y como lo predice la teoría de Gibbs-Donnan, las células se hincharían en condiciones isosmóticas. De acuerdo con esta teoría, en el equilibrio, la concentración total de solutos intracelulares debería ser mayor que la de los extracelulares, lo cual resultaría en una disminución en el potencial químico del agua en el interior, con respecto al exterior celular. Este gradiente de potencial químico del agua causaría un flujo osmótico neto de este solvente hacia el interior celular. Como la membrana de las células animales es distensible, cede ante pequeñas presiones hidrostáticas, por lo que dicho flujo osmótico neto de agua causaría un aumento de volumen y eventual lisis celular. Sin embargo, este aumento de volumen celular coloidosmótico no ocurre y las células mantienen niveles constantes de agua y solutos. Esto indica que aunque las células animales contienen aniones orgánicos impermeantes, los iones inorgánicos permeantes no están distribuidos de acuerdo con la teoría de Gibas-Donnan.

Los mecanismos que previenen el hinchamiento coloidosmótico en condiciones isosmóticas son el transporte de iones a través de vías pasivas y activas, con la generación de cambios en la permeabilidad y en el potencial electroquímico de los mismos. La hipótesis que explica estos mecanismos es la de la “bomba y la fuga” propuesta por Tosteson y Hoffman (1960). El modelo sostiene que la membrana plasmática posee mecanismos de transporte activo que generan un flujo de solutos; el transportador responsable de esto es la bomba de Na^+/K^+. Con la actividad de este transportador se gana Na^+, se pierde K^+ y se genera y mantiene un gradiente electroquímico hacia el interior celular para Na^+ y hacia el exterior para K^+. Con el establecimiento de este gradiente se activa otra serie de transporte de solutos cuyo movimiento ocurre acoplado a los movimientos de Na^+ y K^+. Entre estos están los intercambiadores Na^+/H^+, Na^+/Ca^{2+} y K^+/H^+, y los cotransportadores Na^+/aminoácido, K^+/Cl^- y $Na^+/K^+/Cl^-$ (Cooke y Macknigth,

1984; Hallows y Knauf, 1994; Álvarez-Leefmans y Reuss, 1996). Finalmente, la suma de los flujos de Na^+ hacia el exterior e interior celular es cero, comportamiento esperado si la membrana plasmática fuera totalmente impermeable al Na^+. Lo mismo ocurre para el potasio, considerando la ganancia y pérdida de K^+ el resultado es un movimiento neto de cero, por lo que puede considerarse que la membrana plasmática es impermeable al K^+. Entonces, como el Na^+ y el K^+ no entran en el equilibrio electroquímico de Gibbs-Donnan, las células no se hinchan en condiciones isosmóticas (Hallows y Knauf, 1994; Álvarez-Leefmans y Reuss, 1996).

REGULACIÓN DEL VOLUMEN CELULAR EN CONDICIONES ANISOSMÓTICAS

Cuando una célula animal es expuesta a un medio anisosmótico, inicialmente ocurre un aumento (en un medio hiposmótico) o una disminución (en un medio hiperosmótico) rápido en su volumen debido a la libre entrada de agua. Sin embargo, las células se comportan como osmómetros perfectos sólo al inicio de la exposición a soluciones anisosmóticas, ya que posteriormente se activa una serie de procesos que les permite la recuperación del volumen inicial a pesar de que persistan dichas condiciones. La regulación del volumen celular que ocurre tanto en condiciones hiposmóticas como en hiperosmóticas tiene lugar debido a un cambio, ya sea una disminución o un aumento según sea el caso, en el contenido intracelular de solutos, de manera tal que la presión osmótica tiende a alcanzar el mismo valor que la externa (Fig. 1). Los cambios en el contenido intracelular de solutos incluyen el transporte de solutos intracelulares osmóticamente activos, denominados osmolitos, a través de la membrana plasmática (Hausinger, 1996; Kenneth, 1994; Pasantes-Morales, 1996); así como el aumento o disminución en la concentración de numerosos metabolitos, a través de la activación o inhibición de vías metabólicas lo que se traduce en la síntesis o degradación de macromoléculas, aunque se piensa que este mecanismo tanga una modesta participación (Lang et al., 1998c). Recientemente se ha descrito otro mecanismo importante en el control del volumen el cual consiste en el bombeo activo de agua

en contra de su gradiente de concentración, a través de cotransportadores de osmolitos (Baslow, 1999; Loo et al., 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 los mecanismos reguladores permite a las células mantener la constancia de su volumen frente a una gran variedad de situaciones.

EDEMA CEREBRAL

El término edema proviene del griego oíδημα, hinchazón, el cual se refiere al exceso de líquido en los tejidos y ocurre como consecuencia de una dinámica anormal de los fluidos. En el cerebro hay tres compartimientos que pueden acumular líquidos en exceso: el compartimiento vascular, el celular y el extracelular (espacio intersticial y el que contiene al líquido cefalorraquídeo). La expansión en volumen de cualquiera de estos compartimientos se reflejará en un aumento del volumen cerebral. El edema cerebral tiene importantes implicaciones clínicas, su gravedad y consecuencias son una función directa de la magnitud y amplitud del fenómeno, pero también de las regiones cerebrales comprometidas. Se ha reportado que un incremento por arriba del 5% del volumen cerebral ocasiona un alto grado de morbilidad y mortalidad (Trachtman, 1992). El tejido cerebral tiene muy poco espacio disponible para amortiguar el aumento en volumen debido a que la caja craneana no cede a la distensión. Así, el edema lleva a herniaciones del tejido cerebral a través de espacios de menor resistencia; la herniación a través del *foramen magnum* puede producir la muerte por paro respiratorio y cardiaco debido a la compresión del tallo cerebral (Rosenberg, 1999). El edema cerebral se ha clasificado en dos tipos: celular y vasogénico, aunque esta clasificación no considere al edema intersticial. El edema vasogénico ocurre cuando hay un incremento en la permeabilidad de la barrera hematoencefálica. El edema celular, en el que hay un aumento en el contenido de agua de las células cerebrales, de manera notoria en los astrocitos, puede originarse bajo situaciones de isosmolaridad o de hiposmolaridad, dependiendo

de la condición en un momento dado, del fluido extracelular. Es importante señalar que el desarrollo de algún tipo de edema puede progresar hacia otro; es decir, las situaciones que promueven la aparición de edema vasogénico, pueden inducir el desarrollo de edema celular, así como el edema celular puede provocar daño a la barrera hemotoencefálica con el consiguiente desarrollo de edema vasogénico.

La causa principal del edema hiposmótico es la hiponatremia, en donde este edema ocurre debido a la entrada de agua a las células para alcanzar el equilibrio osmótico. En el edema isosmótico no se presenta una alteración de la osmolaridad del medio externo, sino que ocurre como resultado de un daño primario asociado a diversas patologías que no involucran un cambio en la osmolaridad extracelular, sino una redistribución iónica o la acumulación intracelular de lactato o amonio. Este tipo de edema se presenta en condiciones de epilepsia, isquemia, traumatismo craneoencefálico y encefalopatía hepática.

La hiponatremia es una condición que se presenta como consecuencia de un desequilibrio entre la ingesta y excreción tanto de agua como de electrolitos, y puede ocurrir durante una amplia variedad de padecimientos. El exceso de agua puede ser consecuencia de una ingesta oral excesiva como en la polidipsia psicótica o más comúnmente de una eliminación renal deteriorada como resultado de una falla renal o hepática, o una deficiencia de glucocorticoides, por el uso de diuréticos de tiazida o por una secreción inapropiada de la hormona antidiurética. La pérdida de Na^+ resulta de una deficiencia de mineralocorticoides, o por síndrome nefrótico, diuresis osmótica, diarrea o vómito. La hiponatremia también puede ser una consecuencia de la infusión de soluciones hipotónicas durante el periodo perioperatorio y de una corrección rápida de la uremia por hemodiálisis excesiva (Fall, 2000; Pasantes-Morales et al., 2002). El desarrollo de la hiponatremia también se ha asociado con el uso de la droga éxtasis (Hartung et al., 2002). La aparición de cuadros de hiponatremia es también frecuente entre los corredores en pruebas de larga distancia, como el maratón, en las que el consumo de grandes cantidades de agua, ha sido tradicionalmente considerado como necesario. Una hiponatremia crónica moderada es característica de la vejez y se presenta también durante el embarazo (Law, 1989).

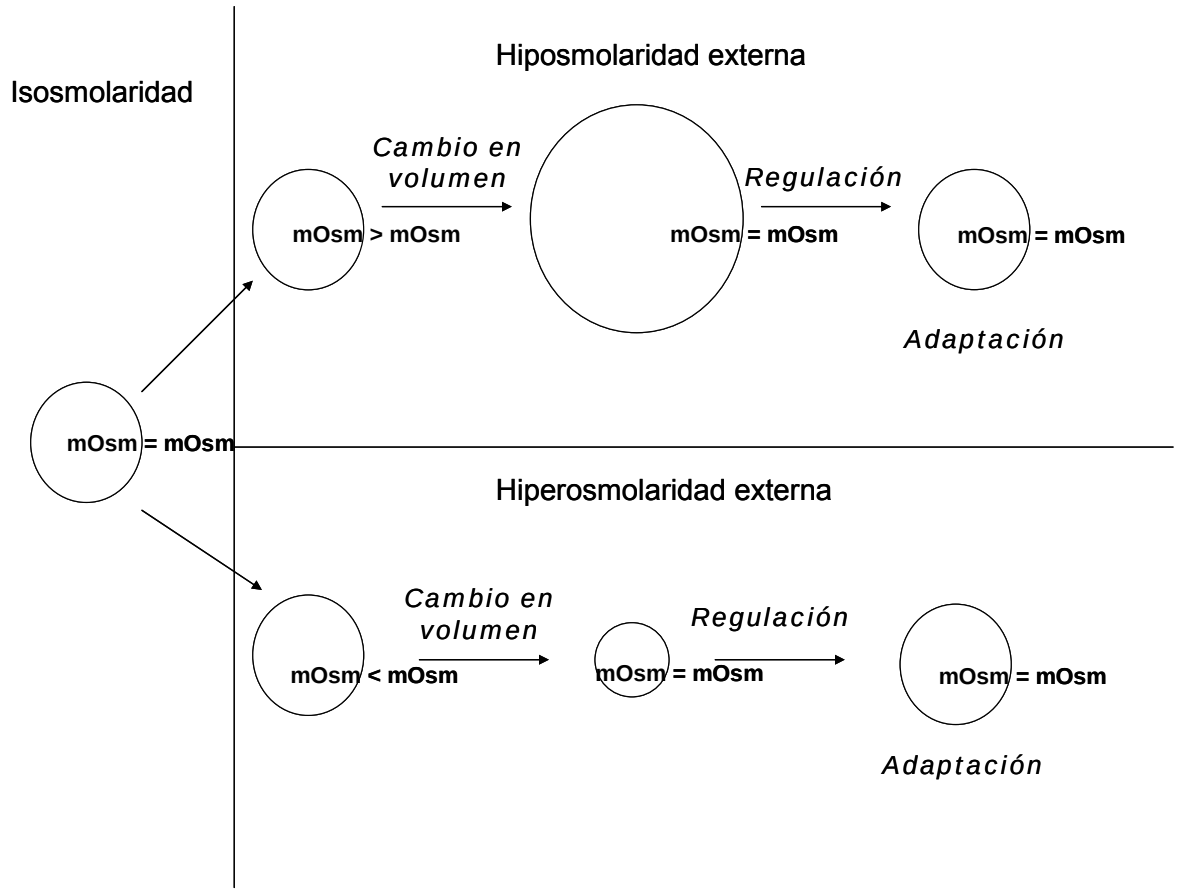

Figura 1. Cambios en el equilibrio osmótico entre el interior y el exterior celular en la exposición a condiciones anisosmóticas. En condiciones de isosmolaridad, existe un equilibrio osmótico entre el citosol y el medio extracelular. En condiciones de hiposmolaridad, inicialmente la osmolaridad celular es mayor que la extracelular, lo que provoca una entrada de agua a la célula y por consiguiente un aumento en su volumen, entonces la osmolaridad intracelular se iguala a la del medio externo (hiposmótico). Seguido del aumento en volumen, se inicia el proceso de regulación que consiste en la pérdida de osmolitos intracelulares acompañados de agua, con lo cual la célula disminuye su volumen. En esta adaptación, la osmolaridad interna y externa continúan en equilibrio. Lo mismo ocurre en condiciones hiperosmóticas, al inicio de la exposición, la osmolaridad interna es menor que la externa, hay una salida de agua de la célula y un encogimiento en su volumen, la osmolaridad de ambos compartimientos se iguala. Se inicia el proceso de regulación con la movilización de osmolitos y agua al interior celular, la célula recupera su volumen y la osmolaridad dentro y fuera de la célula se mantienen en equilibrio.

El edema cerebral hiposmótico ocurre durante la hiponatremia aguda severa, pero no en el curso de la hiponatremia crónica. Esta diferencia probablemente refleja la habilidad de las células cerebrales para adaptarse a la disminución en la osmolaridad externa y a la consiguiente acumulación de agua, cuando el desequilibrio osmótico no sucede de manera repentina. Estudios llevados a cabo en neuronas granulares de cerebelo en cultivo y en rebanadas de hipocampo en donde la estimulación hiposmótica semeja las condiciones de la hiponatremia crónica, es decir, una reducción en la osmolaridad lenta y gradual, muestran que no hay hinchamiento celular (Tuz et al., 2001; Franco et al., 2000).

Recuperación activa del volumen. Mecanismos celulares

La primera respuesta adaptativa de las células cerebrales al afrontar la hiponatremia es un desplazamiento compensatorio de líquido del espacio intersticial al fluido cerebroespinal y, posteriormente, a la circulación sistémica. La segunda respuesta adaptativa consiste en la activación de mecanismos de control del volumen celular cuando éste se ha incrementado debido a la acumulación de agua intracelular como resultado de la hiponatremia. El contenido de agua en las células se regula a través del Decremento Regulador del Volumen (DRV), que se origina por la pérdida de solutos intracelulares y agua osmóticamente obligada. Los osmolitos involucrados en el proceso de regulación son principalmente iones (K^+ y Cl^-) y moléculas orgánicas de bajo peso molecular, entre las que destacan mio-inositol, creatina/fosfocreatina, glicerofosforilcolina, fosfoetanolamina y los aminoácidos más abundantes (taurina, glutamato, glutamina y glicina) (Thurston et al., 1980; Trachtman et al., 1991; Lien et al; 1991; Verbalis y Gullans, 1991; Sterns et al., 1993; Massieu et al., 2004) (Fig. 2). La disminución en el contenido celular de K^+ y Cl^- es transitoria la cual es detectable dentro de 3-24 hrs. del inicio de la hiponatremia (Melton et al., 1987), pero la de los osmolitos orgánicos es sostenida en tanto persista la condición hiponatrémica. Estas diferencias temporales indican que la pérdida de electrolitos es un mecanismo de emergencia para contrarrestar el hinchamiento celular, pero que se reemplaza por la contribución de los osmolitos orgánicos. Esto podría tener una connotación de eficiencia adaptativa,

ya que mientras que la concentración extracelular de iones en el cerebro tiene una profunda influencia en el mantenimiento de la excitabilidad neuronal, algunos de los osmolitos orgánicos son relativamente inocuos; la taurina, en particular, es un osmolito ideal debido a su inercia metabólica. La contribución al cambio en la osmolaridad total cerebral del K^+ y Cl^- y de los osmolitos orgánicos se ha estimado entre 55% y 30%, respectivamente; aunque la pérdida de iones inorgánicos es menor en porcentaje que la de osmolitos orgánicos, aquellos contribuyen más al cambio en la osmolaridad debido a la alta concentración a la que se encuentran en el citosol (Thurston et al., 1987; Lien et al., 1991). Durante la adaptación a pequeños cambios en la osmolaridad, los aminoácidos tienen un papel muy importante, ya que son los que principalmente contribuyen a la regulación del volumen celular (Franco et al., 2000; Tuz et al., 2001).

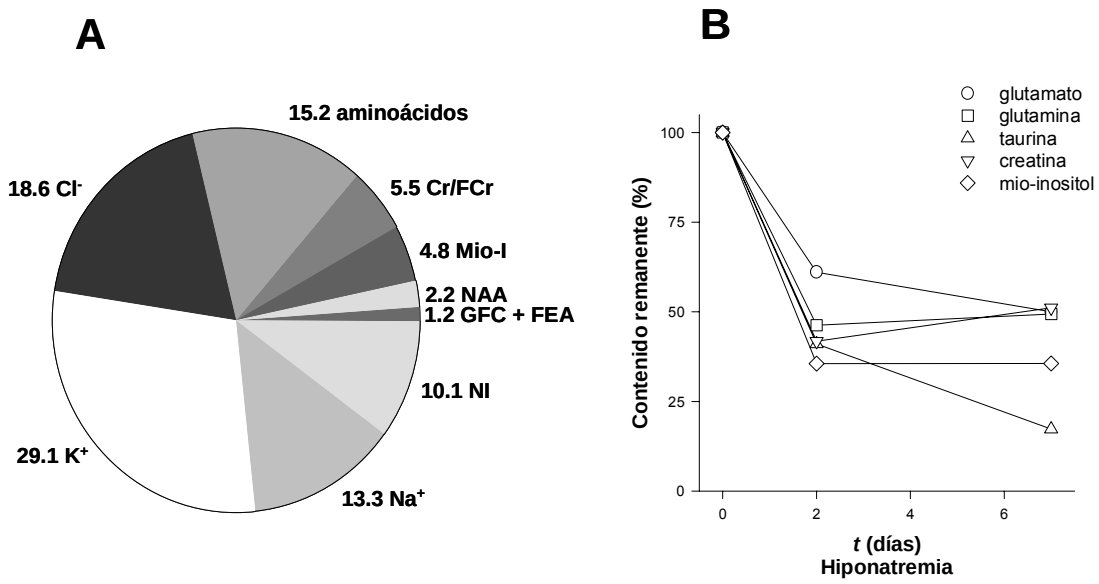

Figura 2. A. Contribución de los iones y osmolitos orgánicos que participan en el mecanismo adaptativo del cerebro a una condición de hiponatremia. B. Decremento en el tiempo de la concentración de algunos osmolitos orgánicos en condiciones de hiponatremia. La taurina es el osmolito que decrece más en términos porcentuales y cuya disminución persiste en tanto esté presente la hiponatremia. Cr/FCr: creatina, fosfocreatina; FEA: fosfoetanolamina; GFC: glicerofosforilcolina; Mio-I: mio-inositol; NAA: N-acetilaspartato; NI: otros osmolitos no identificados. Datos calculados de Verbalis y Gullans, 1991.

La mayoría de los estudios acerca de los mecanismos de hinchamiento y regulación de volumen se han llevado a cabo en cultivos primarios de células cerebrales. En estas preparaciones no se advierten diferencias marcadas entre neuronas y astrocitos, ni en el incremento inducido por la hiposmolaridad ni en la subsiguiente regulación de volumen. Sin embargo, *in vivo*, los astrocitos son las células que predominantemente aumentan su volumen en cualquier condición de edema celular. El hinchamiento preferente de los astrocitos puede ser el resultado de un manejo distinto de osmolitos y/o de un mecanismo intrínseco que prevenga la entrada de agua, como una reducida expresión de aquaporinas. A este respecto, se ha descrito que la aquaporina 4 se expresa preferentemente en los astrocitos y que la expresión de esta proteína aumenta en respuesta a la hiponatremia (Vajda et al., 2000). También se ha visto que en ratones Δ-aquapoina-4, la hiponatremia inducida produce un reducido edema cerebral (Manley et al., 2000), a su vez, se determinó que las rebanadas de cerebro y las vesículas membranales aisladas de estos mismos ratones, presentan una reducida permeabilidad al agua (Papadopoulos et al., 2002). El hinchamiento de los astrocitos sobre las neuronas puede deberse, también, a una redistribución de osmolitos entre neuronas y células gliales, como parte del papel de protección homeostática que los astrocitos tienen en relación con las neuronas. En este sentido, un estudio *in vivo* de Nagelhus y colaboradores (1993) muestra que en el cerebelo de ratas en las que se desarrolló una hiponatremia experimental, reduciendo de 15 a 20% la osmolaridad del plasma, hay una inmediata redistribución del alto contenido celular de taurina de las células de Purkinje a los elementos gliales cercanos en respuesta a la condición hiposmótica. Como resultado, los astrocitos se hinchan, mientras que las neuronas no cambian su volumen. Por otro lado, a pesar de que los cuerpos neuronales son menos sensibles al hinchamiento, no así las distintas regiones neuronales, las terminales nerviosas y las dendritas son particularmente sensibles al hinchamiento hiposmótico e isosmótico (Castejon y Arismendi et al., 2003; Fayuk et al., 2002; Liu et al., 2001; Rensing et al., 2005). En este sentido, se ha visto que en la región CA1 del hipocampo, el *stratum radiatum* y el *stratum oriens* que contienen las

dendritas apicales y basales, respectivamente, responden al hinchamiento hiposmótico de manera más notable que el *stratum piramidale*, formado por los cuerpos celulares (Jonson et al., 2000).

Las neuronas y astrocitos en cultivo al ser expuestos a un medio hiposmótico presentan un hinchamiento rápido seguido de un proceso de regulación de volumen a través de la liberación de K^+, Cl^- y osmolitos orgánicos, con la consiguiente pérdida de agua. Esta es una respuesta adaptativa que les permite recuperar su volumen original aunque la condición hiposmótica persista (Pasantes-Morales et al., 1993; Pasantes-Morales et al., 1994). El proceso de regulación de volumen involucra una cadena compleja de eventos que requiere al menos los siguientes componentes: 1) un sensor que detecte los cambios en el volumen celular, 2) un efector activado que inicie el proceso regulador: vías de flujos transmembranales de osmolitos 3) un sistema de transducción de señales entre el sensor y el efector; y 4) una "memoria" del volumen celular original y la consiguiente inactivación del proceso de regulación (Pasantes-Morales, 1996).

Sensores de cambios en el volumen celular

La regulación del volumen celular requiere de mecanismos que detecten cambios en el volumen los cuales se traducirán en los mecanismos efectores de la regulación. En un principio se pensó en un osmosensor, pero la hipótesis de un sistema sensorial complejo ha ido cobrando fuerza conforme se ha avanzado en el estudio de los posibles osmosensores y su participación en la regulación del volumen celular. A continuación se mencionan algunos de los posibles sistemas osmosensores propuestos a la fecha, los cuales no son excluyentes. El agrupamiento de macromoléculas, cambios en la fuerza iónica total del citoplasma, tensión membranal, alteraciones en el citoesqueleto, la activación de distintas proteínas de la membrana plasmática, como las integrinas y receptores, incluyendo los receptores a factores de crecimiento (Al-Habori, 2001; Cardin et al., 1999; Ding et al., 1998; Fan et al., 1999; Franco et al., 2004; Haussinger et al., 2003; Pedersen et al., 1999; Tilly et al., 1993; Wittels et al., 2000).

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

Cuando una célula detecta que hay un cambio en el volumen, traduce ese cambio en señales específicas que llevan a la activación de los sistemas de transporte involucrados en la regulación de volumen. En condiciones de hinchamiento celular se ha reportado la activación de un sin número de cascadas de señalización. Para muchas de estas vías no se ha determinado el papel que juegan dentro de la respuesta integra de la célula al hinchamiento, es decir, parece que no sólo se activan vías de señalización involucradas en la liberación de los osmolitos, sino que se prenden señales de estrés como un epifenómeno a la regulación de volumen y otras que involucran la expresión de genes preparando a la célula para afrontar una respuesta prolongada a la hiposmolaridad. Hasta el momento no se han determinado las vías de señalización, sólo se han descrito algunos de los bloques de algunas vías; se desconoce la jerarquización y las redes de comunicación que llevan a la activación de las vías de movilización de los osmolitos. A continuación, describiremos algunos de los elementos que intervienen en la activación y/o modulación de la liberación de osmolitos orgánicos, en particular de los aminoácidos, en condiciones de hiposmolaridad.

El hinchamiento hiposmótico induce, en la mayoría de los tipos celulares, un aumento en la concentración de calcio intracelular. Sin embargo, la activación de la liberación de taurina es independiente de Ca^{2+}. Existe evidencia que indica que la activación de la salida de taurina requiere una concentración basal mínima de Ca^{2+} (~40-50 nM) y no un incremento inducido por hiposmolaridad a lo que se denominó requerimiento de “calcio permisivo” (Huang et al., 2001; Mongin et al., 1999; Szucs et al., 1996). En algunos tipos celulares, se ha observado que en condiciones hiposmóticas, se incrementan los niveles de diacilglicerol (DAG) el cual activa a la proteína cinasa C (PKC). En estudios realizados en células aisladas del túbulo colector, hepatoma HTC, células de colangiocarcinoma Mz-ChA-1 y células de cáncer 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 un estrés hiposmótico (Chou et al., 1998; Liu et al., 2003; Roman et al., 1998). Sin embargo, la liberación hiposmótica de taurina no se modula por la actividad de PKC en neuronas granulares de cerebelo (Morales-Mulia et al., 2001) ni en el núcleo supraóptico de la rata (Deleuze et al., 2000). En rebanas de corteza, la liberación hiposmótica de taurina, glutamato y GABA se potencia en presencia de agentes estimuladores de la actividad de la PKC. Sin embargo, en la misma preparación, la queleritrina, un inhibidor de la actividad de la PKC, no modificó la liberación de glutamato y GABA, sólo la de la taurina (Estevez et al., 1999). Estudios realizados en rebanadas de hipocampo muestran que, en condiciones hiposmóticas, la liberación de taurina, glutamato y GABA se modula por la actividad de PKC (Franco et al., 2001; Franco, 2004). Estos resultados sugieren la posible participación de la PKC en la activación de las vías de liberación de osmolitos en respuesta a la hiposmolaridad en algunos tipos celulares.

El hinchamiento hiposmótico induce la activación de varias fosfolipasas, incluyendo a la PLA_2 y a la PLC. La activación de la primera regula la activación de la liberación hiposmótica de taurina en algunos tipos celulares (Basavappa et al., 1998; Hoffmann, 2000; Mitchell et al., 1997; Thoroed et al., 1997; Von Weikersthal et al., 1997). Sin embargo, estos mecanismos de señalización parecen ser específicos del tipo celular, ya que en otros estudios no se han encontrado los mismos resultados (Estevez et al., 1999). Por otro lado, en células cervicales humanas, la inhibición de la PLC aumenta los niveles de hinchamiento hiposmótico e inhibe el proceso de regulación de volumen (Shen et al., 1998), indicando una posible participación de esta fosfolipasa en la regulación de volumen.

Las MAP cinasas se activan por hiposmolaridad en una gran variedad de células (Haussinger y Schliess, 1999). Los mecanismos de activación de esta vía se han descrito para el caso de levaduras (Kultz y Burg, 1998; Widmann et al., 1999). Sin embargo, a pesar de la activación generalizada de la vía de las MAP cinasas por hiposmolaridad, no parece estar involucrada en la regulación de la

liberación de osmolitos en la mayoría de los tipos celulares estudiados. En general, este fenómeno es independiente de la actividad de ERK1/ERK2 inducida por hiposmolaridad. Aunque en astrocitos y en células de cáncer cervico-uterino humano se ha encontrado que la activación de ERK1/ERK2 es necesaria para la inducción de la liberación de algunos osmolitos en respuesta a la hiposmolaridad (Crepel et al., 1998; Shen et al., 2001). La activación hiposmótica de SAP cinasas p38 y JNK también ha sido ampliamente reportada, sin embargo, no se ha descrito correlación alguna entre la activación de estas cinasas y la liberación de osmolitos (Shen et al., 2001; Tilly et al., 1996).

El hinchamiento hiposmótico induce un aumento en los eventos de fosforilación en residuos de tirosina (Tilly et al., 1993). Las cinasas de tirosina activadas por hiposmolaridad, descritas hasta el momento son: p125FAK (de la Paz et al., 2002; Tilly et al., 1996b), p56lck (Lepple-Wienhues et al., 1998), p72SYK y p56LYN (Musch et al., 1999) y no se descarta la posibilidad de la activación de otras cinasas de tirosina no descritas a la fecha. En contraste con lo observado para otras vías de señalización, la actividad de las cinasas de tirosina, inducida por hiposmolaridad, parece ser fundamental para la activación de la liberación de osmolitos, entre ellos Cl^- y taurina y por consiguiente, para el proceso de regulación de volumen. En la mayoría de los tipos celulares estudiados hasta el momento, distintos inhibidores de cinasas de tirosina bloquean la activación de la corriente de Cl^- activada por volumen, mientras que inhibidores de fosfatasas de tirosina, potencian su activación (Crepel et al., 1998; Lepple-Wienhues et al., 1998; Shi et al., 2002; Shuba et al., 2000; Sorota, 1995; Thoroed et al., 1999; Voets et al., 1998; Wei et al., 2003). En el caso de la liberación de taurina activada por hinchamiento hiposmótico, se ha visto que se potencia con inhibidores de fosfatasas de tirosina y se reduce de manera importante con inhibidores de cinasas de tirosina (Deleuze et al., 2000; Mongin et al., 1999b; Morales-Mulia et al., 2001). En la mayoría de los casos, los estudios acerca de la caracterización de las vías de señalización involucradas en la activación y regulación de la liberación de osmolitos en condiciones hiposmóticas, se han enfocado en el Cl^- y la taurina, debido a la suposición de una vía común para la movilización de ambos osmolitos

la cual se ha extrapolado para los osmolitos orgánicos, en particular para los aminoácidos. Sin embargo, se han encontrado diferencias en cuanto a la inhibición de la liberación de los distintos aminoácidos con bloqueadores de cinasas de tirosina, ya que mientras que la liberación de taurina se inhibe de manera notoria en varios tipos celulares con bloqueadores de cinasas de tirosina (de la Paz et al., 2002; Deleuze et al., 2000; Mongin et al., 1999b; Morales-Mulia et al., 2001), la liberación de GABA se inhibe parcialmente (de la Paz et al., 2002) y la liberación de glutamato no se inhibe de manera significativa con estos bloqueadores (de la Paz et al., 2002; Mongin et al., 1999b). Estos resultados sugieren que la liberación de los aminoácidos ocurre a través de diferentes vías y no de una general, como discutiremos más adelante.

Vías de movilización de osmolitos

Aunque aún se desconocen las identidades moleculares de las vías a través de las cuales se movilizan los osmolitos, se han descrito algunas de sus características. Los iones se liberan básicamente a través de canales. Los canales de Cl^- activados por hinchamiento hiposmótico son rectificadores salientes, con una conductancia unitaria intermedia de 40-70 pS a potenciales positivos y de 10-20 pS a potenciales negativos. Se inactivan a potenciales superiores a 60 mV, siendo la cinética de inactivación más rápida a medida que aumenta la concentración intracelular de Mg^{2+}, disminuye el pH extracelular o en presencia de cationes divalentes extracelulares. Su activación requiere la presencia, aunque no la hidrólisis, de ATP (Okada, 1997; Nilius et al., 2000).

El canal de Cl^- sensible a volumen presenta una alta selectividad por aniones sobre cationes, pero muestra una amplia selectividad aniónica, siendo permeable a la mayoría de los aniones monovalentes y algunos aniones grandes como gluconato y metanosulfonato. Se inhibe por los bloqueadores clásicos de canales de Cl^- como DIDS (ácido 4,4'-diisotiocianoestilben-2,2'-disulfónico), SITS (ácido 4-acetamido-4'-isotiocianoestilben-2,2'-disulfónico), NPA (ácido N-fenilantracílico), 9-AC (ácido 9-antraceno-carboxílico) y DPC (ácido difenilamino-2,2'-dicarboxílico). Otros agentes con efectos inhibitorios sobre estos canales son

NPPB [ácido 5-nitro-2-(3-fenilpropilamino) benzoico], DDF (1,9-dideoxiforscolina), ácido niflúmico y ácido flufenámico (Sánchez-Olea et al., 1996). También se inhibe por ácido araquidónico y otros ácidos grasos polinsaturados (Sánchez-Olea et al., 1995).

Los canales de K^+ involucrados en la regulación del volumen pueden ser al menos de dos tipos, uno que se activa por cambios pequeños en la osmolaridad, es dependiente de Ca^{2+} y se inhibe por caribdotoxina, y otro que se activa con cambios en la osmolaridad más pronunciados, es independiente de Ca^{2+} y de voltaje y se inhibe por clofilium (Ordaz et al., 2004).

Con respecto a las vías de movilización de los osmolitos orgánicos, la más estudiada ha sido la de la taurina. Los estudios realizados por Sánchez-Olea y colaboradores (1991) y Schousboe y colaboradores (1991) sugieren un proceso difusional para la liberación de taurina en condiciones hiposmóticas, con la dirección del flujo determinada por el gradiente de concentración y sin contribución de transportadores dependientes de Na^+.

La liberación de Cl^- y taurina presenta una similitud en su sensibilidad farmacológica (Law, 1994; Junankar y Kirk, 2000; Kirk, 1997; Perlman y Goldstein, 1999; Sánchez-Olea et al., 1996) y se ha encontrado un efecto similar de los moduladores de la actividad de cinasas de tirosina en los flujos de taurina y las corrientes de Cl^- sensibles a volumen. Estos se inhiben en presencia de bloqueadores de cinasas de tirosina y se incrementan con inhibidores de fosfatasas de tirosina (Pasantes-Morales y Franco, 2002). Por lo que se propuso una vía común para la liberación de Cl^- y taurina, a la que se denominó canal aniónico activado por volumen. Sin embargo, la liberación de glutamato y GABA también se inhibe con bloqueadores de canales de Cl^- (Bothwell et al., 2001; Estevez et al., 1999b; Pasantes-Morales et al., 1999; Sánchez-Olea et al., 1993) y en estudios electrofisiológicos se han encontrado corrientes de aspartato, glutamato y taurina aniónica a través de un canal aniónico activado por hinchamiento (Banderali y Roy, 1992). De modo que se propuso una vía de amplio espectro para el Cl^- y los osmolitos orgánicos, se piensa que este canal es muy poco selectivo, permitiendo el paso de varios aniones. Sin embargo, en los últimos

años se ha acumulado evidencia que sugiere la existencia de vías independientes. Se ha observado activación del flujo de taurina en condiciones hiposmóticas sin activación de corriente de Cl^- y viceversa, también hay diferencias en los cursos temporales de activación e inactivación de ambos flujos (Junankar y Kirk, 2000). En células de Erlich la liberación osmosensible de taurina se inhibe por DIDS y se potencia por ácido araquidónico, mientras que la liberación de $^{36}Cl^-$ no se afecta por DIDS y se inhibe por ácido araquidónico (Lambert y Hoffmann, 1994). En células HeLa, el flujo de taurina y la corriente de Cl^- sensible a volumen responden con diferente sensibilidad al DIDS (Stutzin et al., 1997). La expresión de canales aniónicos y transportadores en ovocitos de *Xenopus laevis* resulta en la activación de un flujo de taurina en respuesta a la hiposmolaridad, pero la corriente de Cl^- no se modifica (Stegen et al., 2000). La similitud de la acción de varios inhibidores de canales de Cl^- y de moduladores de la actividad de cinasas de tirosina sobre los flujos de taurina y Cl^-, sugieren la existencia de una señal común para las dos vías que se afecta con los mismos inhibidores o que existe una interrelación muy estrecha en el transporte de ambos, de modo que la inhibición de uno de los flujos lleva al bloqueo de los otros.

La taurina

La taurina es un aminoácido azufrado (NH_2-CH_2CH_2-SO_3), producto del catabolismo de la cisteína por la descarboxilasa del sulfinato de cisteína. Aunque en los primates, incluyendo el hombre, su síntesis es reducida y se obtiene de la dieta (Pasantes-Morales et al, 1995). Tiene un pKa1 de 1.5 y un pKa2 de 8.82, por lo que en el intervalo fisiológico se encuentra como zwitterion. Se excreta como tal o unida a sales biliares como el taurocolato. Se acumula en el interior celular a través de un transportador específico dependiente de Na^+ (Huxtable, 1992). La taurina es un aminoácido que no forma parte de las proteínas (Jacobsenn y Smith, 1968) y con excepción de la síntesis del ácido taurocólico en el hígado, no participa en ninguna reacción metabólica. Este aminoácido se encuentra en casi todas las células animales (Jacobsenn y Smith, 1968), constituyendo más del 65% de la poza total de aminoácidos libres (Pasantes-Morales *et al*., 1991). En los

tejidos excitables, alcanza concentraciones de alrededor de 40-60 mM. Así, en la retina se encuentra en concentraciones superiores a 40 mM, en el corazón y músculo estriado se encuentra presente en concentraciones de 10 a 40 mM.

Entre los aminoácidos la taurina destaca como un osmoefector importante, dado que se encuentra en altas concentraciones en la mayoría de las células animales y parece tener propiedades ideales de osmolito ya que no forma parte de las proteínas, es inerte metabólicamente y al ser una molécula electroneutra no altera el potencial de membrana ni otros procesos celulares, además se ha demostrado que su umbral de liberación es de tan sólo una disminución de 2% en la osmolaridad externa (Tuz et al., 2001). Así, la taurina participa como osmolito en gran magnitud sin que modificaciones en su concentración alteren otras funciones celulares.

Otros aminoácidos

Como se ha descrito, la liberación de aminoácidos forma parte importante del proceso compensatorio de regulación de volumen durante el edema cerebral. Algunos de estos aminoácidos como el glutamato y GABA juegan también un papel importante como neurotransmisores. El glutamato es el principal aminoácido excitador en el sistema nervioso central de los mamíferos, mientras que el GABA es el principal neurotransmisor inhibidor. La liberación de neurotransmisores al espacio extracelular en el cerebro y en particular en los sitios donde se lleva a cabo la transmisión sináptica, supondría un riesgo en la excitabilidad neuronal durante la condición hiposmótica.

Sinapsis. Liberación de neurotransmisores

Las sinapsis constituyen el sitio de comunicación entre dos neuronas. Las sinapsis del sistema nervioso central de los mamíferos se encuentran en una gran variedad de formas y tamaños. Algunos pequeños botones están en contacto con espinas dendríticas, como aquellas que se encuentran en las neuronas piramidales del hipocampo, mientras que otras terminan en el soma y contienen múltiples contactos sinápticos. Esta variedad estructural refleja, también, la diversidad

funcional de las sinapsis. Una neurona forma, en promedio, alrededor de 1000 conexiones sinápticas. En el cerebro humano hay al menos 10^{11} neuronas y 10^{14} conexiones sinápticas. A pesar de esta multitud, los mecanismos que operan para controlar la transmisión sináptica en estas conexiones presentan características básicas comunes. Las sinapsis pueden ser eléctricas o químicas. La incidencia de las sinapsis eléctricas es baja. Están formadas por uniones tipo gap que conectan las membranas de las dos células involucradas, de modo que hay una continuidad del citoplasma, y el flujo de corriente iónica que media la transmisión, fluye a través de estos canales, por lo que estas sinapsis están caracterizadas por una alta velocidad de transmisión y por mediar acciones excitatorias. En el caso de las sinapsis químicas, estas median acciones inhibitorias y excitatorias. Las neuronas que forman este tipo de sinapsis no se encuentran conectadas estructuralmente. Las membranas pre y postsinápticas presentan regiones morfológicas especializadas. Las sinapsis químicas están definidas como zonas activas presinápticas o sitios de liberación y sus regiones asociadas de receptores postsinápticos. Al microscopio electrónico, una especialización sináptica de este tipo se puede identificar por una acumulación de vesículas presinápticas, un amplio espacio intercelular denso a los electrones (hendidura sináptica) y una densidad postsináptica la cual está caracterizada por una alta concentración de receptores/canales y usualmente es coextensiva con el sitio presináptico de liberación. Los mecanismos básicos de la liberación vesicular en las sinapsis químicas muestran características comunes. En el mecanismo de transmisión sináptica considerado clásico, el impulso nervioso produce una entrada de Ca^{2+} en la terminal presináptica, a través de canales dependientes de voltaje concentrados en cada sitio de liberación o zona activa la cual está definida como un sitio de entrada de Ca^{2+} y fusión vesicular durante un potencial de acción. Los canales de Ca^{2+} involucrados son miembros de la familia Ca_v2 y cada subtipo conduce corrientes tipo P/Q, N o R. Las corrientes de Ca^{2+} del tipo P son altamente sensibles a la ω-agatoxina IVA de araña (Mintz et al., 1992), mientras que las corrientes tipo Q son menos sensibles (Randall y Tsien, 1995); las corrientes de tipo N se inhiben con ω-conotoxina GVIA de la serpiente marina (McCleskey et al.,

1987) y las corrientes de tipo R son resistentes a los inhibidores orgánicos y peptídicos de canales de Ca^{2+} (Randall y Tsien, 1995; Catterall, 2000). Las vesículas que contienen neurotransmisores se adosan a la zona activa en un primer contacto con la membrana, después pasan por un proceso de maduración que las hace competentes para la rápida fusión con la membrana, la cual se dispara por la entrada de Ca^{2+}. Durante el proceso de maduración, se ensambla un complejo central formado por tres proteínas sinápticas abundantes, llamadas genéricamente SNARE (receptor proteico de unión al factor soluble sensible a N-etilmaleimida). Dos de estas proteínas son de la membrana plasmática [sintaxina y SNAP-25 (proteína de 25 kD asociada al sinaptosoma)] y una de la vesícula sináptica [VAMP(proteína membranal asociada a la vesícula)/sinaptobrevina]. Este complejo forma el anclaje para la cascada de interacciones proteína-proteína que se requieren para la exocitosis (Südhof, 1995). La sintaxina y la SNAP-25 se unen estrechamente una con otra y forman un sitio de unión de alta afinidad para la VAMP/sinaptobrevina, constituyendo un complejo heterotrimérico altamente estable (Hayashi et al., 1994). La importancia de estas proteínas en la exocitosis fue evidente cuando se reconoció que son los sustratos específicos para las neurotoxinas clostridiales las cuales son potentes inhibidores de la liberación de neurotransmisores. Las cadenas ligeras de la toxina tetánica y la toxina botulínica (BoNT) son metaloproteasas que hidrolizan estas proteínas. La toxina tetánica actúa sobre la sinaptobrevina, mientras que la acción de las toxinas botulínicas está asociada con diferentes serotipos, la BoNT A hidroliza SNAP-25; la BoNT B, sinaptobrevina y la BoNT C, sintaxina 1 (Niemann et al., 1994). La acción de las toxinas en las proteínas individuales compromete la estabilidad del complejo y su consecuente habilidad para mediar la exocitosis.

La fusión de las vesículas sinápticas con la membrana produce la liberación de neurotransmisores a la hendidura sináptica, los que difunden a través de esta y se unen a sus receptores localizados en la membrana postsináptica, produciendo un flujo de corriente a través de esta el cual está mediado por canales que pueden estar acoplados directamente a los receptores y/o activarse indirectamente por segundos mensajeros.

La liberación de neurotransmisores es un evento probabilístico. La entrada de Ca^{2+} a la terminal nerviosa eleva la probabilidad de liberación, posiblemente a través de la activación de múltiples sensores a Ca^{2+}. Así, los componentes clave involucrados en la regulación de la probabilidad de liberación dependiente de Ca^{2+} son los canales de Ca^{2+} que permiten la entrada de este catión a la terminal sináptica y los sensores de Ca^{2+} en la maquinaría de fusión que regula la exocitosis. La acción principal del Ca^{2+} es disparar la exocitosis rápida durante un potencial de acción. El Ca^{2+} actúa a altas concentraciones en sitios que requieren la unión de varios iones Ca^{2+} (al menos 3-4) (Goda y Südhof, 1997). Se piensa que el Ca^{2+} puede disparar la liberación rápida de neurotransmisores porque las vesículas adosadas y ancladas se encuentran en un estado tardío y latente en la exocitosis. Las vesículas sinápticas se adosan a la membrana cerca de canales de Ca^{2+} en la zona activa. También se ha visto que las interacciones entre los componentes de la maquinaría de fusión vesicular se regulan por Ca^{2+}, se ha descrito que la sintaxina y la sinaptotagmina, proteínas que participan en el anclaje de las vesículas, se asocian con canales de Ca^{2+} tipo N (Seagar y Takahashi, 1998) y que los canales presinápticos $Ca_v2.1$ y $Ca_v2.2$ interactúan directamente con las proteínas SNARE y con la sinaptotagmina a través de un sitio específico en el asa que conecta los dominios II y III (Sheng et al., 1994; Wiser et al., 1997). La interacción de los canales de Ca^{2+} con el complejo de fusión forma un arreglo que facilita la exocitosis rápida por las altas concentraciones locales de Ca^{2+} que se pueden encontrar durante un potencial de acción (Rettig et al., 1996; 1997). Para disparar el estado final de la reacción de fusión, se requiere un sensor de Ca^{2+} en el sitio de exocitosis. El sensor debe unir Ca^{2+} cooperativamente y sufrir un cambio conformacional dependiente de Ca^{2+}. Hay evidencia que sugiere que las sinaptotagminas funcionan como sensores de Ca^{2+} (Brose et al., 1992; diAntonio et al., 1994; Geppert et al., 1994) las cuales son proteínas vesiculares de unión a Ca^{2+} y presentan dos regiones de unión a este catión, llamados dominios C2A y C2B. La sinaptotagmina se une a fosfolipidos en función de la concentración de Ca^{2+} (Heidelberger et al., 1994), esta unión podría remodelar la membrana durante la exocitosis, transformándola en una membrana fusionada. La

función del complejo de proteínas SNARE se regula por interacciones con numerosas proteínas, que incluyen a la sinaptotagmina. La sintaxina interactúa directamente con sinaptotagmina, la unión del Ca^{2+} al dominio C2A incrementa su afinidad por esta proteína (Chapman et al., 1995). También se ha encontrado que se une de manera estable a la SNAP-25 a través del dominio C2B (Schiavo et al., 1997). En el modelo del complejo de fusión membranal activado por Ca^{2+}, la presencia de Ca^{2+} promueve que las asas de unión a Ca^{2+} de los dominios C2A y C2B de la sinaptotagmina I se inserten en la bicapa. El inositol 4,5-bifosfato localizado en la hoja interna de la sección blanco de la membrana, dirige la penetración de los dominios C2 lo que promueve el acercamiento de las membranas plasmática y vesicular (Bai y Chapman, 2004) (Fig. 3).

La fusión de membranas lipídicas se ha descrito en términos de una progresión de estructuras intermedias de baja energía, las cuales involucran rearreglos de la matriz lipídica, en donde las monocapas proximales se mezclan, formando una estructura altamente curveada parecida a un tallo, con una curvatura negativa neta (los grupos polares en el lado cóncavo), que se encuentra intercalada entre las monocapas distales intactas de las membranas cercanas, y como resultado se obtiene una intermembrana, que define un estado de hemifusión. El tallo se expande hasta que las monocapas distales se fusionan, formando un poro de fusión lipídico de curvatura positiva neta (los grupos polares en el lado convexo) (Szule et al., 2003). Se desconoce que ocurre entre la entrada de Ca^{2+} y la fusión membranal. Se piensa que la fusión requiere el desemsamble del complejo SNARE que ancla la vesícula sináptica a la membrana (Tahara et al., 1998; Zimmerberg et al., 2000).

Además de la entrada de Ca^{2+} a través de canales dependientes de voltaje, otros mecanismos que disparan la liberación de neurotransmisores son: Ca^{2+} liberado a partir de fuentes intracelulares: retículo endoplásmico y/o mitocondria (Adam-Vizi, 1992; Berridge, 1998; Raiteri et al., 2002), también se ha descrito que la fosforilación de proteínas por PKC o PKA potencia la exocitosis dependiente de Ca^{2+} y frecuentemente es suficiente para inducir exocitosis por si misma (Hille et al., 1999; Scepek et al., 1998; Stevens y Sullivan 1998; Yawo, 1999). La PKC es

un importante modulador de la liberación de aminoácidos neurotransmisores de una variedad de preparaciones de cerebro, incluyendo los sinaptosomas (Vaughan et al., 1998). El efecto de PKC puede ocurrir a través de un incremento en la $[Ca^{2+}]_i$ modulando directamente la vía de entrada de Ca^{2+} o puede actuar en algún paso del mecanismo de liberación. Se ha propuesto que la activación de PKC facilita el flujo de glutamato mediante una o más de las siguientes posibilidades: i) reclutamiento de vesículas sinápticas, ii) incremento en la sensibilidad al Ca^{2+} del proceso de secreción y iii) relajación de la red citoesquelética (Vaughan et al., 1998). El mecanismo a través del cual la PKC promueve rearreglos en el citoesqueleto involucra a sus sustratos MARCKS (sustrato de la cinasa C rico en alanina miristoilada) los cuales son importantes en regular las interacciones de la membrana plasmática con la F-actina y posiblemente la exocitosis. En el estado no fosforilado, las MARCKS se unen a la F-actina que está asociada a la membrana plasmática (Aderem, 1995). La fosforilación de las MARCKS resulta en la disociación de estas con la membrana plasmática y en la pérdida de su habilidad para entrecruzar F-actina. Esta fosforilación podría producir un desensamble parcial en la red citoesquelética y por lo tanto permitiría la migración de las vesículas a los sitios de liberación. En el control de la secreción, la PKC también juega un papel importante. La proteína Munc-18 se une a la sintaxina con alta afinidad, esta unión impide que la sintaxina entre en contacto con la SNAP-25 y la sinaptobrevina, y por lo tanto imposibilita la formación del complejo central SNARE de anclaje a la membrana. La PKC fosforila a Munc-18 en las serinas 306 y 313 de manera dependiente de Ca^{2+} y fosfolípidos, esta fosforilación inhibe la interacción Munc-18-sintaxina (Fujita et al., 1996), permitiendo la formación del complejo SNARE y el anclaje de las vesículas a la membrana.

Por otro lado, el método que se usa más frecuentemente para despolarizar y reproducir el evento inicial que conduce a la liberación de neurotransmisores es el uso de una concentración elevada de K^+ extracelular. Con este tipo de estimulación se ha podido estudiar la liberación de neurotransmisores en una gran variedad de preparaciones de tejido nervioso (Bernath, 1992). Aunque este método difiere de la estimulación eléctrica en que una alta concentración de K^+ no

se puede aplicar por una duración comparable a la de los pulsos eléctricos y la liberación de los neurotransmisores estimulada por K^+ no es sensible a la presencia de la tetrodotoxina, lo que indica que esta despolarización no induce la activación de canales de Na^+ dependientes de voltaje. Además, una elevada concentración de K^+ podría causar un hinchamiento considerable de las células, promoviendo la liberación de neurotransmisores como parte de los procesos de regulación de volumen, independientemente de la despolarización (Pasantes-Morales y Schousboe, 1989).

Una preparación que se utilizado hace más de cuarenta años para estudiar el proceso de exocitosis son las terminales nerviosas aisladas, también llamadas sinaptosomas, en las que el proceso de secreción se encuentra bien preservado; la despolarización de estos sinaptosomas induce la liberación dependiente de Ca^{2+} de una amplia variedad de neurotransmisores, aminoácidos, catelcolaminas y neuropéptidos (Ghijsen et al., 2003).

Los sinaptosomas expuestos a hiposmolaridad experimentan un aumento en su volumen y lo regulan a través del DRV (Babila et al., 1990), pero se desconocen los osmolitos implicados en el proceso. Esta preparación constituye un modelo ideal para estudiar la posible liberación de aminoácidos neurotransmisores ante condiciones de hiposmolaridad, con el fin de caracterizar los mecanismos de movilización implicados y definir el papel de esta condición en la excitabilidad neuronal.

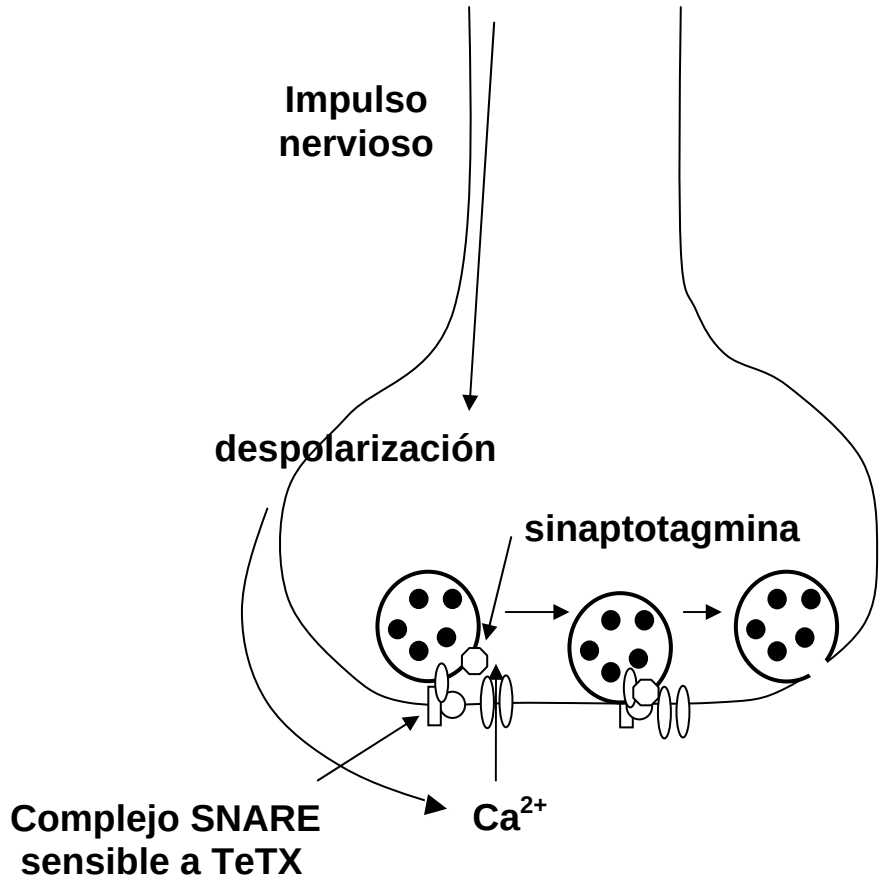

Figura 3. Representación esquemática del mecanismo de exocitosis. La terminal sináptica se despolariza con la llegada del impulso nervioso y se abren canales de Ca^{2+} dependientes de voltaje. El Ca^{2+} promueve la unión del canal de Ca^{2+} con la sinaptotagmina y de esta con el complejo SNARE, las proteínas mencionadas sufren un rearreglo estructural con el cual las membranas vesicular y plasmática se acercan para después fusionarse, en donde se piensa es necesario el desensamble del complejo SNARE.

Consecuencias del edema cerebral. Hiperexcitabilidad

La consecuencia extrema del edema cerebral es, como se ha mencionado, la muerte del individuo por la compresión de los centros cardíaco y respiratorio a consecuencia de la presión del tejido cerebral. En general, se considera que un incremento de tan sólo cinco por ciento en el volumen cerebral implica ya un

riesgo de mortalidad (Tratchtman, 1992). A nivel local y celular, la ruptura de los capilares debida a la compresión craneana, trae como consecuencia anoxia y muerte neuronal. La extensión del daño depende de la localización y magnitud de la causa que genera el edema. La muerte neuronal se produce directa e inmediatamente a consecuencia de la anoxia, pero en las áreas que circundan la zona afectada, la muerte neuronal tiene lugar más tardíamente, al parecer a consecuencia de un fenómeno de excitotoxicidad, que resulta de la liberación del ácido glutámico de los astrocitos hinchados, en conjunto con una función disminuida de los transportadores (Hansson et al., 2000).

Otra complicación asociada al edema celular es la reducción del espacio sináptico con la consecuente aparición de transmisión efáptica y aumento de la concentración extracelular de K^+, condiciones que promueven una situación de hiperexcitabilidad. En particular, se ha descrito que la sincronía en la descarga neuronal en la epilepsia generalizada se debe a este fenómeno y se previene mediante estrategias que reducen el incremento en el volumen celular (Hochman et al., 1995). El incremento en el volumen de las terminaciones nerviosas y la consecuente liberación de osmolitos que funcionan como neurotransmisores podría llevar también a una alteración en la función sináptica, que puede llegar a producir convulsiones. Los mecanismos celulares que conllevan a una alteración de la actividad sináptica durante la hiposmolaridad se desconocen. Se ha descrito que una disminución en la osmolaridad ocasiona un incremento en la duración y amplitud de los potenciales excitadores postsinápticos en rebanadas de hipocampo (Chebabo et al., 1995).

La hiposmolaridad induce la liberación de aminoácidos en células cerebrales, muchos de los cuales funcionan como neurotransmisores y también produce no sólo un aumento en su volumen y el consiguiente proceso de regulación, sino que es un estímulo que promueve una amplia variedad de respuestas. En los sinaptosomas encontramos que la hiposmolaridad produce una despolarización dependiente de Na^+ y un aumento en la concentración de Ca^{2+} citosólico, ambas condiciones que promueven la fusión vesicular. Por lo que, la

hiposmolaridad podría disparar el proceso de exocitosis en las terminales nerviosas, la cual a su vez produciría la liberación de neurotransmisores.

OBJETIVO

Identificar los fenómenos que se generan a nivel sináptico a consecuencia de una reducción en la osmolaridad externa y su efecto sobre la movilización de osmolitos y neurotransmisores de las terminaciones sinápticas aisladas.

Objetivos Particulares

I. Determinar si la hiposmolaridad induce la movilización de aminoácidos neurotransmisores en los sinaptosomas de corteza de la rata.
II. Determinar si el mecanismo de liberación de los aminoácidos corresponde al mecanismo característico de la liberación tipo osmolito o corresponde a una liberación inducida por eventos concurrentes con la hiposmosis, tales como despolarización e incremento de Ca^{2+} en la terminal sináptica, que presente características similares a la liberación de neurotransmisores.
III. Determinar si la hiposmolaridad induce la liberación de la norepinefrina, que no actúa como osmolito.

MATERIAL Y MÉTODOS

Preparación de los sinaptosomas y perfusión

Los sinaptosomas se obtuvieron de la corteza de ratas macho adultas de la cepa Wistar de 180±20 g; se preparó una fracción sinaptosomal cruda por centrifugación diferencial (Whittaker y Barker, 1972), a partir de la cual se obtuvieron los sinaptosomas purificados a través de un gradiente discontinuo de sacarosa (Hajos, 1975). Las suspensiones de los sinaptosomas se incubaron en agitación con ^{3}H-taurina, ^{3}H-glutamato o ^{3}H-GABA (5 µCi/ml) durante 30 min. en medio isosmótico. Para seguir los movimientos de norepinefrina se utilizó un trazador marcado con tritio, los sinaptosomas se incubaron durante 20 min. en la presencia de ^{3}H-norepinefrina (2.5 µCi/ml), pargilina (10 µM), ácido ascórbico (0.2 mg/ml) y álbumina bovina de suero (0.1%) durante 20 min. en medio isosmótico, el cual contiene (en mM): NaCl, 135; KCl, 1; $CaCl_2$, 1; $MgSO_4$, 1.17; KH_2PO_4, 1.7; HEPES, 10 y glucosa, 10. Los medios hiposmóticos (10%, 270 mOsm y 20%, 240 mOsm) se prepararon reduciendo la cantidad necesaria de NaCl. La osmolaridad de todas las soluciones se verificó en un osmómetro de punto de congelación (Osmette A, Precision Systems Inc., Natick, MA, USA). Después de la incubación, los sinaptosomas se separaron de la solución de incubación a través de la filtración rápida en un filtro Millipore (0.45 µm) y se enjuagaron en medio isosmótico. La membrana que contuvo a los sinaptosomas se transfirió a una cámara de perfusión (0.7 ml) y se perfundió con medio isosmótico a una velocidad de flujo de 1 ml/min. Después de un periodo de lavado de 18 minutos, cada minuto se colectaron fracciones del medio perfundido en viales de centelleo. La basal se obtuvo aproximadamente a los tres minutos, después de lo cual el medio isosmótico se reemplazó con medio 10% hiposmótico, 20% hiposmótico o isosmótico conteniendo 20 mM KCl y la perfusión continuó por 7 min. Al final de la perfusión, los sinaptosomas se solubilizaron y la radiactividad de las fracciones colectadas y de aquella que permaneció en los sinaptosomas se determinó por espectrometría de centelleo. Los tiempos de preincubación con las drogas variaron dependiendo del experimento y se indicó en las respectivas leyendas de

figura. Los resultados se expresaron como porcentaje de liberación en cada fracción, de la radiactividad total (radiactividad en las muestras más radiactividad remanente en los sinaptosomas al final de la perfusión).

Determinación de los cambios en el potencial de membrana

Los cambios en el potencial de membrana se determinaron por espectrofluorometría con el fluoróforo bisoxonol DiSBAC2(3) (150 nM), en un espectrómetro de luminiscencia Aminco-Bowman (series 2, SLM AMINCO; Rochester, NY, USA) equipado con un agitador magnético. El bisoxonol se adicionó a 2 ml de medio isosmótico en una cuveta de cuarzo y se agregaron los sinaptosomas (40-50 µg proteína, determinada por el método de Bradford; Bradford, 1976). Después de 30 segundos, el medio se diluyó hasta alcanzar 20% de hiposmolaridad y los cambios en la fluorescencia se siguieron durante 60 seg. Las longitudes de onda de excitación y emisión del fluoróforo fueron de 540 y 580 nm, respectivamente. Los resultados se expresaron como unidades arbitrarias de fluorescencia.

Determinación de la concentración de Ca^{2+} intrasinaptosomal

Los sinaptosomas se incubaron con el indicador fluorescente de Ca^{2+} fura-2/AM (2 µM), en agitación durante 60 minutos. Después de este periodo, los sinaptosomas se centrifugaron a 25 000 *g* durante 5 minutos para remover el indicador extrasinaptosomal. El pellet sinaptosomal se resuspendió en medio isosmótico y se mantuvo en agitación durante 30 min. Después, la preparación se centrifugó (25 000 *g*, 5 min.) cuatro veces y el pellet se resuspendió en medio isosmótico. Los cambios en la [Ca^{2+}] se determinaron en un espectrómetro de luminiscencia Aminco-Bowman equipado con un agitador magnético. La longitud de onda de excitación se alternó entre 340 y 380 nm y la intensidad de fluorescencia se monitoreo a 510 nm. Cada experimento se calibró individualmente para obtener un máximo de fluorescencia después de permeabilizar los sinaptosomas con tritón X-100 (10%), y la fluorescencia mínima después de atrapar el Ca^{2+} en el medio con EGTA (250 mM). Los valores obtenidos a través de este procedimiento se

utilizaron para calcular la concentración intrasinaptosomal de Ca^{2+} de acuerdo a las ecuaciones publicadas previamente por Grynkiewicz y colaboradores (1985).

Medición de la exocitosis

La exocitosis se determinó por espectrofluorometría como se describe en Guatimosim y colaboradores (1997), utilizando el fluoróforo FM1-43. Los sinaptosomas se incubaron con FM1-43 (4 µM) durante 10 minutos en medio isosmótico y después durante 1 minuto en medio isosmótico conteniendo 40 mM KCl para permitir la incorporación del fluoróforo a las vesículas exocitóticas. Los sinaptosomas se lavaron tres veces con medio isosmótico más albúmina bovina de suero (1 mg/ml) a través de ciclos de centrifugación y resuspensión y se transfirieron a una cuveta de cuarzo para el ensayo fluorométrico. El experimento se llevó a cabo en un espectrómetro de luminiscencia con agitación continua. Las muestras se excitaron a 488 nm y la fluorescencia de emisión se registró a 560 nm.

Análisis estadístico

Las diferencias significativas en los valores de las medias se calcularon utilizando la prueba *t* de Student no direccional.

RESULTADOS

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

European Journal of Neuroscience, Vol. 19, pp. 916–924, 2004

Depolarization, exocytosis and amino acid release evoked by hyposmolarity from cortical synaptosomes

Karina Tuz, Claudia Peña-Segura, Rodrigo Franco and Herminia Pasantes-Morales
Department of Biophysics, Institute of Cell Physiology, National University of Mexico, Mexico

Keywords: glutamate, nerve endings, swelling, taurine, volume regulation

Abstract

External osmolarity reduction (20%) led to labelled glutamate, GABA and taurine release from rat brain cortical synaptosomes. A Cl^--independent, Na^+-dependent, La^{3+}-sensitive and tetrodotoxin (TTX) reduced depolarization of synaptosomes occurred upon hyposmolarity, suggestive of Na^+ entry through nonselective cation channels. This depolarization, together with cytosolic Ca^{2+} ($[Ca^{2+}]_i$) increase, resulted in exocytosis, monitored by FM1-43. The release fraction resulting from these phenomena was estimated, by its decrease, by La^{3+}, EGTA-AM and tetanus toxin (TeTX), as 34–44% for glutamate, 21–29% for GABA and 18–22% for taurine. Protein kinase C (PKC) activation by phorbol-12-myristate-13-acetate (PMA) increased the hyposmolarity-elicited exocytosis and this activation increased glutamate (80%), GABA (51%) and taurine (42%) hyposmotic efflux. Inhibition by chelerythrine reduced glutamate, GABA and taurine efflux by 64%, 50% and 24%, respectively. The Na^+-dependence of amino acid release (glutamate 63%, GABA 46% and taurine 29%) may result from both, prevention of the depolarization-exocytosis efflux, and blockade of the carrier reversal operation. Carrier blockade by DL-threo-β-benzyloxy aspartate (TBOA) and NO-711 resulted in 37% and 28% reduction of glutamate and GABA release, respectively. Contribution of the osmolyte leak pathway to amino acid release, estimated by the influence of Cl^- (NPPB) and tyrosine kinase (AG18) blocker, was up to 55% for taurine, but only 10–18% for GABA, with apparently no contribution for glutamate. The predominant osmolyte-type mechanism of taurine release suggest its function in volume control in nerve endings, while glutamate and GABA respond to events concurrent with hyposmolarity by a neurotransmitter-like release mechanism. The hyposmolarity-induced amino acid efflux from nerve endings may have consequences for neuronal excitability during hyponatremia.

Introduction

The ability to regulate volume after hyposmotic swelling has been preserved in most cells, even when surrounded by an external environment of controlled osmolarity (Lang *et al*., 1998). This persistent ability may be necessary for cells to correct local and transient volume changes associated with normal functions and metabolic reactions as well as in pathological situations. Such a requirement to keep cell volume constant has been proven critical in brain, where oedema is a most severe sequel event in numerous pathologies (Andrew, 1991; Kimelberg, 1999). In brain cells, as in many other cells, hyposmolarity leads to rapid swelling followed by a volume regulatory process, via the extrusion of K^+, Cl^- and some organic molecules, primarily amino acids (Kimelberg *et al*., 1990; Pasantes-Morales *et al*., 1993; Pasantes-Morales *et al*., 1994a,b). Less is known about the occurrence of such mechanisms in intracellular compartments or in specialized regions of neurons, such as dendrites or nerve endings, despite the fact that dendrites are particularly sensitive to swelling and nerve endings are continuously exposed to osmotic gradients due to the constant influx and efflux of ions and neurotransmitters. Work by Babila *et al*. (1990) showed that isolated nerve endings exposed to hyposmotic solutions first swell and then rapidly recover their original volume. A more recent report (Mongin *et al*., 1997) shows an increase in $[Ca^{2+}]_i$ as a consequence of swelling in synaptosomes but the influence of Ca^{2+} on the osmolyte corrective fluxes has not been addressed.

In the present study we investigated whether hyposmolarity, and the resulting swelling, activate amino acid extrusion from synaptosomes. Taurine acts as an osmolyte in many cell types, including brain cells, and may also play this role in synaptic endings, where it is present in high concentrations, in spite of its marginal function as neurotransmitter (Kontro *et al*., 1980). Other amino acids, such as GABA and glutamate – with a prominent role as neurotransmitters – are also involved in volume regulation mechanisms in brain cells and slices. The large concentrations (mM) of these amino acids in nerve endings (Lahdesmaki *et al*., 1977), largely exceeding those required for a synaptic transmitter function, is suggestive of an osmolyte role in nerve endings too. A hyposmolarity-evoked release of amino acids from synaptosomes may occur either by the leak pathway, well characterized in cultured cells, or by events concurrent with hyposmolarity, particularly depolarization, that in turn trigger other mechanisms of release. The first possibility was tested by the extent of inhibition by Cl^- channel blockers and by the dependence on tyrosine kinase reactions, as markers for the osmolyte-type release. As for the second possibility, as we found that hyposmolarity indeed results in synaptosome depolarization, we examined two possible mechanisms for release: (i) a Ca^{2+}-dependent exocytosis and (ii) the reversal of the carrier. All of these mechanisms may operate and contribute to the total release observed.

Correspondence: Dr Herminia Pasantes-Morales, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70–253, 04510 México D.F., México.
E-mail: hpasante@ifc.unam.mx

Received 22 July 2003, revised 12 December 2003, accepted 16 December 2003

doi:10.1111/j.1460-9568.2004.03209.x

Materials and methods

Materials

Salts (NaCl, KCl, KH_2PO_4, $CaCl_2$, $MgSO_4$ and $MgCl_2$), *N*-2-hydroxyethyl piperazine-*N*′-(2-ethanesulphonic acid) (HEPES) and glucose were from T. J. Baker (Xalostoc, Mexico); wortmannin, tyrphostin AG18, phorbol-12-myristate-13-acetate (PMA), chelerythrine chloride, cytochalasin E, 5-nitro-3-(3-phenylpromylamino)benzoic acid (NPPB), ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester (EGTA-AM) and tetanus toxin (TeTX) were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA); fura-2 acetoxymethyl ester (fura-2/AM), FM1-43 and bis-(1–3diethylthiobarbituric acid) trimethine oxonol [DiSBAC2(3)] (bisoxonol) were from Molecular Probes (Eugene, OR, USA). Tetrodotoxin (TTX) was from Alomone Laboratories (Jerusalem, Israel). [^{3}H]Taurine, [^{3}H]L-glutamate and [^{3}H]GABA were from New England Nuclear (Boston, MA, USA). NO-711 hydrochloride, DL-threo-β-benzyloxy aspartate (TBOA), 4,4′-diisothiocyanato-stilbene-2,2′ disulphonic acid (DIDS), bovine serum albumin (BSA), triton X-100, choline chloride and gluconate salts were from Sigma Chemical Co. (St. Louis, MO, USA).

Synaptosomal preparation and superfusion

Cerebral cortex was excised from male Wistar adult rats of about 180, after decapitation. A crude synaptosomal fraction was prepared by differential centrifugation according to Whittaker & Barker (1972). Purified synaptosomes were obtained as described elsewhere (Hajos, 1975) in a sucrose discontinuous gradient. Synaptosome suspensions were incubated under shaking with [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA (5 μCi/mL) during 30 min in isosmotic medium containing (in mM): NaCl, 135; KCl, 1; $CaCl_2$, 1; $MgSO_4$, 1.17; KH_2PO_4, 1.7; HEPES, 10 and glucose, 10. Hyposmotic media (10%, 270 mOsm; 20%, 240 mOsm) were prepared by reducing the necessary amount of NaCl. Osmolarity of all solutions was measured in a freezing point osmometer (Osmette A, Precision Systems Inc., Natick, MA, USA). After incubation, synaptosomes were separated from the loading solution by rapid filtration through a Millipore filter (pore size 0.45 μm) and rinsed with isosmotic medium. The filter membrane containing synaptosomes was transferred to a superfusion chamber (0.7 mL) and superfused with isosmotic medium at a flow rate of 1 mL/min. After a wash period of 15 min, fractions of the perfusate medium were collected at 1 min intervals into scintillation vials. The baseline was attained at about 3 min, after which the isosmotic medium was replaced by 20% or 10% hyposmotic medium and superfusion continued for further 12 min. At the end of the superfusion, synaptosomes were solubilized and the radioactivity of collected fractions and that remaining in synaptosomes was measured by scintillation spectrometry. Preincubation times with drugs varied depending on the experiment and are indicated in the corresponding figure legends. When required, controls always contained the vehicle. Results are expressed in percentage release in each fraction of the total radioactivity (i.e., radioactivity in samples plus radioactivity remaining in synaptosomes at the end of superfusion). The radioactivity of the hyposmotic superfusion fractions representing authentic GABA and glutamate was found to be 82.7% and 74.3%, respectively, as assessed by paper chromatography.

Depolarization measurement

Depolarization was measured by spectrofluorometry using an Aminco-Bowman luminescence spectrometer (series 2, SLM AMINCO; Rochester, NY, USA), equipped with a magnetic ministirrer and the fluorophore bisoxonol DiSBAC2(3) (150 nM). Bisoxonol was added to 2 mL of isosmotic medium in a quartz cuvette and the reaction started by addition of synaptosomes (40–50 μg protein, determined by the Bradford method; Bradford, 1976). After 30 s, the medium was diluted to reach 20% hyposmolarity and fluorescence changes were followed during further 60 s. Fluorescence intensity of the dye was recorded at excitation and emission of 540 and 580 nm, respectively (5 nm slits for both excitation and emission wavelengths). Bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values as the valinomycin nullpoint method (Waggoner, 1976) could not be applied due to the formation of complexes between the lipophilic anion bisoxonol and the positively charged molecule of valinomycin.

Determination of synaptosomal $[Ca^{2+}]_i$

Synaptosomes were incubated under shaking with the fluorescent Ca^{2+} indicator, fura-2/AM (2 μM) during 60 min. After this period, synaptosomes were spun at 25 000 ***g*** for 5 min to remove the extracellular dye. The synaptosomal pellet was resuspended in isosmotic medium and maintained with shaking during 30 min. Then, the preparation was centrifuged (25 000 ***g*** for 5 min) four times and the pellet resuspended in isosmotic medium. Changes in $[Ca^{2+}]_i$ were determined in an Aminco-Bowman luminescence spectrometer equipped with a magnetic ministirrer. Excitation wavelength was alternated between 340 and 380 nm and fluorescence intensity was monitored at 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the synaptosomes with 10% triton X-100, and the minimum fluorescence obtained after buffering the Ca^{2+} in the solution with 250 mM EGTA. The values obtained through this procedure were used to calculate the intracellular Ca^{2+} concentration according to previously published equations (Grynkiewicz *et al*., 1985).

Exocytosis measurements

Exocytosis was assayed by spectrofluorimetry as described by Guatimosim *et al*. (1997) using the probe FM1-43. Synaptosomes were incubated with 4 μM FM1-43 for 10 min in isosmotic medium and then during 1 min in medium containing 40 mM KCl. Loaded synaptosomes were washed three times with isosmotic medium plus BSA (1 mg/mL) by centrifugation and resuspension cycles, and transferred to a quartz cuvette for the fluorimetric assay. The experiment was performed on a luminescence spectrometer with continuous stirring. Samples were excited at 488 nm and the fluorescence emission was recorded at 560 nm.

Statistical analysis

Significance of differences in mean values was calculated using the two-tailed Student's *t*-test.

Results

Efflux of amino acids in response to hyposmolarity

Synaptosomes loaded with [^{3}H]taurine, [^{3}H]GABA, or [^{3}H]glutamate, and superfused with isosmotic medium, exhibited a basal efflux corresponding to 1–1.3% of the total radioactivity accumulated during loading (Fig. 1). Stimulation with 20% hyposmotic medium (−60 mOsm), led to an immediate increase in amino acid efflux, which reached a peak 2–3 min after the stimulus. At this time, the percentage efflux increased to a maximal of 5.8%, 5.5% and 3.7% for [^{3}H]glutamate, [^{3}H]taurine, and [^{3}H]GABA, respectively. After this maximal, the amino acid efflux decreased towards the initial values of release, despite the persistence of the hyposmotic condition. The time course of the osmosensitive release was rather similar for the three amino acids but with a more rapid inactivation for glutamate efflux

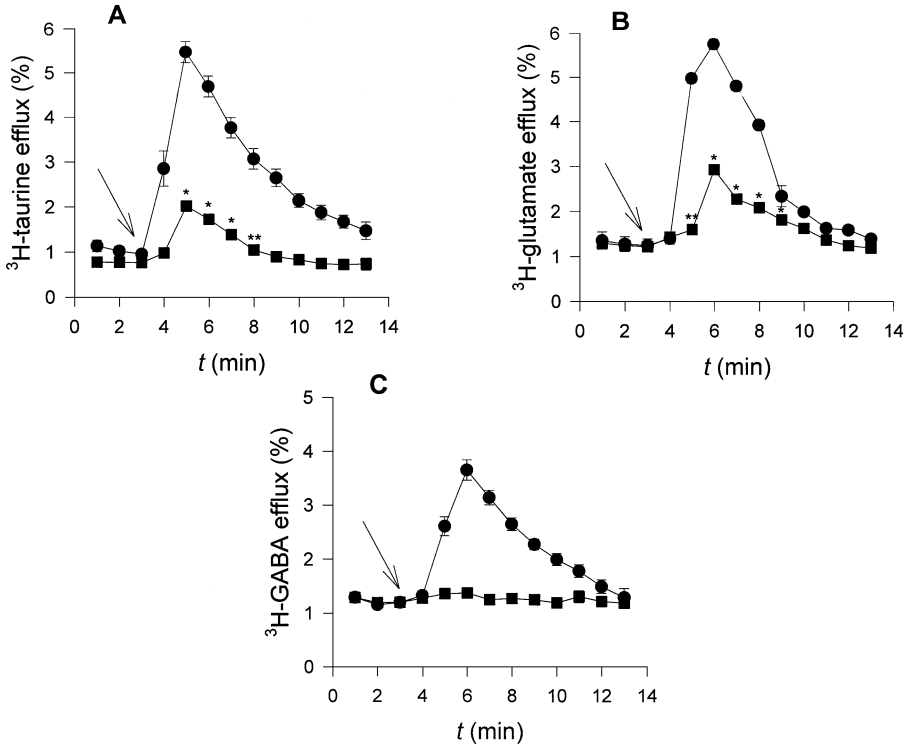

FIG. 1. Amino acid release from rat cerebral cortex synaptosomes exposed to 20% or 10% hyposmotic medium. Synaptosomes were preloaded with [^{3}H]taurine (A), [^{3}H]glutamate (B) or [^{3}H]GABA (C), washed and prepared for superfusion as described in Methods. Then, synaptosomes were superfused (1 mL/min) during 3 min with isosmotic medium to obtain a constant basal efflux. At the arrow, the medium was replaced by 20% (●), or 10% (■) hyposmotic solutions, and superfusion continued for 10 min. Fractions were collected every minute. Results are expressed as radioactivity released per min as a percentage of the total radioactivity incorporated. Data are means of 6–15 experiments. SE is represented as vertical bars when they exceed the size of symbols. $^*P < 0.001$, $^{**}P < 0.05$ with respect to the last point in isosmotic.

(Fig. 1A–C). The total amount released during the 10 min of stimulus was 28%, 27% and 19%, for [^{3}H]glutamate, [^{3}H]taurine and [^{3}H]GABA, respectively. A significant increase in glutamate and taurine efflux, but not in GABA efflux, was elicited by 10% hyposmotic medium (−30 mOsm relative to normal levels) (Fig. 1A–C). Amino acid release is a consequence of the osmolarity reduction and not of the decrease in NaCl, as no amino acid efflux was observed in solutions with low NaCl but made isosmotic with sucrose or mannitol. It is neither due to synaptosomal damage as no leakage of lactate dehydrogenase (LDH) was observed (results not shown).

Hyposmolarity-induced depolarization

Exposure to 20% hyposmotic medium resulted in a marked depolarization of nerve endings (Fig. 2). Depolarization may result from Cl^- efflux as part of the volume corrective process. However, depolarization was not affected by a prolonged exposure to Cl^--free medium (replaced by gluconate) nor by blockade of Cl^- fluxes by NPPB (Fig. 2A). In contrast, external Na^+ depletion essentially abolished it, suggesting that Na^+ influx caused depolarization. TTX (5 μM) decreased the hyposmolarity-induced depolarization by about 43% (Fig. 2B). Depolarization was unaffected in Ca^{2+}-free medium conditions (results not shown). Addition of 100 μM La^{3+} suppressed depolarization (Fig. 2B), implicating nonselective cation channels as a possible mechanism.

To evaluate the contribution of the hyposmolarity-induced depolarization to hyposmotic amino acid efflux, the effects of Na^+-free medium (NaCl replaced by choline Cl) or of La^{3+}, were then examined. In the presence of 100 μM La^{3+}, the efflux of glutamate, GABA and taurine decreased 44, 29 and 22%, respectively (Fig. 3A). In Na^+-free medium, glutamate, GABA and taurine release was reduced by 63%, 46%, 29%, respectively (Fig. 3B).

Effect of carrier blockers

The Na^+-dependence of amino acid efflux may represent either the release fraction responding to depolarization, or that occurring by reversal of the carrier operation. This later possibility was examined in synaptosomes treated with competitive, nontransportable blockers of the GABA and glutamate carriers. Figure 3C shows the effect of NO-711 and of TBOA, nontransportable blockers of GABA and glutamate transporters, respectively, on the hyposmotic release of amino acids. NO-711 reduced GABA release by 28% and TBOA decreased glutamate release by almost 37% (Fig. 3C). Blockade of taurine transport was not performed due to lack of nontransportable carrier inhibitors.

Ca^{2+} and PKC

Hyposmotic swelling significantly increased $[Ca^{2+}]_i$ in nerve endings as reported previously by Mongin *et al*. (1997). Upon 20% reduction in osmolarity, the initial $[Ca^{2+}]_i$ of 365 nM increased to 580 nM. This $[Ca^{2+}]_i$ elevation is due predominantly to an influx of external Ca^{2+}, as in Ca^{2+}-free medium containing 0.1 mM EGTA plus 10 mM $MgCl_2$, hyposmolarity elicited only a marginal increase in $[Ca^{2+}]_i$. Under this condition, the basal $[Ca^{2+}]_i$ levels showed a marked decrease to 230 nM (Fig. 4A). In synaptosomes treated with EGTA-AM, $[Ca^{2+}]_i$ basal levels further decreased to 105 nM and no increase was induced by the hyposmotic condition (Fig. 4A). The effect of $[Ca^{2+}]_i$ reduction was examined in the hyposmotic release of amino acids in synaptosomes treated with EGTA-AM. A decrease in the efflux of all amino acids was observed, with glutamate the most sensitive to this treatment

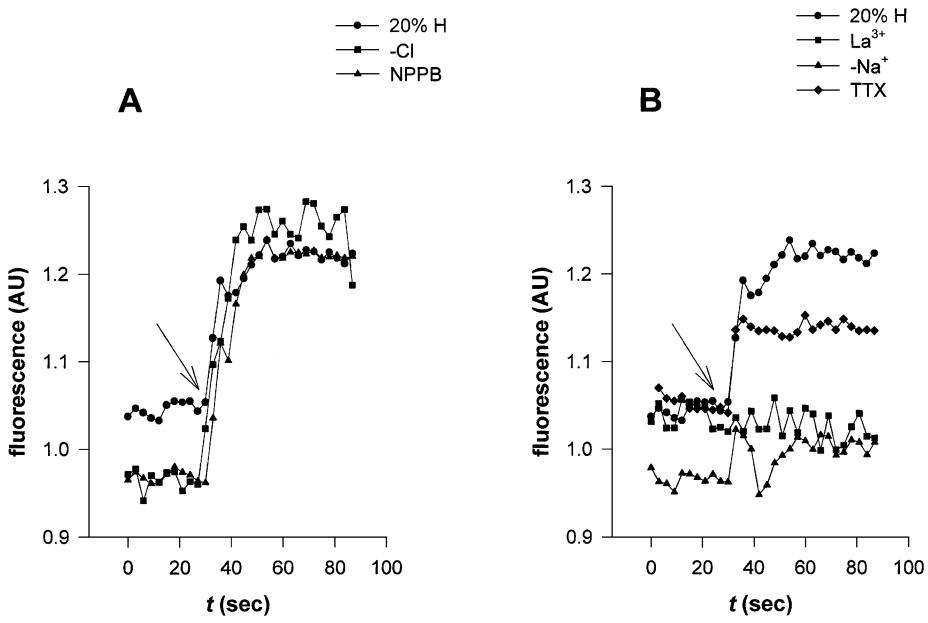

FIG. 2. Depolarization elicited by hyposmotic medium. Membrane potential was monitored by changes in fluorescence in synaptosomes loaded with bisoxonol, as described in Methods. Fluorescence was measured in isosmotic medium (30 s) and at the arrow, the medium was diluted up to reach 20% hyposmolarity, and fluorescence changes were followed for a further 60 s. (A) Control, 20% hyposmotic (●). Cl^- free medium (Cl^- replaced by gluconates) (-Cl^-; ■); 100 μM NPPB (NPPB; ▲). (B) Control, 20% hyposmotic (●). Na^+-free medium (Na^+ replaced by choline chloride) (–Na^+ ▲); 100 μM La^{3+} (La^{3+} ■); 5 μM TTX (TTX; ◆). Synaptosomes were exposed to the Cl^- or Na^+-free solutions during 15 min before and through all the experiment and during 10 min to TTX. NPPB and La^{3+} were added together with water at the hyposmotic stimulus. Values represent fluorescence arbitrary units (AU), after subtracting the dilution factor. Representative results of 3–4 experiments.

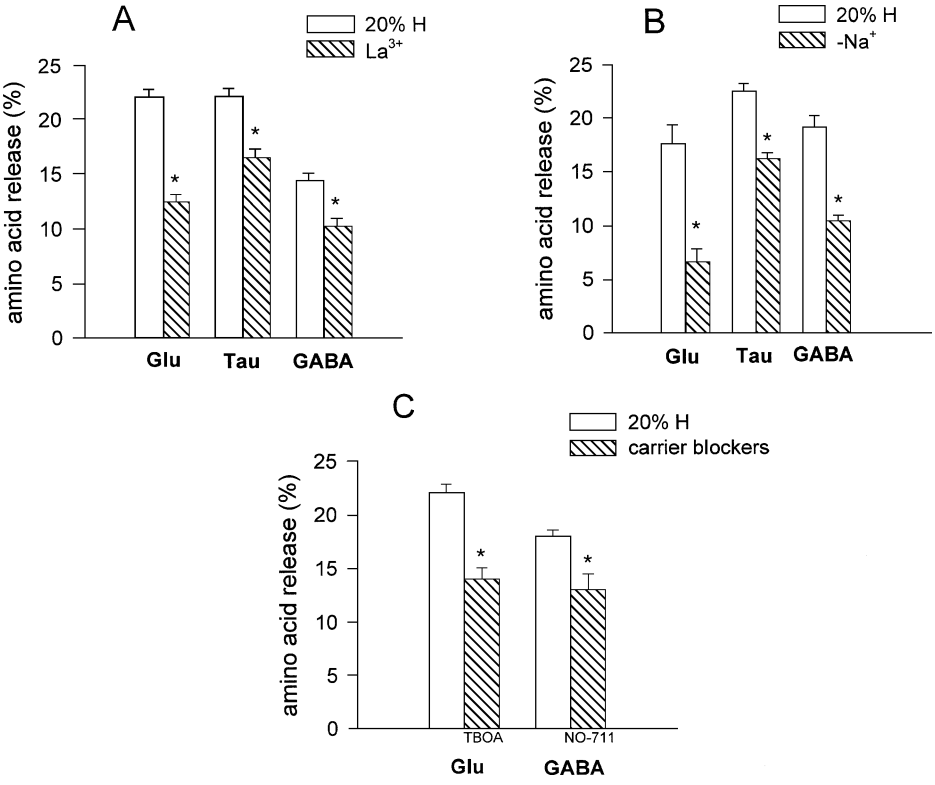

FIG. 3. Effects of Na^+-free medium, La^{3+} and carrier blockers on the hyposmotic release of amino acid from synaptosomes. Synaptosomes were preloaded with [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA and superfused as in Fig. 1. Bars represent the radioactivity released (%) at the peak release fractions (4–8 for taurine and 5–9 for glutamate and GABA). Empty and dashed bars correspond to control and experimental conditions, respectively. (A) Effect of 100 μM La^{3+} (La^{3+}). La^{3+} was present only in the hyposmotic medium. (B) Effect of Na^+-free medium (–Na^+). Synaptosomes were exposed to the Na^+-free medium (NaCl replaced by choline chloride, starting at washing and through all the experiment). (C) Effect of TBOA (100 μM) or NO-711 (20 μM). Synaptosomes were treated with the carrier blockers during 15–20 min prior to superfusion and were present throughout the experiment. Data are means ± SE of 6–17 experiments. $^*P < 0.001$ with respect to controls.

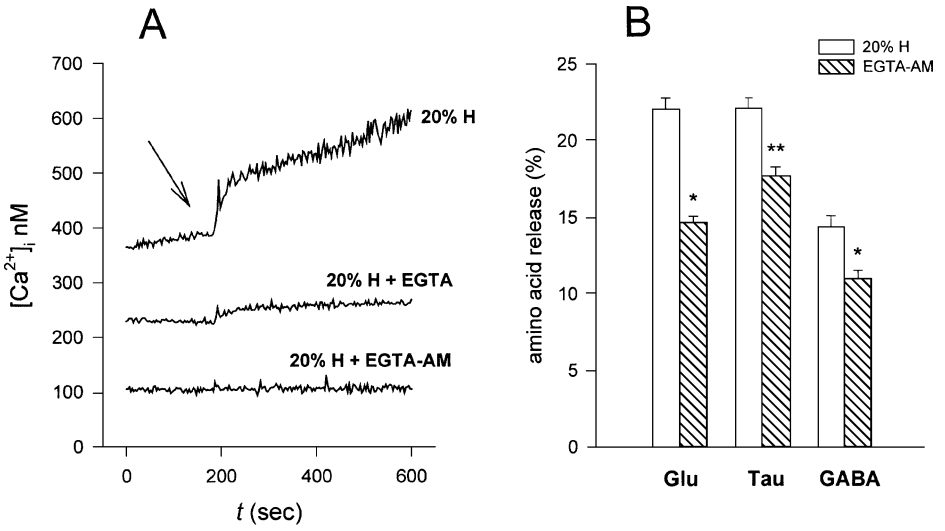

FIG. 4. Change in synaptosomal $[Ca^{2+}]_i$, elicited by hyposmolarity and its effect on amino acid efflux. (A) Synaptosomes were loaded with fura-2/AM and $[Ca^{2+}]_i$ determined as described in Methods. $[Ca^{2+}]_i$ was measured in isosmotic medium in synaptosomes under continuous stirring. At the arrow, the medium was diluted up to reach 20% hyposmolarity (20% H). In 20% H + EGTA, the experiment was carried out in Ca^{2+}-free medium containing 10 mM $MgCl_2$ plus 0.1 mM EGTA. In 20% H + EGTA-AM, synaptosomes were preincubated (15 min) with 50 μM EGTA-AM in a Ca^{2+}-free medium. Fluorescence units were transformed into Ca^{2+} concentration as described in Methods. Curves representative of 3–5 separate experiments. (B) Effect of EGTA-AM (dashed bars) on the hyposmotic (20% H, empty bars) release of amino acids. Data are means ± SE of 4–17 experiments. $^{*}P < 0.001$, $^{**}P < 0.01$.

(34% decrease). GABA and taurine efflux decreased 24% and 20%, respectively (Fig. 4B).

The effect of modulating PKC activity, i.e. inhibition by chelerythrine and activation by PMA, was examined on the hyposmotic amino acid efflux. Chelerythrine (2.5 μM) reduced (> 64%) the efflux of [^{3}H]glutamate and PKC activation by PMA (100 nM) increased it almost 80% (Fig. 5A). Chelerythrine reduced (50%) the efflux of GABA, and PMA increased it by 51%. The efflux of taurine was decreased by chelerythrine (24%) and enhanced by PMA (42%; Fig. 5A). Potentiation by PKC of glutamate release was essentially abolished in synaptosomes treated with EGTA-AM (Fig. 5B). Replacement of Na^+ by choline Cl or *N*-methyl-D-glucamine, also suppressed the stimulatory effect of PKC (Fig. 5B) (taurine and GABA were not examined).

Hyposmolarity-induced exocytosis

Exocytosis associated with hyposmolarity was monitored by the decrease in fluorescence release of FM1-43 dye, previously incorporated into synaptic vesicles by synaptosomal exposure to 40 mM KCl. The accumulated dye is released into the extracellular space, where it looses its fluorescence when the vesicle lumen is exposed to the extracellular medium. Release of FM1-43 is then detected as a fluorescence decrease, reflecting the exocytosis extent. As shown in Fig. 6, hyposmolarity induced a rapid decrease in FM1-43 fluorescence intensity, which exceeded the change in isosmotic conditions, due to medium dilution. Hyposmotic-induced drop in FM1-43 fluorescence was prevented by preloading synaptosomes with EGTA-AM, and in free-Na^+ conditions, reflecting both Ca^{2+} and Na^+-dependence of

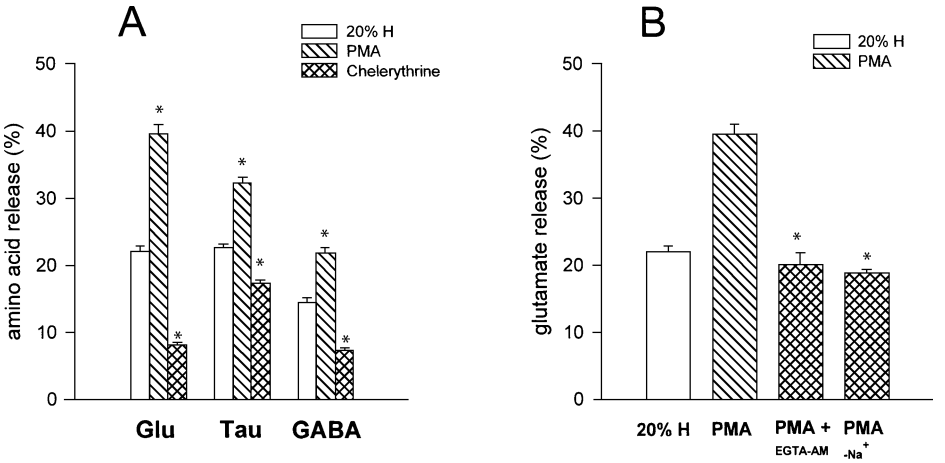

FIG. 5. Effect of PKC activity modulation on hyposmolarity induced amino acid release from synaptosomes. (A) Effects of 100 nM PMA and 2.5 μM chelerythrine (15 min preincubation and throughout the experiment) on [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA release. Data expressed as in Fig. 3, are means ± SE of 4–8 experiments. Significantly different from 20% H without additions, by $^{*}P < 0.001$. (B) Effect of Ca^{2+}- and Na^+-free media on the PMA-potentiated 20% hyposmotic release of glutamate. Sodium substitution, PMA, -Na^+ and EGTA-AM conditions were as described in Methods. In all cases, bars represent the radioactivity released by hyposmolarity as in Fig. 3. Data are means ± SE of 4–8 experiments. $^{*}P < 0.001$ with respect to PMA-treated condition.

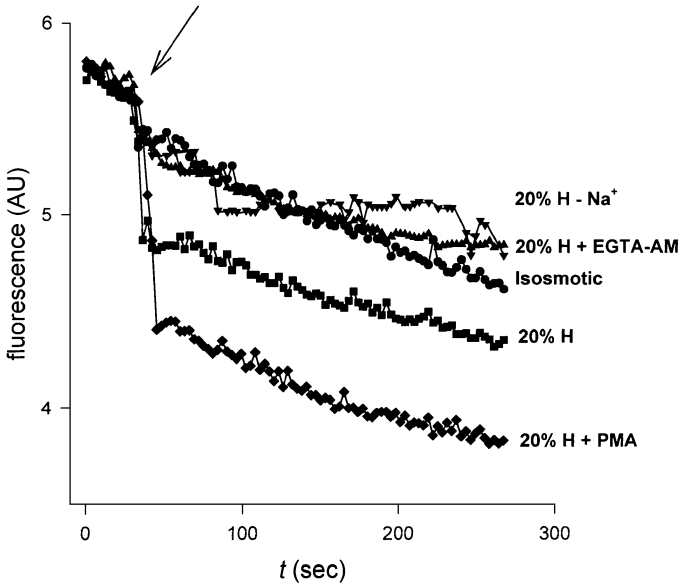

FIG. 6. Hyposmolarity-elicited exocytosis in synaptosomes. Exocytosis was monitored by FM1-43 fluorescence decrease as described in Methods. After 30 s in isosmotic condition (arrow), the medium was diluted to reach 20% hyposmolarity (■). Control condition (isosmotic) (●) was made using the same volume addition of isosmotic medium. The Na^+-free (▼) EGTA-AM (▲) and PMA (◆) pretreatment was as described in Methods. Data are expressed as fluorescence arbitrary units (AU). Each trace is representative of three separate experiments.

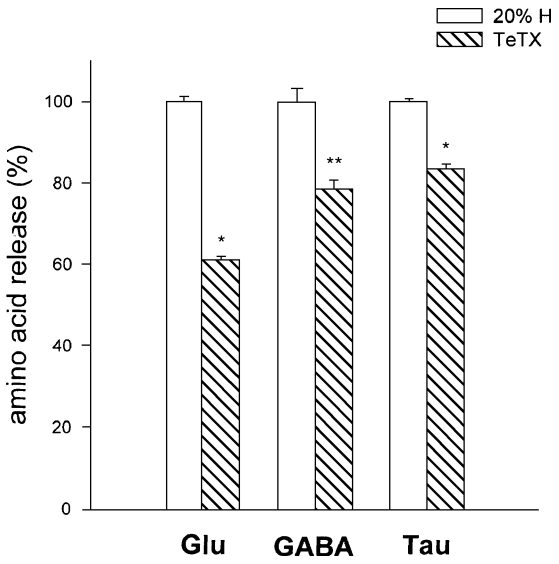

FIG. 7. Effect of TeTX on hyposmotic amino acid release from synaptosomes. Synaptosomes preloaded with [^{3}H]glutamate, [^{3}H]GABA and [^{3}H]taurine were preincubated during 90 min in isosmotic medium in the presence of 50 nM TeTX with 0.1% BSA. Thereafter, synaptosomes were superfused as in Fig. 1. Empty bars represent net release (hyposmotic minus isosmotic release) as 100%. Dashed bars correspond to percentage decrease by the toxin. Data are means ± SE of three to six experiments. $^*P < 0.001$, $^{**}P < 0.01$.

hyposmolarity-induced exocytosis. PMA pretreatment markedly potentiated hyposmolarity-evoked exocytosis (Fig. 6).

The effect of tetanus toxin (TeTX) on amino acid release

The above results raise the possibility of vesicular release as the mechanism for a fraction of the hyposmotic amino acid efflux. To test this hypothesis, the efflux of GABA, glutamate and taurine was measured in synaptosomes treated with tetanus toxin (50 nM). The treatment resulted in a significant reduction of 39%, 21% and 18% for glutamate, GABA and taurine, respectively (Fig. 7). The effect of this toxin could not be tested directly on exocytosis due to interference of the toxin with the fluorophore used.

Effect of Cl^- channel blockers and of inhibition of protein tyrosine kinase

In a large variety of cells, including neurons in culture, the efflux of taurine activated by hyposmolarity occurs through a leak pathway markedly sensitive to anion channel blockers (Pasantes-Morales & Schousboe, 1997). Two of those agents, NPPB and DIDS, were tested on the osmosensitive release of amino acids from synaptosomes, at concentrations that effectively reduce hyposmotic taurine efflux in cultured neurons. The release of taurine was reduced (53–55%) by 100 μM NPPB and 600 μM DIDS. These agents decreased GABA efflux by 18% and 15% but only DIDS decreased glutamate efflux by 12% (Table 1).

Osmosensitive fluxes of taurine in neurons and in other cell types are markedly dependent on the activity of tyrosine kinases, being reduced particularly by tyrphostins (Pasantes-Morales *et al*., 2000). The effect of the tyrosine kinase general blocker, tyrphostin AG18 (50 μM) was tested on amino acid fluxes from synaptosomes and results are shown in Table 1. Marked differences were observed in the effect of this agent between the three amino acids. Whereas taurine efflux was decreased (41%), that of [^{3}H]GABA was reduced only by about 10%, and that of [^{3}H]glutamate was unaffected (Table 1).

Discussion

Mild decreases in osmolarity led to taurine, GABA and glutamate efflux from synaptosomes. This release may occur by the well characterized leak pathway, through which organic osmolytes are extruded in response to hyposmolarity, as part of the volume corrective process (Kimelberg *et al*., 1990; Pasantes-Morales *et al*., 1993; Junankar & Kirk, 2000; van der Wijk *et al*., 2000). Alternately, or in addition, the efflux of amino acids, particularly that of GABA and glutamate – that play a prominent role as neurotransmitters – may result in part, from activation of synaptic mechanisms triggered by the hyposmolarity-induced depolarization found in our study. The present results showed that these two mechanisms are indeed operating during amino acid release, with differences in contribution for each amino

TABLE 1 Effect of the Cl^- channel blockers NPPB and DIDS, and the tyrosine kinase blocker tyrphostin AG18 on hyposmolarity-evoked amino acid release

	Amino acid release (%)		
Blockers (μM)	Glutamate	GABA	Taurine
Control hyposmotic	22.0 ± 0.71	14.3 ± 0.71	21.3 ± 0.72
NPPB (100)	21.9 ± 1.16	11.7 ± 0.75**	9.5 ± 0.71*
DIDS (600)	19.3 ± 1.12	12.1 ± 0.90**	10.0 ± 0.93*
AG18 (50)	23.6 ± 0.35	12.0 ± 0.23**	12.7 ± 0.26*

Synaptosomes preloaded with [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA were preincubated during 30 min in isosmotic medium in the presence of the blockers, and superfused as in Fig. 1. The blockers were present in all solutions throughout the experiment. The numbers correspond to radioactivity released at the peak release fractions as in Fig. 3 and are means ± SE of 4–11 experiments. $^*P < 001$, $^{**}P < 0.05$ with respect to controls.

acid. The reversal operation of the carrier also contributes to GABA and glutamate release.

Osmosensitive amino acid release mediated by depolarization and exocytosis

Hyposmolarity caused a large depolarization in synaptosomes. Depolarization evoked by hyposmolarity occurs in a variety of cell types and is mediated by activation of Cl^- exit through conductive channels (Diener & Scharrer, 1995; Hallows & Knauf, 1994; Kinard *et al*., 2001), or by cation influx, mainly Na^+ (Kim & Fu, 1993; Welsh *et al*., 2000). Depolarization in synaptosomes was unrelated to Cl^- efflux but resulted from Na^+ influx, possibly through nonspecific cation channels, as suggested by its prevention in Na^+-free medium or by La^{3+}. Activation of nonselective cation channels by hyposmolarity has been described in smooth muscle cells (Welsh *et al*., 2000), in atrial cells (Kim & Fu, 1993; Crumb *et al*., 1995) and in the A6 epithelial cell line (Li *et al*., 1998). Opening of these channels leads to membrane depolarization, carried by Na^+. This type of channel may be responsible for the swelling-induced depolarization in synaptosomes. This initial depolarization probably activates voltage-dependent Na^+ channels, as shown by partial TTX inhibition, which will further contribute to the observed depolarization. An interesting finding in the present study was the occurrence of hyposmolarity-induced exocytosis, which was depolarization- and $[Ca^{2+}]_i$-dependent. A fraction of amino acid extrusion in hyposmotic conditions occurs via this depolarization-exocytosis mechanism, as indicated by: (i) its reduction by preventing depolarization, namely in the absence of Na^+ and in the presence of La^{3+} (ii) its decrease in conditions of low $[Ca^{2+}]_i$, which abolished the hyposmolarity-induced exocytosis, and (iii) its reduction by TeTX. The contribution of this mechanism to amino acid release can be estimated by the magnitude of the Ca^{2+}-dependent fraction and the extent of reduction by La^{3+} and TeTX. The decrease in Na^+-free medium is not conclusive for this estimation, as in this condition, the carrier-mediated release is also affected. According to these estimations, the fraction responding to depolarization-exocytosis ranges form 34 to 40% for glutamate, 21 to 29% for GABA and 18 to 22% for taurine (Table 2). PKC activation by PMA increased the hyposmolarity-evoked exocytosis, a finding in line with the facilitatory action of PKC on vesicular release described in several preparations, including nerve endings (Majewski & Iannazzo, 1998; Vaughan *et al*., 1998). Thus, the influence of PKC is also indicative of the importance of exocytosis as a mechanism for amino acid release. In agreement with the different contribution of exocytosis as a mechanism for amino acid release, i.e. higher for glutamate and lower for taurine, glutamate efflux was the most sensitive and taurine the least sensitive to manipulations of PKC activity. It was found that PKC activation increased the hyposmotic efflux of glutamate, GABA and taurine by 80%, 51% and 42%, respectively, while PKC blockade by chelerythrine decreased the amino acid hyposmotic efflux as follows: 64% for glutamate, 50% for GABA and 24% for taurine. PKC modulates hyposmotic glutamate (but not of taurine) efflux in hippocampal slices (Franco *et al*., 2001), and some influence of PKC on hyposmotic amino acid release has been also reported in brain *in vivo* (Estévez *et al*., 1999b).

Altogether, these results suggest a chain of events for the hyposmolarity-induced release of amino acids with the following sequential steps: (i) hyposmolarity activation of nonspecific cation channels; (ii) Na^+ entry and subsequent nerve ending depolarization; (iii) Na^+- and Ca^{2+}-dependent exocytosis and (iv) PKC-modulated exocytotic release of amino acids. The initial step in this cascade of phenomena elicited by hyposmolarity seems to be the activation of nonselective cation channels, but the mechanism of this activation has not been explored in detail in the present study. Swelling-activated nonselective cation channels have been reported in atrial cells (Kim & Fu, 1993) and in renal epithelial A6 cells (Marunaka *et al*., 1997). In cerebrovascular smooth muscle, cation channels mediating depolarization have been found activated by swelling or intravascular pressure (Welsh *et al*., 2000).

The hyposmotic amino acid release occurs in part by the carrier reversal operation

The Na^+ entry which seems to occur upon hyposmolarity may activate the reversal operation of the carrier, a mechanism responsible for amino acid release in a variety of conditions, including ischemia and energetic failure (Attwell *et al*., 1993; Katsumori *et al*., 1999; Rossi *et al*., 2000). A fraction of 28% of the release of GABA and 37% for glutamate, implicates this mechanism, as shown by the efflux reduction in the presence of the competitive, non transportable carrier blockers NO-711 and TBOA. The contribution of this mechanism to taurine efflux could not be investigated due to the lack of reliable blockers of taurine transport.

Contribution of the osmolyte leak pathway

The depolarization-/exocytosis-independent fraction of glutamate, GABA and taurine osmosensitive release may occur through the well known leak pathway, typical for organic osmolyte extrusion. This pathway is characteristically sensitive to Cl^- channel blockers and is modulated by tyrosine kinase activity (Junankan & Kirk, 2000; van der Wijk *et al*., 2000; Pasantes-Morales & Franco, 2002). Considering these two properties as markers for the leak pathway, we present here results suggesting that a substantial amount of taurine efflux (up to 55%) occurs through this pathway, while this mechanism appears to contribute only about 10–18% to the release of GABA (Table 2). The insensitivity of glutamate release to tyrphostins (Mongin *et al*., 1999) and to NPPB, suggests that the leak pathway is not implicated. The decrease in glutamate release by DIDS may be due to an effect of this agent on the nonselective cation channel activity (Welsh *et al*., 2000).

These results suggest that the release of taurine from synaptosomes mainly reflects its osmolyte role, which is widely documented in numerous brain preparations including neurons and astrocytes in culture (Pasantes-Morales & Schousboe, 1997), brain slices (Oja & Saransaari, 1992; Deleuze *et al*., 1998; Franco *et al*., 2001) as well as in brain *in vivo* (Estévez *et al*., 1999a). An osmolyte role for taurine in nerve endings is further suggested by its high concentrations in

TABLE 2. Contribution of depolarization-exocytosis, leak pathway and carrier reversal to the hyposmolarity evoked release of glutamate, GABA and taurine

	Fraction release (%)		
	Glutamate	GABA	Taurine
Depolarization-exocytosis†	34–44	21–29	18–22
Leak pathway‡	–	10–18	41–55
Carrier reversal§	37	28	ND

†The contribution of depolarization-exocytosis was estimated by the effect reducing amino acid release of La^{3+}, EGTA-AM and TeTX. ‡Estimated by the inhibitory effect of Cl^- channel- and tyrosine kinase-blockers. §Estimated by the reduction observed in the presence of the competitive, nontransportable blockers NO-711 for GABA and TBOA for glutamate. ND, not determined. As described in Methods, the efflux of labelled GABA and glutamate after the time required to complete the experiments, corresponds to about 80% of the nonlabelled amino acids. Thus, the fraction of release through the different routes, illustrated in this Table may have been overestimated, but as the labelled/unlabelled ratio was found essentially constant in all conditions, the relative contribution of each pathway likely remains the same.

synaptosomes and in synaptic vesicles, despite its negligible function as neurotransmitter (Lahdesmaki *et al*., 1977; Kontro *et al*., 1980). Also along this line is the presence of taurine in the purely cholinergic nerve endings of the *Torpedo* electric organ (Vyas & Bradford, 1987) and the corelease of taurine and glutamate from cerebellar granule neurons (Holopainen *et al*., 1989). Thus, the synaptic taurine pool may be released in connection with swelling events concurrent with synaptic function. Even the taurine release fraction associated with exocytosis may result from a vesicular pool involved in the osmotic control of the vesicular compartment. The significance of the glutamate and GABA osmosensitive release is less clear. The mechanism of this release shows the characteristic features of vesicular release, but the amino acid concentrations in nerve endings largely exceed those required for a neurotransmitter function (Kontro *et al*., 1980), and some implication in an osmolyte role cannot be excluded.

Consequences of the osmosensitive release of amino acids from nerve endings

Hyposmotic swelling in brain occurs in numerous pathologies associated with hyponatremia. This condition occurs either from water excess or from a Na^+ deficit. Water excess may come from excessive oral intake, as in psychotic polydipsia, or more commonly from impaired renal water elimination as a consequence of inappropriate secretion of antidiuretic hormone, glucocorticoid deficiency, hypothyroidism, and renal or hepatic failure. In addition, a variety of diseases or conditions such as head trauma, brain tumour and cerebrovascular accidents result in hyponatremia associated with the syndrome of inappropriate secretion of antidiuretic hormone or the cerebral salt wasting syndrome. Hyponatremia also results from Na^+ loss due to mineralocorticoid deficiency, nephrotic syndrome, osmotic diuresis, vomiting or diarrhea, or during rapid correction of uremia by excessive haemodialysis and by infusion of hypotonic solutions in the perioperative period. Hyponatremia is a common state in the elderly and during pregnancy (Law, 1989; Verbalis, 1998; Fall, 2000). Fatal hyponatremia-induced cerebral oedema has been recently associated with 'ecstasy' use (Holmes *et al*., 1999).

The extent of swelling in different brain cell types or in discrete neuronal regions has not been examined in detail, although swelling in dendrites has been observed in hyposmotic conditions (Andrew *et al*., 1997). Nerve ending swelling has been reported in head trauma (Castejon *et al*., 1995), kainate-induced seizures (Sperk *et al*., 1983) and kindling (Langmeier *et al*., 1982). It may occur also in hyponatremia, as suggested by studies in hippocampal slices, where osmolarity reduction causes an increase in amplitude of evoked field potentials and of excitatory postsynaptic potentials which is inversely related to osmolarity (Chebabo *et al*., 1995; Schwartzkroin *et al*., 1998). Hyposmolarity does not affect cell properties such as resting membrane potential, cell input resistance and action potential threshold and duration, suggesting a hyperfunction of excitatory synapses (Chebabo *et al*., 1995). Hyposmolarity similarly affects the inhibitory postsynaptic potentials, with notably less effect, though, than on the excitatory potentials (Andrew, 1991; Chebabo *et al*., 1995; Huang *et al*., 1997). The release of glutamate and GABA from nerve terminals documented here may be responsible for these effects. This finding may also contribute to explain the generation of epileptiform activity and increased susceptibility to seizures in chronic and acute hyponatremia (Andrew, 1991).

The swelling-evoked taurine release from nerve endings, and probably also from synaptic vesicles, is an interesting finding as taurine has a marginal (if any) role as a neurotransmitter. The presence of this swelling-responsive synaptic taurine pool may reflect a need for volume correction mechanisms in the nerve terminal and/or in synaptic vesicles under physiological conditions, resulting from ion redistribution related to synaptic activity. In pathological conditions, the release of taurine may have a dual benefit, relieving swelling first, and once translocated into the extracellular space, acting as neuroprotectant, a well documented action of this amino acid (Saransaari & Oja, 2000).

Acknowledgements

This study was supported by grants No. IN206403-3 from DGAPA, UNAM and 3586-N and 34886-M from CONACyT.

References

Andrew, R.D. (1991) Seizure and acute osmotic change: clinical and neurophysiological aspects. *J. Neurol. Sci.*, **101**, 7–182.

Andrew, R.D., Lobinowich, M.E. & Osehobo, E.P. (1997) Evidence against volume regulation in cortical brain cells during acute osmotic stress. *Exp. Neurol.*, **143**, 300–312.

Attwell, D., Barbour, B. & Szatkowski, M. (1993) Nonvesicular release of neurotransmitter. *Neuron*, **11**, 401–407.

Babila, T., Atlan, H., Fromer, I., Schwalb, H., Uretzky, G. & Lichtstein, D. (1990) Volume regulation of nerve terminals. *J. Neurochem.*, **55**, 2058–2062.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Castejon, O.J., Valero, C. & Diaz, M. (1995) Synaptic degenerative changes in human traumatic brain edema. An electron microscopic study of cerebral cortical biopsies. *J. Neurosurg. Sci.*, **39**, 47–65.

Chebabo, S.R., Hester, M.A., 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.

Crumb, W.J. Jr, Pigott, J.D. & Clarkson, C.W. (1995) Description of a nonselective cation current in human atrium. *Circ. Res.*, **77**, 950–956.

Deleuze, C., Duvoid, A. & Hussy, N. (1998) Properties and glial origin of osmotic-dependent release of taurine from the rat supraoptic nucleus. *J. Physiol. (Lond.)*, **507**, 463–471.

Diener, M. & Scharrer, E. (1995) Swelling-activated conductances for chloride, potassium and amino acids in the rat colon: a whole-cell study. *Exp. Physiol.*, **80**, 411–428.

Estévez, A.Y., O'Regan, M.H., Song, D. & Phillis, J.W. (1999a) Effects of anion channels blockers on hyposmotically induced amino acid release from the *in vivo* rat cerebral cortex. *Neurochem. Res.*, **24**, 447–452.

Estévez, A.Y., O'Regan, M.H., Song, D. & Phillis, J.W. (1999b) Hyposmotically induced amino acid release from the rat cerebral cortex: role of phospholipases and protein kinases. *Brain Res.*, **844**, 1–9.

Fall, P.J. (2000) Hyponatremia and hypernatremia. A systematic approach to causes and their correction. *Postgrad. Med.*, **107**, 75–85.

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

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

Guatimosim, C., Romano-Silva, M.A., Cruz, J.S., Beirao, P.S., Kalapothakis, E., Morales-Santos, T., Cordeiro, M.N., Diniz, C.R., Gomez, M.V. & Prado, M.A. (1997) A toxin from the spider *Phoneutria nigriventer* that blocks calcium channels coupled to exocytosis. *Br. J. Pharmacol.*, **122**, 591–597.

Hajos, F. (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.*, **93**, 485–489.

Hallows, K.R. & Knauf, P.A. (1994) Regulatory volume decrease in HL-60 cells: importance of rapid changes in permeability of Cl^- and organic solutes. *Am. J. Physiol.*, **267**, C1045–C1056.

Holmes, S.B., Banerjee, A.K. & Alexander, W.D. (1999) Hyponatraemia and seizures after ecstasy use. *Postgrad. Med. J.*, **75**, 32–33.

Holopainen, I., Kontro, P. & Oja, S.S. (1989) Release of taurine from cultured cerebellar granule cells and astrocytes: co-release with glutamate. *Neuroscience*, **29**, 425–432.

Huang, R., Bossut, D.F. & 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.

Junankar, P.R. & Kirk, K. (2000) Organic osmolyte channels: a comparative view. *Cell. Physiol. Biochem.*, **10**, 355–360.

Katsumori, H., Baldwin, R.A. & Wasterlain, C.G. (1999) Reverse transport of glutamate during depolarization in immature hippocampal slices. *Brain Res.*, **819**, 160–104.

Kim, D. & Fu, C. (1993) Activation of a nonselective cation channel by swelling in atrial cells. *J. Membr. Biol.*, **135**, 27–37.

Kimelberg, H.K. (1999) Astrocytic edema in CNS trauma. *J. Neurotrauma*, **9**, S71–S81.

Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S. & Waniewsky, R.A. (1990) Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J. Neurosci.*, **10**, 1583–1591.

Kinard, T.A., Goforth, P.B., Tao, Q., Abood, M.E., Teague, J. & Satin, L.S. (2001) Chloride channels regulate HIT cell Volume but cannot fully account for swelling-induced insulin secretion. *Diabetes*, **50**, 992–1003.

Kontro, P., Marnela, K.M. & Oja, S.S. (1980) Free amino acids in the synaptosome and synaptic vesicle. Fractions of different bovine brain areas. *Brain Res.*, **184**, 129–141.

Lahdesmaki, P., Karppinen, A., Saarni, H. & Winter, R. (1977) Amino acids in the synaptic vesicle fraction from calf brain: content, uptake and metabolism. *Brain Res.*, **138**, 295–308.

Lang. F., Busch. G.L., Ritter. M., Volkl. H., Waldegger. S., Gulbins. E. & Haussinger, D. (1998) Functional significance of cell volume regulatory mechanism. *Physiol. Rev.*, **78**, 247–306.

Langmeier, M., Fischer, J. & Mares, J. (1982) Ultrastructural changes in cortical synapses shortly after termination of a seizure during kindling. *Physiol. Bohemoslov.*, **31**, 213–216.

Law, R.O. (1989) Effects of pregnancy on the contents of water, taurine, and total amino nitrogen in rat cerebral cortex. *J. Neurochem.*, **53**, 300–302.

Li, J., De Smet, P., Hans, D., Simaels, J. & Van Driessche, W. (1998) Swelling-activated cation-selective channels in A6 epithelia are permeable to large cations. *Am. J. Physiol.*, **275**, C358–C366.

Majewski, H. & Iannazzo, L. (1998) Protein kinase C: a physiological mediator of enhanced transmitter output. *Prog. Neurobiol.*, **55**, 463–475.

Marunaka, Y., Shintani, Y., Downey, G.P. & Niisato, N. (1997) Activation of Na^+-permeant cation channel by stretch and cyclic AMP-dependent phosphorylation in renal epithelial A6 cells. *J. General Physiol.*, **110**, 327–336.

Mongin, A.A., Aksentsev, S.L., Orlov, S.N. & Konev, S.V. (1997) Hypoosmotic shock activates Ca^{2+} channels in isolated nerve terminals. *Neurochem. Int.*, **31**, 835–843.

Mongin, A.A., Reddi, J.M., Charniga, C. & 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.

Oja, S.S. & Saransaari, P. (1992) Taurine release and swelling of cerebral cortex slices from adult and developing mice in media of different ionic compositions. *J. Neurosci. Res.*, **32**, 551–561.

Pasantes-Morales, H., Alavez, 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.

Pasantes-Morales, H., Cardin. V. & Tuz, K. (2000) Signaling events during swelling and regulatory Volume decrease. *Neurochem. Res.*, **25**, 1301–1314.

Pasantes-Morales, H. & Franco, R. (2002) Influence of protein tyrosine kinases on taurine release. *Cerebellum*, **1**, 103–109.

Pasantes-Morales, H., Murray, R.A., Lilja, L. & Morán, J. (1994a) Regulatory Volume decrease in cultured astrocytes. I. Potassium- and chloride-activated permeability. *Am. J. Physiol.*, **266**, C165–C171.

Pasantes-Morales, H., Murray, R.A., Sánchez-Olea, R. & Morán, J. (1994b) Regulatory volume decrease in cultured astrocytes. II. Permeability pathway to amino acids and polyols. *Am. J. Physiol.*, **266**, C172–C178.

Pasantes-Morales, H. & Schousboe, A. (1997) Role of taurine in osmoregulation in brain cells: Mechanisms and functional implications. *Amino Acids*, **12**, 281–292.

Rossi, D.J., Oshima, T. & Attwell, D. (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature*, **403**, 316–321.

Saransaari, P. & Oja, S.S. (2000) Taurine and neural cell damage. *Amino Acids*, **19**, 509–526.

Schwartzkroin, P.A., Baraban, S.C. & Hochman, D.W. (1998) Osmolarity, ionic flux, and changes in brain excitability. *Epilepsy Res.*, **32**, 275–287.

Sperk, G., Lassmann, H., Baran, H., Kish, S.J., Seitelberger, F. & Hornykiewicz, O. (1983) Kainic acid induced seizures: neurochemical and histopathological changes. *Neuroscience*, **10**, 1301–1315.

Vaughan, P.F., Walker, J.H. & Peers, C. (1998) The regulation of neurotransmitter secretion by protein kinase C. *Mol. Neurobiol.*, **18**, 125–155.

Verbalis, J.G. (1998) Adaptation to acute and chronic hyponatremia: implications for symptomatology, diagnosis, and therapy. *Semin. Nephrol.*, **18**, 3–19.

Vyas, S. & Bradford, H.F. (1987) Co-release of acetylcholine, glutamate and taurine from synaptosomes of *Torpedo* electric organ. *Neurosci. Lett.*, **82**, 58–64.

Waggoner, A. (1976) Optical probes of membrane potential. *J. Membr. Biol.*, **27**, 317–334.

Welsh, D.G., Nelson, M.T., Eckman, D.M. & Brayden, J.E. (2000) Swelling-activated cation channels mediate depolarization of rat cerebrovascular smooth muscle by hyposmolarity and intravascular pressure. *J. Physiol.*, **527**, 139–148.

Whittaker, V.P. & Barker, L.A. (1972) The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In Fried, R., (ed.) *Methods of Neurochemistry.* Marcel Dekker, New York.

van der 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.

European Journal of Neuroscience, Vol. 22, pp. 1636–1642, 2005

Hyposmolarity evokes norepinephrine efflux from synaptosomes by a depolarization- and Ca^{2+}-dependent exocytotic mechanism

K. Tuz and H. Pasantes-Morales
Department of Biophysics, Institute of Cell Physiology, National University of Mexico, UNAM, Apartado Postal 70–253, 04510 México D.F., Mexico City, Mexico

Keywords: exocytosis, hyposmolarity, nerve endings, norepinephrine

Abstract

Osmolarity reduction (20%) elicited ^{3}H-norepinephrine (NE) efflux from rat cortical synaptosomes. The hyposmotic NE release resulted from the following events: (i) a Na^+-dependent and La^{3+}-, Gd^{3+}- and ruthenium red-sensitive depolarization; (ii) a cytosolic Ca^{2+} ($[Ca^{2+}]_i$) rise with contributions from external Ca^{2+} influx and internal Ca^{2+} release, probably through the mitochondrial Na^+–Ca^{2+} exchanger; and (iii) activation of a $[Ca^{2+}]_i$-evoked, tetanus toxin (TeTX)-sensitive, PKC-modulated NE efflux mechanism. This sequence was established from results showing a drop in the hyposmotic $[Ca^{2+}]_i$ rise by preventing depolarization with La^{3+}, and by the inhibitory effects of Ca^{2+}-free medium (EGTA; 50%), CGP37157 (the mitochondrial Na^+–Ca^{2+} exchanger blocker; 48%), EGTA + CGP37157 or by EGTA-AM (> 95% in both cases). In close correspondence with these effects, NE efflux was 92% decreased by Na^+ omission, 75% by La^{3+}, 47% by EGTA, 50% by CGP37157, 90% by EGTA + CGP37157 and 88% by EGTA-AM. PKC influenced the intracellular Ca^{2+} release and, mainly through this action, modulated NE efflux. TeTX suppressed NE efflux. The K^+-stimulated NE release, studied in parallel, was unaffected by Na^+ omission, or by La^{3+}, Gd^{3+} or ruthenium red. It was fully dependent on external Ca^{2+}, insensitive to CGP37157 and abolished by TeTX. These results suggest that the hyposmotic events, although different from the K^+-evoked depolarization and $[Ca^{2+}]_i$ rise mechanisms, are able to trigger a depolarization-dependent, Ca^{2+}-dependent and TeTX-sensitive mechanism for neurotransmitter release.

Introduction

The osmolarity of the fluids surrounding animal cells is tightly controlled in physiological conditions but it may be altered in pathological states, particularly in those with associated hyponatremia. Severe chronic hyponatremia leads to cellular brain oedema which, in turn, evokes a complex syndrome including coma, a dazing state and higher susceptibility to seizures. This suggests an effect of the hyposmotic condition on brain excitability, a notion supported by studies *in vitro* in hippocampal slices showing an increase in amplitude and duration of excitatory postsynaptic potentials (Chebabo *et al*., 1995). This effect suggests an increase in glutamate efflux during the hyposmolar condition. This was examined in our previous study in rat cortical synaptosomes, in which we found a hyposmolarity-induced release of glutamate, GABA and taurine (Tuz *et al*., 2004). These three amino acids participate as osmolytes in brain *in vivo* as well as in neurons and astrocytes in culture (Verbalis & Gullans, 1991; Pasantes-Morales *et al*., 1993). Glutamate and GABA, on the other hand, have a role as main neurotransmitters. In our study we found that hyposmolarity induces a Na^+-dependent depolarization, $[Ca^{2+}]_i$ rise and increased exocytosis. A fraction of the hyposmotically induced release of amino acids, particularly large for glutamate, was found to be depolarization- and Ca^{2+}-dependent and blocked by tetanus toxin (TeTX), suggesting that this release occurs by exocytosis (Tuz *et al*., 2004). All these results indicate that hyposmolarity elicits in nerve endings, a number of processes which mimic those of depolarization–secretion exocytosis, and raise the question of whether other neurotransmitters, not involved as osmolytes in the response to swelling, may also be responsive to hyposmolarity. To test this possibility we examined the effect of hyposmolarity on the release of norepinephrine (NE) from rat cortical synaptosomes, the same preparation used in our previous study. Norepinephrine is an important synaptic transmitter in rat cerebral cortex, and its release fits well with the typical depolarization–secretion vesicular process. The effect of hyposmolarity and the features of the NE efflux in this condition were studied in parallel with the release evoked by depolarizing concentrations of KCl (20 mM), a well studied process which follows the classical vesicular release pattern.

Correspondence: Dr Herminia Pasantes-Morales, as above.
E-mail: hpasante@ifc.unam.mx

Received 11 May 2005, revised 25 July 2005, accepted 26 July 2005

Materials and methods

Materials

Salts ($CaCl_2$, KCl, KH_2PO_4, $MgCl_2$, $MgSO_4$ and NaCl), 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES) and glucose were from T. J. Baker (Xalostoc, Mexico); ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester (EGTA-AM), TeTX and Gö6976 were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA); fura-2 acetoxymethyl ester (fura-2 AM), FM1-43 and bis-(1,3diethylthiobarbituric acid) trimethine oxonol (bisoxonol) were

doi:10.1111/j.1460-9568.2005.04344.x

from Molecular Probes (Eugene, OR, USA). L-[7,8–^{3}H]-norepinephrine (NE) was from Amersham (UK). Bovine serum albumin (BSA), triton X-100, pargyline, ascorbic acid, ruthenium red, $LaCl_3$, $GdCl_3$, choline chloride, gluconate salts, phorbol-12-myristate-13-acetate (PMA), ω-conotoxin MVIIC, thapsigargin and dantrolene were from Sigma Chemical Co. (St Louis, MO, USA). Nimodipine and nitrendipine were from RBI (Natick, MA, USA) and CGP 37157 was from Tocris (Ellisville, MO, USA).

Synaptosomal preparation and superfusion

Cerebral cortex was excised from decapitated male Wistar adult rats of ~180 g. A crude synaptosomal fraction was prepared by differential centrifugation according to Whittaker & Barker (1972). Purified synaptosomes were obtained as described elsewhere (Hajos, 1975) in a sucrose discontinuous gradient. Synaptosome suspensions were incubated under shaking with ^{3}H-norephinephrine (2.5 μCi/mL) in the presence of 10 μM pargyline, 0.1% BSA and 0.0002 g/mL ascorbic acid, for 20 min in isosmotic medium containing (in mM): NaCl, 135; KCl, 1; $CaCl_2$, 1; $MgSO_4$, 1.17; KH_2PO_4, 1.7; HEPES, 10; and glucose, 10. Hyposmotic media (−20%, 240 mOsm) were prepared by reducing the necessary amount of NaCl. Osmolarity of all solutions was measured in a freezing-point osmometer (Osmette A; Precision Systems Inc., Natick, MA, USA). After incubation, synaptosomes were separated from the loading solution by rapid filtration through a Millipore filter (pore size 0.45 μm) and rinsed with isosmotic medium. The filter membrane containing synaptosomes was transferred to a superfusion chamber (0.7 mL) and superfused with isosmotic medium at a flow rate of 1 mL/min. After a wash period of 18 min, fractions of the perfusate medium were collected at 1-min intervals into scintillation vials. The baseline was attained at ≈3 min, after which the isosmotic medium was replaced by 20% hyposmotic or isosmotic medium plus 20 mM KCl and superfusion continued for a further 7 min. At the end of the superfusion, synaptosomes were solubilized and the radioactivity of collected fractions and that remaining in synaptosomes was measured by scintillation spectrometry. Preincubation time with drugs varied depending on the experiment and are indicated at the corresponding figure legends. When required, controls contained the vehicle. Results are expressed as percentage release in each fraction of the total radioactivity (i.e. radioactivity in samples plus radioactivity remaining in synaptosomes at the end of superfusion).

Depolarization measurement

Depolarization was measured by spectrofluorometry using an Aminco-Bowman luminescence spectrometer (series 2; SLM Aminco, Rochester, NY, USA), equipped with a magnetic ministirrer and the fluorophore bisoxonol (150 nM). The fluorophore was added to 2 mL of isosmotic medium in a quartz cuvette and the reaction started by addition of synaptosomes (40–50 μg protein, determined by the Bradford method). After 30 s, the medium was diluted up to reach −20% hyposmolarity and fluorescence changes were followed for a further 60 s. Fluorescence intensity of the dye was recorded at excitation and emission of 540 and 580 nm, respectively (5-nm slits for both excitation and emission wavelengths). Bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values because the valinomycin nullpoint method (Waggoner, 1976) could not be applied due to the formation of complexes between the lipophilic anion bisoxonol and the positively charged molecule of valinomycin. Therefore, results of depolarization are expressed in arbitrary units (AU).

Determination of synaptosomal $[Ca^{2+}]_i$

Synaptosomes were incubated under shaking with the fluorescent Ca^{2+} indicator fura-2 AM (2 μM) for 60 min. After this period, synaptosomes were spun at 25 000 ***g*** for 5 min to remove the extracellular dye. The synaptosomal pellet was resuspended in isosmotic medium and maintained with shaking for 30 min. The preparation was then centrifuged (25 000 ***g*** for 5 min) four times and the pellet resuspended in isosmotic medium. Changes in $[Ca^{2+}]_i$ were determined in an Aminco-Bowman luminescence spectrometer equipped with a magnetic ministirrer. Excitation wavelength was alternated between 340 and 380 nm and fluorescence intensity was monitored at 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the synaptosomes with 10% triton X-100, and the minimum fluorescence obtained after buffering the Ca^{2+} in the solution with 250 mM EGTA. The values obtained through this procedure were used to calculate the intracellular Ca^{2+} concentration according to previously published equations (Grynkiewicz *et al*., 1985).

Exocytosis measurements

Exocytosis was assayed by spectrofluorimetry as described by Guatimosim *et al*. (1997) using the probe FM1-43. Synaptosomes were incubated with 4 μM FM1-43 for 10 min in isosmotic medium and then for 1 min in medium containing 40 mM KCl. Loaded synaptosomes were washed three times with isosmotic medium plus BSA (1 mg/mL) by centrifugation and resuspension cycles, and transferred to a quartz cuvette for the fluorimetric assay. The experiment was performed on a luminescence spectrometer with continuous stirring. Samples were excited at 488 nm and the fluorescence emission was recorded at 560 nm.

Statistical analysis

Significance of differences in mean values was calculated using the two-tailed Student's *t*-test.

Results

Efflux of NE in response to hyposmolarity

Synaptosomes loaded with ^{3}H-NE were washed and superfused with isosmotic medium and, after a short period, when the basal efflux was attained, superfusion continued with a 20% hyposmotic medium (−60 mOsm) or with isosmotic medium containing 20 mM KCl. Hyposmolarity elicited a rapid increase in NE efflux, with a peak attained 2 min after the stimulus. After this maximum, efflux slowly inactivated despite the persistence of hyposmolarity, decreasing efflux towards basal levels (Fig. 1A). The net NE release, i.e. hyposmotic minus isosmotic release over the 5 min immediately following the stimulus is shown in the inset bars (Fig. 1A). Upon stimulation with a depolarizing concentration of KCl, NE release showed an increase similar in magnitude to that evoked by hyposmolarity, with a maximal efflux after 3 min and a faster inactivation phase (Fig. 1B).

Hyposmolarity-induced depolarization and NE release

Hyposmolarity induces depolarization in synaptosomes as shown in our previous study on hyposmolarity-evoked amino acid efflux (Tuz *et al*., 2004). This depolarization was independent of Cl^- but strictly dependent on the presence of Na^+. Figure 2A shows the effect of La^{3+} or Gd^{3+} (100 μM) in preventing depolarization. These results suggest a

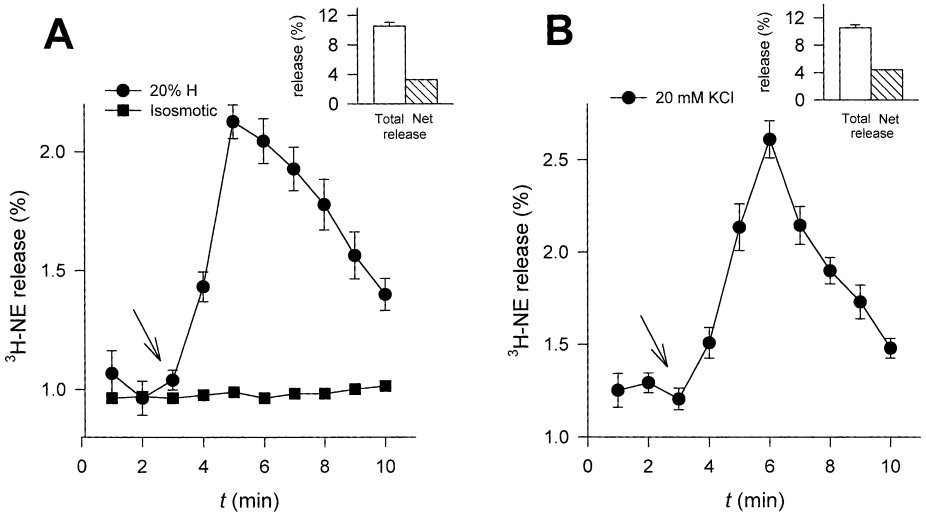

FIG. 1. Norepinephrine release from synaptosomes of rat cerebral cortex exposed to 20% hyposmotic or 20 mM KCl medium. Synaptosomes were incubated with ^{3}H-NE, washed and prepared for superfusion as described in Materials and Methods. Then, synaptosomes were superfused at a flow rate of 1 mL/min with isosmotic medium (■) to obtain a constant basal efflux and, at the arrow, the medium was replaced by (A) 20% hyposmotic (●) or (B) 20 mM KCl. Results are expressed as radioactivity released per min as a percentage of the total radioactivity incorporated. Insets show total release (the sum of release in the higher five points after the stimulus) (open bars) and net release (total release minus basal release; hatched bars). Data are means of 3–9 experiments.

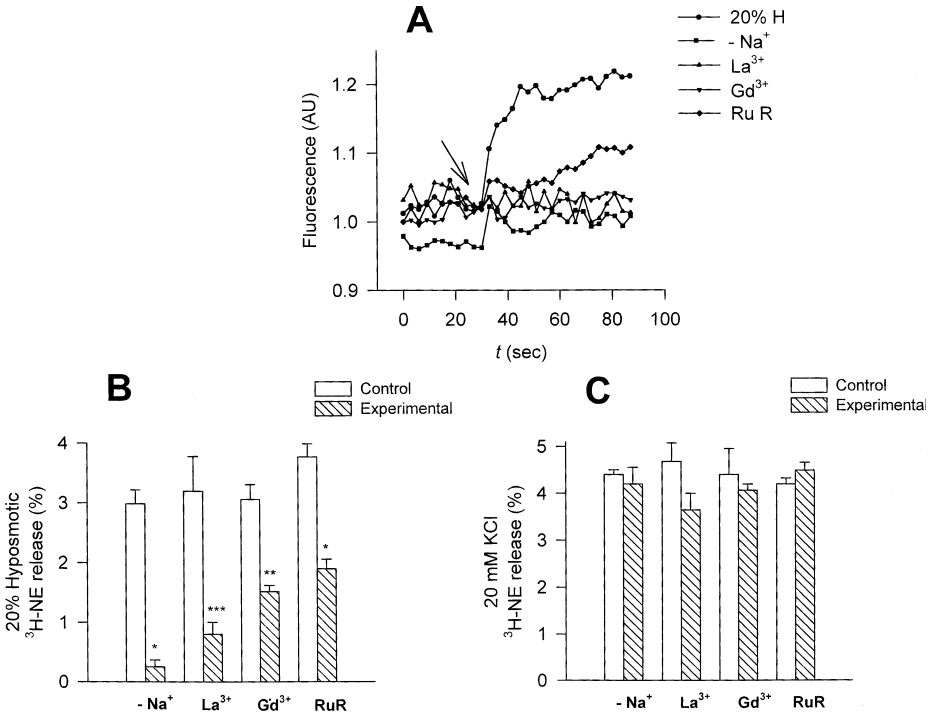

FIG. 2. Hyposmolarity-induced depolarization and its effect on NE release. (A) Depolarization was measured by bisoxonol fluorescence as described in Materials and Methods. Fluorescence was measured in isosmotic medium and, at the arrow, medium was diluted up to reach −20% hyposmolarity. Fluorescence changes were followed for a further 60 s. Control, 20% hyposmotic (●); Na^+ free medium (Na^+ replaced by choline chloride; $-Na^+$ ■), 100 μM La^{3+} (La^{3+} ▲), 100 μM Gd^{3+} (Gd^{3+} ▼) and 1 μM ruthenium red (RuR; ◆). Synaptosomes were exposed to the different conditions for 15 min before and throughout the experiment. Values represent fluorescence arbitrary units (AU) after subtracting the dilution factor. Representative results of three experiments. (B and C) Effects of Na^+-free medium, La^{3+}, Gd^{3+} and RuR on the NE release elicited by (B) 20% hyposmotic or (C) 20 mM KCl. Synaptosomes were preloaded with ^{3}H-NE and superfused as in Fig. 1. Bars represent the radioactivity released (%) in five fractions after the stimulus minus the basal release in the same time. Empty and hatched bars correspond to control and experimental conditions, respectively. Data are means ± SEM of 3–6 experiments. *$P < 0.001$, **$P < 0.01$ and ***$P < 0.02$ with respect to controls.

nonselective cation channel as a possible mechanism for this hyposmolarity-evoked, Na^+-dependent depolarization. Nonspecific cation channels of the transient receptor potential (TRP) channel family may be candidates for this role because one subtype, the TRVP4, is present in brain, is activated by stretch and by hyposmolarity, and is sensitive to La^{3+} and Gd^{3+} (Gunthorpe *et al.*, 2002). The activity of this TRP channel subtype is also reduced by ruthenium red. Figure 2A shows that this agent markedly decreased the hyposmolarity-induced

depolarization, in further support of the possible involvement of nonspecific cation channels in this phenomenon. Next, the effect of these conditions or agents in reducing the hyposmotic depolarization was tested on NE efflux. Figure 2B shows that hyposmotic NE efflux was essentially prevented in a Na^+-free medium and was also notably decreased by La^{3+} (75%) and reduced (51%) by Gd^{3+}. Ruthenium red decreased it by 50%. In contrast, NE release evoked by 20 mM KCl was unaffected by the Na^+-free condition, was slightly reduced by La^{3+} and was insensitive to Gd^{3+} and ruthenium red (Fig. 2C).

The influence of Ca^{2+} and protein kinase C (PKC)

The hyposmotic condition is known to increase $[Ca^{2+}]_i$ in most cell types (Pasantes-Morales & Morales-Mulia, 2000). This also occurs in synaptosomes as previously reported by Mongin *et al.* (1997) and confirmed in our previous study (Tuz *et al.*, 2004). Upon 20% reduction in osmolarity, the initial $[Ca^{2+}]_i$ of 345 nM increased to 580 nM. In conditions which were nominally externally Ca^{2+}-free (no Ca^{2+} plus EGTA), the increase in $[Ca^{2+}]_i$ was markedly reduced but not abolished (Fig. 3A). In synaptosomes treated with EGTA-AM, $[Ca^{2+}]_i$ basal levels markedly dropped to 125 nM and no increase was induced by the hyposmotic condition (Fig. 3A). These results suggest that the $[Ca^{2+}]_i$ increase results from influx through membrane channels as well as from release from internal stores. The hyposmotic Ca^{2+} influx was insensitive to L-type voltage-gated Ca^{2+} channel blockers nimodipine and nitrendipine, but was reduced by $\approx$30% by ω-conotoxin MVIIC, a blocker of the P/Q type channels (Fig. 3B).

The internal Ca^{2+} released by hyposmolarity seems to come essentially from the mitochondrial store, involving the Na^+–Ca^{2+} exchanger as a mechanism for release. This is suggested by the effect of the mitochondrial Na^+–Ca^{2+} exchange blocker CGP37157, decreasing by 48% the $[Ca^{2+}]_i$ rise (Fig. 3A). In the absence of external Ca^{2+}, CGP37157 fully suppressed the $[Ca^{2+}]_i$ elevation (Fig. 3A). La^{3+}, at the same concentration that prevented depolarization (100 μM), markedly reduced the $[Ca^{2+}]_i$ increase (Fig. 3B).

The effect of these treatments and agents on NE release was next examined. The osmosensitive NE efflux was ~47% reduced by external Ca^{2+} omission, was insensitive to the dihydropyridines but was markedly reduced by EGTA-AM, suggesting an important contribution of Ca^{2+} from internal sources (Fig. 3C). Treatment with thapsigargin or dantrolene did not reduce NE efflux (control, 3.65 ± 0.02; dantrolene, 3.69 ± 0.09; thapsigargin, 3.52 ± 0.08, $n = 3$). The mitochondrial Ca^{2+} which, as shown above, substantially contributed to the hyposmolarity-induced $[Ca^{2+}]_i$ elevation, significantly supported NE efflux as shown by its 50% reduction when the mitochondrial Na^+–Ca^{2+} exchanger was blocked with CGP37157. The effect of external Ca^{2+} omission plus that of CGP37157 were additive and, when the two conditions were tested together, NE efflux was abolished as shown in Fig. 3C. The K^+-stimulated NE release exhibited features different from those of the hyposmolarity-sensitive release regarding the Ca^{2+} influence. The K^+-induced efflux was almost 90% inhibited in the absence of external Ca^{2+} or by treatment with EGTA-AM. It was 43–55% reduced by the dihydropyridines, while it was unaffected by CGP37157 (Fig. 3D).

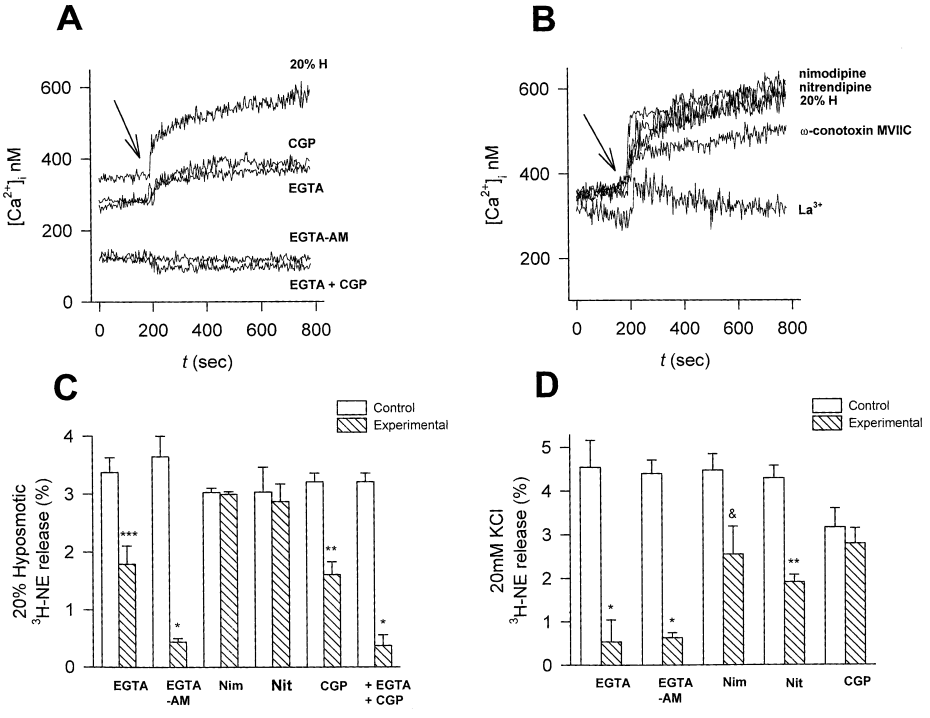

FIG. 3. Changes in $[Ca^{2+}]_i$ elicited by hyposmolarity and its effect on NE efflux. (A) Synaptosomes were loaded with fura-2 AM and $[Ca^{2+}]_i$ was estimated as described in Materials and Methods. $[Ca^{2+}]_i$ was measured in isosmotic medium and, at the arrow, the medium was diluted up to reach −20% hyposmolarity (20% H). EGTA, synaptosomes treated with a Ca^{2+}-free medium containing 0.1 mM EGTA plus 10 mM $MgCl_2$; EGTA-AM, synaptosomes 15 min preincubated with 50 μM EGTA-AM in Ca^{2+}-free medium; CGP, incubation with 10 μM CGP37157; CGP + EGTA, treatment with CGP37157 in a Ca^{2+}-free medium. Fluorescence units were transformed into Ca^{2+} concentration as described in Materials and Methods. (B) Effect of nimodipine, nitrendipine, ω-conotoxin MVIIC and La^{3+} on $[Ca^{2+}]_i$ rise evoked by 20% hyposmolarity. Nim, nimodipine, 10 μM; Nit, nitrendipine, 10 μM; ω-conotoxin MVIIC, 100 nM; La^{3+}, 100 μM. The blockers were present for 15 min before and throughout the experiment. Curves representative of three or four separate experiments. (C and D) Effects of treatments and agents described in A and B (except ω-conotoxin MVIIC and La^{3+}) on (C) the hyposmolarity- or (D) the KCl-stimulated NE release. Data are expressed as in Fig. 2 and are means ± SEM of 3–6 experiments. $^*P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.02$ and $^{\&}P < 0.05$ with respect to controls.

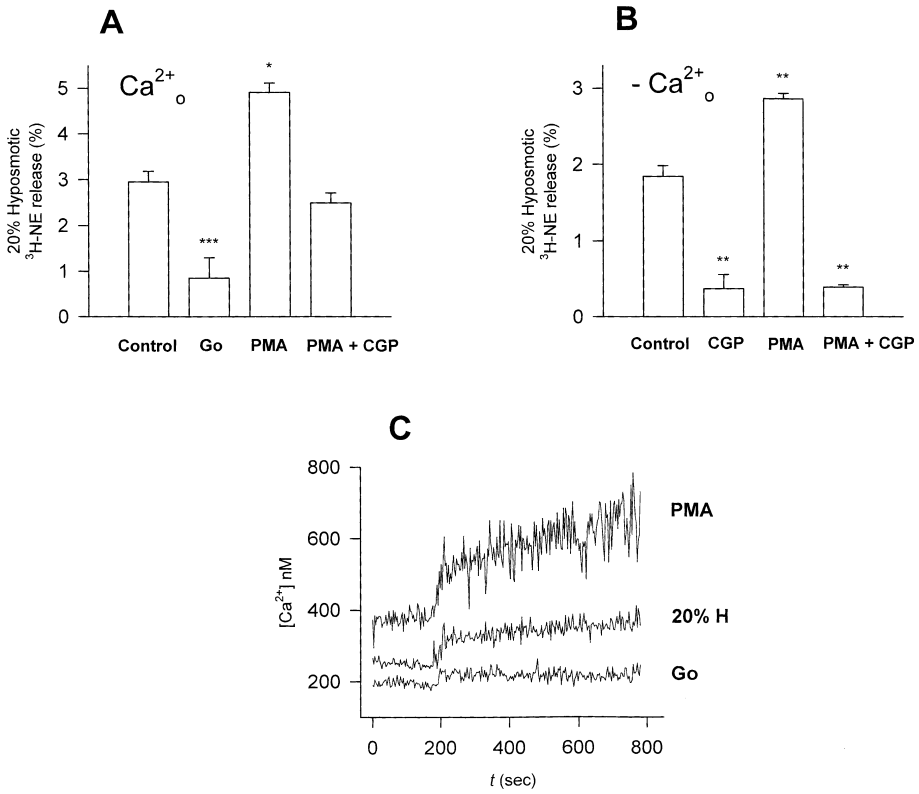

FIG. 4. Effect of modifying PKC activity on the $[Ca^{2+}]_i$ changes and NE release elicited by hyposmolarity. (A) Effect of Gö6976, PMA or CGP37157 on NE release evoked by 20% hyposmolarity in the presence of external Ca^{2+}. PMA + CGP, 75 nM PMA plus 10 μM CGP37157. (B) Effect of PMA and/or CGP37157 on NE release elicited by hyposmolarity in a Ca^{2+}-free medium. Synaptosomes were preloaded with ^{3}H-NE and superfused as in Fig. 1. The agents were present for 15 min before (except PMA, 3.5 min) and throughout the experiment. Data are expressed as in Fig. 2 and are means ± SEM of 3–8 experiments. $*P < 0.001$, $**P < 0.01$ and $***P < 0.02$ with respect to controls. (C) Effect of Gö6976 and PMA on hyposmotic $[Ca^{2+}]_i$ changes in a Ca^{2+}-free medium (prepared as in Fig. 3). Synaptosomes were loaded with fura-2 AM and $[Ca^{2+}]_i$ was measured as described in Fig. 3. Go, 1 μM Gö6976 (15 min preincubated); PMA, 75 nM PMA (3.5 min preincubated).

The influence of PKC on the hyposmotic NE release is shown by the effect of the PKC blocker Gö6976, reducing the release by 72%, and the stimulatory effect of PMA, increasing NE efflux by ~66% (Fig. 4A). PKC increased the hyposmotic NE efflux in both the presence and absence of Ca^{2+} (Fig. 4A and B). In the two conditions, CGP37157 markedly inhibited the PKC-induced NE efflux increase (Figs 3C and 4B). PKC seems to influence NE efflux through an effect on the mechanism of internal Ca^{2+} release because, in the absence of external Ca^{2+}, the hyposmotic $[Ca^{2+}]_i$ elevation was markedly reduced by Gö6976 and potentiated by PMA (Fig. 4C).

Increased exocytosis by hyposmolarity and the effect of TeTX on NE efflux

In our previous study (Tuz *et al*., 2004) we found an effect of hyposmolarity in enhancing exocytosis in synaptosomes, as monitored by the decrease in the fluorescent dye FM1-43 from previously loaded synaptosomes (Guatimosim *et al*., 1997; Fig. 5A). The hyposmotic-induced exocytosis was prevented in Na^+-free medium (Na^+ replaced by choline chloride) and in synaptosomes treated with EGTA-AM (Fig. 5B), and it was increased by PMA (Fig. 5B). All these conditions, as shown above, modified the hyposmotic NE efflux, suggesting an exocytotic mechanism for its release. To further support this possibility, synaptosomes were treated with TeTX, an agent known to disrupt the vesicular release. Figure 5C shows that this toxin decrease the hyposmotic NE efflux by >90%. NE release evoked by high K^+ concentration was even more reduced by treatment with the toxin (Fig. 5D).

Discussion

The present results show a hyposmolarity-evoked NE efflux from synaptosomes, resulting from a series of events which reproduce those of the excitation–secretion process, i.e. depolarization, $[Ca^{2+}]_i$ rise and vesicular exocytosis. The trigger for all these reactions eventually resulting in NE release seems to be the La^{3+}-, Gd^{3+}- and ruthenium red-sensitive depolarization, probably due to Na^+ influx through nonspecific cation channels, possibly of the TRP class. A subtype of this family of channels, the TRPV4, is almost exclusively present in the nervous tissue, is osmotically and mechanically sensitive and is blocked by Gd^{3+}, La^{3+} and ruthenium red (Gunthorpe *et al*., 2002).

The event subsequent to the hyposmotic depolarization is a $[Ca^{2+}]_i$ increase. The fact that preventing depolarization with La^{3+} abolished this Ca^{2+} response established the link between these two events. The hyposmotic $[Ca^{2+}]_i$ increase was characterized in the present study and found to be different in some respects from that evoked by depolarizing K^+ concentration. The mechanisms and sources contributing to the increase in $[Ca^{2+}]_i$ are different in the two paradigms. While in the K^+ model $[Ca^{2+}]_i$ elevation was, as expected, essentially dependent on external Ca^{2+}, elevation elicited by hyposmolarity resulted from some external Ca^{2+} entry as well as from a significant contribution of Ca^{2+} released from internal sources. The mechanism of

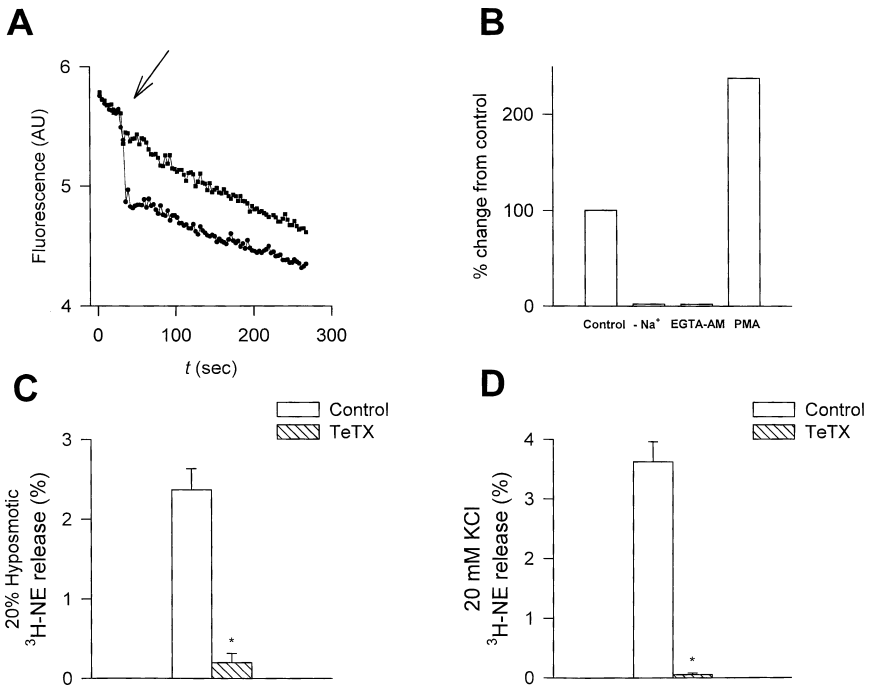

FIG. 5. Hyposmolarity-induced exocytosis and effect of TeTX on NE release from synaptosomes. (A) Exocytosis was monitored by changes in fluorescence in synaptosomes loaded with 4 μM FM1-43 for 10 min in isosmotic medium and 1 min in 40 mM KCl to internalize the fluorophore, as described in Materials and Methods. After 30 s in isosmotic conditions, the medium was diluted up to reach −20% hyposmolarity (arrow) and the FM1-43 release in this condition (●) was compared with that under a continuous isosmotic medium (■). Data are expressed as fluorescence arbitrary units (AU). The figure is a representative of three experiments. (B) Effect of Na^+-free medium (–Na^+), EGTA-AM and PMA on the hyposmolarity-induced exocytosis. (C and D) Effect of TeTX on NE efflux evoked by (C) hyposmolarity or (D) depolarizing K^+ concentrations. Synaptosomes preloaded with ^{3}H-NE were incubated for 90 min in isosmotic medium in the presence of 50 nM TeTX with 0.1% BSA. Thereafter, synaptosomes were superfused as in Fig. 1. Open bars, controls; hatched bars, TeTX. Data are expressed as in Fig. 2 and are means ± SEM of three experiments. *$P < 0.01$ with respect to controls.

external Ca^{2+} influx also showed marked differences between the two stimulatory conditions, with null participation of the voltage-gated L-type Ca^{2+} channels in the hyposmotic $[Ca^{2+}]_i$ increase, in contrast to their significant role in the high-K^+ condition. A $[Ca^{2+}]_i$ elevation evoked by hyposmolarity has been reported in hippocampal pyramidal neurons (Borgdorff *et al*., 2000), with contributions of extracellular Ca^{2+} as well as of Ca^{2+} released from internal sources. In that study, the external Ca^{2+} influx is attributed to the NaCl decrease in the hyposmotic medium, whereas it is proposed that the internal Ca^{2+} is elicited by stretch or swelling. In the present study in synaptosomes we further explored the mechanisms of the two sources of hyposmotic $[Ca^{2+}]_i$ elevation. Similar to the study in neurons, we found a contribution of external Ca^{2+} influx occurring not via the voltage-gated L-type Ca^{2+} channels but partly (30%) through P/Q type channels. The mechanism of Ca^{2+} influx insensitive to the voltage-gated channel blockers was not identified in the present study. As for the internal source of Ca^{2+} released by hyposmolarity, our results suggest the mitochondrial pool, with the release involving the mitochondrial Na^+–Ca^{2+} exchanger, which is activated by an increase in cytosolic Na^+ (Adam-Vizi, 1992; Hayasaki-Kajiwara *et al*., 1999; Raiteri *et al*., 2002). This is the notion suggested by the effect of the mitochondrial Na^+–Ca^{2+} exchanger blocker CGP37157 reducing by 48% the hyposmotic $[Ca^{2+}]_i$ rise and fully preventing it when tested in the absence of external Ca^{2+}.

The hyposmotic NE release responded in full correspondence to each one of the precedent events, i.e. depolarization and $[Ca^{2+}]_i$ elevation. Preventing or reducing depolarization by Na^+ omission, or by La^{3+}, Gd^{3+} or ruthenium red, consequently reduced NE efflux. Also, NE release closely paralleled the changes in $[Ca^{2+}]_i$ in the different conditions tested. Thus, ∼53% of the hyposmotic efflux persisted in the absence of external Ca^{2+}, 50% remained after blockade of the internal Ca^{2+} release (CGP37157) and >90% was inhibited when the two conditions were tested together.

PKC was found to modulate NE efflux, which was inhibited by PKC blockade (Gö6976) and potentiated by PKC activation (PMA). The kinase activation may result from the hyposmotic simulation of phospholipases (Thoroed *et al*., 1997; Tomassen *et al*., 2004) or from an increase in cytosolic Na^+ (Hayasaki-Kajiwara, 1999). Once activated, PKC may act directly on the exocytotic process (Vaughan *et al*., 1998), by recruiting vesicles or/and by increasing Ca^{2+} affinity for the vesicular release (Keenan & Kelleher, 1998; Hille *et al*., 1999). PKC may also act by enhancing the Ca^{2+} mobilization from the mitochondrial reservoir through an action on the Na^+–Ca^{2+} exchanger, as has been recently described in motoneurons (Yang *et al*., 2003). Even though the two possibilities are plausible, our results favour a major effect on the mitochondrial Ca^{2+} release because: (i) PKC activation increased and PKC inhibition decreased the hyposmolarity-induced $[Ca^{2+}]_i$ rise in the absence of external Ca^{2+} and (ii) blockade of the mitochondrial Ca^{2+} release by CGP37157 in either the presence or absence of Ca^{2+} prevented the PKC potentiation of NE efflux.

The events inducing the hyposmotic NE efflux, i.e. depolarization and $[Ca^{2+}]_i$ rise, are typical of the vesicular mechanism by which neurotransmitters are released by exocytosis. The contribution of this mechanism to NE release is suggested by the effect of TeTX in preventing hyposmotic NE efflux. The fact that this toxin similarly affected the K^+-stimulated NE efflux, known to occur by vesicular exocytosis, further supports this notion.

In summary, the present results suggest the following serial events evoked by hyposmolarity, ultimately leading to NE efflux: (i) the Na^+-dependent depolarization of the terminal, probably mediated by

osmotically or stretch sensitive nonspecific cation channels; (ii) the activation of voltage-dependent Na^+ and Ca^{2+} channels; (iii) a consequent $[Ca^{2+}]_i$ elevation resulting from both Ca^{2+} entry and a PKC-modulated, Na^+–Ca^{2+} exchange-mediated Ca^{2+} release from mitochondrial stores; (iv) the $Ca^{2+}{}_i$-evoked TeTx-sensitive release of NE. As above discussed, the pathways of hyposmolarity-triggered depolarization and $[Ca^{2+}]_i$ elevation differ in some respects from those of the classical depolarization–secretion here exemplified by the K^+-stimulated NE release, but the resulting $[Ca^{2+}]_i$ increase seems to trigger in both cases a TeTX-sensitive mechanism which may not differ from the vesicular exocytosis characteristic of neurotransmitter release.

In a previous study (Tuz *et al*., 2004) we found a hyposmotic release of glutamate, the major excitatory neurotransmitter. Glutamate release was partly Ca^{2+}-dependent and TeTX-sensitive, suggesting the contribution of exocytosis, but the reversal operation of the carrier was contributing as well. The glutamate release may explain the effect of hyposmolarity increasing the amplitude and duration of excitatory postsynaptic potentials (Chebabo *et al*., 1995), suggesting that the hyposmotic stimulus might potentiate the impulse-evoked release, acting probably via an additive mechanism. This could be also the case for NE, because the differences observed between the K^+- and the hyposmolarity-evoked release may result in additive effects of the two stimulatory conditions. Although this was not explored in the present study in which the hyposmolarity effects were examined only on the basal NE efflux, the potentiation of synaptic transmission by hyposmolarity and/or swelling opens an interesting avenue for future studies on the intriguing possibility of a modulation of synaptic function by conditions leading to volume increase in nerve terminals, in both pathological and physiological conditions.

Acknowledgements

This work was supported in part by grant No. IN204900 from DGAPA, UNAM and 4646 5-4 from CONACyT.

Abbreviations

$[Ca^{2+}]_i$, cytosolic Ca^{2+}; bisoxonol, bis-(1,3diethylthiobarbituric acid) trimethine oxonol; BSA, bovine serum albumin; fura-2 AM, fura-2 acetoxymethyl ester; NE, norepinephrine; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; TeTX, tetanus toxin; TRP, transient receptor potential.

References

Adam-Vizi, V. (1992) External Ca^{2+}-independent release of neurotransmitters. *J. Neurochem.*, **58**, 395–405.

Borgdorff, A.J., Somjen, G.G. & Wadman, W.J. (2000) Two mechanisms that increase free intracellular calcium in rat hippocampal neurons during hyposmotic and low NaCl treatment. *J. Neurophysiol.*, **83**, 81–89.

Chebabo, S.R., Hester, M.A., 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.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

Guatimosim, C., Romano-Silva, M.A., Cruz, J.S., Beirao, P.S., Kalapothakis, E., Morales-Santos, T., Cordeiro, M.N., Diniz, C.R., Gomes, M.V. & Prado, M.A. (1997) A toxin from the spider *Phoneutria nigriventer* that blocks calcium channles coupled to exocytosis. *Br. J. Pharmacol.*, **122**, 591–597.

Gunthorpe, M.J., Benham, C.D., Randall, A. & Davis, J.B. (2002) The diversity in the vanilloid (TRPV) receptor family of ion channels. *Trends Pharmacol. Sci.*, **23**, 183–191.

Hajos, F. (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.*, **93**, 485–489.

Hayasaki-Kajiwara, Y., Kitano, Y., Iwasaki, T., Shimamura, T., Naya, N., Iwaki, K. & Nakajima, M. (1999) Na^+ influx via Na^+/H^+ exchange activates protein kinase C isozymes delta and epsilon in cultured neonatal rat cardiac myocytes. *J. Mol. Cell Cardiol.*, **31**, 1559–1572.

Hille, B., Billard, J., Babcock, D.F., Nguyen, T. & Koh, D.S. (1999) Stimulation of exocytosis without a calcium signal. *J. Physiol. (Lond.)*, **520**, 23–31.

Keenan, C. & Kelleher, D. (1998) Protein kinase C and the cytoskeleton. *Cell Signal*, **10**, 225–232.

Mongin, A.A., Aksentsev, S.L., Orlov, S.N. & Konev, S.V. (1997) Hypoosmotic shock activates Ca^{2+} channels in isolated nerve terminals. *Neurochem. Int.*, **31**, 835–843.

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

Pasantes-Morales, H. & Morales-Mulia, S. (2000) Influence of calcium on regulatory volume increase: role of potassium channels. *Nephron*, **86**, 414–427.

Raiteri, L., Stigliani, S., Zedda, L., Raiteri, M. & Bonanno, G. (2002) Multiple mechanisms of transmitter release evoked by 'pathologically' elevated extracellular $[K^+]$: involvement of transporter reversal and mitochondrial calcium. *J. Neurochem.*, **80**, 706–714.

Thoroed, S.M., Lauritzen, L., Lambert, I.H., Hansen, H.M. & Hoffmann, E.K. (1997) Cell swelling activates phospholipase A2 in Ehrlich ascites tumor cells. *J. Membr. Biol.*, **160**, 47–58.

Tomassen, S.F.B., van der Wijk, T., de Jonge, H.R. & Tilly, B.C. (2004) Activation of phospholipase D by osmotic cell swelling. *FEBS Lett.*, **566**, 287–290.

Tuz, K., Peña-Segura, C., Franco, R. & Pasantes-Morales, H. (2004) Depolarization, exocytosis and amino acid release evoked by hyposmolarity from cortical synaotosomes. *Eur. J. Neurosci.*, **19**, 916–924.

Vaughan, P.F.T., Walker, J.H. & Peers, C. (1998) The regulation of neurotransmitter secretion by protein kinase C. *Mol. Neurobiol.*, **18**, 125–155.

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.

Waggoner, A. (1976) Optical probes of membrane potential. *J. Membr. Biol.*, **27**, 317–334.

Whittaker, V.P. & Barker, L.A. (1972) The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In Fried, R. (ed.), *Methods of Neurochemistry*. Marcel Dekker, New York.

Yang, F., Xiang-Ping, H., Russell, J. & Lu, B. (2003) Ca^{2+} influx-independent synaptic potentiation mediated by mitochondrial Na^+–Ca^{2+}exchanger and protein kinase C. *J. Cell Biol.*, **163**, 511–523.

DISCUSIÓN

Los aminoácidos tienen un papel importante en el proceso de regulación del volumen celular, ya que su movilización en respuesta a un incremento de volumen contribuye al proceso de adaptación a la condición hiposmótica externa. Esta liberación es, además, ubicua, ya que se presenta en casi todos los tipos celulares. En el sistema nervioso en particular, los aminoácidos contribuyen de manera predominante a la adaptación del cerebro en condiciones de hiponatremia crónica. La liberación de aminoácidos en condiciones de hinchamiento hiposmótico se ha estudiado en distintos tipos de preparaciones nerviosas, incluyendo células en cultivo. Con frecuencia se considera a la vía de liberación de la taurina como representativa para la de los aminoácidos y aún para otros osmolitos orgánicos. Se postula que se trata de una vía difusional, en la que el osmolito se mueve siguiendo la dirección del gradiente de concentración, una vez que la vía ha sido activada. Esta vía se caracteriza por ser sensible a bloqueadores de canales de Cl^- y requiere de la actividad de cinasas de tirosina (Junankar y Kirk, 2000; Pasantes-Morales y Franco, 2002; Perlman y Goldstein, 1999; van der Wijk et al., 2000).

A pesar de que en la mayor parte de las células en las que se ha estudiado, la liberación del ácido glutámico comparte estas características, éste parece no ser el caso para las células nerviosas. A la fecha, existe evidencia que indica que la liberación hiposmótica de glutamato en el tejido nervioso no presenta las características de la vía difusional típica. En astrocitos en cultivo expuestos a condiciones hiposmóticas, se observa un incremento en la liberación de taurina y glutamato. En estas células, aunque la liberación de taurina se inhibe casi totalmente en presencia de SITS (Kimelberg et al., 1990), la de glutamato sólo se inhibe de manera parcial (Kimelberg et al., 1990; Rutledge et al., 1998). En otras preparaciones, que incluyen células en cultivo, rebanadas de cerebro, así como estudios *in vivo*, también se han descrito resultados similares; la liberación de taurina se inhibe marcadamente con los inhibidores de canales de Cl^- (Estevez et al., 1999b; Junankar y Kirk, 2000; Law, 1994; Kirk, 1997; Perlman y Goldstein,

1999; Sánchez-Olea et al., 1996) y la liberación de glutamato se inhibe de manera parcial ante estos mismos bloqueadores (Estevez et al., 1999b; Law, 1996; Pasantes-Morales et al., 1999; Song et al., 1998). En el caso del GABA, se ha reportado que en retina y corteza cerebral, la liberación hiposmótica de este aminoácido no se inhibe en una fracción significativa por bloqueadores de canales de Cl^- (Pasantes-Morales et al., 1999; Law, 1996; Estevez et al., 1999b). En general, la liberación de taurina es más sensible a decrementos en la osmolaridad del medio, así como a la presencia de inhibidores de canales de Cl^-, en comparación con otros aminoácidos, aún en células no excitables (Basavappa et al., 1996; Grant et al., 2000; Haynes y Goldstein, 1993; Miyata et al., 1997; Pasantes-Morales et al., 1996; Sánchez-Olea et al., 1991b; Sánchez-Olea et al., 1995b). En el caso de la participación de las cinasas de tirosina en la movilización de aminoácidos estimulada por volumen, la liberación de taurina requiere de la actividad de estas cinasas y se inhibe significativamente en presencia de tirfostinas (Cardin et al., 2003; Huang et al., 2001; Mongin et al., 1999b; Morales-Mulia et al., 2001; de la Paz et al., 2002; Pedersen et al., 2002; Shen et al., 2001), las cuales son análogos del ATP, diseñados para competir por los sitios específicos de unión a ATP de receptores membranales con actividad intrínseca de cinasa de tirosina (Al-Obeidi et al., 1998; Levitzki y Bohmer, 1998; Levitzki y Gazit, 1995; Fry et al., 2003). En algunos tipos celulares, también se ha visto que la liberación hiposmótica de taurina se inhibe por otros agentes, distintos de las tirfostinas, que inhiben a las cinasas de tirosina (Huang et al., 2001; Pedersen et al., 2002). En el caso de la liberación de los aminoácidos glutamato y GABA, esta no se inhibe con las tirfostinas (Franco et al., 2001; Mongin et al., 1999b; de la Paz et al., 2002), excepto en un estudio efectuado en retina, en donde se observa una modesta disminución en la liberación de GABA activada por volumen en presencia de la tirfostina A23 (de la Paz et al., 2002). Las diferencias descritas en cuanto a la liberación de taurina y de los aminoácidos neurotransmisores en condiciones hiposmóticas, reflejan vías distintas de liberación.

La respuesta celular que involucra la liberación de aminoácidos como el GABA y el glutamato en condiciones de hiposmolaridad, es de especial

importancia en el sistema nervioso, ya que estos aminoácidos tienen un importantísimo papel como neurotransmisores. En el sistema nervioso central, el glutamato media la mayor parte de la transmisión sináptica excitatoria y se encuentra almacenado en grandes cantidades. El GABA es el neurotransmisor inhibidor más importante en el sistema nervioso central y se encuentra presente en altas concentraciones en muchas regiones cerebrales. Estas características hacen que la liberación de estos aminoácidos neurotransmisores inducida por una condición hiposmótica pueda llevar a alteraciones en la excitabilidad neuronal. Por ello resultan de especial interés las preguntas que nos planteamos inicialmente en esta investigación que son, en primer lugar, si la liberación de estos aminoácidos con el doble papel de osmolitos y neurotransmisores ocurre en el sitio de la sinapsis, es decir, de las terminaciones sinápticas y en segundo, cuál es el mecanismo de esta liberación.

En esta investigación demostramos por primera vez, que en efecto, los aminoácidos neurotransmisores se movilizan de las terminaciones nerviosas aisladas (sinaptosomas) en respuesta a un decremento en la osmolaridad externa. Demostramos también que el mecanismo de movilización difiere, sobre todo en el caso del glutamato, del clásico descrito para los osmolitos, en tanto que la taurina se moviliza en su mayor parte mediante este mecanismo. La liberación de taurina, reportada aquí, ocurre a través de la vía típica difusional en una fracción importante, de más del 55%; mientras que en el caso del GABA, la contribución es de alrededor del 18; y en el de glutamato, ausente.

Cambios en la terminación sináptica inducidos por hiposmolaridad

Durante la caracterización de la vía de movilización de los aminoácidos en respuesta a la hiposmolaridad en los sinaptosomas, encontramos que esta condición desencadena una serie de fenómenos que inciden directamente en los mecanismos de liberación de los neurotransmisores. Observamos en primer lugar, que la hiposmolaridad produce despolarización de la membrana. Esta ocurre por un mecanismo independiente de Cl^- y dependiente de Na^+. La entrada de Na^+ es la responsable de la despolarización, que se previene consecuentemente en un

medio libre de Na^+. La entrada de Na^+ parece ser a través de canales catiónicos no selectivos, si se considera el efecto inhibidor de La^{3+} y Gd^{3+}, que son bloqueadores de estos canales. Se ha reportado que la hipotonicidad activa canales catiónicos no selectivos en otros tipos celulares (Kim y Fu, 1993; Marunaka et al., 1997; Welsh et al., 2000), pero el mecanismo de activación se desconoce. La presencia de un subtipo de estos canales en los sinaptosomas, el TRPV4, podría vincular a los canales TRP con la despolarización inducida por hiposmolaridad. A consecuencia de la despolarización hay un incremento en la concentración de Ca^{2+} citosólico. Este incremento puede deberse a la apertura de canales de Ca^{2+} sensibles a voltaje y entrada de Ca^{2+} extracelular por una parte, pero también debido a la activación del intercambiador Na^+/Ca^{2+} mitocondrial y liberación de Ca^{2+} al citosol. Finalmente, este aumento en el Ca^{2+} citosólico incrementa la exocitosis, y con ello, potencialmente, puede incrementarse la liberación de neurotransmisores. La exocitosis inducida por hiposmolaridad es sensible a TeTX, dependiente de Ca^{2+} y modulada por PKC. Se ha visto que el hinchamiento hiposmótico puede inducir un aumento en los procesos de fusión vesicular con la membrana plasmática en muchos tipos celulares, incluyendo la liberación vesiculada de varias hormonas (Bacova et al., 2006; Najvirtova et al., 2003; Strbak y Greer, 2000) y por otra parte, la hipertonicidad inhibe el proceso de exocitosis (Rizoli et al, 2000). Esto sugiere que los cambios en el volumen pueden estar asociados a la modulación de las tasas de exocitosis/endocitosis.

Un punto importante que hay que resaltar, es que en los sinaptosomas, la exocitosis de los aminoácidos en condiciones hiposmóticas es dependiente de Ca^{2+} de pozas internas, particularmente de la mitocondria, aunque si hay un aumento significativo en la concentración de Ca^{2+} citosólico de origen externo. La exocitosis dependiente de Ca^{2+} extracelular se considera clásica, pero se ha descrito que el Ca^{2+} liberado a partir de fuentes intracelulares también es capaz de disparar exocitosis. Este Ca^{2+} puede liberarse de la mitocondria a través de la activación del intercambiador Na^+/Ca^{2+} mitocondrial, una vez que el Na^+ entra a la terminal sináptica con la llegada del potencial de acción. Además, hay subdominios del retículo endoplásmico que presentan grupos de receptores a

rianodina e inositol trifosfato que se encuentran muy cerca de la mitocondria, el Ca^{2+} de origen mitocondrial puede activar estos receptores y producir una liberación de Ca^{2+} del retículo inducida por Ca^{2+} (Adam-Vizi, 1992; Berridge, 1998; Raiteri et al., 2002). El incremento en la concentración intraterminal de Ca^{2+}, proveniente de ambas fuentes, es localizado y los cationes pueden encontrarse muy cerca de los sitos activos de liberación vesicular promoviendo la exocitosis. La contribución predominante de Ca^{2+} de origen mitocondrial en el incremento evocado por la condición hiposmótica que mostramos en este trabajo, argumenta en contra de un posible mecanismo de liberación vesicular sináptica típica. En el caso de la rebanada de hipocampo, la osmo-exocitosis de los aminoácidos no está mediada por incrementos en la concentración de Ca^{2+} intracelular y en ese caso se ha sugerido una fusión vesicular mediada por PKC (Franco et al., 2001). La 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 en casi todos los tipos de neuronas, incluyendo colinérgicas, dopaminérgicas, noradrenérgicas, GABAérgicas, gutamatérgicas y serotoninérgicas (Majewski y Iannazzo, 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: incremento en la sensibilidad al Ca^{2+} de la maquinaria de fusión, modulación de la conductancia de canales iónicos, 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 presináptica incrementando el número de vesículas disponibles para la liberación (Bouron, 2001; Iannazzo, 2001; Thomson, 2000; Vaughan et al., 1998); y un mecanismo de reciente descripción, en el que la PKC aumenta la movilización de Ca^{2+} de la mitocondria a través de un efecto en el intercambiador Na^{+}/Ca^{2+} (Yang et al., 2003). En un estudio realizado en rebanadas de hipocampo (Franco et al., 2001) se propone un mecanismo exocitótico independiente de Ca^{2+} para la

liberación de glutamato y GABA activada por volumen y posiblemente la PKC dispare la exocitosis hiposmótica de estos neurotransmisores (Franco et al., 2001; Franco, 2004). En los sinaptosomas, nuestros resultados sugieren que la PKC participa en el proceso osmo-exocitótico a través de un efecto en la liberación de Ca^{2+} mitocondrial, aunque no se descarta la participación de esta enzima en el reclutamiento vesicular.

Mecanismos de movilización hiposmótica de los aminoácidos en los sinaptosomas

En esta investigación documentamos la existencia de al menos dos vías de movilización de los aminoácidos de la terminación sináptica en respuesta a la hiposmolaridad: la vía difusional y la liberación vesicular. Sin embargo, nuestros resultados indican que la contribución de estas vías a la liberación de los aminoácidos varía dependiendo de cada uno de ellos y que la vía difusional no es común para todos los aminoácidos. Así, a través de la vía difusional se libera el 55% de la taurina, el 18% del GABA y el glutamato prácticamente no se libera por este mecanismo. Esta proporción de hecho se invierte cuando se considera el mecanismo de liberación vesicular. A través de esta vía se libera el 44% del glutamato, el 30% del GABA y el 20% de la taurina. Es importante hacer notar que la liberación vesicular de aminoácidos en condiciones de hinchamiento hiposmótico, parece presentarse principalmente en el tejido nervioso, ya que en otros tipos celulares, la liberación de taurina y glutamato presentan las características descritas para una liberación a través de la vía difusional (Sánchez-Olea et al., 1995b; Shennan y McNeillie, 1995).

Una fracción de la liberación de los aminoácidos puede también ocurrir a través de los cotransportadores dependientes de la energía generada por la disipación del gradiente de Na^{+} trabajando en sentido inverso. En nuestra investigación, utilizando bloqueadores competitivos no transportables específicos de los transportadores, pudimos demostrar que este mecanismo está operando, en efecto, para el caso del GABA y el glutamato en una proporción importante, de 28% y 37%, respectivamente. Aunque no se pudo comprobar esta condición para

la taurina, debido a que los inhibidores de su transporte no son del tipo alostérico, es posible que este mecanismo sea responsable de la fracción restante no identificada.

Hiposmolaridad y liberación de neurotransmisores

La secuencia de eventos generada por la hiposmolaridad, como son la despolarización, el incremento en el Ca^{2+} citosólico, activación de PKC, y aumento en la exocitosis, son condiciones generales para la liberación de neurotransmisores, independientemente de que funcionen o no como osmolitos. Esto abre la posibilidad de que en el curso de la hiposmolaridad, dado que la hipotonicidad promueve la fusión vesicular, se liberen otros neurotransmisores de la terminal sináptica, aunque no tengan una función como osmolitos. Esta hipótesis fue comprobada en esta tesis al demostrar la liberación vesicular de norepinefrina en condiciones de hinchamiento hiposmótico en los sinaptosomas. A diferencia de lo encontrado para los aminoácidos neurotransmisores, en el caso de la norepinefrina el total de la liberación se lleva a cabo a través de un mecanismo exocitótico dependiente de Ca^{2+}, sin que haya ninguna intervención de vías de tipo difusional o de operación en reversa de los cotransportadores. El aumento en la concentración de Ca^{2+} proviene, como ya se mencionó, tanto del medio externo como de pozas internas, en particular, de la mitocondria. A su vez, una fracción de la liberación vesicular de norepinefrina depende de Ca^{2+} externo, y otra, de Ca^{2+} mitocondrial. Nuestros resultados son el primer reporte de liberación de un neurotransmisor no aminoácido en condiciones hiposmóticas. Estudios recientes del laboratorio demuestran que la hiposmolaridad también induce una liberación de serotonina, al parecer a través de mecanismos similares a los de norepinefrina. Es importante mencionar que estos dos neurotransmisores no tienen una función como osmolito, obviamente por las concentraciones tan bajas presentes en las células (Buu y Debinski, 1990), de modo que no tienen un papel en el proceso de regulación del volumen, por lo que la liberación vesicular inducida por la hiposmolaridad significa simplemente que esta condición trae aparejada una

serie de fenómenos similares a los que ocurren durante la estimulación fisiológica de la terminación sináptica.

Liberación de osmolitos y neurotransmisores al espacio sináptico. Importancia

Como se discutió en párrafos anteriores, la liberación de los aminoácidos estimulada por hiposmolaridad sucede a través de diferentes mecanismos, aunque es notoria su distinta participación en la liberación de cada uno de ellos. La movilización de taurina sucede a través de la vía difusional en mayor proporción y a través de la vía osmo-exocitótica en menor proporción, en tanto que la liberación de glutamato es exocitótica sin contribución de la vía difusional, y la liberación de GABA es intermedia con la participación de ambas vías. En el caso de los aminoácidos, hay que diferenciar la función de la taurina por una parte, y la de los aminoácidos que tienen la doble acción de osmolitos y neurotransmisores, por otra. La taurina en su mayor parte no está contribuyendo como neurotransmisor sino preponderantemente como osmolito. Sus propiedades muy particulares, tales como el que no forma parte de las proteínas, no se metaboliza y no participa en ninguna reacción celular (exceptuando la formación del ácido taurocólico), indican que su función es básicamente la de un osmolito, pudiendo movilizarse en respuesta a un estrés hiposmótico, sin modificar otras funciones celulares (Huxtable, 1992). A pesar de que no participa prácticamente como neurotransmisor, la taurina se encuentra en concentraciones muy elevadas en la terminación sináptica, así como en las propias vesículas sinápticas. En ellas, coincide seguramente con los neurotransmisores, como lo apunta el hallazgo de que en las vesículas sinápticas del órgano eléctrico de Torpedo en el que las terminaciones sinápticas son puramente colinérgicas, la taurina se encuentra en una alta concentración, que excede en un 50-60% la de la acetilcolina (Vyas y Bradford, 1987). Es posible, entonces, que la taurina esté desempeñando una función reguladora del volumen tanto en la terminal sináptica propiamente como en las vesículas. La continua generación de microgradientes osmóticos a consecuencia de los movimientos iónicos que acompañan a la función sináptica,

así como la recaptura de neurotransmisores y el llenado y vaciamiento de las vesículas, hacen necesaria una regulación estricta del volumen en los distintos compartimientos de la terminal. La taurina puede entonces, tener un papel fundamental en este fenómeno.

En cuanto a los otros aminoácidos, su función principal es la de neurotransmisores pero no puede descartarse un papel secundario en el control del volumen sinaptosomal y vesicular. En cualquier caso, la liberación de neurotransmisores durante el hinchamiento hiposmótico, e independientemente de si ésta ocurre a través de la vía difusional o si es una liberación vesicular, puede originar alteraciones importantes en la excitabilidad del tejido nervioso. La liberación de aminoácidos excitadores como el glutamato en la terminal sináptica, como demostramos en esta investigación, puede ser la responsable del incremento en la amplitud de los potenciales excitadores postsinápticos (PEPS) observado en rebanadas de hipocampo expuestas a medios de baja osmolaridad (Chebabo et al., 1995; Huang et al., 1997). La hiposmolaridad también produce un aumento en los potenciales inhibidores postsinápticos, con un efecto menos notable que aquél que produce en los excitadores y nuestros resultados sobre la liberación hiposmótica del GABA podrían explicar este efecto (Huang et al., 1997). Asimismo, la liberación de glutamato al espacio sináptico en condiciones de hiposmolaridad podría explicar, al menos en parte, el hecho de que durante el curso de la hiponatremia se genera actividad epileptiforme y hay un aumento en la susceptibilidad a convulsiones (Andrew, 1991).

La norepinefrina tiene un papel importante como neurotransmisor excitador en el sistema nervioso central. Su liberación en respuesta a la hiposmolaridad, al igual que la de glutamato, puede explicar en parte el aumento en la excitabilidad neuronal que se observa durante la hiponatremia (Andrew et al., 1991) y el incremento en la duración y amplitud de los PEPS reportado para la rebanada de hipocampo en un medio de baja osmolaridad (Chebabo et al., 1995; Huang et al., 1997). Las características de liberación exocitótica de glutamato y norepinefrina durante la hiposmolaridad son distintas de aquella que sucede durante el impulso nervioso, en cuanto al origen del Ca^{2+} que dispara la fusión vesicular, ya que la

liberación osmo-exocitótica sí requiere de Ca^{2+} mitocondrial; lo que sugiere que el estímulo hiposmótico podría potenciar la liberación de neurotransmisores inducida por el impulso nervioso.

Además de los efectos debidos al incremento en volumen de la terminal sináptica aquí descritos y su consecuencia en la excitabilidad neuronal, la condición hiposmótica podría afectar la excitabilidad nerviosa a través de otro mecanismo. El incremento en el volumen celular y la consecuente reducción en el espacio extracelular podría generar fenómenos de transmisión electrotónica y actividad epileptogénica (Andrew et al., 1989; Roper et al., 1992). A este respecto, se ha descrito que el hinchamiento de las dendritas favorece la propagación electrotónica originando fenómenos de depresión propagada de la excitabilidad neuronal (Baraban et al, 1997; Chebabo et al., 1995). También se ha observado que la sincronía en la descarga neuronal en la epilepsia generalizada se debe a una disminución en el espacio sináptico y se previene consecuentemente, mediante estrategias que reducen el incremento en el volumen celular (Hochman et al., 1995). En condiciones hiposmóticas, se genera la sincronización de disparos, debido a que la disminución en el espacio extracelular favorece la actividad epiloptogénica. 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 tienen el efecto opuesto (Somjen, 2002). La gama de situaciones descritas, promovida por la hiposmolaridad, produce una modificación en la excitabilidad neuronal, lo que podría explicar, al menos en parte, el que durante el curso de la hiponatremia se facilite la aparición de convulsiones y se genere actividad epileptiforme (Andrew, 1991).

En conclusión, los resultados de nuestra investigación sobre el efecto de la condición hiposmótica en la liberación de neurotransmisores, independientemente de su vínculo con mecanismos de regulación de volumen, que describimos en este

trabajo, sugiere que la hiposmolaridad es una condición que puede modificar la transmisión nerviosa y que puede influir de manera más general en la función sináptica.

REFERENCIAS

Adam-Vizi V (1992) External Ca(2+)-independent release of neurotransmitters. J Neurochem. 58(2): 395-405.

Aderem A (1995) The MARCKS family of protein kinase-C substrates. Biochem Soc Trans. 23(3): 587-591.

Al-Habori M (2001) Macromolecular crowding and its role as intracellular signalling of cell volume regulation. Int J Biochem Cell Biol. 33: 844-864.

Al-Obeidi FA, Wu JJ, Lam KS (1998) Protein tyrosine kinases: structure, substrate specificity, and drug discovery. Biopolymers. 47(3):197-223

Álvarez-Leefmans FJ, Reuss L (1996) Operación orquestada de los sistemas de transporte a través de la membrana plasmática: regulación y mantenimiento del volumen celular. Capitulo 7, en: Biofísica y fisiología celular. Latorre R, López-Barneo J, Bezanilla F, Llinás R (editores). Universidad de Sevilla pp 178-212.

Andrew RD (1991) Seizure and acute osmotic change: clinical and neurophysiological aspects. J Neurol Sci. 101(1): 7-18.

Andrew RD, Fagan M, Ballyk BA, Rosen AS (1989) Seizure susceptibility and the osmotic state. Brain Res. 498(1): 175-180.

Babila T, Atlan H, Fromer I, Schwalb H, Uretzky G, Lichtstein D (1990) Volume regulation of nerve terminals. J Neurochem. 55(6): 2058-2062.

Bacova Z, Baqi L, Benacka O, Payer J, Krizanova O, Zeman M, Smrekova L, Zorad S, Strbak V (2006) Thyrotropin-releasing hormone in rat heart: effect of swelling, angiotensin II and renin gene. Acta Physiol. 187(1-2): 313-319.

Bai J, Chapman ER (2004) The C2 domains of synaptotagmin-partners in exocytosis. Trends Biochem Sci. 29(3): 143-151.

Ballyk BA, Quackenbush SJ, Andrew RD (1991) Osmotic effects on the CA1 neuronal population in hippocampal slices with special reference to glucose. J Neurophysiol. 65: 1055-1066.

Banderali U, Roy G (1992) Anion channels for amino acids in MDCK cells. Am J Physiol. 263: C1200-c1207.

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(Pt 3): 679-689.

Basavappa S, Huang CC, Mangel AW, Lebedev DV, Knauf PA, Ellory JC (1996) Swelling-activated amino acid efflux in the human neuroblastoma cell line CHP-100. J Neurophysiol. 76(2): 764-769.

Basavappa S, Pedersen SF, Jorgensen NK, Ellory JC, Hoffmann EK (1998) Swelling-induced arachidonic acid release via the 85-kDa $cPLA_2$ in human neuroblastoma cells. J Neurophysiol. 79: 1441-1449.

Baslow MH (1999) The existence of molecular water pumps in the nervous system: a review of the evidence. Neurochem Int. 34(1): 77-90.

Bernath S (1991) Calcium-independent release of amino acid neurotransmitters: fact or artifact? Prog Neurobiol. 38(1): 57-91.

Berridge MJ (1998) Neuronal calcium signaling. Neuron. 21(1): 13-26.

Bothwell JH, Rae C, Dixon RM, Styles P, Bhakoo KK (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(6): 613-635.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248-254.

Brose N, Petrenko AG, Sudhof TC, Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science. 256(5059):1021-1025.

Buu NT, Debinski W (1990) Vesicular and ganglionic norepinephrine in the rat: progressive increase with age. J Neurochem. 54(2): 620-626.

Cardin V, Lezama R, Torres-Marquez ME, 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.

Cardin V, Peña-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.

Castejon OJ, Arismendi GJ (2003) Morphological changes of dendrites in the human edematous cerebral cortex. A transmission electron microscopic study. J Submicrosc Cytol Pathol. 35(4): 395-413.

Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. Ann Rev Cell Dev Biol. 16: 521-555.

Chapman ER, Hanson PI, An S, Jahn R (1995) Ca^{2+} regulates the interaction between synaptotagmin and syntaxin 1. J Biol Chem. 270(40): 23667-23671.

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(2): 203-216.

Chou CY, Shen MR, Hsu KS, Huang HY, Lin HC (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.

Cooke KR, Macknigth AD (1984) Effects of medium acetate on cellular volume in rabbit renal cortical slices. J Physiol. 349: 135-156.

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.

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.

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. 523: 291-299.

DiAntonio A, Schwarz TL (1994) The effect on synaptic physiology of synaptotagmin mutations in Drosophila. Neuron. 12(4):909-920.

Ding M, Eliasson C, Betsholtz C, Hamberger A, Pekny M (1998) Altered taurine release following hypotonic stress in astrocytes from mice deficient for GFAP and vimentin. Brain Res Mol Brain Res. 62: 77-81.

Dudek FE, Obenaus A, Tasker JG (1990) Osmolality-induced changes in extracellular volume alter epileptiform bursts independent of chemical synapses in the rat: importance of non-synaptic mechanisms in hippocampal epileptogenesis. Neurosci Lett. 120(2): 267-270.

Dunkelberg JC, Feranchak AP, Fitz JG (2001) Liver cell volume regulation: size matters. Hepatology 33(6): 1349-1352.

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.

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

Fajuk D, Aitken PG, Somjen GG, Turner DA (2002) Two different mechanisms underlie reversible, intrinsic optical signals in rat hippocampal slices. J Neurophysiol. 87: 1924-1937.

Fall PJ (2000) Hyponatremia and hypernatremia. A systematic approach to causes and their correction. Postgrad Med. 107(5): 175-82.

Fan SF, Christ GJ, Melman A, Brink PR (1999) A stretch-activated Cl^- channel in human corpus cavernosal myocytes. Int J Impot Res. 11: 1-7.

Franco R (2004) Control del volumen celular en condiciones anisosmóticas: vías de movilización de osmolitos y cascadas de señalización involucradas. Tesis. Doctorado en Ciencias Biomédicas, Instituto de Fisiología Celular, UNAM. pp 1-171.

Franco R, Lezama R, Ordaz B, Pasantes-Morales H (2004) Epidermal growth factor receptor is activated by hyposmolarity and is an early signal modulating osmolyte efflux pathway in Swiss 3T3 fibroblasts. Pflugers Arch. 447(6): 830-839.

Franco R, Quesada O, Pasantes-Morales H (2000) Efflux of osmolyte amino acids during isovolumic regulation in hippocampal slices. J Neurosci Res. 61(6): 701-711.

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.

Fry DW (2003) Mechanism of action of erbB tyrosine kinase inhibitors. Exp Cell Res. 284(1): 131-9.

Fujita Y, Sasaki T, Fukui K, Kotani H, Kimura T, Hata Y, Sudhof TC, Scheller RH, Takai Y (1996) Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. J Biol Chem. 271: 7265-7268.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Sudhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell. 79(4): 717-727.

Ghijsen WE, Leenders AG, Lopes da Silva FH (2003) Regulation of vesicle traffic and neurotransmitter release in isolated nerve terminals. Neurochem Res. 28(10):1443-1452.

Goda Y, Sudhof TC (1997) Calcium regulation of neurotransmitter release: reliably unreliable? Curr Opin Cell Biol. 9(4): 513-8.

Grant AC, Thomson J, Zammit VA, Shennan DB (2000) Volume-sensitive amino acid efflux from a pancreatic beta-cell line. Mol Cell Endocrinol. 162(1-2): 203-210.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem. 206: 3440-3450.

Guarner V (1996) The hydro-mineral equilibrium in the superior adult mammalian. Capitulo 8, en: Ontogeny and phylogeny of the functions. Información Profesional Especializada, S.A de C.V. pp 125-137.

Guatimosim C, Romano-Silva MA, Cruz JS, Beirao PS, Kalapothakis E, Morales-Santos T, Cordeiro MN, Diniz CR, Gomez MV, Prado MA (1997) A toxin from the spider *Phoneutria nigriventer* that blocks calcium channels coupled to exocytosis. Br J Pharmacol. 122: 591-597.

Hajos F (1975) An improved method for the preparation of synaptosomal fraction in high purity. Brain Res. 93: 485-489.

Hallows KR, Knuaf PA (1994) Regulatory volume decrease in HL-60 cells: importance of rapid changes in permeability of Cl^- and organic solutes. Am J Physiol. 267(4 Pt 1): C1045-C1056.

Hansson E, Muyderman H, Leonova J, Allansson L, Sinclair J, Blomstrand F, Thorlin T, Nilsson M, Ronnback L (2000) Astroglia and glutamate in physiology and pathology: aspects on glutamate transport, glutamate-induced cell swelling and gap-junction communication. Neurochem Int. 37: 317-329.

Hartung TK, Schofield E, Short AI, Parr MJ, Henry JA (2002) Hyponatraemic states following 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') ingestion. QJM 95(7): 431-437.

Haussinger D (1996) Regulation and functional significance of liver cell volume. Prog Liver Dis. 14: 29-53.

Haussinger D, Kurz AK, Wettstein M, Graf D, Vom Dhal S, Schliess F (2003) Involvement of integrin and Src in tauroursodeoxycholate-induced and swelling-induced choleresis. Gastroenterology. 124: 1476-1487.

Haussinger D, Schliess F (1999) Osmotic induction of signaling cascades: role in regulation of cell function. Biochem Biophys Res Com. 255: 551-555.

Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, Südhof TC, Niemann H (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. EMBO J. 13:5051-5061.

Haynes JK, Goldstein L (1993) Volume-regulatory amino acid transport in erythrocytes of the little skate, Raja erinacea. Am J Physiol. 265(1 Pt 2): R173-R179.

Heidelberger R, Heinemann C, Neher E, Matthews G (1994) Calcium dependence of the rate of exocytosis in a synaptic terminal. Nature. 371(6497):513-515.

Hille B, Billiard J, Babcock DF, Nguyen T, Koh DS (1999) Stimulation of exocytosis without a calcium signal. J Physiol. 520 (Pt 1) :23-31.

Hochman DW, Baraban SC, Owens JW, Schwartzkroin PA (1995) Dissociation of synchronization and excitability in furosemide blockade of epileptiform activity. Science. 270(5233): 99-102.

Hoffman EK (2000) Intracellular signalling involved in volume regulatory decrease. Cell Physiol Biochem. 10: 273-288.

Huang CC, Chang CB, Liu JY, Basavappa S, Lim PH (2001) Effects of calcium, calmodulin, protein kinase C and protein tyrosin kinases on volume-activated taurine efflux in human erythroleukemia cells J Cell Physiol 189: 316-322.

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(5): 2349-2359.

Huxtable RJ (1992) Physiological actions of taurine. Physiol Rev. 72: 101-163.

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(11): 901-904.

Jacobsen JG, Smith LH (1968) Biochemistry and physiology of taurine derivatives. Physiol Rev. 48: 424-511.

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

Junankar PR, Kirk K (2000) Organic osmolyte channels: a comparative view. Cell Physiol Biochem. 10: 355-360.

Kenneth RHP (1994) Principles of cell volume regulation. En: Cellular and molecular physiology of cell volume regulation. Strange K (editores).

Kim D, Fu C (1993) Activation of a nonselective cation channel by swelling in atrial cells. J Membr Biol. 135(1): 27-37.

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(5): 1583-1591.

Kirk K (1997) Topical review: Swelling-activated organic osmolyte channels. J Membr Biol. 158: 1-16.

Kultz D, Burg M (1998) Evolution of osmotic stress signaling via MAP kinase cascades J Exp Biol. 201: 3015-3021.

Lambert IH, Hoffman EK (1994) Cell swelling activates separate taurine and chloride channels in Ehrlich mouse ascites tumor cells. J Membr biol. 142: 289-298.

Lang F, Busch GL, Ritter M, Völkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev. 78(1): 247-306.

Lang F, Busch GL, Völkl H (1998c) The diversity of volume regulatory mechanism. Cell Physiol Biochem. 8: 1-45.

Lang F, Lepple-Wienhues A, Paulmichl M, Szabo I, Siemen D, Gulbins E (1998b) Ion channels, cell volume, and apoptotic cell death. Cell Physiol Biochem. 8(6): 285-92.

Lang F, Ritter M, Gamper N, Huber S, Fillon S, Tenneur V, Lepple-Wienhues A, Szabo I, Gulbins E (2000) Cell volume in the regulation of cell proliferation and apoptotic cell death. Cell Physiol Biochem. 10(5-6): 417-428.

Law RO (1989) Effects of pregnancy on the contents of water, taurine, and total amino nitrogen in rat cerebral cortex. J Neurochem. 53(1): 300-302.

Law RO (1994) Effects of extracellular bicarbonate ions and pH on volume-regulatory taurine efflux from rat cerebral cortical slices in vitro: evidence for separate neutral and anionic transport mechanisms. Biochim Biophys Acta. 1224(3): 377-383.

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(1-2): 34-42.

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

Levitzki A, Bohmer FD (1998) Altered efficacy and selectivity of tyrosine kinase inhibitors of the activated states of protein tyrosine kinases. Anticancer Drug Des. 13: 731-734.

Levitzki A, Gazit A (1995) Tyrosine kinase inhibition: an approach to drug development. Science. 267(5205): 1782-1788.

Lien YH, Shapiro JI, Chan L (1991) Study of brain electrolytes and organic osmolytes during correction of chronic hyponatremia. Implications for the pathogenesis of central pontine myelinolysis. J Clin Invest. 88(1): 303-9.

Liu K-F, Li F, Tatlisumak T, Garcia JH, Sotak CH, Fisher M, Fenstermacher JD (2001) Regional variations in the apparent diffusion coefficient and the intracellular distribution of water in rat brain during acute focal ischemia. Stroke. 32: 1897-1905.

Liu X, Zhang MIN, Peterson LB, O'Neil RG (2003) Osmomechanical stress selectively regulates translocation of protein kinase C isoforms. FEBS Lett. 538: 101-106.

Loo DD, Wright EM, Zeuthen T (2002) Water pumps. J Physiol. 542(Pt 1): 53-60.

Majewski H, Iannazzo L (1998) Protein kinase C: a physiological mediator of enhanced transmitter output. Prog Neurobiol. 55(5): 463-475.

Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P, Verkman AS (2000) Aquaporin-4 deletion in mice reduces brain edema after water intoxication and ischemic stroke. Nat Med. 6: 159-163.

Marunaka Y, Shintani Y, Downey GP, Niisato N (1997) Activation of Na^+-permeant cation channel by stretch and cyclic AMP-dependent phosphorylation in renal epithelial A6 cells. J Gen Physiol. 110(3): 327-36.

Massieu L, Montiel T, Robles G, Quesada O (2004) Brain amino acids during hyponatremia in vivo: clinical observations and experimental studies. Neurochem Res. 29(1): 73-81.

McCleskey EW, Fox AP, Feldman DH, Cruz LJ, Olivera BM (1987) ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Proc Natl Acad Sci. USA 84: 4327-4331.

Melton JE, Patlak CS, Pettigrew KD, Cserr HF (1987) Volume regulatory loss of Na, Cl, and K from rat brain during acute hyponatremia. Am J Physiol. 252(4 Pt 2): F661-F669.

Mintz IM, Adams ME, Bean BP (1992) P-type calcium channels in rat central and peripheral neurons. Neuron. 9: 85-95.

Mitchell CH, Zhang JJ, Wang L, Jacob TCJ (1997) Volume-sensitive chloride current in pigmented ciliary epithelial cells: role of phospholipases. Am J Physiol. 272: C212-C222.

Miyata S, Matsushima O, Hatton GI (1997) Taurine in rat posterior pituitary: localization in astrocytes and selective release by hypoosmotic stimulation. J Comp Neurol. 381(4): 513-23.

Mongin AA, Cai Z, Kimelberg HK (1999) Volume-dependent taurine release from cultured astrocytes requires permissive $[Ca^{2+}]_i$ and calmodulin. Am J Physiol. Cell Physiol. 277: C823-C832.

Mongin AA, Reddi JM, Charniga C, Kimelberg HK (1999b) [3H]taurine and D-[3H]aspartate release from cultured astrocytes are differently regulated by tyrosine kinases. Am J Physiol. 276: C1226-C1230.

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.

Morán J, Hernández-Pech X, Merchant-Larios H, Pasantes-Morales H (2000) Release of taurine in apoptotic cerebellar granule neurons in culture. Pflugers Arch 439(3):271-277.

Morgan JM (1984) Osmoregulation and water stress in higher plants. Ann Rev Plant Physiol. 35: 299-319.

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

Nagelhus EA, Lehmann A, Ottersen OP (1993) Neuronal-glial exchange of taurine during hypo-osmotic stress: a combined inmunocytochemical and biochemical analysis in rat cerebral cortex. Neuroscience. 54: 615-631.

Najvirtova M, Greer SE, Greer MA, Baqi L, Benicky J, Strbak V (2003) Cell volume induced hormone secretion: studies on signal transduction and specificity. Cell Physiol Biochem. 13(2): 113-22. Neuroscientist. 8(3): 254-267.

Nicholls DG (1998) Presynaptic modulation of glutamate release. Prog Brain Res. 116: 15-22.

Niemann H, Blasi J, Jahn R (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. Trends Cell Biol. 4(5): 179-85.

Nilius B, Eggermont J, Droogmans G. The endothelial volume-regulated anion channel, VRAC. Cell Physiol Biochem.10:313-320.

Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9(7): 484-496

O'Neil WC (1999) Physiological significance of volume-regulatory transporters. Am J Physiol. 276(5 Pt 1): C995-C1011.

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.

Ordaz B, Vaca L, Franco R, Pasantes-Morales H (2004) Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K^+ channels. Am J Physiol Cell Physiol. 286(6): C1399-C1409.

Papadopoulos MC, Krishna S, Verkman AS (2002) Aquaporin water channels and brain edema. Mt Sinai J Med. 69: 242-248.

Pasantes-Morales H (1996) Volume regulation in brain cells: cellular and molecular mechanisms. Metab Brain Dis. 11(3): 187-204.

Pasantes-Morales H, Franco R (2002) Influence of protein tyrosine kinases on cell volume change-induced taurine release. Cerebellum. 1: 103-109.

Pasantes-Morales H, García J, Sánchez-Olea R (1991) Hyposmolarity-sensitive release of taurine and free amino acids from human lymphocytes. Biochem Pharmacol. 41: 303-307.

Pasantes-Morales H, Lopez I, Ysunza A (1995) Taurine content in breast milk of Mexican women from urban and rural areas. Arch Med Res. 26(1): 47-52.

Pasantes-Morales H, Maar T, Morán J (1993) Cell volume regulation in cultured cerebellar granule neurons. J Neurosci Res. 34(2): 219-224.

Pasantes-Morales H, Murray LA, Lilja L, Morán J (1994) Regulatory volume decrease in cultured astrocytes. I. Potassium- and chloride-activated permeability. Am J Physiol. 266: C165-C171.

Pasantes-Morales H, Ochoa de la Paz LD, Sepulveda J, Quesada O (1999) Amino acids as osmolytes in the retina. Neurochem Res. 24: 1339-1346.

Pasantes-Morales H, Pena Segura C, Garcia O, Morales Mulia MM, Sanchez Olea R, Moran J (1996) Characterization of the volume-activated taurine pathway in cultured cerebellar granule neurons. Adv Exp Med Biol. 403: 393-400.

Pasantes-Morales H, Schousboe A (1989) Release of taurine from astrocytes during potassium-evoked swelling. Glia. 2(1): 45-50.

Pasantes-Morales, Franco R, Ordaz B, Ochoa LD (2002) Mechanisms counteracting swelling in brain cells during hyponatremia. Arch Med Res. 33(3): 237-244.

Pedersen SF, Beisner KH, Hougaard C, Willumsen BM, Lambert IH, Hoffman EK (2002) Rho family GTP binding proteins are involved in the regulatory volume decrease process in NIH3T3 mouse fibroblasts. J Physiol. 541: 779-796.

Perlman DF, Goldstein L (1999) Organic osmolyte channels in cell volume regulation in vertebrates. J Exp Zool. 283: 725-733.

Raiteri L, Stigliani S, Zedda L, Raiteri M, Bonanno G (2002) Multiple mechanisms of transmitter release evoked by "pathologically" elevated extracellular [K+]: involvement of transporter reversal and mitochondrial calcium. J Neurochem. 80(4): 706-714.

Randall A, Tsien RW (1995) Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. J Neurosci. 15: 2995-3012.

Rensing N, Ouyang Y, Yang X-F, Yamada KA, Rothman SM, Wong M (2005) In vivo imaging of dendritic spines during electrographic seizures. Ann Neurol. 58: 888-898.

Rettig J, Heinemann C, Ashery U, Sheng ZH, Yokoyama CT, Catterall WA, Neher E (1997) Alteration of Ca^{2+} dependence of neurotransmitter release by disruption of Ca^{2+} channel/syntaxin interaction. J Neurosci. 17(17): 6647-6656.

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP, Catterall WA (1996) Isoform-specific interaction of the alpha1A subunits of brain Ca^{2+} channels with the presynaptic proteins syntaxin and SNAP-25. Proc Natl Acad Sci. USA 93(14):7363-7368.

Rizoli SB, Rotstein OD, Parodo J, Phillips MJ, Kapus A (2000) Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling. Am J Physiol Cell Physiol. 279: C619-C633.

Roman RM, Bodily KO, Wang Y, Reymond JR, Fitz JG (1998) Activation of protein kinase C alpha couples cell volume to membrane Cl^- permeability in HTC hepatoma and Mz-ChA-1 cholangiocarcinoma cells. Hepatology. 28: 1073-1080.

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

Rosen AS, Andrew RD (1990) Osmotic effects upon excitability in rat neocortical slices. Neuroscience. 38: 579-590.

Rosenberg GA (1999) Ischemic brain edema. Prog Cardiovasc Dis. 402(3): 209-216.

Rutledge EM, Aschner M, Kimelberg HK (1998) Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. Am J Physiol. 274(6 Pt 1): C1511-1520.

Sánchez-Olea R, Fuller C, Benos D, Pasantes-Morales H (1995b) Volume-associated osmolyte fluxes in cell lines with or without the anion exchanger. Am J Physiol. 269(5 Pt 1): C1280-1286.

Sánchez-Olea R, Morales M, García O y 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.

Sánchez-Olea R, Morales-Mulia M, Morán J, Pasantes-Morales H (1995) Inhibition by polyunsaturated fatty acids of regulatory volume decrease and osmolyte fluxes in astrocytes in culture. Am J Physiol. 269: C96-C102.

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

Sánchez-Olea R, Pasantes-Morales H, Lazaro A, Cereijido M (1991b) Osmolarity-sensitive release of free amino acids from cultured kidney cells (MDCK). J Membr Biol. 121(1): 1-9.

Sánchez-Olea R, Peña C, Morán 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.

Scepek S, Coorssen JR, Lindau M (1998) Fusion pore expansion in horse eosinophils is modulated by Ca2+ and protein kinase C via distinct mechanisms. EMBO J. 17(15): 4340-4345.

Schiavo G, Stenbeck G, Rothman JE, Sollner TH (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. Proc Natl Acad Sci. USA. 94(3): 997-1001.

Schousboe A, Sánchez-Olea R, Morán J, 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.

Seagar M, Takahashi M (1998) Interactions between presynaptic calcium channels and proteins implicated in synaptic vesicle trafficking and exocytosis. J Bioenerg Biomembr. 30(4): 347-56.

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

Shen M-R, Chou C-Y, Wu M-L, Huang K-E (1998) Differential osmosensing signalling pathways and G-protein involvement in human cervical cells with different tumour potential. Cell Signal. 10: 113-120.

Sheng Z-H, Retting J, Takahashi M, Catterall WA (1994) Identification of a syntaxin-binding site on N-type calcium channels. Neuron. 13: 1303-1313.

Shennan DB, McNeillie SA (1995) Volume-activated amino acid efflux from term human placental tissue: stimulation of efflux via a pathway sensitive to anion transport inhibitors. Placenta. 16(3): 297-308.

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.

Shuba YM, Prevarskaya N, Lemonnier L, Van Coppenolle F, Kostyuk PG, Mauroy b, Skryma R (2000) Volume-regulated chloride conductance in the LNCaP human prostate cancer cell line. Am J Physiol. 279: C1144-C1154.

Somjen GG (2002) Ion regulation in the brain: implications for pathophysiology.

Song D, O'Regan MH, Phillis JW (1998) Amino acid release during volume regulation by cardiac cells: cellular mechanisms. Eur J Pharmacol. 341(2-3): 273-280.

Sorota S (1995) Tyrosine protein kinase inhibitors prevent activation of cardiac swelling-induced chloride current. Pflugers Arch. 431: 178-185.

Stegen C, Matskevich I, Wagner CA, Paulmichl M, Lang F, Broer S (2000) Swelling-induced taurine release without chloride channel activity in Xenopus

laevis oocytes expressing anion channels and transporters. Biochim Biophys Acta. 1467(1): 91-100.

Sterns RH, Baer J, Ebersol S, Thomas D, Lohr JW, Kamm DE (1993) Organic osmolytes in acute hyponatremia. Am J Physiol. 264(5 Pt 2): F833-F886.

Stevens CF, Sullivan JM (1998) Regulation of the readily releasable vesicle pool by protein kinase C. Neuron. 21(4): 885-893.

Strbak V, Greer MA (2000) Regulation of hormone secretion by acute cell volume changes: Ca^{2+}-independent hormone secretion. Cell Physiol Biochem 10(5-6): 393-402.

Stutzin A, Eguiguren AL, Cid LP y Sepúlveda FV (1997) Modulation by extracelular Cl of volume-activated organic osmolyte and halide permeabilities in HeLa cells. Am J Physiol. 273: C999-C1007.

Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature. 375(6533): 645-53.

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.

Szule JA, Coorssen JR (2003) Revisiting the role of SNAREs in exocytosis and membrane fusion. Biochim Biophys Acta. 1641(2-3): 121-135.

Tahara M, Coorssen JR, Timmers K, Blank PS, Whalley T, Scheller R, Zimmerberg J (1998) Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion. J Biol Chem. 273(50): 33667-33673.

Thomson AM (2000) Facilitation, augmentation and potentiation at central synapses. Trends Neurosci. 23(7): 305-312.

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

Thoroed SM, Lauritzen L, Lambert IH, Hansen HS, Hoffman EK (1997) Cell swelling activates phospholipase A_2 in Ehlrich ascites tumor cells. J Membr Biol. 160: 47-58.

Thurston HJ, Hauhart RE, Nelson JS (1987) Adaptative decreases in amino acids (taurine in particular) creatine and electrolytes, prevent cerebral edema in chronically hyponatremic mice: rapid correction (experimental model of central pontine myelinolysis) causes dehydration and shrinkage of brain. Metab Brain Dis. 2: 223-241.

Thurston JH, Hauhart RE, Dirgo JA (1980) Taurine: a role in osmotic regulation in mammalian brain and possible clinical significance. Life Sci. 26: 1561-1568.

Tilly BC, Edixhoven MJ, Tertoolen LG, Morri N, Saitoh Y, Narumiya S, de Jonge HR (1996b) Activation of the osmosensitive chloride conductance involves p21rho and is accompanied by a trasient reorganization of the F-actin cytoskeleton. Mol Biol Cell. 7: 1419-1427.

Tilly BC, Gaestel M, Engel K, Edixhoven MJ, De Jonge HR (1996) Hyposmotic cell swelling activates de p38 MAP kinase signaling cascade. FEBS Lett. 395: 133-136.

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

Tosteson DC, Hoffman JF (1960) Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J Gen Physiol. 44: 169-194.

Trachtman H (1992) Cell volume regulation: a review of cerebral adaptive mechanisms and implications for clinical treatment of osmolal disturbances: II. Pediatr Nephrol. 6(1): 104-112.

Trachtman H, Futterweit S, Hammer E, Siegel TW, Oates P (1991) The role of polyols in cerebral cell volume regulation in hypernatremic and hyponatremic states. Life Sci. 49(9):677-88.

Tuz K, Ordaz B, Vaca L, Quesada O, Pasantes-Morales H (2001) Isovolumetric regulation mechanisms in cultured cerebellar granule neurons. J Neurochem. 79(1): 143-151.

Vajda Z, Promeneur D, Doczi T, Sulyok E, Frokiaer J, Ottersen OP, Nielsen S (2000) Increased aquaporin-4 inmunoreactivity in rat brain in response to systemic hyponatremia. Biochem Biophys Res Commun. 270: 495-503.

van der Wijk T, Tomassen SF, de Jonge HR, Tilly BC (2000) Signalling mechanisms involved in volume regulation of intestinal epithelial cells. Cell Physiol Biochem. 10(5-6): 289-296.

Vaughan PF, Walker JH, Peers C (1998) The regulation of neurotransmitter secretion by protein kinase C. Mol Neurobiol. 18(2): 125-155.

Verbalis JG, Gullans SR (1991) Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res. 567(2): 274-82.

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. 506: 341-352.

Von Weikersthal SF, Hickman ME, Hladsky SB, Barrand MA (1997) Hypotonicity-induced changes in anion permeability of cultured rat brain endothelial cells. Biochim Biophys Acta. 1325: 99-107.

Vyas S, Bradford HF (1987) Co-release of acetylcholine, glutamate and taurine from synaptosomes of Torpedo electric organ. Neurosci Lett. 82: 58-64.

Wei H, Mei YA, Sun JT, Zhon HQ, Zhang ZH (2003) Regulation of swelling-activated chloride channels in embryonic chick heart cell. Cell Res. 13: 21-28.

Welsh DG, Nelson MT, Eckman DM, Brayden JE (2000) Swelling-activated cation channels mediate depolarization of rat cerebrovascular smooth muscle by hyposmolarity and intravascular pressure. J Physiol. 527(Pt 1): 139-48.

Whittaker VP, Barker LA (1972) The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. En: Methods of neurochemistry. Fried R (editor). Marcel Dekker, NY.

Widmann C, Gibson S, Jarpe M, Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 79: 143-180.

Wiser O, Tobi D, Trus M, Atlas D (1997) Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel. FEBS Lett. 404: 203-207.

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

Yang F, He XP, Russell J, Lu B (2003) Ca2+ influx-independent synaptic potentiation mediated by mitochondrial Na(+)-Ca2+ exchanger and protein kinase C. J Cell Biol. 163(3): 511-523.

Yawo H (1999) Two components of transmitter release from the chick ciliary presynaptic terminal and their regulation by protein kinase C. J Physiol. 516 (Pt 2): 461-470.

Zimmerberg J, Blank PS, Kolosova I, Cho MS, Tahara M, Coorssen JR (2000) A stage-specific preparation to study the Ca(2+)-triggered fusion steps of exocytosis: rationale and perspectives. Biochimie. 82(4): 303-314.

APÉNDICE

OTROS ARTÍCULOS PUBLICADOS DURANTE EL DOCTORADO

ARTÍCULO 1

Isovolumetric regulation mechanisms in cultured cerebellar granule neurons.

ARTÍCULO 2

Osmolytes and mechanisms involved in regulatory volume decrease under conditions of sudden or gradual osmolarity decrease.

ARTÍCULO 3

Mechanisms of cell volume regulation in hypo-osmolarity.

ARTÍCULO 4

Volume changes in neurons: hyperexcitability and neuronal death.

Journal of Neurochemistry, 2001, **79**, 143–151

Isovolumetric regulation mechanisms in cultured cerebellar granule neurons

Karina Tuz,* Benito Ordaz,* Luis Vaca,† Octavio Quesada* and Herminia Pasantes-Morales*

*Departments of *Biophysics and †Cell Biology, Institute of Cell Physiology, National University of Mexico, Mexico City, Mexico*

Abstract

Cultured cerebellar granule neurons exposed to gradual reductions in osmolarity (− 1.8 mOsm/min) maintained constant volume up to − 50% external osmolarity (π_o), showing the occurrence of isovolumetric regulation (IVR). Amino acids, Cl^-, and K^+ contributed at different phases of IVR, with early efflux threshold for [^{3}H]taurine, D-[^{3}H]aspartate (as marker for glutamate) of π_o − 2% and − 19%, respectively, and more delayed thresholds of − 30% for [^{3}H]glycine and − 25% and − 29%, respectively, for Cl^- (^{125}I) and K^+ (^{86}Rb). Taurine seems preferentially involved in IVR, showing the lowest threshold, the highest efflux rate (five-fold over other amino acids) and the largest cell content decrease. Taurine and Cl^- efflux were abolished by niflumic acid and ^{86}Rb by 15 mM Ba^{2+}. Niflumic acid essentially prevented IVR in all ranges of π_o. Cl^--free medium impaired IVR when π_o decreased to − 24% and Ba^{2+} blocked it only at a late phase of − 30% π_o. These results indicate that in cerebellar granule neurons: (i) IVR is an active process of volume regulation accomplished by efflux of intracellular osmolytes; (ii) the volume regulation operating at small changes of π_o is fully accounted for by mechanisms sensitive to niflumic acid, with contributions of both Cl^- and amino acids, particularly taurine; (iii) Cl^- contribution to IVR is delayed with respect to other niflumic acid-sensitive osmolyte fluxes (osmolarity threshold of − 25% π_o); and (iv), K^+ fluxes do not contribute to IVR until a late phase ($< -$ 30% π_o).

Keywords: swelling, taurine, volume regulation.

J. Neurochem. (2001) **79**, 143–151.

Cell volume 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 normal cell functioning (Lang *et al*. 1998). Osmolyte fluxes activated by hypo-osmotic swelling and accomplishing the regulatory volume decrease (RVD) have been extensively examined in a variety of cells, including brain cells (Nilius *et al*. 1997; Pasantes-Morales and Schousboe 1997). However, in most studies, cells are exposed to abrupt and large decreases in external osmolarity that, although useful in understanding some basic features of volume regulation, only rarely occur, even in pathological situations. Less is known about the osmolyte movements after cell swelling in iso-osmotic conditions or after small and gradual reductions in external osmolarity, an approach more likely reproducing the changes in osmolarity in pathological conditions such as hyponatremia and ischemia. An early report in renal cells (Lohr and Grantham 1986) showed unchanged volume in cells gradually exposed to small decreases in external osmolarity, and this response was named 'isovolumetric regulation' (IVR). This term may appear somewhat misleading, but Lohr and Grantham emphasized the regulatory nature of the process by experiments showing that cells gradually exposed to the hypo-osmotic gradient shrink when suddenly returned to iso-osmotic conditions (Lohr and Grantham 1986). IVR in the hypo-osmotic range has been found so far in only two types of renal cells (Lohr and Grantham 1986; Lohr 1990; Van Driessche *et al*. 1997), and with partial efficiency in glioma C6 cells and myocytes (Lohr and Yohe 2000; Souza *et al*. 2000). The osmolytes involved in this process and the nature of the translocation pathways activated are not well known. In the renal epithelial cell line A6 and in myocytes, K^+ appears to play a predominant role in IVR, while taurine also

Received June 8, 2001; revised manuscript received July 16; accepted July 19, 2001.

Address correspondence and reprint requests to Dr Herminia Pasantes-Morales, Instituto de Fisiología Celular, UNAM, Ap. Postal 70-253, México, 04510, D.F., México.

E-mail: hpasante@ifisiol.unam.mx

Abbreviations used: IVR, isovolumetric regulation; RVD, regulatory volume decrease.

contributes in myocytes (Van Driessche *et al*. 1997; Souza *et al*. 2000). IVR is absent in trout erythrocytes, which respond to osmolarity gradients neither by cell volume recovery nor by releasing K^+ or taurine in sufficient amounts (Godart *et al*. 1999). Clearly, studies aimed to characterize IVR in different cell types are necessary to determine, first, if this is a widespread mechanism for cell volume control and, second, whether its features are cell specific or respond to basic mechanisms common to all cell types. The mechanisms subserving IVR in brain cells are not well known and it is not unlikely that in these cells IVR exhibits features different from those in non excitable cells. IVR associated with amino acids, but not with K^+ efflux, has been described in hippocampal slices (Franco *et al*. 2000), but in such integrated preparation, in comparison with a cell monolayer, it is difficult to identify the cell type(s) involved, as well as to look in detail to the activated mechanisms.

In the present work in cultured cerebellar granule neurons, we examined the occurrence of IVR and the involvement of amino acids, K^+, and Cl^- in this process. It is known that these cells exhibit RVD (Pasantes-Morales *et al*. 1994), accomplished by the efflux of inorganic ions (K^+ and Cl^-) and organic osmolytes, preferentially amino acids (Sánchez-Olea *et al*. 1996; Morales-Mulia *et al*. 1998).

Materials and methods

Cell cultures

Primary cell cultures of cerebellar granule neurons were prepared as previously described (Morán and Patel 1989). Briefly, the dissociated cell suspensions from 8-day-old rat cerebellum were plated at a density of 265×10^3 cell/cm^2 in plastic dishes, previously coated with poly-L-lysine (5 μg/mL). The culture medium consisted of basal Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. Culture dishes were kept at 37°C in a humidified atmosphere (5% CO_2/95% air). The enrichment of cultures with neurons was assessed by counting immunostained positives cells to a neuron-specific enolase polyclonal antibody or to an antiglial fibrillar acidic protein antibody (Dakopatts, Carpinteria, CA, USA), giving a ratio of 95 : 5 between neurons and GFAP-positive cells. For measurements of cell volume, cerebellar granule neurons were plated at lower density (200×10^3 cell/cm^2) and growth on glass coverslips. Otherwise, conditions were the same as described above.

Amino acids, ^{125}I and ^{86}Rb efflux

Cultured cells were incubated during 60 min in culture medium containing the labeled amino acids: D-[^{3}H]aspartate (0.5 μCi/mL), [^{3}H]taurine (3.0 μCi/mL), [^{3}H]glycine (3.0 μCi/mL) or [^{3}H]alanine (5.0 μCi/mL), or ^{125}I (5.0 μCi/mL), as a tracer for Cl^-, or ^{86}Rb (1.0 μCi/mL), as a tracer for K^+. After the loading period, culture dishes were superfused (2 mL/min) with warmed (37°C), iso-osmotic medium containing (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, iso-osmotic, pH 7.4). Hypo-osmotic solutions [210 mOsm/L (H-30%) and 150 mOsm/L (H-50%)], were prepared by reducing [NaCl] accordingly. Final osmolarities were verified with a freezing-point osmometer (Osmette A, Precision Systems Inc., Natick, MA, USA). After 18 min (washing), a stable baseline efflux was usually attained. From hereafter, samples were collected every minute and after 5–6 min of basal release, the osmolarity of the perfusion medium was linearly reduced at a rate of 1.8 mOsm/min with a gradient-generating system, as that described in detail by Lohr and Grantham (1986) and 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 iso-osmotic medium an the second with the same volume of H-50% medium. Media were kept at 39°C placing the cylinders on a temperature-controlled hot plate with stirring. Perfusion medium was pulled from the first container with a polystaltic pump, allowing the H-50% medium to begin to enter this cylinder, mixing gradually and continuously with the iso-osmotic medium. In this way, a linear osmotic gradient is produced, which at the end of the experiment (82 min later) reached 150 mOsm/L (50% hypo-osmotic). The gradient linearity was verified in a freezing-point osmometer.

Labeled amino acid, ^{125}I, and ^{86}Rb fluxes were calculated as efflux rate constants, i.e. the amount of radioactivity released in any given fraction, divided by the total amount of label present in cells at that moment. Efflux curves from cells exposed to iso-osmotic solution or to the hypo-osmotic gradient are shown in all cases.

Determination of K^+ content

After exposing cell cultures to the osmotic gradient, dishes were prepared for potassium content measurement. Cells were scrapped from dishes and suspended in 1 mL of water. Cell suspensions were digested in boiling HCl (0.1 M, 30 min), then filtered and the K^+ content determined by atomic absorption spectrometry (Varian 460). Protein content was measured by the Bradford assay.

Cell volume changes

Changes in cell volume were determined by a spectrofluorometric method, based on that described by Crowe *et al*. (1995) and by Altamirano *et al*. (1998). Cerebellar granule neurons grown on glass coverslips were used for these determinations. Cell cultures were incubated in iso-osmotic medium (above detailed) containing 1 μM calcein-AM (Molecular Probes, Eugene, Oregon, USA) for 15 min at room temperature. After this period, dishes were rinsed twice with fresh medium and a coverslip placed on a superfusion chamber and observed under an epifluorescence inverted microscope (Nikon Diaphot TMD, Japan), attached to a spectrofluorometer (SLM Aminco DMX-1000, Urbana, IL, USA). Excitation wavelength was set at 497 nm. Images were recorded from a small region of the cell (9% of the total area), defined by a pinhole at the image plane, and transferred through a CCD camera (C2400, Hamamatzu, Japan) to a personal computer, where the fluorescence images were analyzed with the BIOLASE Imaging System software (Newton, MA, USA). Cells were exposed to one of two hypo-osmotic stimulation paradigms. In the first one, different coverslips were first superfused with iso-osmotic medium and then exposed to hypo-osmotic media (H-30%) during 15 min. Fluorescence images were collected every 0.5 min until minute 7 and then every 5 min. In the second system, tissues were initially superfused with

iso-osmotic medium and then with increasingly hypo-osmotic medium (− 1.8 mOsm/L/min) until external osmolarity reached 150 mOsm/L (50% hypo-osmotic). Fluorescence images were collected every 10 min. In both paradigms, control coverslips were superfused with iso-osmotic medium. Cell volume changes (V_t/V_0) were computed from calculated changes in relative fluorescence (F_0/F_t), according to the following equation (Crowe *et al*. 1995):

$$[(F_0/F_t) - F_{bkg}]/(1 - F_{bkg}) = V_t/V_0$$

where F_0 is the fluorescence from one cell in iso-osmotic medium, having an osmotic pressure π_0; F_t is the fluorescence of the same cell in a medium of osmotic pressure π_t; F_{bkg} is the background fluorescence (see below); V_0 is the volume of the cell in a medium with an osmotic pressure π_0 and V_t the volume of the cell in a medium with an osmotic pressure π_t. F_{bkg} includes the intrinsic cell fluorescence plus the osmotically insensitive component of the fluorescence in dye-loaded cells. F_{bkg} was calculated as described by Crowe *et al*. (1995), from the *y*-intercept of a plot F_0/F_t versus π_0/π_t, exposing different coverslips during 15 min to media with 450, 390, 300, 210 and 150 mOsm/L (not shown). The numerical value obtained was 0.6. To quantify changes in cell volume due to the exposure to the osmotic gradient, F_0 and F_t were initially determined, measuring at different times the fluorescence of cells superfused with iso-osmotic medium and with the osmotic gradient, respectively. The experimental points were then fitted to a linear regression to correct the drift in the fluorescence signal due to dye leakage and photobleaching. Then, solving the equation for each time point with the corrected data of F_0 and F_t gives V_t/V_0.

Results

Evidence of IVR in cerebellar granule neurons

Figure 1 shows the change in cell volume in neurons exposed to aniso-osmotic conditions (a and b) or to gradual decreases in osmolarity (c). Relative volume changes (V_t/V_0) were obtained from the calculated changes in relative fluorescence (F_0/F_t) as described in Materials and methods. In external osmolarity (π_o) of − 30% and − 50% (210 and 150 mOsm/L, respectively) neurons exhibited the characteristic increase in cell volume which was maximal at 2 min after the stimulus, followed by the regulatory volume

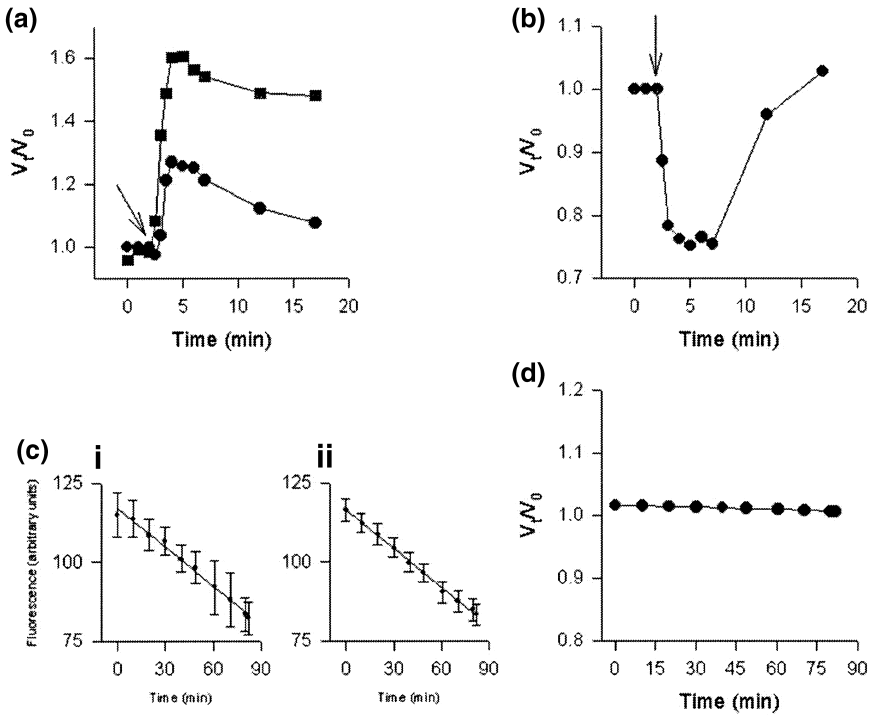

Fig. 1 Cell volume of cerebellar granule neurons exposed to sudden or gradual changes in external osmolarity. Cells were incubated with 1 μM calcein-AM, rinsed and superfused with iso-osmotic medium and subsequently (arrow) with hypo-osmotic or hyperosmotic solutions. Fluorescence measurements were transformed into cell volume data as described in Materials and methods. (a) Relative cell volume (V_t/V_0) change of cells ($n = 11$) exposed to 30% (●) and 50% (■) hypo-osmotic medium. (b) Cell volume change in 30% hyperosmotic medium ($n = 9$ cells). (c) Raw fluorescence data from cells exposed to iso-osmotic medium (ci), or superfused with a continuous hypo-osmotic gradient (cii). Points represents averages ± SE of four (ci) or 14 cells (cii). Lines are the linear regressions ci: $r^2 = 0.991$; (cii): $r^2 = 0.997$. (d) Relative cell volume (V_t/V_0) from cells superfused with a continuous hypo-osmotic gradient. The plot was built with the data shown in (ci) and (cii).

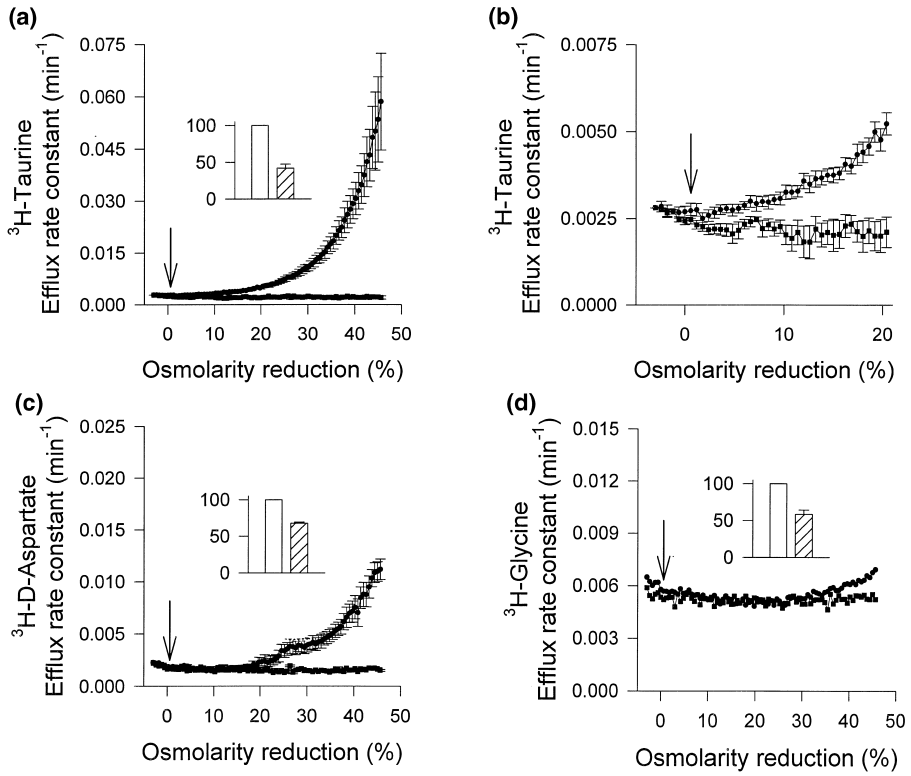

Fig. 2 Amino acid release from cerebellar granule neurons exposed to gradual reductions in external osmolarity. Cells were preloaded with [^{3}H]taurine (a and b), D-[^{3}H]aspartate (c), and [^{3}H]glycine (d), as described in Materials and methods. After loading, cells were washed and superfused (rate 2.0 mL/min) during 6 min with iso-osmotic medium. At the indicated time (arrow) in (●) π_o was decreased at a rate of 1.8 mOsm/min and superfusion continued up to π_o reduction of − 50% (final osmolarity, 150 mOsm/L). In (b) the data from (a) up to π_o 240 mOsm/L (− 20%) are plotted at a magnified scale. Controls (■) were continuously superfused with iso-osmotic medium. Fractions were collected every min. Data are expressed as efflux rate constants (min^{-1}) and are means of 5–10 experiments ± SE represented by vertical bars if exceeded the size of symbols. Insets in each case show the percentage decrease of total radioactivity in cells at the end of the experiment. Empty bars, iso-osmotic condition; dashed bars, hypo-osmotic gradient.

decrease (Fig. 1a) (Pasantes-Morales *et al*. 1994). In hyper-osmotic solutions (350 mOsm/L), the corresponding cell shrinkage was observed (Fig. 1b). These responses, typical of cells exposed to aniso-osmotic conditions, validate the method used to estimate the changes in cell volume in our preparation. Figure 1(c) shows the similar fluorescence change in neurons superfused with iso-osmotic medium (Fig. 1ci) and with an osmotic gradient, allowing π_o changes of − 1.8 mOsm/min (Fig. 1cii). After 82 min, π_o has decreased to 150 mOsm/L. Despite this marked osmolarity reduction, there is no noticeable change in cell volume as calculated from the fluorescence measurements (Fig. 1d). These results document for the first time the occurrence of IVR in cerebellar granule neurons.

Amino acid efflux during IVR in cerebellar granule neurons

Figure 2 shows the efflux from neurons of labeled tracers of taurine, glutamate (D-aspartate) and glycine, in response to gradual decreases in π_o at a rate of 1.8 mOsm/min. The labeled amino acid efflux from cells exposed to the osmotic gradient was compared at each point with the release from cells bathed with iso-osmotic medium. The hypo-osmotic gradient elicited the efflux of taurine, glutamate and glycine (Fig. 2) but not of alanine (not shown). Figure 2(a and b) shows the efflux pattern of taurine release in iso-osmotic medium and in the hypo-osmotic gradient. [^{3}H]Taurine in the iso-osmotic solution slightly decreased with time, whereas that from cells exposed to the osmotic gradient was almost immediately increased, with an early threshold efflux of 293 mOsm/L (− 2% π_o) and continued to increase in parallel with π_o reduction (Figs 2a and b). At the end of the experiment, when π_o is 50% reduced, the [^{3}H]taurine efflux rate was more than 22-fold higher than that in iso-osmotic medium. The D-[^{3}H]aspartate efflux activation threshold was observed at 243 mOsm/L (− 19%) with a maximal five-fold increase over iso-osmotic medium. The release of [^{3}H]glycine was notably more delayed

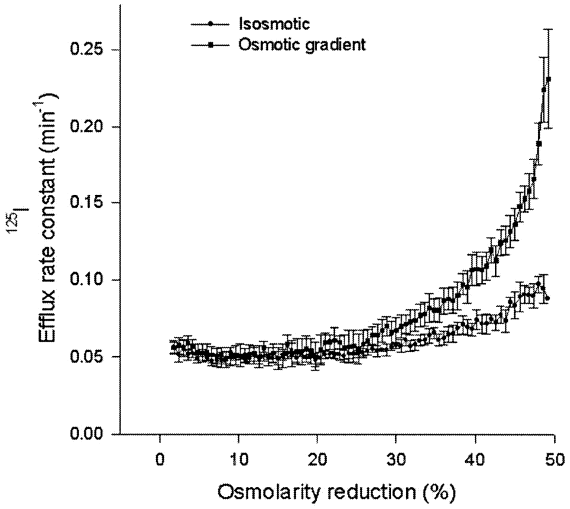

Fig. 3 ^{125}I efflux from cerebellar granule neurons in response to the osmotic gradient. Cells were preloaded with 5 μCi of ^{125}I, and then treated as in Fig. 2. (●) Controls in iso-osmotic medium. (■) Osmotic gradient of − 1.8 mOsm/min. Data are means of eight experiments ± SE.

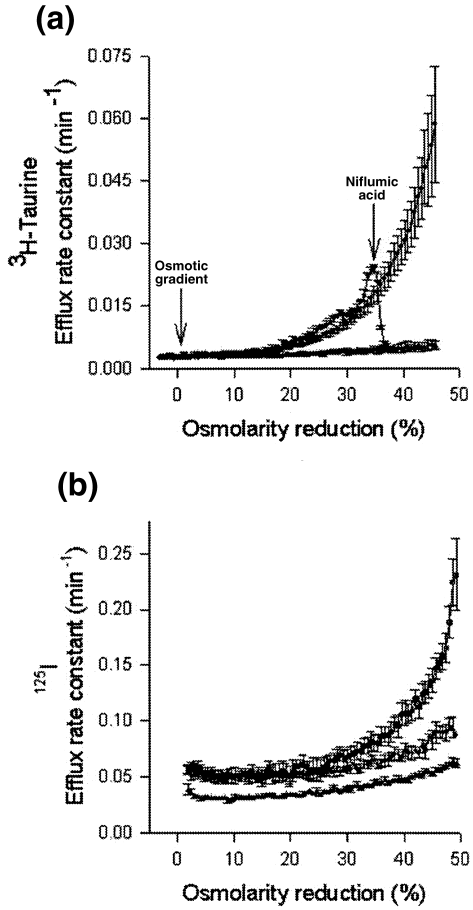

Fig. 4 Effects of niflumic acid on [^{3}H]taurine and ^{125}I efflux during IVR. Loading conditions and basal collection were as in Fig. 2. (a) [^{3}H]Taurine release elicited by the osmotic gradient (●); effect of 600 μM niflumic acid present throughout the experiment (▲) or added (arrow) at π_o (− 35%) (▼). (b) ^{125}I efflux in iso-osmotic (●), osmotic gradient (■) or osmotic gradient containing 600 μM niflumic acid (▲). Data are expressed as rate constants (min^{-1}) and are means ± SE represented by vertical bars if exceeded the size of symbols ($n = 4$).

(210 mOsm/L, − 30%). Throughout all the superfusion period, the efflux rates for [^{3}H]taurine were always higher (more than five-fold) than those for D-[^{3}H]aspartate (Figs 2a and c). About 62% of the labeled taurine pool in cells was released during the whole range of π_o decrease. During the same period, 32% and 42% of D-aspartate and glycine were released, respectively (Fig. 2, insets). The decrease in Na^+ and Cl^- may impair the operation of the amino acid Na^+-dependent carrier, which at least for taurine is also Cl^--dependent. To test this possibility, taurine efflux was measured in solutions where all NaCl was replaced by choline chloride. No change in either efflux rate or threshold were observed under these conditions (results not shown).

Efflux of ^{125}I as marker of Cl^-

Cerebellar granule neurons loaded with ^{125}I exhibited a continuous release of the tracer in iso-osmotic conditions. ^{125}I release from cells superfused with the osmotic gradient was higher than the efflux in iso-osmotic medium when π_o decreased to 225 mOsm/L (− 25%) (Fig. 3). The rate of ^{125}I release increased with decreasing osmolarity, being about three-fold higher than in iso-osmotic medium at the end of the experiment (Fig. 3).

Effect of niflumic acid on osmolyte release

Niflumic acid is a potent blocker of taurine and ^{125}I osmosensitive release from cerebellar granule neurons in models of sudden decrease in osmolarity (Sánchez-Olea *et al*. 1996). This agent also strongly reduced taurine and ^{125}I efflux in the osmotic gradient model. The presence of 600 μM niflumic acid from the beginning of the superfusion prevented the osmolarity-dependent taurine efflux. If the blocker was added once the release has been already activated, an immediate inhibition occurred (Fig. 4a). Niflumic acid markedly decreased the release of ^{125}I in iso-osmotic conditions, and abolished the efflux activated by the osmotic gradient (Fig. 4b).

^{86}Rb efflux and changes in intracellular K^+ content during IVR

Potassium, together with an accompanying anion, functions as a predominant osmolyte in essentially all cells exposed to abrupt changes in osmolarity, including cerebellar granule neurons (Morales-Mulia *et al*. 1998). The role of this cation in IVR was examined measuring the unidirectional efflux of ^{86}Rb and the intracellular K^+ content during exposure to gradually decreased π_o. The efflux of ^{86}Rb was unchanged

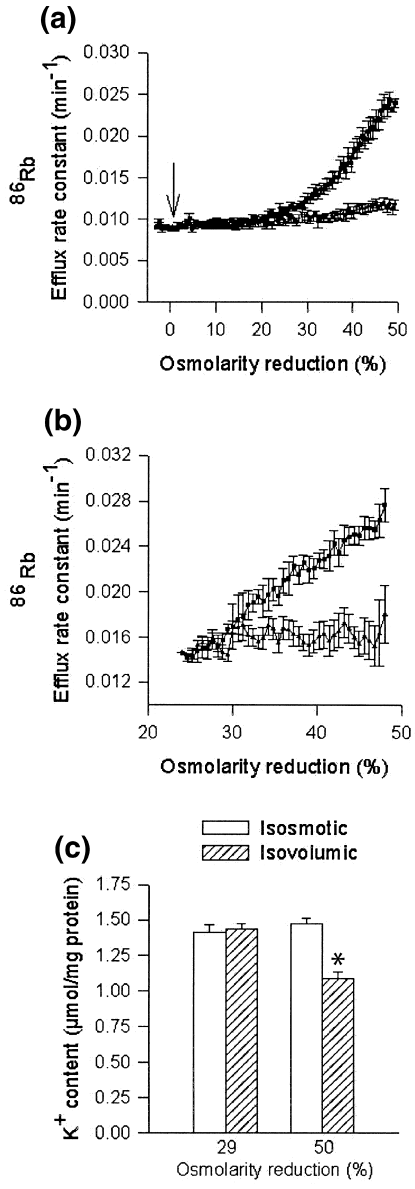

Fig. 5 ^{86}Rb release and changes in K^+ cell content in cerebellar granule neurons exposed to gradual or sudden decreases in osmolarity. (a) Cells preloaded with ^{86}Rb were superfused as in Fig. 2 with medium of gradually decreased osmolarity (■) or with iso-osmotic medium (●). At the end of the experiment π_o was 150 mOsm/L (− 50%). Data are means of eight experiments ± SE represented by vertical bars if exceeded the size of symbols. (b) Effect of 15 mM Ba^+ (▲) on ^{86}Rb efflux plotted from π_o − 24%. (c) Cell K^+ content after superfusion with the osmotic gradient. The intracellular concentration was measured at π_o − 29% (corresponding to the ^{86}Rb efflux threshold) and at the end of the experiment, π_o − 50%. Cell K^+ content was measured by atomic absorption spectrometry. Results are means of six experiments ± SE. *$p < 0.005$, Student's *t*-test.

as compared with the release in iso-osmotic medium until π_o decreased to 212 mOsm/L (− 29%). From this point on, the rate of ^{86}Rb efflux increased progressively and did not inactivate for the remaining of the experiment (π_o 150 mOsm/L, − 50%) (Fig. 5a). The efflux of ^{86}Rb activated during IVR was insensitive to 4-AP (1 mM), TEA (10 mM), charybdotoxin (20 nM), cesium (5 mM), and gadolinium (10 µM) (results not shown). Barium (Ba^{2+}), 5–10 mM did not affect ^{86}Rb efflux (not shown) but at 15 mM clearly reduced the release (Fig. 5b). In accordance with results using the radioactive tracer, the intracellular K^+ content was unchanged up to π_o 212 mOsm/L, i.e. just before the activation threshold of ^{86}Rb release, but at the end of the superfusion, when π_o is reduced to 150 mOsm/L, the cell K^+ content decreased 26% (Fig. 5c).

Change in cell volume in the presence of niflumic acid and Ba^{2+} and in Cl^--free medium

The effects on IVR of agents or conditions which blocked the efflux of amino acids, Cl^- and K^+ are shown in Fig. 6. In the presence of 600 µM niflumic acid, IVR was prevented (Fig. 6a). Cell swelling was apparent from the first point estimated (π_o 282 mOsm/L), in medium containing the blocker, and increased continuously to attain a 25% swelling when π_o was reduced to 210 mOsm/L (− 30%) (Fig. 6a). This increase in cell volume is close to that in cells suddenly exposed to 30% hypo-osmotic solutions, as shown in Fig. 1(a), and indicated by a triangle in Fig. 6(a). At the end of the experiment, when π_o is − 50%, cell swelling was about 52%, while the maximal volume increase is 60% in cells after an abrupt exposure to 50% hypo-osmotic medium (Fig. 1a, triangle in Fig. 6a). When all Cl^- in the solutions was replaced by gluconate, a marginal (5%) swelling occurred in the π_o range of 300–246 mOsm/L, showing that this condition did not affect IVR within this range of hyposmolarity. At π_o 228 mOsm/L, cell volume increased to 11% and further continued to reach 29% at the end of the experiment at π_o − 50%. In the presence of 15 mM Ba^{2+}, no swelling was observed up to a π_o reduction of 210 mOsm (− 30%) (Fig. 6b). Maximal swelling in the presence of Ba^{2+} at − 50% π_o was about 30%.

Discussion

The present results document the occurrence of IVR in neurons, since volume in cerebellar granule neurons remained unchanged up to 50% osmolarity reduction. The mechanisms responsible for IVR have not been investigated in detail, although it is assumed that the osmolytes involved are essentially those implicated in RVD, i.e. K^+, Cl^- and organic osmolytes. However, the relative contribution of these different osmolytes to IVR is still unclear. In cerebellar granule neurons, K^+ and Cl^- appear to participate only during the late phase of IVR, as supported by the

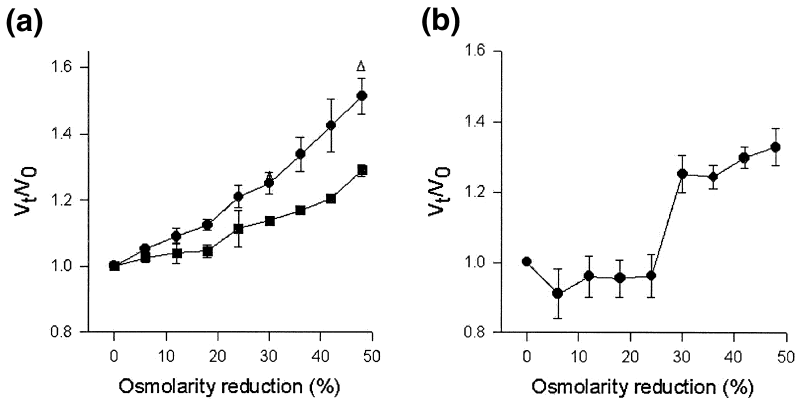

Fig. 6 Impairment of IVR by niflumic acid, Ba^{2+} and Cl^--free medium. Cell volume was measured as in Fig. 1. (a) Cell volume measured in cells exposed to the osmotic gradient in the presence of 600 μM niflumic acid (●), or Cl^--free medium (Cl^- replaced by gluconates) (■). The triangle in this figure (△) indicates the increase in cell volume elicited by sudden exposure to 30% or 50% hypo-osmotic medium. (b) Change in cell volume in the presence of 15 mM Ba^{2+} (●). Cell volume was measured every 10 min, corresponding to decreases in π_o of 18 mOsm/L. Data are means ± SE of three or four separate experiments.

present results showing: (i) a delayed osmolarity threshold of K^+ and Cl^- efflux; (ii) a late decrease in intracellular K^+ levels; and (iii) an effect of Ba^{2+} and Cl^- free medium impairing IVR only at this same late phase. Studies in A6 renal cell line and in cardiomyocytes (Van Driessche *et al*. 1997; Souza *et al*. 2000), similar to our present results, document a delayed release of ^{86}Rb during IVR, detectable only at a threshold of − 29% π_o. The amount of K^+ lost of about 27% is remarkably similar in the three cell types (Van Driessche *et al*. 1997; Souza *et al*. 2000 and present results). In contrast to results in isolated cells, K^+ efflux could not be detected in hippocampal slices, due probably to buffering systems present in such integrated preparation (Franco *et al*. 2000). The release of K^+ is likely accompanied by Cl^-, which has not been previously examined, and that in our study occurs with an efflux threshold earlier than that of K^+, but still at a late phase of IVR. The efflux of amino acids, in particular taurine, exhibited a notably more sensitive response to the small changes in osmolarity, suggestive of their involvement in earlier phases of IVR. An early efflux of taurine has also been observed in hippocampal slices exposed to an osmotic gradient (Franco *et al*. 2000)

The contribution of the different osmolytes to IVR can be estimated from the effect on cell volume of agents or conditions which block their release. In cerebellar granule neurons what we observed is that in the presence of Ba^{2+} which abolished K^+ fluxes, there was no change in cell volume until π_o was reduced to − 24%, coincident with the threshold of ^{86}Rb efflux activation and the intracellular K^+ decrease. After this time, when K^+ efflux are fully activated, Ba^{2+} prevented IVR, increasing cell volume by 30%. Cell treatment with solutions where all Cl^- was replaced by gluconate did not significantly affect cell volume during the first phase of IVR, corresponding to π_o decreases of − 6% to − 18%. At − 24% π_o, swelling started, and continued progressively to reach a maximum of 29% at − 50% π_o, an increase similar to that attained by K^+ efflux blockade.

In the presence of niflumic acid, which is a general blocker of organic osmolyte fluxes (Lang *et al*. 1998), including taurine (as shown in the present work), swelling was observed from the first minutes of superfusion with the osmotic gradient and this increase continued progressively, to reach a maximum of 50% over cell volume in iso-osmotic conditions. The 29% volume increase reached in niflumic acid-treated cells, when π_o is − 30%, is almost identical to the 30% attained after sudden exposure to a similar (− 30%) hypo-osmotic medium. This finding indicates that the early phase of volume regulation is entirely accomplished by niflumic acid-sensitive mechanisms. A comparison between swelling in Cl^- free medium which affects only Cl^- (and not organic osmolyte fluxes), and in the presence of niflumic acid, which blocks both Cl^- and organic osmolyte fluxes, may give an approximate estimation of the contribution of organic osmolytes at all phases of IVR. The difference between the two curves in Fig. 6(a) suggests an early, continuous and substantial contribution of organic osmolytes throughout all the osmotic gradient. Cell swelling induced by sudden exposure of cerebellar granule neurons to 50% hypo-osmotic solutions is about 60%, thus leaving a difference of about 10% with the maximal swelling observed by impairing IVR with blockers. This difference may be accounted for by electroneutral transporters not affected by niflumic acid or by a fraction of K^+ release independent of Cl^- efflux.

The question often raised about whether IVR is due to restricted swelling was largely clarified in the present work showing that if present at all, this restricted swelling represents only a minimal proportion of the IVR. Our results with niflumic acid and Cl^--free conditions clearly show that IVR is not equivalent to absence of cell swelling. Rather it is an active process of volume regulation, accomplished by niflumic acid-sensitive pathways for osmolyte extrusion, possibly activated by mechanisms similar to those operating in RVD. The cell shrinkage in renal cells returned to iso-osmotic medium after IVR reported by Lohr and Grantham (1986) further supports this view. Taurine and Cl^- efflux elicited by sudden exposure to hyposmolarity appear triggered by a decrease in intracellular ionic strength

subsequent to water entry (Emma *et al*. 1997; Nilius *et al*. 1998; Cardin *et al*. 1999). This may also be a signal for osmolyte extrusion during IVR if the external hyposmolarity even when gradual, leads to water influx immediately activating the corrective osmolyte fluxes.

The present results emphasize the role of osmolyte amino acids in the early phase of IVR and underline the role of taurine in this process. Taurine was the amino acid preferentially released, with the lowest threshold, the highest efflux rate and the largest cell content decrease. Taurine efflux rate was 4–10-fold higher than those of glycine and glutamate (D-aspartate), at essentially all osmolarities. A similar preferential involvement of taurine as compared with other amino acids in the paradigm of osmotic gradual changes, has been observed in hippocampal slices (Franco *et al*. 2000). This remarkable difference may reflect either higher permeation through the transport pathway or/and more availability of taurine cell pools to face the osmotic stress. Taurine is not involved in metabolic reactions, and is found essentially free in the cytosol, while glycine and glutamate, which are highly active in cell metabolism and act as synaptic transmitters, might be sequestered in metabolic or physical compartments, restricting their availability to function as osmolytes.

The release of taurine occurs at osmolarity drops observed in brain in pathologies, such as chronic hyponatremia or ischemia, i.e. drops of about 12–16% (Trachtman 1991; McManus and Churchwell 1994; Fraser and Arieff 1997). Studies *in vivo* underline the critical role of organic osmolytes in the maintenance of normal brain water content and survival in chronic hyponatremia. Thurston *et al*. (1987) report that the total decrease in brain organic osmolyte content in chronic hyponatremic mice is, on a molar basis, greater than the reduction in K^+. Taurine appears to be a main contributor to the osmotic adjustment, first, because it is the most abundant amino acid in mouse brain and, second, because it showed the largest reduction (70%) facing the hyponatremic condition, a result remarkably similar to that found in the present study. Similarly, in cultured astrocytes exposed to a 24-h period of hyponatremia, while cell volume remains constant, the taurine pool was essentially depleted whereas no change in glutamate nor in K^+ content is observed (Olson 1999). Altogether, those findings and the present results point out to a prominent role of taurine as an element counteracting brain cell edema. It should be noted, however, that the decrease in taurine and other amino acids observed during IVR is clearly insufficient to equilibrate the imposed osmotic gradient when K^+ and Cl-fluxes have not yet been activated. Therefore, an additional contribution of other osmolytes seems required to compensate for this deficit. Organic molecules such as myo-inositol, glycerophosphorylcholine, betaine, phosphoetanolamine, creatine, phosphocreatine, and *N*-acetyl aspartate (Gotoh *et al*. 1997) may provide important contributions in this phase of cell volume adjustment.

The present results in neurons suggest that organic and inorganic osmolytes may differently contribute to volume regulation depending on the severity of the hypo-osmotic challenge, preferentially handling organic osmolytes over K^+ and Cl^- at lower decreases in osmolarity, similar to what is observed in physiological and pathological conditions. This may be attributed to the key role played by K^+ and Cl^- in the control of neuronal excitability. Extracellular accumulation of these ions, by causing excitability imbalance may be incompatible with normal neuronal functioning and survival. It remains to be determined whether the apparently restricted function of K^+ and Cl^- as osmolytes is a characteristic of excitable cells not shared by other cell types. In this respect, it is noteworthy that, unlike neurons, bird erythrocytes appear devoid of the mechanisms for IVR (Godart *et al*. 1999). Clearly, the occurrence of IVR and its properties should be examined in other cell types to have a more accurate picture of the relevance and mechanisms of this phenomenon.

Acknowledgements

We acknowledge the technical help of Ms Claudia Peña. We thank Karla Hernández-Fonseca for her assistance in some phases of this study and to Dr Rafael Moreno from the Institute of Cardiology, Mexico, for measurements of K^+ concentration. This research was supported in part by grants IN-204900 from DGAPA-UNAM and 34886-M from CONACYT, Mexico.

References

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.

Cardin V., Peña-Segura C. and 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.

Crowe W. E., Altamirano J., Huerto L. and Alvarez-Leefmans F. J. (1995) Volume changes in single N1E-115 neuroblastoma cells measured with a fluorescent probe. *Neuroscience* **69**, 283–296.

Emma F., McManus M. and Strange K. (1997) Intracellular electrolytes regulate the volume set point of the organic osmolyte/anion channel VSOAC. *Am. J. Physiol.* **272**, C1766–C1775.

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.

Fraser C. L. and Arieff A. I. (1997) Epidemiology, pathophysiology and management of hyponatremic encephalopathy. *Am. J. Med.* **102**, 67–77.

Godart H., Ellory J. C. and Motais R. (1999) Regulatory volume response of erythrocytes exposed to a gradual and slow decrease in medium osmolarity. *Pflugers Arch. Eur. J. Physiol.* **437**, 776–779.

Gotoh M., Davies S. E. and Obrenovitch T. P. (1997) Brain tissue acidosis: effects on the extracellular concentration of N-acetylaspartate. *J. Neurochem.* **69**, 655–661.

Lang F., Busch G. L., Ritter M., Völki H., Waldegger S., Gulbins E. and

Häussinger D. (1998) Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* **78**, 247–306.

Lohr J. W. (1990) Isovolumetric regulation of renal proximal tubules in hypotonic medium. *Renal Physiol. Biochem.* **13**, 233–240.

Lohr J. W. and Grantham J. J. (1986) Isovolumetric regulation of isolated S_2 proximal tubules in anisotonic media. *J. Clin. Invest.* **78**, 1165–1172.

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.

McManus M. L. and Churchwell K. B. (1994) Clinical significance of cellular osmoregulation, in: *Cellular and Molecular Physiology of Cell Volume Regulation* (Strange K., ed), pp. 63–80. CRC Press, London.

Morales-Mulia S., Ordaz B., Quesada O. and Pasantes-Morales H. (1998) Ca^{2+} changes and ^{86}Rb efflux activated by hyposmolarity in cerebellar granule neurons. *J. Neurosci. Res.* **53**, 626–635.

Morán J. and Patel A. J. (1989) Stimulation of the *N*-methyl-D-aspartate receptor promotes the biochemical differentiation of cerebellar granule neurons and not astrocytes. *Brain Res.* **486**, 15–25.

Nilius B., Eggermont J., Voets T., Buyse G., Manolopoulos V. and Droogmans G. (1997) Properties of volume-regulated anion channels in mammalian cells. *Progr. Biophys. Molec. Biol.* **68**, 69–119.

Nilius B., Prenen J., Voets T., Eggermont J. and Droogmans G. (1998) Activation of volume-regulated chloride currents by reduction of intracellular ionic strength in bovine endothelial cells. *J. Physiol.* **506**, 353–361.

Olson J. E. (1999) Osmolyte contents of cultured astrocytes grown in hypoosmotic medium. *Biochim. Biophys. Acta.* **1453**, 175–179.

Pasantes-Morales H. and Schousboe A. (1997) Role of taurine in osmoregulation: mechanisms and functional implications. *Amino Acids.* **12**, 281–292.

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.

Sánchez-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.

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.

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

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

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.

Neurochemical Research, Vol. 29, No. 1, January 2004 (© 2004), pp. 65–72

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

Benito Ordaz,[1] Karina Tuz,[1] Lenin D. Ochoa,[1] Ruth Lezama,[1] Claudia Peña-Segura,[1] and Rodrigo Franco[1,2]

(Accepted July 28, 2003)

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.

KEY WORDS: Swelling; volume regulation; osmolytes; isovolumetric regulation; astrocytes; taurine.

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

* Special issue dedicated to Dr. Herminia Pasantes-Morales.

[1] Institute of Cell Physiology, Department of Biophysics, National University of Mexico, Mexico City, Mexico.
[2] Address reprint requests to: Rodrigo Franco, Instituto de Fisiología Celular, UNAM, Insurgentes Sur, Ciudad Universitaria, D.F. Apartado Postal 70-253, 04510, México D.F., Mexico. Tel: (5255) 5622-5608; Fax: (5255) 5622-5607; E-mail: rofranco@ifc.unam.mx

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 intracellular 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

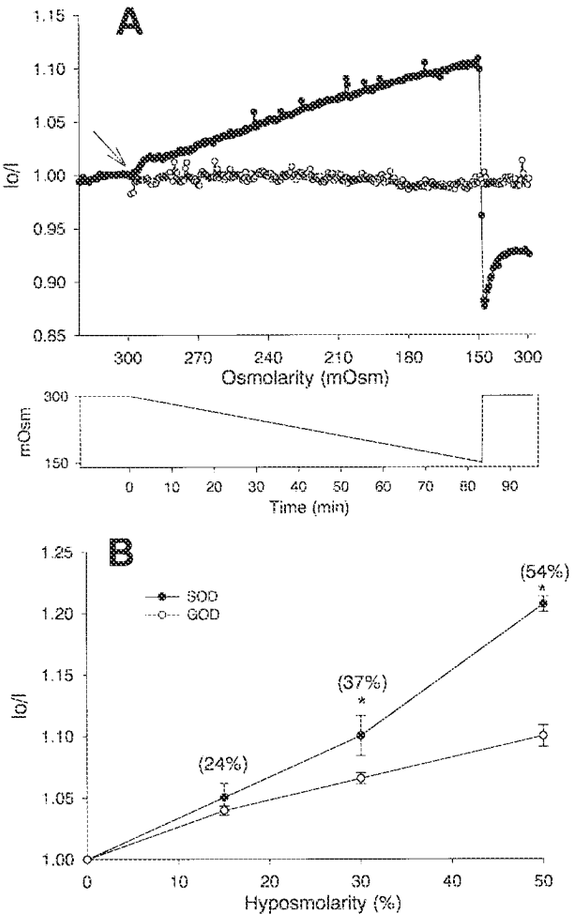

Fig. 1. Cell volume changes of cerebellar astrocytes exposed to gradual changes in external osmolarity. Estimation of relative cell volume was performed using large-angle light scattering as described by McManus et al., (35). Cells seeded on rectangular coverslips were placed on a superfusion chamber 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^-

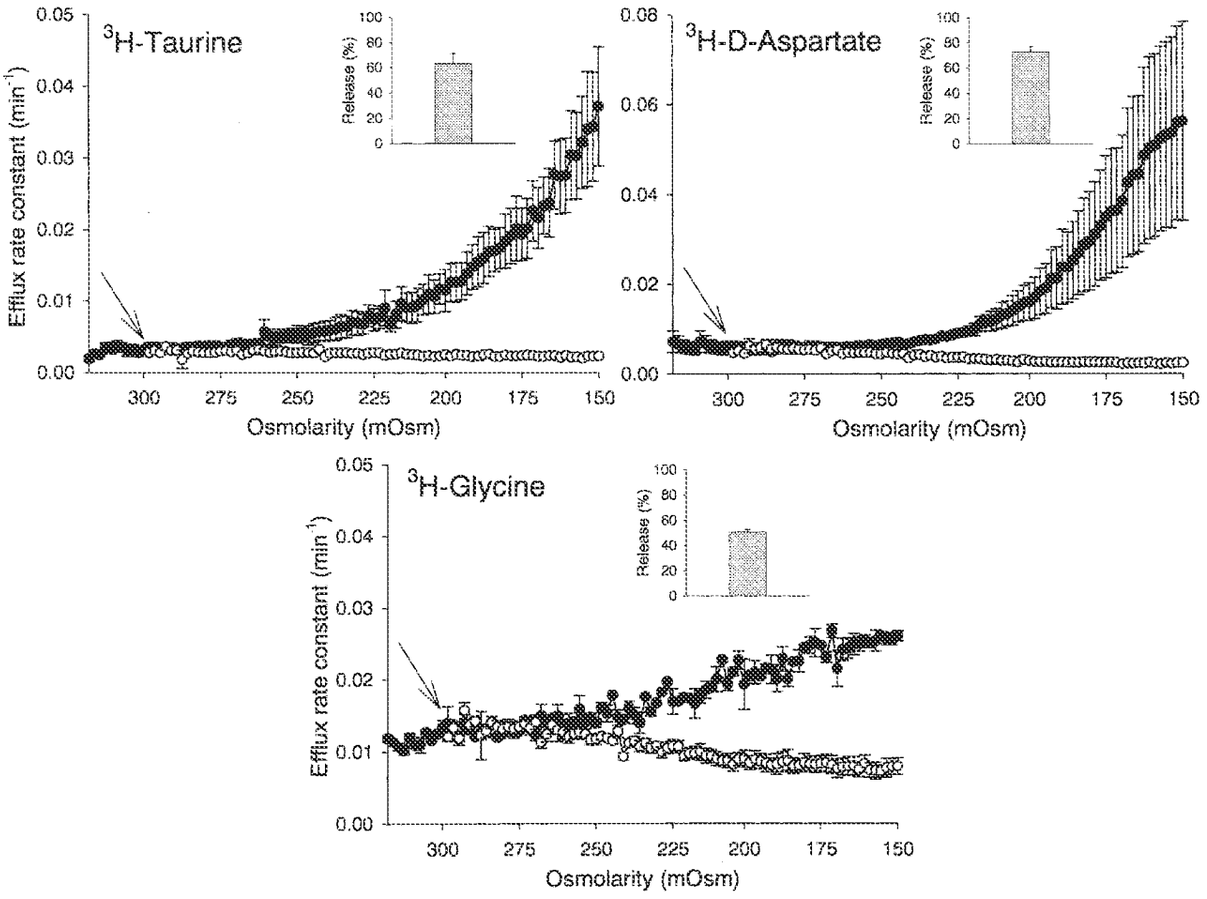

Fig. 2. Amino acid release from cerebellar astrocytes exposed to gradual reductions in external osmolarity. Cells were preloaded with ^{3}H-taurine, ^{3}H-D-aspartate, and ^{3}H-glycine as described in Tuz et al. (30) After the loading period, cells were washed and superfused (rate 2.0 ml/min) during 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.

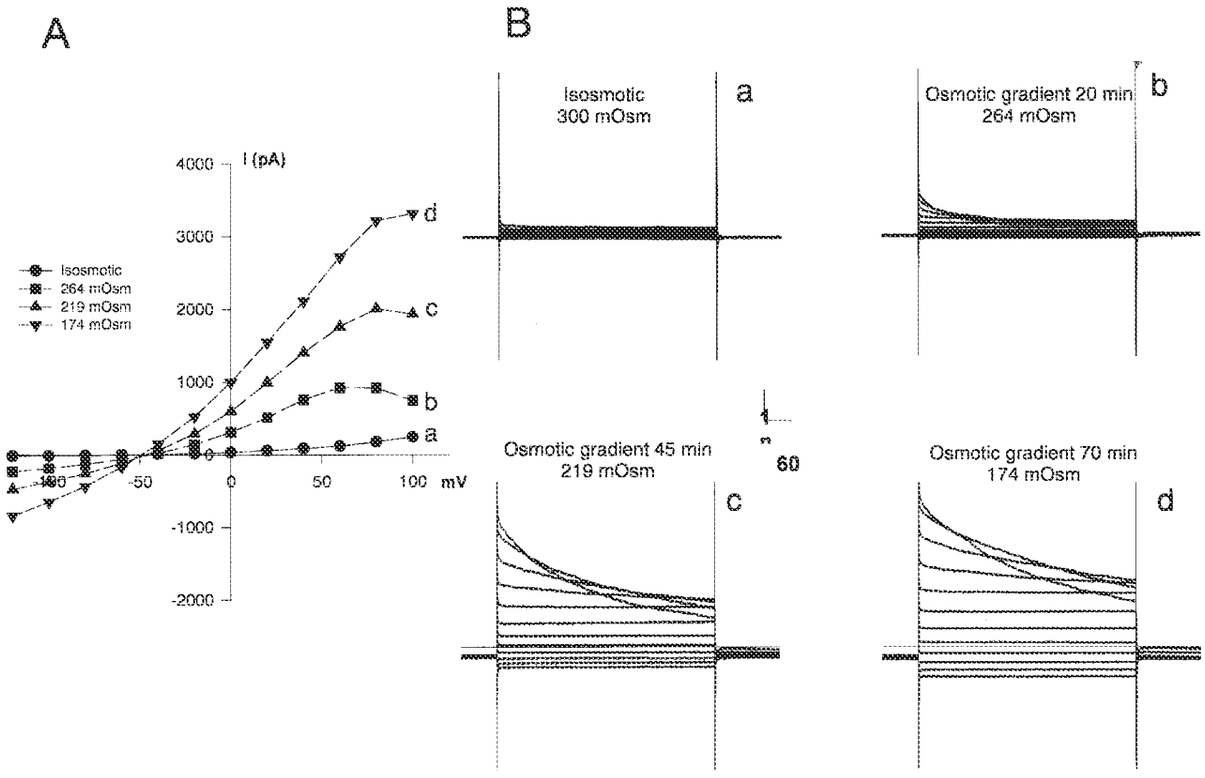

Fig. 3. Osmotic gradient–induced activation of whole-cell currents in cerebellar astrocytes. Astrocytes were seeded in 35-mm petri dishes at a low density of 0.3 mill/ml. Currents were monitored with Axopatch 200 patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell membrane currents 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).

ACKNOWLEDGMENTS

This work was supported in part by grants No. 32886-M and 35806 from CONACYT and IN204900 from DGAPA, UNAM.

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.

The American Journal of Medicine (2006) Vol 119 (7A), S4–S11

ELSEVIER

THE AMERICAN JOURNAL *of* MEDICINE®

Mechanisms of Cell Volume Regulation in Hypo-osmolality

Herminia Pasantes-Morales, PhD,* Ruth A. Lezama, PhD, Gerardo Ramos-Mandujano, PhD, Karina L. Tuz, PhD

Department of Biophysics, Institute of Cell Physiology, National University of Mexico, Mexico City, Mexico

ABSTRACT

Cells respond to a condition of hypo-osmolality by rapid swelling followed by an adaptive response that tends to recover the normal cell volume despite the persistence of the hypo-osmotic condition. This is an active process accomplished by the extrusion of intracellular osmolytes, essentially K^+, Cl^-, and small organic molecules. This regulatory process operates through a chain of events that essentially consists of a sensor or sensing mechanism to detect changes in cell volume, a signaling cascade to amplify the sensing signal and orient it to activate pathways for osmolyte extrusion, and a memory of the original cell volume, which sets the timing for inactivation of the volume-regulatory process. This article presents a brief overview of recent progress in these different aspects of the volume-regulatory process, including (1) the mechanisms and/or candidate molecules serving the role of volume sensors, (2) the osmosignaling network and the interplay and hierarchy of the different elements in this chain, and (3) the nature and properties of the osmolyte extrusion pathways. Emphasis is placed on some of the main unsolved questions concerning different aspects of the volume-regulatory process. Recent findings regarding the effect of hypo-osmolality on synaptic function are briefly discussed in terms of the possible molecular basis for the neurologic symptoms induced by hyponatremia.

KEYWORDS: Osmosensitive channels; Osmotransduction; Taurine; Tyrosine kinases; Volume sensor

The maintenance of a constant volume is a homeostatic imperative in animal cells. Changes in cell water content, by affecting the concentration of messenger molecules, disturb the complex signaling network that is crucial for cell functioning and intercellular communication. Although under physiologic conditions the extracellular fluid has a highly controlled osmolality, the intracellular volume constancy is continuously compromised by the generation of local and transient osmotic microgradients associated with uptake of nutrients, exocytosis of cellular products, cytoskeletal remodeling, and transsynaptic ionic gradients. Cell volume is also disturbed in a variety of pathologic conditions, notably hyponatremia, and the cells respond to this challenge by active mechanisms of volume adjustment.[1] This ability to regulate cell volume is an ancient conserved trait that is present in essentially all species throughout evolution. Cell volume changes have particularly dramatic consequences in the brain. The limits to expansion imposed by the rigid skull allow narrow margins for the buffering of intracranial volume changes.[1] As expansion occurs, compression of small vessels generates episodes of ischemia, infarct, excitotoxicity, and neuronal death. In extreme conditions, caudal herniation of the brain parenchyma through the tentorium and foramen magnum compresses brainstem nuclei, resulting in death by respiratory and cardiac arrest.

CELLULAR AND MOLECULAR MECHANISMS OF CELL VOLUME REGULATION

In general, cells are highly permeable to water, and, in the absence of significant hydrostatic pressure gradients, the water flow across the membrane is directed by the osmotic pressure gradient. Therefore, in a hypo-osmotic condition, cells initially behave as almost perfect osmometers and swell in a magnitude proportional to the osmolality reduction. Immediately thereafter, an active volume correction is

This work was supported by Grant No. IN206403 from Dirección General de Asuntos del Personal (DGAPA), Grant No. 46465 from Consejo Nacional de Ciencia y Tecnología (CONACYT), and by a grant from the National University of Mexico (UNAM).

*Requests for reprints should be addressed to Herminia Pasantes-Morales, PhD, Instituto de Fisiología Celular, UNAM, Ciudad Universitaria, Circuito Exterior, 04510 México DF, México.

E-mail address: hpasante@ifc.unam.mx.

0002-9343/$ -see front matter
doi:10.1016/j.amjmed.2006.05.002

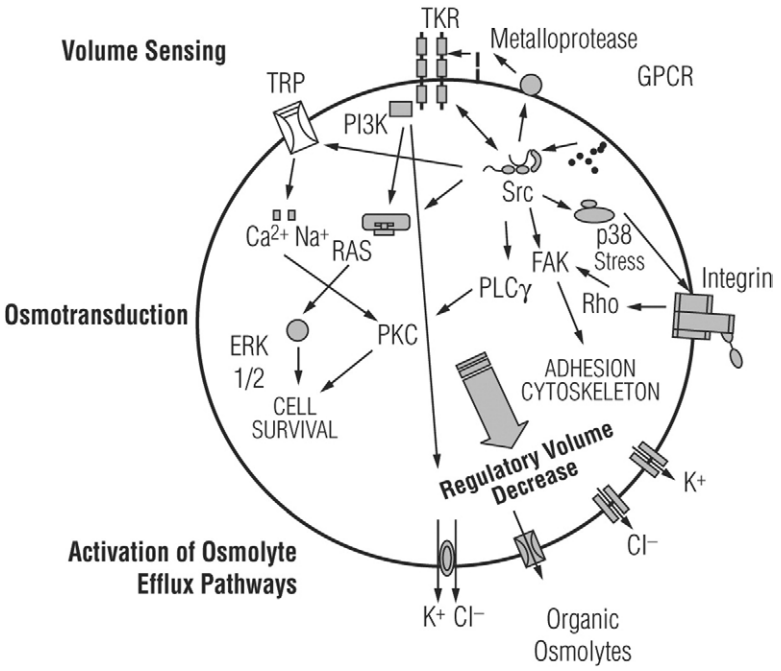

Figure 1 Three main steps are necessary for cell volume recovery after hypo-osmotic swelling. Illustrated here are the elements involved in each of the steps required for cell volume adjustment and their suggested interrelations. ERK1/2 = extracellular signal–regulated kinases 1 and 2; FAK = focal adhesion kinase; GPCR = G-protein coupled receptors; PI3K = phosphatidylinositol-3 kinase; PKC = protein kinase C; PLCγ = phospholipase Cγ; RAS and Rho = members of the RAS family of small guanosine triphosphatases; Src = sarcoma oncogene; TKR = tyrosine kinase receptor; TRP = transient receptor potential channel.

Table 1 Properties of the Cl^- channels activated by hyposmotic swelling (Cl^-_{swell})

- Moderate outward rectifiers
- Intermediate unitary conductance (40–78 picosiemens)
- Inactivating at potentials ≥+60 mV
- High selectivity for anions over cations
- Permeable to most monovalent anions
- Largely Ca^{2+} independent
- Require ATP, but not its hydrolysis
- Blocked by DIDS, SITS, 9-AC, DPC, NPPB, DDF, niflumic acid, and flufenamic acid
- Expressed in most animal cell types

9-AC = 9-anthracene-carboxylic acid; ATP = adenosine triphosphate; DDF = 1,9-dideoxy-forskolin; DIDS = 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPC = diphenylamine-2,2′-dicarboxylic acid; NPPB = 5-nitro-2-(3-phenylpropylamino)benzoic acid; SITS = 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

initiated, which is based on the extrusion of intracellular solutes together with osmotically obligated water, thereby tending to normalize cell volume. This adaptive mechanism is known as regulatory volume decrease (RVD) in cell size. The osmolytes involved in the adaptive process are the main intracellular ions, K^+ and Cl^-, and a number of organic molecules, including amino acids, polyalcohols, and amines. RVD is a complex chain of events that at minimum requires the following steps: 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 (**Figure 1**).

Osmosensitive Cl^- and K^+ Channels

The volume-activated fluxes of K^+ and Cl^- occur in most cell types but often through separate channels. Swelling-activated Cl^- channels (Cl^-_{swell}) have been characterized in numerous cell types and show similar electrophysiologic and biophysical features in most of them. Cl^-_{swell} typically are outward rectifiers, with an intermediate unitary conductance of 40 to 78 picosiemens, inactivating at potentials of ≥+60 mV (**Table 1**). They have a high selectivity for anions over cations but broad anion selectivity, being permeable to most monovalent anions, including large anions such as gluconate and methanesulfonate. Activation of Cl^-_{swell} requires adenosine triphosphate, but not its hydrolysis. The channel is sensitive to the

following general Cl^- channel blockers, which exhibit different potencies according to the cell type: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; 9-anthracene-carboxylic acid; diphenylamine 2-carboxylate, 5-nitro-2-(3-phenylpropylamino) benzoate, 1,9-dideoxyforskolin; niflumic acid; and flufenamic acid. Arachidonic acid and other polyunsaturated fatty acids are also potent Cl^-_{swell} blockers.[2] Despite the numerous Cl^- channels recently cloned, some of which are osmolality sensitive, none exhibits the profile of Cl^-_{swell}. Therefore, the molecular identity of Cl^-_{swell} is as yet unknown. Some molecules with Cl^- permeability properties, such as I_{cln} and the P-glycoprotein, have been suggested to play a role in osmosensitive Cl^- transport, either as a primary Cl^- pathway (a possibility that recently came into question) or as regulating elements of the functional Cl^- channels.[3]

Some of the unsolved questions about Cl^-_{swell} include:

- What is the molecular identity of Cl^-_{swell}?
- Is Cl^-_{swell} the same in terms of molecular identity in all cell types?
- What are the similar or dissimilar effects of elements of the signaling cascades, including tyrosine kinases, on Cl^-_{swell} activity in different cell types?
- What factors or situations determine the operation of different types of osmosensitive Cl^- channels and other anion-permeating molecules in the same cell?

In contrast to the similarities in the properties of Cl^-_{swell} in different types of cells, suggestive of the involvement of essentially 1 type of Cl^- channel in RVD, a variety of different K^+ channels appear to participate in cell volume regulation (**Table 2**).[4] These channels may have other functions in the cell but are activated in response to signals evoked by swelling. Depolarization and increases in the concentration of cytosolic Ca^{2+}, or $[Ca^{2+}]_i$, are among these signals. Swelling evokes depolarization in neurons, astrocytes, and other cell types, likely owing to the rapid activation of Cl^-_{swell} and the subsequent Cl^- efflux. Depolarization then activates voltage-gated K^+ channels (Kv), which participate in volume regulation. Implication of Kv channels in volume regulation has been documented in lymphocytes and in hippocampal pyramidal neurons, although not all the Kv subtypes are responsive.[4] The increase in $[Ca^{2+}]_i$ is a most consistent response to hyposmolality.[4] In epithelial cells, but not in all cells, this $[Ca^{2+}]_i$ elevation activates Ca^{2+}-dependent K^+ channels, which then play a prominent role in RVD. The pharmacologic profile of these channels shows sensitivity to tetraethylammonium, Ba^{2+}, and charybdotoxin, and their large conductance implicates the calcium-activated potassium channels in the volume-sensitive K^+ efflux. K^+ channels that are directly gated by hypo-osmolality or swelling (K^+_{swell}) have been recently described.[5] They are insensitive to typical K^+ channel blockers, such as tetraethylammonium and 4-aminopyridine, but are inhibited by clofilium. Mechanogated channels are stretch-activated K^+ channels, insensitive to typical blockers, but inhibited by gadolinium and quinidine. The K^+_{swell} and the mechanogated K^+ channels are possibly members of the 2-pore potassium channel family. Among them, only TREK-1 is outwardly rectifying, and therefore is the only subtype allowing K^+ efflux. It is still unclear whether these various types of K^+ channels operate in the same cell but activate at different osmolality thresholds. However, support for this possibility has been described in a glial cell line, in which a Ca^{2+}-dependent, charybdotoxin-sensitive K^+ channel was activated after small decreases (12%) in osmolality, while after larger decreases (>15%), a Ca^{2+}-independent, clofilium-sensitive K^+ channel was activated.[6]

K^+ fluxes during RVD may be carried by the electroneutral K^+/Cl^- cotransporter, but the contribution of this transporter to volume regulation is notably lower than that of the conductive pathways. Instead, its main role may be related to regulation of intracellular Cl^-.

The following are some unanswered questions about the K^+ efflux pathways involved in RVD:

- What is the distribution of the K^+_{swell}? Is it present in most cell types, or do some cells have an efficient adaptation to volume changes in the absence of these channels?
- What is the molecular identity of the K^+_{swell}?
- What is the influence of the type of K^+ channel predominantly involved in the cell response to swelling in conferring Ca^{2+}-dependence or independence to the whole volume regulation process?
- What is the relative contribution of conductive or electroneutral pathways to RVD in different cell types or swelling conditions?

VOLUME-SENSITIVE PATHWAYS FOR ORGANIC OSMOLYTES

The organic osmolyte pool involved in volume regulation is formed by a heterogeneous group of small molecules, including amino acids (taurine, glutamate, glycine, alanine), polyalcohols (myoinositol, sorbitol), and other compounds such as creatine, phosphocreatine, phosphoethanolamine, glycerophosphorylcholine, and *N*-acetyl-aspartate. Because the swelling-activated efflux of organic osmolytes occurs through release pathways with at least some common properties for the various organic osmolytes,[7] it has been suggested that there may be a shared volume-sensitive pathway for all of these molecules. Remarkably, the organic osmolyte efflux has a pharmacologic profile similar to that of the Cl^-_{swell}, a finding leading to the proposal of an anion channel-like molecule as the common route for extrusion of both Cl^- and organic osmolytes. Although the notion of a common pathway has been challenged by recent experimental evidence,[6,8] this does not explain the consistently reported inhibitory effect of Cl^- channel blockers on the osmolyte efflux pathway, which reflects an influence of Cl^- fluxes and/or intracellular Cl^- levels on organic osmolyte

Table 2 K^+ channels activated by hyposmotic swelling

- Kv channels
  - —Activated by depolarization subsequent to hyposmotic-evoked Cl^- efflux
  - —Are outward rectifiers
  - —Blocked by TEA, noxiustoxin, argiotoxin, and dendrotoxins
  - —Activated by hypo-osmolarity in lymphocytes and in hippocampal pyramidal neurons
- KB channels
  - —Activated by the hypo-osmolarity-induced cytosolic Ca^{2+} elevation
  - —Large unitary conductance (100–200 picosiemens)
  - —Blocked by TEA, charybdotoxin, iberiotoxin, and paxilline
  - —Linked to volume recovery in most epithelial cell types
- K^+_{swell} channels
  - —Directly gated by swelling
  - —Intermediate unitary conductance
  - —Independent of Ca^{2+} and voltage
  - —Insensitive to classic K^+ channel blockers
  - —Blocked by clofilium
  - —Described in HeLa and Ehrlich ascitic cells
- Stretch-activated K^+ channels
  - —Mechanogated channels
  - —Insensitive to the classic K^+ channel blockers
  - —Blocked by quinidine and gadolinium
  - —May belong to the K_{2P} channels
  - —TREK-1 is the only outward rectifier of K_{2P} channels

K^+_{swell} = swelling-activated K^+ channels; K_{2P} = 2-pore domain K channels; KBI = Ca^{2+} activated, large-conductance K^+ channels; Kv = voltage-gated K^+ channels; TEA = tetraethylammonium.

permeation. The importance of organic osmolytes in mammalian cells is evident from the work of Verbalis and colleagues[9] on brain adaptation to chronic hyponatremia. This study and others show a marked efflux of K^+ and Cl^- elicited by the change in osmolality, representing an acute RVD, followed by a sustained adaptation via progressive decreases in the brain content of the organic osmolytes,[9,10] to almost complete depletion as in the case of taurine.

Taurine may have a prominent role as an osmolyte in conditions such as chronic hyponatremia, in which the osmolality changes are small and gradual. Experiments reproducing this condition in animals have shown highly efficient counteraction of swelling in cells that respond to the hyposmotic challenge by an early and robust efflux of taurine.[9] Glycine, γ-aminobutyric acid, glutamate, and aspartate also contribute to counteracting the swelling induced in chronic hyponatremia. However, in organs like the brain, this may result in a risk for enhanced excitability due to the prominent role of these amino acids as synaptic transmitters.

Unresolved questions about the contribution and mechanisms of organic osmolytes involved in regulatory volume decrease include:

- What is the molecular characterization and identity of the organic anion efflux pathway?
- How are Cl^- and anion fluxes linked?
- What are the relative contributions of the various osmolytes to RVD in different cell types?

VOLUME SENSING AND OSMOTRANSDUCTION

Volume Sensing

How cells sense volume changes is an initial and critical step in the chain of reactions activated for volume correction, and yet this important question thus far remains unanswered. It is still unclear whether the sensing mechanism predominantly involves just 1 type of molecule, or whether a variety of molecules interact to ultimately detect the change in cell volume. Candidates proposed to play this role include transmembrane molecules such as integrins, receptors of growth factors with intrinsic tyrosine kinase activity, and the transient receptor potential channels (TRPC), all of which activate in response to hypo-osmolality and are well positioned to transmit changes in the external environment or within the membrane. On the intracellular side, the dilution of cytosolic macromolecules or a decrease in intracellular ionic strength, both of which are consequent to water entry, have been proposed as signals for detecting a change in cell volume. Cytoskeleton rearrangements or changes in the concentration of signaling molecules such as Ca^{2+} or Mg^{2+} have also been implicated in volume-sensing mechanisms. The mechanisms underlying volume sensing are also relevant to the mechanisms of inactivation of osmolyte fluxes when cell volume moves back toward normal levels. This is still an essentially unexplored aspect of RVD.

Tyrosine kinase receptors (TKRs) have received considerable attention as possible candidates participating in volume-sensing mechanisms. TKRs have a molecular architecture consisting of an extracellular domain, a transmembrane element, and an intracellular domain with intrinsic sites for protein tyrosine kinase and phosphorylation.[11] Thus, they are well structured to serve as a link between the external environment, including changes in the fluids surrounding cells or at the extracellular matrix proteins, and the intracellular compartment. Moreover, intracellularly, TKRs are convergence sites of a variety of downstream signaling pathways, ultimately regulating the expression and activity of numerous cell functions.[11] Upon interaction with ligands, or by other stimuli, TKRs activate by dimerization and subsequent autophosphorylation by the intrinsic tyrosine kinase sites in the dimerized receptors. Tyrosine phosphorylation of TKRs is triggered by their specific ligands, the growth factors, but ligand-independent activation also occurs in response to various stimuli including radiation, oxidative stress, or membrane stretch.[12] Changes in external osmolality are also sensed by TKRs. Recent reports[13,14] show a robust activation of several types of TKR by hypo-osmolality, which may be the initial step for the operation of numerous cytosolic tyrosine kinases and tyrosine kinase targets that are activated by hypo-osmolality (see below) and have a close connection and interplay with TKR receptors (Figure 1 and **Figure 2**).[15] A connection of TKRs with integrins is also feasible, as integrins are known to cooperate with an extensive repertoire of TKRs. Other adhesion molecules such as focal adhesion kinase (FAK) are linked to TKR phosphorylation. Signaling pathways also converge at TKRs, including the mitogen-activated protein (MAP) kinase pathway and the antiapoptotic pathway formed by phosphatidylinositol-3 kinase (PI3K) and Akt (Figure 2). Although a conclusive role for these receptors in volume-sensor mechanisms remains to be established, their role as early signals in the osmotransduction process seems now to be well recognized.

Other candidates for volume sensing are the transient receptor potential channels (TRPCs). Transient receptor potential vanilloid–4 (TRPV4), a member of this family of TRPCs, is a cation channel activated by hypo-osmolality and by membrane stretch. Hypotonic stress results in rapid tyrosine phosphorylation of TRPV4, possibly mediated by Src kinases, and a robust increase in $[Ca^{2+}]_i$.[16,17] This effect is dependent on tyrosine kinase phosphorylation at the channel molecule, and a critical phosphorylation site mapped through mutagenesis studies has been identified at Tyr-253. Point mutation of this site abolishes the hypo-osmolality-dependent $[Ca^{2+}]_i$ increase.[17] Therefore, this channel is likely involved in the mechanism of volume regulation when this process is Ca^{2+} dependent. However, in spite of the almost universal elevation of Ca^{2+} in response to hypo-osmolality, volume regulation in some cells is Ca^{2+} independent.[4] In these cells, it is unclear whether TRPV4 or another member of the TRPC family represents a crucial element in osmotransduction. Clarifying this question will require further identification of TRPV4 links with other elements of the hypo-osmolality-activated signaling chain. The high expression of TRPV4 in the kidney and its uneven distribution in this organ suggest its role in systemic osmoregulation,[16] which is currently under intensive investigation.

Integrins are another group of molecules with the potential to participate in osmosensing.[18] They establish cell adhesion in the extracellular matrix and connect at the cytoplasmic site with molecules of the osmotic signaling network such as p38 and Src kinases, as well as with elements implicated in dynamic changes in the cytoskeleton.

Many questions remain about the sensing mechanism of volume changes, including:

- Is volume sensing accomplished by only 1 type of molecule, or is it a complex mechanism involving several different signaling molecules?
- Is the volume-sensing mechanism the same in all cell types, or do different mechanisms operate in different cells?
- Is the volume-sensing domain located in the cell membrane, the extracellular matrix, or the intracellular compartment?
- In the case of hypo-osmotically-induced volume change, is the sensor detecting a change in cell volume or in internal osmolality?

Osmotransduction Signaling

The osmotransduction signaling pathways have well-recognized elements such as an increase in $[Ca^{2+}]_i$ and the activation of protein tyrosine kinases. Elevation of $[Ca^{2+}]_i$ is among the most consistent cell responses to hyposmotic swelling, and Ca^{2+} is therefore considered a good candidate to participate in osmotransduction. However, in a large variety of cell types, the volume-sensitive Cl^- and K^+ channels and the organic osmolyte efflux pathways are Ca^{2+} independent, and consequently RVD is Ca^{2+} independent as well; in others, such as in most epithelial cells, RVD and K^+ efflux are Ca^{2+} dependent.[4] As previously mentioned, in these cells, the magnitude of the hypo-osmolality-evoked $[Ca^{2+}]_i$ elevation is sufficient to activate the Ca^{2+}-dependent large conductance K^+ channels, which predominantly contribute to RVD.[4]

As for tyrosine kinases, it is known that a number of these enzymes are activated by hypo-osmolality.[13–15] The MAP kinases, extracellular signal–regulated kinases 1 and 2 (ERK1/2) and p38, FAK, members of the Src family, and tyrosine-phosphorylated growth factor receptors all activate in response to hypo-osmolality (Figure 2), but an integrated scheme of the hierarchy, interplay, and influence of these enzymes in the different processes associated with RVD is still incomplete. Cell swelling and RVD are complex processes in which a variety of cell responses occur in addition to those directly involved in volume adjustment. Among

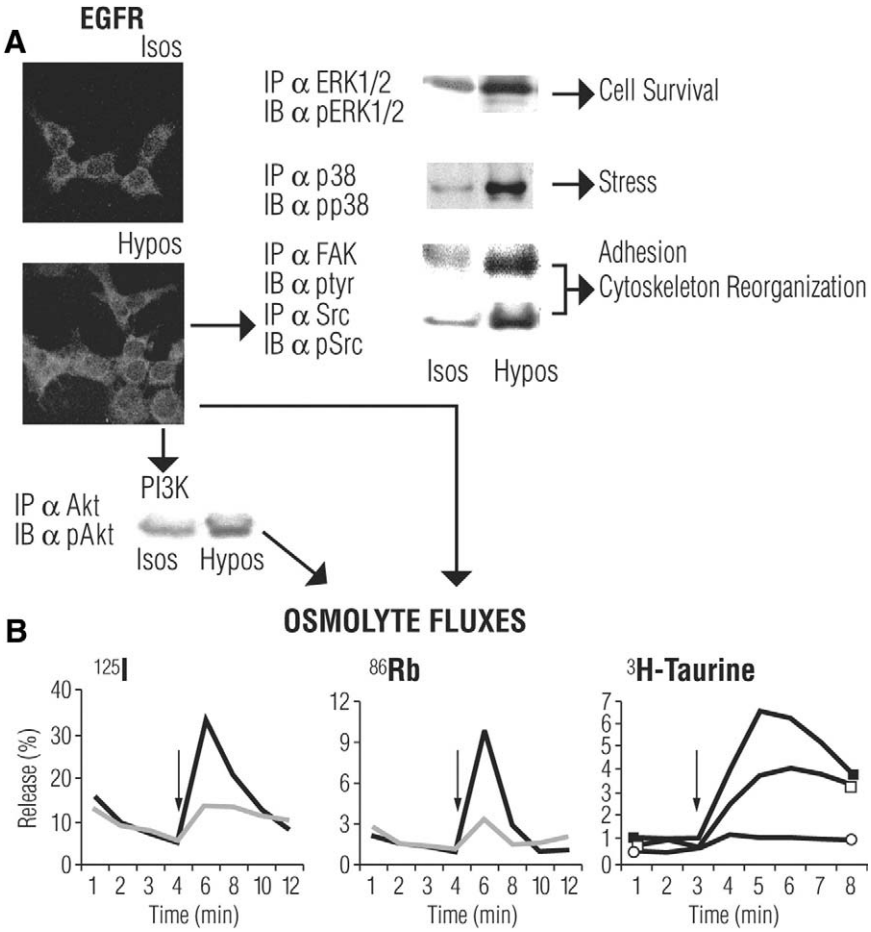

Figure 2 (*A*) Hypo-osmolality activates numerous protein tyrosine kinases. Swiss 3T3 fibroblasts, serum starved for 24 hours, were treated for 3 minutes at the indicated conditions. The hyposmotic epidermal growth factor receptor (EGFR) activation was analyzed by immunofluorescence (EGFR is a member of TKR). After exposure to isosmotic or hyposmotic conditions, cells were fixed and incubated with phosphorylated EGFR antibody, then incubated with a secondary fluorescent antibody, and visualized by confocal microscopy (*left panels*). To evaluate the phosphorylation levels of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, focal adhesion kinase (FAK), Src, and phosphatidylinositol-3 kinase (PI3K) cell lysates were obtained after the hyposmotic stimulus, immunoprecipitated (IP) overnight with the indicated antibody, and analyzed by Western blotting (IB) with the appropriate phosphorylated antibody. Arrows indicate the suggested role of the tyrosine kinases in cell responses evoked by hypo-osmolality. (*B*) Tyrosine kinase activity regulates the osmolyte fluxes during volume adjustment. Cell volume recovery after hyposmolarity-induced swelling is accomplished by the extrusion of intracellular osmolytes. K^+ and Cl^- and organic molecules, particularly amino acids, are the main osmolytes involved in volume regulation. Illustrated is the influence of tyrosine phosphorylation on the efflux of Cl^-, K^+, and taurine. Taurine is one of the most abundant amino acids and its particular features make it an ideal osmolyte; it is often considered representative of the organic osmolytes. The graphs represent the effect of hyposmolarity increasing the Cl^- (traced by ^{125}I), K^+ (traced by ^{86}Rb), and ^3H-taurine efflux as well as the effect of agents affecting the protein tyrosine phosphorylation in cells. Tyrphostin A23 is a tyrosine kinase blocker and reduces osmolyte efflux, whereas *O*-vanadate is a tyrosine phosphatase blocker that increases tyrosine phosphorylation in cells and potentiates the osmolyte fluxes. Cells preloaded with ^{125}I, ^{86}Rb (Intestine-407 cell line), or ^3H-taurine (cerebellar granule neurons) were washed and superfused with isosmotic medium for 4 minutes and then with 20% hyposmotic medium (*arrows*) for 8 minutes. Points represent the radioactivity released per minute, expressed as a percentage of the total loaded. *Open squares* = hyposmolarity-evoked release; *solid squares* = release in the presence of the tyrosine phosphatase blocker *O*-vanadate; *circles* = the effect of the tyrosine kinase blocker tyrphostin A-23187 (depicted in the ^3H-taurine graph). Cells were preincubated for 30 minutes with the blockers, which were then present in all solutions throughout the experiment. Hypos = hyposmotic medium (30%) made by equiosmolar reduction of NaCl; Isos = isosmotic medium, 300 mOsm (135 mmol/L NaCl); p = phosphorylated; tyr = tyrosine. (Adapted from *Pflugers Arch Eur J Physiol*[13] and *J Biol Chem.*[15])

them are those related to cell adhesion to the extracellular matrix, reorganization of the cytoskeleton to cope with the changes in cell volume, temporal deformation and remodeling of the membrane elements, and reactions related to detect a stressful situation and to alert the cells to respond in consequence. In agreement with these multiple effects, hyposmotic activation of some of these tyrosine kinases can be linked to these phenomena, and not directly to cell volume regulation. Thus, FAK seems related to adhesion reaction, whereas p38, a stress-sensitive kinase, may act as a stress sensor. ERK1/2 could be in charge of activating responses for cell survival. The surface TKR may, besides being

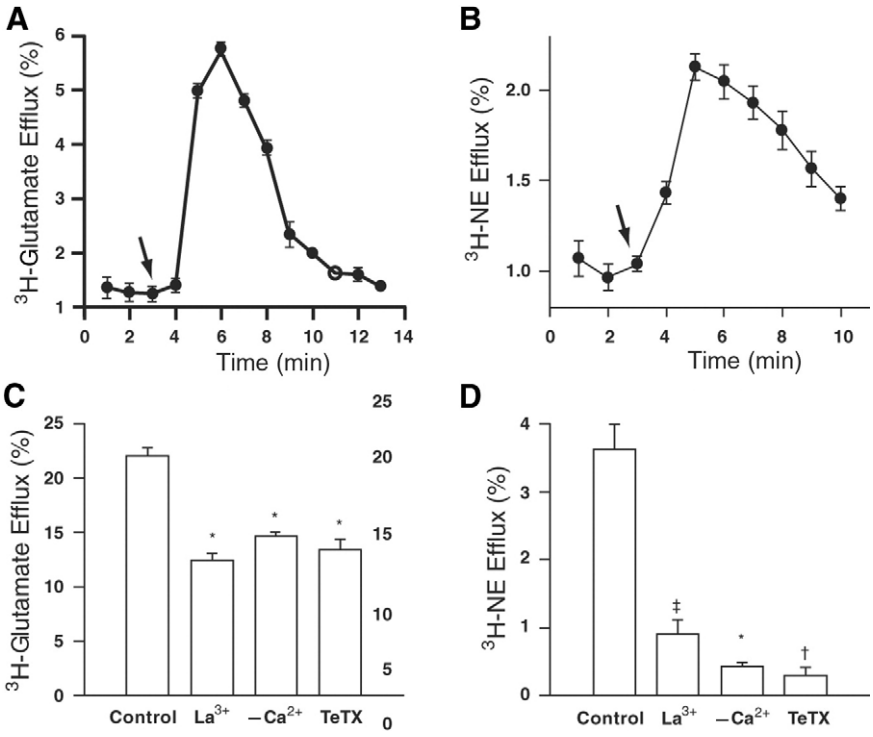

Figure 3 Hyposmolality elicits the efflux of glutamate and norepinephrine from isolated nerve endings. Nerve endings (synaptosomes) prepared from rat cerebral cortex were incubated with (*A*) ^{3}H-glutamate or (*B*) ^{3}H-norepinephrine (^{3}H-NE), washed, and superfused at a flow rate of 1 mL/min with isosmotic medium (changed to a 20% hyposmotic solution where indicated by the arrow). (*C and D*) Effects of La^{3+}, ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester (EGTA-AM), and tetanus toxin on hyposmolality-stimulated neurotransmitter release are shown. Synaptosomes were treated for 15 minutes before superfusion with 100 μmol/L La^{3+} (La^{3+}), 50 μmol/L EGTA-AM in a Ca^{2+} free medium (– Ca^{2+}), or 50 nmol/L tetanus toxin plus 0.1% bovine serum albumin (TeTX). Bars represent the percent radioactivity released in 5 fractions after the stimulus minus the basal release in the same time. Data are means of 3 to 9 experiments. $^*P < 0.001$; $^\dagger P < 0.01$; and $^\ddagger P < 0.02$. (Adapted from *Eur J Neurosci*.[21,22])

possibly involved in the volume-sensing mechanisms, activate antiapoptotic pathways via PI3K, which is also responsive to hypo-osmolality (Figure 2). A signaling pathway directly involved in osmolyte-corrective fluxes is that formed by TKR, epidermal growth factor receptor (EGFR), and PI3K.[13,14]

The cytosolic tyrosine kinase Src could also be a convergent point of osmotransduction signaling pathways.[19] The Src family is composed of 9 tyrosine kinases, some of which (cSrc, FYN, and YES) are ubiquitously expressed. Src kinases are connected with most of the tyrosine kinases activated by hypo-osmolality. Activated Src phosphorylates the EGFR and is reciprocally phosphorylated by this receptor. Src is connected with FAK, p38, and the MAP kinase pathway. Besides this intense interplay with membrane and cytosolic tyrosine kinases, Src appears to influence some of the most important pathways for osmolyte fluxes. K^+ channels of the various types involved in RVD are modulated by Src. Operation of the volume-sensitive Cl^- channel is also under the influence of Src, as has been clearly shown in lymphocytes, and the pathway for organic amino acid fluxes seems to be similarly regulated by Src. Although further investigation is necessary to clarify these ubiquitous influences of Src, the evidence so far suggests a key role of these kinases as an element of confluence of osmotransduction signaling and effector operation.[19]

ROLE OF PHOSPHOLIPASES, ARACHIDONIC ACID, AND REACTIVE OXYGEN SPECIES IN OSMOTRANSDUCTION

Activation of phospholipases (PLAs), particularly PLA_2 and PLD, and the subsequent mobilization of arachidonic acid and lysophosphatidylcholine (LPC) are events that occur in association with hyposmotic swelling. The contribution of these 2 types of phospholipases, or of the different members or isoforms of the PLA_2 family, to the cell response to hypo-osmolality is still under investigation, but there is now information about the influence of the reaction products of these enzymes on osmotransduction signaling.[8] Arachidonic acid helps regulate the activity of K^+ and Cl^- channels as well as the operation of the organic osmolyte efflux pathway. In addition, both arachidonic acid and LPC are known to generate reactive oxygen species (e.g., oxygen radicals, hydrogen peroxides and superoxides, hydroxyl radicals), which may in turn act as signaling elements in osmotransduction by modifying the redox state of proteins involved in the signaling cascade.[8] This notion is based on results showing, first, that hyposmotic swell-

ing generates reactive oxygen species, and second, that preventing this reaction attenuates the phosphorylation of osmotically activated tyrosine kinases.[8] A proposed interpretation of these effects is that the free radicals may impair the function of tyrosine phosphatases, thus increasing the phosphorylation state of proteins phosphorylated by tyrosine kinases involved in osmotransduction.

HYPO-OSMOLALITY AND NEURONAL EXCITABILITY

Severe, acute hyponatremia is associated with a complex neurologic syndrome that includes altered level of consciousness, coma, and increased susceptibility to seizures, suggesting an effect of the hyposmotic condition on brain excitability. Studies in hippocampal slices in vitro show an increase in amplitude and duration of the excitatory postsynaptic potentials under hypo-osmolar conditions, supporting this possibility.[20] This effect could be caused by an increase in glutamate efflux during the hypo-osmolar condition, as has been recently reported in isolated nerve endings.[21] In this study, hypo-osmolality was found to elicit a chain of events beginning with Na^+-dependent depolarization, followed by a rise in intracellular $[Ca^{2+}]_i$, and increased exocytosis. These phenomena may be responsible for the release of glutamate, which, as shown in **Figure 3**,[22] is indeed released by hypo-osmolality. A large proportion of this response is dependent on both depolarization and Ca^{2+} and can be blocked by tetanus toxin, suggesting exocytosis as the efflux mechanism.[21] These results indicate that hypo-osmolality activates a number of processes in nerve endings, many of which mimic those of depolarization-secretion exocytosis. The findings further raise the question of whether other neurotransmitters, not involved as osmolytes, might also be responsive to hypo-osmolality. In support of this possibility, studies using the same isolated nerve ending preparation have shown a significant increase in the release of norepinephrine, an important synaptic transmitter in the cerebral cortex.[22] The properties of this release fit well with a typical depolarization-secretion vesicular process, which is characteristic of neurotransmitter efflux. These results open the intriguing possibility of modulation of synaptic transmission by conditions causing swelling in nerve terminals under both pathologic and possibly physiologic conditions.

References

1. Lang F, Busch GL, Völkl H. The diversity of volume regulatory mechanisms. *Cell Physiol Biochem*. 1998;8:1–45.
2. Okada Y, Maeno E. Apoptosis, cell volume regulation and volume-regulatory chloride channels. *Comp Biochem Physiol A Mol Integr Physiol*. 2001;130:377–383.
3. Okada Y. Volume expansion-sensing outward-rectifier Cl^- channel: fresh start to the molecular identity and volume sensor. *Am J Physiol*. 1997;273(pt 1):C755–C789.
4. Pasantes-Morales H, Morales Mulia S. Influence of calcium on regulatory volume decrease: role of potassium channels. *Nephron*. 2000; 86:414–427.
5. Niemeyer MI, Cid LP, Sepulveda FV. K^+ conductance activated during regulatory volume decrease: the channels in Ehrlich cells and their possible molecular counterpart. *Comp Biochem Physiol A Mol Integr Physiol*. 2001;130:565–575.
6. Ordaz B, Vaca L, Franco R, Pasantes-Morales H. Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K^+ channels. *Am J Physiol Cell Physiol*. 2004;286:C1399–C1409.
7. Junankar PR, Kirk K. Organic osmolyte channels: a comparative view. *Cell Physiol Biochem*. 2000;10:355–360.
8. Lambert IH. Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. *Neurochem Res*. 2004;29:27–63.
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. Melton JE, Patlak CS, Pettigrew KD, Cserr HF. Volume regulatory loss of Na, Cl, and K from rat brain during acute hyponatremia. *Am J Physiol*. 1987;252:F661–F669.
11. Hackel PO, Zwick E, Prenzel N, Ullrich A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol*. 1999;11:184–189.
12. Dent P, Yacoub A, Contessa J, et al. Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res*. 2003;159:283–300.
13. Franco R, Lezama R, Ordaz B, Pasantes-Morales H. Epidermal growth factor receptor is activated by hyposmolarity and is an early signal modulating osmolyte efflux pathways in Swiss 3T3 fibroblasts. *Pflugers Arch Eur J Physiol*. 2004;447:830–839.
14. Lezama R, Ortega A, Ordaz B, Pasantes-Morales H. Hyposmolarity-induced ErbB4 phosphorylation and its influence on the non-receptor tyrosine kinase network response in cultured cerebellar granule neurons. *J Neurochem*. 2005;93:1189–1198.
15. 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.
16. Cohen DM. TRPV4 and the mammalian kidney. *Pflugers Arch Eur J Physiol*. 2005;451:168–175.
17. Xu H, Zhao H, Tian W, Yoshida K, Roullet J-B, Cohen DM. Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation: Src family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress. *J Biol Chem*. 2003;278:11520–11527.
18. vom Dahl S, Schliess F, Reissmann R, et al. Involvement of integrins in osmosensing and signaling toward autophagic proteolysis in rat liver. *J Biol Chem*. 2003;278:27088–27095.
19. Cohen DM. SRC family kinases in cell volume regulation. *Am J Physiol Cell Physiol*. 2005;288:C483–C493.
20. 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.
21. Tuz K, Peña-Segura C, Franco R, Pasantes-Morales H. Depolarization, exocytosis and amino acid release evoked by hyposmolarity from cortical synaptosomes. *Eur J Neurosci*. 2004;19:916–924.
22. Tuz K, Pasantes-Morales H. Hyposmolarity evokes norepinephrine efflux from synaptosomes by a depolarization- and Ca^{2+}-dependent exocytotic mechanism. *Eur J Neurosci*. 2005;22:1636–1642.

Lang F (ed): Mechanisms and Significance of Cell Volume Regulation.
Contrib Nephrol. Basel, Karger, 2006, vol 152, pp 221–240

Volume Changes in Neurons: Hyperexcitability and Neuronal Death

Herminia Pasantes-Morales, Karina Tuz

Department of Biophysics, Instituto de Fisiología Celular, Universidad Nacional, Autónoma de México, Mexico City, Mexico

Abstract

Hyponatremia propitiates and increases susceptibility to seizure episodes. In vitro, hyposmolarity induces hyperexcitability and epileptiform activity and increases the amplitude of excitatory postsynaptic potentials. Synaptic (increased glutamate vesicular release) and non-synaptic (swelling-induced extracellular space shrinkage and ephaptic interactions) might be responsible for the hyposmolarity effects on brain excitability. Neuronal volume constancy in hyponatremia is preserved by the isovolumetric regulation, relying importantly on organic osmolytes. Changes in cell volume are closely linked to neuronal death: swelling characterizes necrotic death as in acute ischemic episodes or brain trauma, whereas volume decrease is typical of apoptotic death. Swelling in necrotic death results from the intracellular Na^+ increase followed by Cl^- and water influx. Na^+ accumulation is due initially to the Na^+/K^+ ATPase dysfunction and subsequently from the Na^+ influx through the overactivated ionotropic glutamate receptors. A second wave of swelling generates by excitotoxic derived formation of reactive oxygen species, membrane lipoperoxidation and further ion overload. Excessive swelling contributes to membrane rupture and release of cell debris, propagating the damage to adjacent cells. Apoptotic death is characterized by cell volume decrease termed apoptotic volume decrease, which in neurons seems to occur by mechanisms remarkably similar to those operating in the hyposmotic swelling activated volume regulatory volume decrease, i.e. channel-mediated efflux of K^+ and Cl^-. A variety of K^+ channels, depending on the cell type and the volume-regulated anion channel are the channels involved in apoptotic volume decrease. K^+ has a protagonic role as an early element in neuronal apoptosis: an outwardly rectifying K^+ current IK_{DR} is enhanced by apoptosis, prior to the caspase activation, increased extracellular K^+ and IK_{DR} blockers attenuate apoptosis and intracellular K^+ loss through ionophores induces apoptosis. Volume-regulated anion channel participates as well in the Cl^- efflux although its role and hierarchy in the apoptotic program are not well defined. Efflux of organic osmolytes, such as taurine participate as well in apoptotic volume decrease.

Swelling in hyposmotic or isosmotic conditions is closely linked to ion movements. Regulatory volume decrease (RVD) activated by hyposmotic swelling relies on the outflow of K^+, Cl^- and small organic osmolytes. Swelling in isosmotic conditions occurs by ion redistribution, modifying the ionic homeostasis in the cell. All these changes are of crucial importance in brain, because the extracellular/intracellular ionic equilibrium determines the resting potential and the discharge pattern of neurons, as excitatory and inhibitory synaptic events are driven by ionic gradients. Besides, some of the organic osmolytes released in connection with volume recovery play in the brain a prominent role as synaptic transmitters. Moreover, changes in cell volume, swelling or shrinkage, may be critical signals in directing the cell death type to necrosis or apoptosis.

Brain Cells Volume and Hyperexcitability

The influence of changes in cell volume on brain excitability was suggested by the seizure occurrence during acute hyponatremia, and the increased susceptibility to seizures in chronic hyponatremia or psychotic polydipsia. That the hyperexcitable condition was due to cell swelling and not to changes in the Na^+ concentration in plasma, was demonstrated by seizure attenuation by infusion of hypertonic solutions or by water restriction. At the cellular level, also hyposmolarity induces hyperexcitability and increases evoked epileptiform activity as shown in CA3 neurons of hippocampal slices and in neocortical pyramidal neurons [Rosen and Andrew, 1990; Saly and Andrew, 1993]. Hyposmolarity also affects excitatory synaptic transmission, increasing the amplitude of excitatory postsynaptic potentials. These effects may result from events connected with cell swelling and volume recovery, occurring in both neurons and astrocytes. Cell swelling may increase brain excitability by one or both of these factors: (i) swelling-induced release of excitatory neurotransmitters, notably glutamate, (ii) reduction in the size of the extracellular space, propitiating ephaptic interactions and restraining the diffusion of neurotransmitters and depolarizing agents [Schwartzkroin et al., 1998].

The first possibility, i.e. a swelling-evoked release of excitatory neurotransmitters is documented in a variety of brain preparations [de la Paz et al., 2002; Franco et al., 2001; Kimelberg et al., 1990; Saransaari and Oja, 1999]. Interestingly, the glutamate efflux in hippocampal slices has features which deviates from the release of typical organic osmolytes represented by taurine. The efflux of taurine inactivates slowly, is sensitive to the Cl^- channel blockers, NPPB and niflumic acid; and is markedly reduced by tyrosine kinase blockers. Glutamate efflux, in contrast, is rapidly inactivated, is insensitive to all the agents which affected taurine, being only decreased by DIDS [Franco et al., 2001].

Hyposmotic efflux of taurine and glutamate is also observed in rat cortical isolated nerve endings (synaptosomes) [Tuz et al., 2004] (fig. 1a, b). In the case of glutamate, the release results from a chain of events triggered by hyposmolarity and ultimately leading to an increase in synaptic vesicle discharge. The initial event is a Na^+-dependent depolarization, sensitive to La^{3+}, Gd^{3+} and ruthenium red, mediated possibly by TRP channels. A subtype of this family of channels, the TRPV4 is almost exclusively present in the nervous tissue, it is osmotically- and mechano-sensitive and is blocked by Gd^{3+}, La^{3+} and ruthenium red [Gunthorpe et al., 2002]. Depolarization is followed by a Na^+-dependent, La^{3+} sensitive, PKC-modulated $[Ca^{2+}]_i$ rise, originated mostly from internal stores. The mitochondrial Ca^{2+} pool released by activation of the mitochondrial Na^+/Ca^{2+} exchanger, as result of the increase in cytosolic Na^+ has an important contribution. $[Ca^{2+}]_i$ rise evoked by hyposmolarity has been reported in cerebellar granule neurons and in hippocampal pyramidal neurons, with contributions of extracellular Ca^{2+} as well as of Ca^{2+} released from internal sources [Borgdorff et al., 2000; Pasantes-Morales and Morales-Mulia, 2000]. Depolarization and $[Ca^{2+}]_i$ rise evoked by hyposmolarity leads to enhanced exocytosis in the nerve endings, which is Ca^{2+}-dependent and prevented by tetanus toxin (TeTX) (fig. 1). This vesicular release is the mechanism responsible for a fraction of the glutamate release from nerve endings, which accordingly, is La^{3+}- and Ca^{2+}-dependent, PKC-modulated and blocked by TeTX. Another fraction of this release occurs via the reversal operation of the carrier and is consequently suppressed by the transporter blockers [Tuz et al., 2004]. Hyposmolarity also increases the efflux of taurine and at a lesser extent, that of GABA (fig. 1b). Noteworthy, the hyposmotic release of taurine does not occur via the Ca^{2+}-dependent vesicular release but it has the features of the volume-activated diffusion pathway, characteristic of the organic osmolyte outflow found in most cell types, i.e. reduced by Cl^- channel blockers and modulated by tyrosine kinase phosphorylation [Tuz et al., 2004]. The hyposmolarity-induced vesicular synaptic release of glutamate from nerve endings may explain in part, the increased in amplitude of spontaneous or evoked excitatory postsynaptic potentials found in CA3 cells in the hippocampus and in neocortical pyramidal neurons. Since hyposmolarity has no effect on the neuronal intrinsic properties such as resting membrane potential, cell input resistance, action potential threshold and duration, its effect on the excitatory postsynaptic potentials must reflect a synaptic phenomenon [Baraban and Schwartzkroin, 1998; Rosen and Andrew, 1990; Saly and Andrew, 1993].

The effects evoked by hyposmolarity resulting ultimately in enhanced exocytosis predict a more general effect of this condition on neurotransmitter release, even when the transmitter is unrelated to an osmolyte function. This was confirmed by data showing an increase in hyposmotic norepinephrine

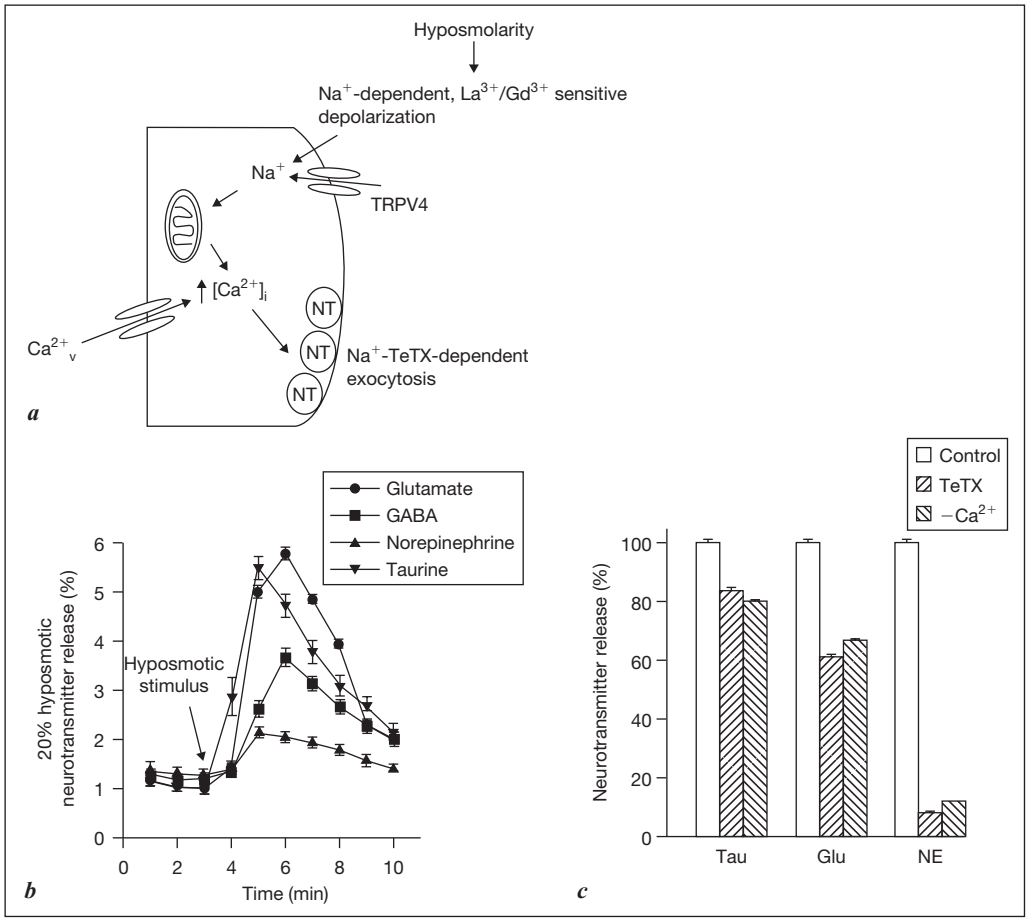

Fig. 1. Hyposmolarity and neurotransmitter release. ***a*** Events sequence of the hyposmolarity-induced release of neurotransmitters from isolated nerve endings (synaptosomes). The initial event is a Na^+-dependent, La^{3+}/Gd^{3+} sensitive depolarization, followed by $[Ca^{2+}]_i$ increase from external and internal sources and activation of a PKC-dependent exocytotic release of neurotransmitters. ***b*** Time course of neurotransmitter release evoked by hyposmolarity from isolated nerve endings. Synaptosomes were prepared and loaded with ^{3}H-glutamate (●), ^{3}H-GABA (■), ^{3}H-norepinephrine (▲) or ^{3}H-taurine (▼), washed and superfused (1 ml/min) during 3 min with isosmotic medium to obtain a constant basal efflux. At the arrow, the medium was replaced by 20% hyposmotic medium, and superfusion continued for 7 min. Results (radioactivity released per min) are expressed as percentage of the total radioactivity incorporated. SE is represented as vertical bars when they exceed the size of symbols. ***c*** Effect of EGTA-AM and TeTX on hyposmotic neurotransmitter release from synaptosomes obtained and treated as in (***b***). Bars represent the radioactivity released (%) at the peak release fractions (5–9). Empty bars represent net release (hyposmotic – isosmotic release) as 100%. Dashed bars correspond to percentage decrease by EGTA-AM or TeTX.

release from isolated nerve endings, which is fully dependent on depolarization, $[Ca^{2+}]_i$ rise and exocytosis [Tuz and Pasantes-Morales, 2005] (fig. 1c). In comparison with the norepinephrine release evoked by depolarizing concentration of K^+, the hyposmotic norepinephrine is more dependent on intracellular than on extracellular sources for $[Ca^{2+}]_i$ rise and is insensitive to blockers of the voltage-dependent L-type Ca^{2+} channels (fig. 1). Altogether these results confirm that despite the differences in the mechanism to induce depolarization and the source of the $[Ca^{2+}]_i$ increase, the synaptic events elicited by hyposmolarity result at the end on a TeTX-sensitive mechanism not different from the vesicular exocytosis characteristic of the classical excitation–secretion coupling release of neurotransmitters. This opens the intriguing possibility of a modulatory effect of changes in cell volume in synaptic transmission.

Swelling may affect neuronal excitability also by non-synaptic mechanisms. Swelling of neurons, but particularly of astrocytes, leads to narrowing of the extracellular space. Astrocyte swelling occurs either in hyponatremia or during clearance of the high extracellular K^+ resulting from intense neuronal activity. As cells expand, there is a reduction in the size of the extracellular space, enhancing ephaptic interactions. This is likely in the origin of the non-synaptic mechanisms of the hypersynchronous behavior of cortical neurons typical of seizures and of the synchronization of epileptiform activity. The influence of cell swelling and the mirrored extracellular space decrease may be of particular importance in brain regions such as the hippocampus where the tight packing of the cell somata restricts the size of the extracellular space. In line with this notion, hyperosmotic solutions block the high K^+ induced epileptiform activity [Dudek et al., 1998] and furosemide suppresses the neuronal synchronized activity generated by episodes of electrically evoked afterdischarges, which had induced astrocyte swelling and extracellular space shrinkage, both of which were prevented by the blocker [Hochman et al., 1995].

Neuronal Protection in Hyponatremia

Exposure of cultured neurons, astrocytes or brain slices, to hyposmotic solutions has been a current experimental device to simulate hyponatremia in vivo. The typical response characterized by rapid swelling followed by return towards the original volume, i.e. the RVD, observed when cells are exposed to

Synaptosomes were 15 min preincubated with 50 μM EGTA-AM in Ca^{2+} free medium containing 0.1 mM EGTA + 10 mM $MgCl_2$. or during 90 min in isosmotic medium in the presence of 50 nM TeTX. Other experimental details in Tuz et al. [2004, 2005]. Glu = Glutamate, NE = norepinephrine, tau = taurine.

sudden and large osmolarity reductions, has been useful to magnify the process and facilitate the identification of signals and mechanisms responsible for this adaptive cell response. However, changes of this magnitude probably never occur in brain under physiological conditions nor even in pathological situations. When the external osmolarity is gradually reduced (−2.2 mOsm/l) the typical steps of RVD are not observed, but depending on the cell type, there is some volume increase of degree varying in different cells. This is what was named isovolumetric regulation by Lohr and Grantham [1986] who first showed this phenomenon in renal proximal tubule cells. Exposed to gradual changes in osmolarity, renal cells were able to maintain a constant volume within a broad range of osmolarities. The term 'isovolumetric regulation' reflects the active nature of this process, as the unchanged volume is not due to the absence of swelling, but to a continuous volume adjustment accomplished by the extrusion of intracellular osmolytes. This paradigm has been applied to other cell types and marked differences have been found with respect to the efficiency of the process. Interestingly, these differences appear to be related to the contribution of amino acids.

Cerebellar granule neurons, as renal cells, respond to the gradual decrease in external osmolarity by a constancy in cell volume even if osmolarity reductions reached up to 50% [Tuz et al., 2001] (fig. 2a). Glioma C6 cells and cultured astrocytes exhibit some swelling, but significantly lower that if an osmotic stimulus of the same magnitude is suddenly imposed [Ordaz et al., 2004a; 2004b] (fig. 2a). Finally, trout erythrocytes respond with similar swelling to gradual or sudden exposure to hyposmotic solutions [Godart et al., 1999].

The osmolytes involved in volume corrective mechanisms during isovolumetric regulation are the same as in RVD, i.e. K^+, Cl^- and organic molecules. The activation threshold of the osmolyte fluxes appears related to the efficiency of the different cell types to counteract the changes in external osmolarity. A correlation is observed between the efflux threshold of taurine and glutamate and the extent of swelling during gradual osmolarity changes. In cerebellar granule neurons, which show the typical isovolumetric regulation, the efflux of taurine and glutamate activates very early after the hyposmolarity reduction, as early as 2% for taurine [Tuz et al., 2001]. In astrocytes and C6 cells the efflux is delayed up to 15 and 39% for taurine and glutamate, respectively [Ordaz et al., 2004a; 2004b], and in trout erythrocytes which do not exhibit isovolumetric regulation, the efflux of taurine occurs in low amounts and is very delayed [Godart et al., 1999]. The higher ability of neurons as compared to astrocytes to resist to changes in external osmolarity, which seems based primarily on the contribution of organic osmolytes, may represent a protective mechanism to spare neurons from the deleterious consequences of swelling. In line with this notion is the interesting observation by Nagelhus et al. [1993] in the cerebellum

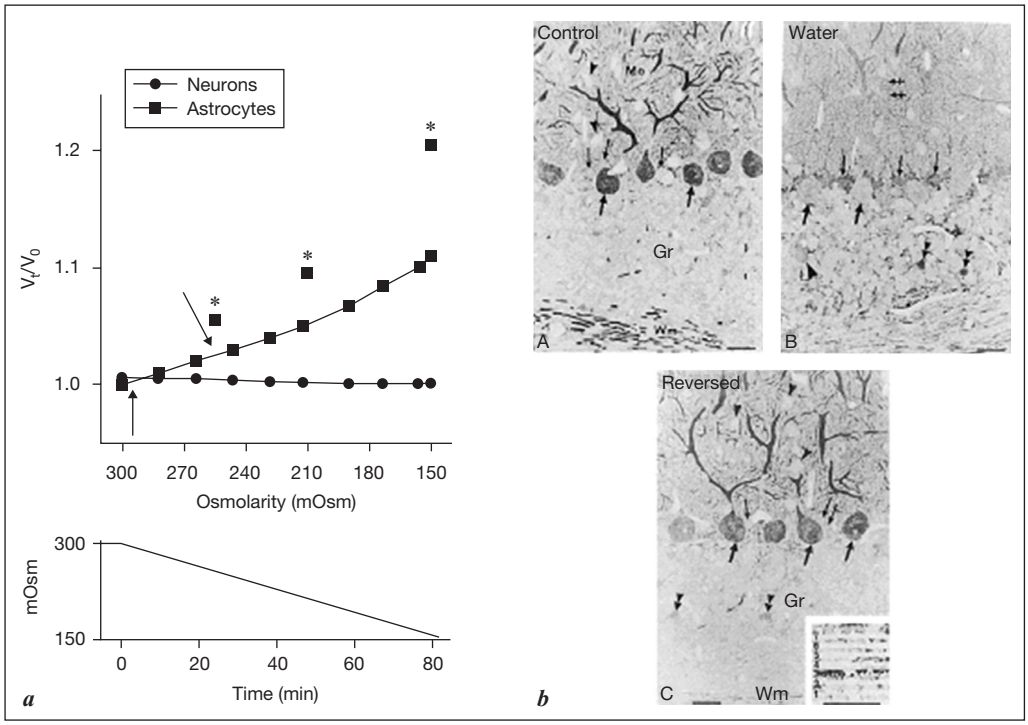

Fig. 2. ***a*** Cell volume constancy i.e. isovolumetric regulation, in cultured cerebellar granule neurons (●) exposed to small and gradual changes in osmolarity (−1.8 mOsm/min). The same treatment in astrocytes (■) increases cell volume but not at the extent observed when the osmotic change is suddenly imposed (■*). For experimental details see Tuz et al. [2001] and Ordaz et al. [2004a, b]. ***b*** Redistribution of taurine-like immunoreactivity in rat cerebellar cortex following water loading. Details of the experiment are in Nagelhus et al. [1993]. In control, isosmotic conditions, taurine is found accumulated in Purkinje cells (large arrows). Upon water loading taurine is transferred to the adjacent glial elements (arrows) which contain very low taurine in isosmotic conditions. As consequence of this redistribution, astrocytes swell while neurons are spared. The original distribution is restored after hyposmolarity correction. Reproduced from Naghelhus et al. [1993] with permission.

of water loaded rats. In the isosmotic condition, the cerebellar Purkinje cells contain high concentrations of taurine, while the nearby astrocytes contain essentially no taurine. In response to the osmolarity reduction there is a remarkable change in the location of taurine: all taurine is lost from the Purkinje cells and is then accumulated in astrocytes (fig. 2b). As consequence of this redistribution, astrocyte swells and neurons are spared. This notable protective action of astrocytes suggests the importance that for neuronal function may have the

maintenance of cell volume, not only in the soma but also in dendrites and axons. Special mechanisms developed for preserving intact the cytoarchitecture of neurons appear to involve importantly organic osmolytes.

Swelling and Necrotic Neuronal Death

Brain cell swelling occurs during pathological conditions such as ischemia, epilepsies, trauma and hepatic encephalopathy. Vasogenic as well as cellular edema are found coincidently in these pathologies. Cellular swelling occurs in all those pathologies resulting in hyponatremia. Hyposmotic swelling results from a decrease in the external osmolarity and the subsequent water flow tending to establish a new osmotic equilibrium. Isosmotic (cytotoxic) swelling is generated by redistribution of ions or molecules responding to phenomena inherent to the pathology, such as the energetic failure and dissipation of Na^+ gradient during hypoxia/ischemia, the increase of extracellular K^+ during ischemia and epilepsies, or the ammonium accumulation during hepatic encephalopathy. Hyposmotic swelling, even if drastic, rarely results in cell death, while cytotoxic swelling commonly ends in excitotoxicity and necrotic death.

It is generally accepted that cytotoxic swelling, occurs mainly in astrocytes whereas neurons are less affected. This is in line with the known essential role of astrocytes in protecting neurons from the disturbing effects of changes in the composition of the extracellular space, or from excessive concentration of potentially toxic molecules. Typical examples of this role of astrocytes is the K^+ clearance from the extracellular space by spatial buffering or the glutamate uptake persisting in astrocytes longer than in neurons due to their ability of facing the energy failure by generating ATP via the glycolitic pathway [rev. in Pasantes-Morales and Franco, 2005]. When the pathological conditions are too severe or prolonged, the protective mechanisms in the astrocyte are exceeded and neurons could then be affected in several ways, including swelling occurrence.

The mechanisms of swelling-induced necrotic neuronal death will be discussed essentially for the ischemic condition. During trauma, vasogenic is the predominant type of edema, while cellular edema is consequent to the ischemic conditions. Swelling in hepatic encephalopathy affects essentially astrocytes, since ammonia detoxification, which is the primary swelling inductor, occurs only in astrocytes [Butterworth, 2002].

The chain of events responsible for cytotoxic swelling and necrotic cell death in ischemia are initiated by the energy failure and the consequent Na^+/K^+ ATPase dysfunction. The resultant elevation of intracellular Na^+ and extracellular K^+ is followed by depolarization and glutamate release. High extracellular

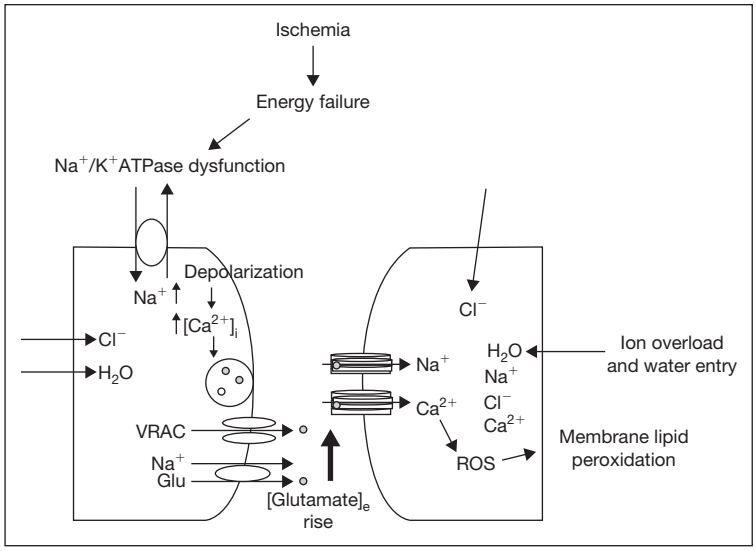

Fig. 3. Swelling inductors in ischemic necrotic neuronal cell death. The energy failure produced by ischemia provokes Na^+/K^+ ATPase dysfunction, intracellular Na^+ increase, and depolarization. This is followed by Ca^{2+}-dependent glutamate release, both from exocytosis, reversal of the transporter and activation of VRAC. The overfunction of ionotropic glutamate receptors further increases intracellular Na^+ followed again by Cl and water. The massive Ca^{2+} influx entry promotes production of ROS, membrane lipid peroxidation and a new wave of swelling due to ion overload through the injured membranes. Excessive swelling contributes to membrane rupture and release of cell debris, propagating the damage to adjacent cells.

glutamate levels resulting from depolarization cannot be removed by the Na^+-driven glutamate transporters since the Na^+ gradient is dissipated. The transporters may even operate in a reverse mode, further increasing extracellular glutamate. Overfunction of ionotropic glutamate receptors leads to massive Na^+ influx. Then, Cl^- and water influx driven by the intracellular high concentration of Na^+ generates a first wave of cytotoxic swelling. Further glutamate release via the swelling-activated glutamate efflux pathway also contributes to the increase and persistence of glutamate in the extracellular space (fig. 3). A second wave of swelling occurs as consequence of Ca^{2+} influx through the ionotropic glutamate receptors. This $[Ca^{2+}]_i$ rise produces reactive oxygen species (ROS) through the activation of prooxidant mechanisms, such as phospholipases, xantine oxidase and nitric oxid synthase. The free fatty acid release and the generation of several free radical species damages membrane integrity and favors ion overload and a further wave of Na^+ and Ca^{2+} increase (fig. 3). There is almost general agreement on that elevation of $[Ca^{2+}]_i$ is the primary

cause of excitotoxic neuronal death, mediated by proteases of the family of calpains and cathepsins At this state, swelling contributes to necrotic neuronal death propagation propitiating plasma membrane rupture and cell lysis. The release of cellular content and necrotic debris to the extracellular space precipitates damage to cells in the vicinity, via the chain of events involving ROS, membrane lipid peroxidation and Na^+ and Ca^{2+} overload through the affected membranes.

Lactacidosis generated in ischemia and trauma is another factor of swelling and neuronal death. Lactate is formed during the operation of the glycolitic pathway in astrocytes, which provides an alternate source of energy as far as there is residual glucose. Cytotoxic swelling by lactacidosis occurs preferentially in astrocytes but there is evidence of neuronal swelling in vivo as well in cultured neurons exposed to extracellular lactate levels as those present in ischemia [Alojado et al., 1996; Staub et al., 1993]. Swelling is a function of the severity of acidosis and duration of the exposure, although not all neurons appear to be equally susceptible [Cronberg et al., 2005].

How neurons respond to cytotoxic swelling has not been examined in detail. Neurons exhibit a complex morphology, with specialized functions in soma, dendrites, axon or nerve terminals. Then, swelling may not be homogeneous and its functional consequences will depend on the affected area. There is no clear evidence of active volume recovery in neurons after cytotoxic swelling, but a low efficiency or inability is predictable, in view of the nature of the swelling inductors above discussed. Cell volume regulation relies on the expulsion of intracellular osmolytes, which occurs in general, via diffusion pathways or channels through which the osmolytes move following the gradient direction. In ischemia and epilepsies, the excessive extracellular K^+ concentration resulting from the Na^+/K^+ ATPase dysfunction and neuronal overexcitability will limit the operation of the volume-activated K^+ channels. The ATP drop impairs also the volume-sensitive Cl^- channel which characteristically requires nonhydrolytic ATP binding. Organic osmolytes also have this ATP requirement [Okada et al., 2006]. In addition, the volume-sensitive Cl^- channel seems impaired by lactacidosis [Mori et al., 2002]. Altogether, these conditions accentuate the difficulty of brain cells, including neurons, to accomplish the set of reactions necessary to restore the normal cell volume in conditions of ischemic cytotoxic swelling.

Cell Volume and Apoptosis in Neurons

Apoptosis is a highly regulated process of cell deletion directed to eliminate a definite group of cells at a precise time, for preserving the optimal

operation of tissues and organs. In the developing brain, apoptosis is an essential process to reach the numerical match of neuronal populations and their targets, for organizing the regional cytoarchitecture, and to establish functional synapses and building of neural circuitry. In the adult brain, apoptosis has some contribution to neuronal loss in acute neuropathologies, in some neurodegenerative disorders and in brain aging. Apoptosis occurs according to a strictly ordered set of biochemical events, one of which is a characteristic reduction in cell volume, termed apoptotic volume decrease (AVD).

When the temporary resolution of apoptosis was established, it was clear that AVD is an early event in the program, occurring before the surge of other characteristic traits such as caspase 3 cleavage, cytochrome c release and translocation, endonuclease activation and DNA fragmentation. Moreover, it is now considered that AVD may be part of the causal signals and not only consequence of apoptosis [Bortner and Cidlowski, 2002, 2004]. Noteworthy, AVD is not followed by any compensatory mechanism directed to counteract the cell volume loss. There is no evidence of regulatory volume increase, which is apparently inhibited or overridden by an as yet unknown signal [Bortner and Cidlowsly, 2004]. Although AVD occurs in isosmotic conditions, it relies on the same mechanisms of RVD, i.e. the active translocation of intracellular osmolytes and osmotically obligated water flow. K^+ and Cl^- are the main intracellular osmotically active solutes and are natural candidates to accomplish AVD. Some organic molecules also participate.

K^+ Efflux and AVD in Neurons. K^+ Channels Involved

The importance of K^+ efflux and AVD as part of the apoptotic signaling chain is now well established, and there is reasonable agreement on that K^+ outflow occurs via K^+ channels [Bortner and Cidlowski, 2004; Burg et al., 2006; Yu, 2003]. Studies in mice cultured cortical neurons undergoing apoptosis by treatment with staurosporin or ceramide have shown AVD, K^+ loss and the selective and early enhancement of a voltage-dependent delayed rectifier current (IK_{DR}) in the apoptotic neurons. Decreasing cell K^+ by the ionophores valinomycin and beauvericin induced apoptosis and accordingly, high external K^+ levels suppressed apoptosis. The antiapoptotic effect of increasing external K^+ was Ca^{2+}- and caspase-independent [Yu et al., 1997, 1999]. IK_{DR} blockade by tetraethyl ammonium or clofilium also attenuates apoptosis. These results suggest an early location of K^+ efflux and AVD in the apoptotic signaling chain in mice cortical neurons. However, a study in rat cortical neurons, showed that staurosporine-induced apoptosis is not reduced by tetraethyl ammonium, clofilium or high external K^+ but it is prevented by SITS [Small et al., 2002]. These differences may be due to the higher concentration of staurosporine used in this study, which may induce both apoptosis and necrosis.

Cerebellar granule neurons require for survival a depolarizing environment provided by 25 mM K^+ or NMDA receptor agonists. Cell death prevention in this case depends on the Ca^{2+} influx through voltage-gated channels [Alavez et al., 2003]. In conditions of low K^+, cells die by apoptosis which occurs concomitant to the increase of an outwardly rectifying K^+ current named standing outward K^+ current (IK_{so}) [Lauritzen et al., 2003].

Apoptosis is induced by staurosporine in neuronal progenitor cells generated from mice striatal stem cells [Hribar et al., 2004]. Similar to mice cultured cortical neurons, a delayed rectifier K^+ current already expressed in the neuronal precursors, is enhanced by staurosporine since the first days of differentiation. IK_{DR} enhancement precedes the activation of caspase 3 and increasing external K^+ reduced IK_{DR} and attenuates apoptosis. These results are similar to those found in cortical neurons but in the neuronal progenitor cells the amplitude of IK_{DR} is reduced by caspase blockers.

Apoptotic cell death concurs with necrotic cell death in some neurodegenerative disorders as well as in ischemia and trauma. Necrotic neuronal death predominates at the ischemic focus while apoptotic cell death prevails in the perifocal area. Apoptosis is triggered and sustained by multiple factors concurrent with the ischemic condition: acidosis, increased expression of death receptors or proapoptotic molecules, activation of MAP kinases and generation of nitric oxid and peroxynitrite. Activation of apoptosis directly by nitric oxide in mice cortical neurons led to IK_{DR} enhancement, K^+ outflow and cell K^+ loss, all resistant to the general caspase blocker zVAD [Bossy-Wetzel, 2004]. The same set of reactions occurs in cortical neurons in a thiol-oxidant model of apoptosis [Aizenman et al., 2000]. The β-amyloid peptide linked to Alzheimer disease, enhances an outward K^+ current in cortical neurons in conjunction with apoptosis [Yu et al., 1998]. In cerebellar Purkinje cells apoptosis associated to lurcher gene occurs after activation of a voltage-gated channel, suggesting the involvement of K^+ in the apoptotic program in this neurodegenerative condition [Norman et al., 1995].

The molecular identity of the ADV-linked K^+ channels is now extensively investigated and from the diversity of cell types examined, including neurons, an important point emerged: there is apparently not a specific channel, devoted exclusively to permeate K^+ for reducing cell volume during apoptosis. This function seems accomplished by K^+ channels present in the non-apoptotic cell, and performing a variety of tasks in the physiological condition. The type of apoptosis-activated K^+ channel may be cell specific and could be different according to the apoptotic inductor. K^+ channels linked to AVD and apoptosis include: Kv channels (isoforms Kv1.1, Kv1.3, Kv1.5, Kv2.1), K2P channels (TASK-1 and TASK-3), HERG and BK Ca^{2+} channels. The K_{ATP} channels are involved in the intrinsic mid-and late phase of apoptosis [Yu, 2003]. Kv channels

are those linked to AVD in cortical neurons. These channels fit an important requirement for the K^+ exit route i.e. large K^+ conductance and slow inactivation during depolarization. The Kv2.1 isoform carries a significant fraction of the enhanced IK_{DR} in apoptotic cortical neurons. This is concluded by the effect of two dominant negative mutant forms of Kv2.1 decreasing IK_{DR}, and complementary, the apoptosis attenuation in neurons deficient in functional Kv2.1 [Pal et al., 2003]. In cultured cerebellar granule neurons, the standing outward K^+ current (IK_{so}) which increases with cell maturation in the culture could is suggested as the apoptotic K^+ exit route. IK_{so} has biophysical, pharmacological and regulation properties characteristic of the TASK-1 and TASK-3 2P-domain K^+ channels (K2P) [Lauritzen et al., 2003]. The following evidence connects IK_{so} and K2P channels to apoptosis: (i) young cerebellar neurons lacking IK_{so} are resistant to low K^+-induced cell death, (ii) conditions or agents decreasing K2P prevent apoposis in mature neurons, (iii) apoptosis occurs in hippocampal neurons lacking IK_{so} after viral-induced expression of TASK-1 or TASK-3 channels (iiii) inactivation of endogenous TASK channels by expression of the dominant-negative loss of function TASK mutants, protects neurons from the low-K^+ induced apoptosis [Lauritzen et al., 2003]. In some conditions, glutamate induces apoptosis and, similar to other apoptogenic models, K^+ efflux is linked to apoptotic death. Kv channels, increased K^+ permeability through the activated receptors or activation of high conductance Ca^{2+}-activated K^+ channels are all proposed routes for K^+ exit [Isaacson and Murphy, 2001; Yu, 2003]. Most studies on AVD in neurons have addressed to the early phase of the apoptotic program and the K^+ efflux driven by plasmalemmal K^+ currents. However, K^+ channels may also be involved in the mid- and late phases characterized by mitochondrial depolarization and cytochrome c release. K_{ATP} channels are those commonly associated with these phenomena.

AVD and Cl^- fluxes

K^+ efflux is a characteristic trait of apoptosis in neurons as in many other cell types, but in order to effectively contribute to water outflow and AVD, it has to occur in conjunction with Cl^- exit. Activation of Cl^- currents in CD95-induced apoptosis was first shown by Lang and coworkers in Jurkat cells [Lang et al., 2005; Szabo et al., 1998]. This was confirmed thereafter in other cell types in apoptosis driven by staurosporine, FAS ligand, TNFa, ceramide or doxorubicin [Maeno et al., 2000; Okada et al., 2006]. The apoptosis-linked anion currents have properties in all similar to those carried by the volume-regulated anion channel (named VRAC) which plays a prominent role in RVD, but noteworthy this activation occurs in isosmotic conditions or even in shrinking, not swelling cells. The mechanism(s) or signals for VRAC activation are as yet unknown, neither in RVD nor in AVD. Cl^- channel blockers, DIDS, NPPB or

phloretin prevent staurosporine-induced apoptosis in neural PC12 and in neuroblastoma NG108–15 cells [Maeno et al., 2000], and SITS is effective in hippocampal neurons undergoing apoptosis by ischemia/reperfusion [Inoue et al., 2005]. However, a study in cortical neurons shows that DIDS, NPPB or phloretin provide only limited protection against apoptosis induced by staurosporine, ceramide or serum deprivation, while K^+ channel blockers supply complete protection [Wei et al., 2004]. This failure of Cl^- channel blockers has been attributed to the low concentration of the blockers used in the cortical neurons preparation due to their toxic effect at higher concentrations [Okada et al., 2006].

In addition to VRAC, the voltage-dependent anion channel (VDAC) is proposed to participate in AVD. VDAC is a large conductance anion channel (300–400 pS), located at the outer mitochondrial membrane. In staurosporine-induced apoptosis in the hippocampal cell line HT-22 and in the human neuroblastoma cell line SK-N-MC, VDAC was found functionally expressed in the cell membrane in 48% of apoptotic cells and its blockade by functional antibodies or by high concentrations of sucrose decrease the number of apoptotic cells [Elinder et al., 2005]. In physiological conditions, membranal VDAC may function as an NADH-reductase involved in transmembrane redox regulation. The Cl^-/HCO_3^- exchanger is also suggested as mechanism for Cl^- outflow in apoptosis based on the strong effect of the typical anion exchanger blocker DIDS, in suppressing the apoptotic program. This has been challenged by experiments showing no change in the antiapoptotic effect of DIDS in the absence of bicarbonate, a condition reducing the exchanger operation [Okada et al., 2006].

Signals for Activation of Ion Fluxes in Apoptosis

The signals in the apoptotic process activating Cl^- and K^+ fluxes for AVD remain largely unknown. Since various types of K^+ channel are involved, a common signal may not operate. The stress-reacting MAPK p38 is suggested as a signal [McLaughlin et al., 2001] under the upstream influence of another MAPK, the MAPKKK also called ASK1 (apoptosis signal-regulating kinase). Inactive ASK-1 or expression of a dominant-negative form of ASK-1 suppresses IK_{DR} and prevents apoptosis in these cells [Aras and Aizenman, 2005]. Tyrosine kinases may also participate in the AVD-linked K^+ fluxes in cortical neurons, since the tyrosine kinase general blockers herbimycin or lavendustin reduce IK_{DR} in cortical neurons [Yu et al., 1999]. Tyrosine kinases may be directly or indirectly associated with p38. Another proposed mechanism for apoptotic IK_{DR} upregulation is the membrane insertion of preformed, endogenous channels as shown for Kv2.1 channels in cortical neurons [Pal et al., 2006]. This translocation requires t-SNARE proteins, the same involved in the

exocytotic mechanism of neurotransmitter release. Disruption of SNARE proteins by botulin neurotoxin suppresses the apoptotic enhanced IK_{DR} [Pal et al., 2006].

Cl^- outflow seems to occurs via the same anion channel in RVD and AVD, in spite of the different, even opposite volume of cells in the two conditions, suggesting a change in the volume set point for the channel gating. The mechanism for this adjustment in the set point is unknown. Reactive oxygen species or tyrosine kinases such as the Src kinase p56Lck might be involved [Lambert, 2004; Leppe-Wienhues et al., 1998]. A role for the cytotolic concentration of ATP has also been considered [Okada et al., 2006].

Volume Decrease or Ionic Homeostasis: Which is the Apoptotic Signal?

Cell volume decrease has been the key morphological trait to distinguish between necrotic and apoptotic death, but it is only in the last decade that insertion of AVD in the signaling chain of the apoptotic process has been demonstrated. Clearly AVD results essentially from K^+ and Cl^- outflow, but the question remains of whether the relevant point in terms of apoptotic program, is the cell volume reduction or the decrease in the intracellular ion concentrations. If K^+ or Cl^- at physiological levels could have an inhibitory influence on factors or reactions of the apoptotic chain, the activation of an efflux pathway and the consequent decrease in cell levels would relieve this inhibition. AVD would then be just the consequence of the ion extrusion. This hypothesis is supported by two types of results: (i) preventing K^+ loss by increasing extracellular K^+ interrupts apoptosis even when the K^+ exit route is fully active and (ii) decreasing cell K^+ concentrations with no activation of the apoptogen-induced K^+ currents is sufficient to set in motion the apoptotic machinery. Caspases and nucleases may be the sites of K^+ influence as suggested by the inhibitory effect of physiological K^+ concentrations on the activity of caspase 3. The K^+ threshold concentration for activating the apoptotic reactions has not been precisely defined [Bortner and Cidlowski, 2004]. A possible influence of Cl^- levels on the apoptotic steps has not being examined in detail. It is also unclear if K^+ and Cl^- have equal importance as a relevant signal, or if only one of them is actively expelled as part of the apoptotic signaling chain, and the other one is just passively carried to support the persistence and magnitude of the active ion outflow. That Cl^- may play this passive role is suggested by a study in staurosporine-induced apoptosis in cortical neurons showing that DIDS prevents AVD but not caspase-3 nor DNA fragmentation, whereas K^+ channel blockers have a full inhibitory action on all the apoptotic events [Wei et al., 2004]. The temporal sequence of apoptosis-linked Cl^- and K^+ fluxes has not been examined in detail. In RVD the order of K^+ and Cl^- efflux activation is dependent on the cell type. Thus, Cl^- channels activate prior to K channels in

most epithelial cells and the opposite is found in non-epithelial cells [Pasantes-Morales and Morales-Mulia, 2000].

A role for Na^+ in AVD and apoptosis has recently raised attention. An early and transient increase in intracellular Na^+ has been detected in apoptotic Jurkat cells [Bortner and Cidlowki, 2003] which if suppressed, prevents K^+ loss, cell shrinkage and DNA degradation. Na^+ influx is a hallmark of necrotic death and therefore, if Na^+ entry is also crucial for apoptosis, the influx mechanisms and their activation and inactivation signals must be critical for deciding the ultimate direction of the cell death type.

AVD and Taurine Efflux

Organic osmolytes contribute to RVD and they could also participate in AVD. Taurine efflux concurrent with apoptosis has been shown in a variety of cell types including cerebellar granule neurons. The mechanism of the apoptotic taurine release is not well characterized. In cerebellar granule neurons as well as in Jurkat cells, the Cl^- channel blockers which are very efficient in preventing taurine efflux in RVD do not decrease but increase the AVD-linked taurine efflux [Lang et al., 2000; Morán et al., 2000]. Also, the tyrosine kinase influence observed in taurine efflux in RVD is not found in AVD. Raising external taurine up to 20 mM, a condition likely preventing taurine efflux or/and cell taurine loss, attenuates apoptosis in cortical neurons [Huang et al., 2006]. Thus, the arguments raised about the key role of K^+ cell loss and not AVD, are also valid for taurine. A consistent observation is that in cells treated with high external taurine, the apoptotic step corresponding to the assembly of apoptosomes and the further activation of caspase 9 is prevented [Takatani et al., 2004], suggesting an inactivating role of physiological concentrations of taurine in certain apoptotic signals. In line with this interpretation is the apoptotic death of photoreceptors in taurine transporter knockout mice [Heller-Stilb et al., 2002].

Final Comments

Excitability, hyperexcitability, hypersynchrony, survival and death, all have a link with one of the most ancient and preserved traits of the cell biology: the cell volume control. Even in the highly specialized neuron, cell volume influences its life cycle, from rescue to death, from proliferation to extinction, from rest to excitation. More question than answers still characterize our knowledge of this essential biological function. Among the most intriguing, refer to the crossing points between RVD and AVD while regulatory volume increase becomes protagonist by its apparently programmed repression. The nature of a

volume sensor finely tuned to detect in RVD, the imperative of activating osmolyte outflow routes for rescue and in AVD, activating apparently similar routes for death. The set point adjustment allowing the activation of the same channels when cells are in opposite conditions, in swollen or shrinked cells, the similarities or dissimilarities between the signaling cascades in RVD and AVD, connecting the same sensors and the same effectors, the sequence, consequence and interdependence of K^+ and Cl^- fluxes leading cells to normality or to death. Solving these and many other questions will add to our knowledge of the seminal topic of neuronal death and survival in pathologies related to changes in cell volume.

Acknowledegement

The work of the authors was supported by grants No.IN206403 from DGAPA UNAM and 46465 from CONACYT.

References

Aizenman E, Stout AK, Hartnett KA, Dineley KE, McLaughlin B, Reynolds IJ: Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. J Neurochem 2000;75: 1878–1888.

Alavez S, Pedroza D, Morán J: Mechanisms of cell death by deprivation of depolarizing conditions during cerebellar granule neurons maturation. Neurochem Int 2003;43:581–590.

Alojado ME, Morimoto Y, Morimoto Y, Kemmotsu O: Mechanism of cellular swelling induced by extracellular lactic acidosis in neuroblastoma-glioma hybrid (NG108–15) cells. Anesth Analg 1996;83:1002–1008.

Aras MA, Aizenman E: Obligatory role of ASK1 in the apoptotic surge of K^+ currents. Neurosci Lett 2005;387:136–140.

Baraban SC, Schwartzkroin PA: Effects of hyposmolar solutions on membrane currents of hippocampal interneurons and mossy cells in vitro. J Neurophysiol 1998;79:1108–1112.

Borgdorff AJ, Somjen GG, Wadman JW: Two mechanisms that raise free intracellular calcium in rat hippocampal neurons during hypoosmotic and low NaCl treatment. J Neurophysiol 2000;83:81–89.

Bortner CD, Cidlowski JA: Apoptotic volume decrease and the incredible shrinking cell. Cell Death Differ 2002;9:1307–1310.

Bortner CD, Cidlowski JA: Uncoupling cell shrinkage from apoptosis reveals that Na^+ influx is required for volume loss during programmed cell death. J Biol Chem 2003;278:39176–39184.

Bortner CD, Cidlowski JA: The role of apoptotic volume decrease and ionic homeostasis in the activation and repression of apoptosis. Pflugers Arch 2004;448:313–318.

Bossy-Wetzel E, Talantova MV, Lee WD, Schölzke MN, Harrop A, Mathews E, Götz T, Han J, Ellisman MH, Perkins GA, Lipton SA: Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p-38-activated K^+ channels. Neuron 2004;41: 351–365.

Burg ED, Remillard CV, Yuan JX-J: K^+ channels in apoptosis. J Membr Biol 2006;209:1–18.

Butterworth MF: Pathophysiology of hepatic encephalopathy: a new look at ammonia. Metab Brain Dis 2002;17:221–227.

Cronberg T, Jensen K, Rytter A, Wieloch T: Selective sparing of hippocampal CA3 cells following in vitro ischemia is due to selective inhibition by acidosis. Eur J Neurosci 2005;22:310–316.

de La Paz LD, Lezama R, Torres-Marquez ME, Pasantes-Morales H: Tyrosine kinases and amino acid efflux under hyposmotic and ischaemic conditions in the chicken retina. Pflugers Arch 2002;445:89–96.

Dudek FE, Yasumura T, Rash JE: 'Non-synaptic' mechanisms in seizures and epileptogenesis. Cell Biol Int 1998;22:793–805.

Elinder F, Akanda N, Tofighi R, Shimizu S, Tsujimoto Y, Orrenius S, Ceccatelli S: Opening of plasma membrane voltage-dependent anion channels (VDAC) precedes caspase activation in neuronal apoptosis induced by toxic stimuli. Cell Death Differ 2005;12:1134–1140.

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

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

Gunthorpe MJ, Benham CD, Randall A, Davies JB: the diversity in the vallinoid (TRPV) receptor family of ion channels. Trends Pharmacol Sci 2002;23:183–191.

Heller-Stilb B, van Roeyen C, Rascher K, Hartwig HG, Huth A, Seeliger MW, Warskulat U, Haussinger D: Disruption of the taurine transporter gene (taut) leads to retinal degeneration in mice. FASEB J 2002;16:231–233.

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

Hribar M, Bloc A, Medilanski J, Nüsch L, Eder-Colli L: Voltage-gated K^+ current: a marker for apoptosis in differentiating neuronal progenitor cells? Eur J Neurosci 2004;20:635–648.

Huang Z, Hou Q, Cheung NS, Li QT: Neuronal cell death caused by inhibition of intracellular cholesterol trafficking is caspase dependent and associated with activation of the mitochondrial apoptosis pathway. J Neurochem 2006;97:280–291.

Inoue H, Mori S, Morishima S, Okada Y: Volume-sensitive chloride channels in mouse cortical neurons: characterization and role in volume regulation. Eur J Neurosci 2005;21:1648–1658.

Isaacson JS, Murphy GI: Glutamate-mediated extrasynaptic inhibition: direct coupling of NMDA receptors to $Ca(^{2+})$-activated K^+ channel. Neuron 2001;31:1027–1034.

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

Lambert IH: Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. Neurochem Res 2004;29:27–63.

Lang F, Föller M, Lang KS, Lang PA, Ritter M, Gulbins E, Vereninov A, Huber SM: Ion channels in cell proliferation and apoptotic cell death. J Membr Biol 2005;205:147–157.

Lang F, Madlung J, Siemen D, Ellory C, Lepple-Wienhues A, Glubins 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.

Lauritzen I, Zanzouri M, Honoré E, Duprat F, Ehrengruber MU, Lazdunski M, Patel AJ: K^+-dependent cerebellar granule neuron apoptosis. J Biol Chem 2003;34:32068–32076.

Lepple-Wienhues A, Szabo I, Laun T, Kaba NK, Gulbins E, Lang F: The tyrosine kinase p561ck mediates activation of swelling-induced chloride channels in lymphocytes. J Cell Biol 1998;141: 281–286.

Lohr JW, Grantham JJ: Isovolumetric regulation of isolated S2 proximal tubules in anisotonic media. J Clin Invest 1986;78:1165–1172.

Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. Proc Natl Acad Sci USA 2000;1997: 9487–9492.

McLaughlin B, Pal S, Tran MP, Parsons AA, Borone FC, Erhardt, JA, Aizenman E: p38 activation is required upstream of potassium current enhancement and caspase cleavage in thiol oxidant-induced neuronal apoptosis. J Neurosci 2001;21:3303–3311.

McManus M, Fischbarg J, Sun A, Hebert S, Strange K: Laser light-scattering system for studying cell volume regulation and membrane transport processes. Am J Physiol 1993;265:C562–C570.

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

Mori S, Morishima S, Takasaki M, Okada Y: Impaired activity of volume-sensitive anion channel during lactacidosis-induced swelling in neuronally differentiated NG108–15 cells. Brain Res 2002;957: 1–11.

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.

Norman DJ, Feng L, Cheng SS, Gubbay J, Chan E, Heintz N: The lurcher gene induces apoptotic death in cerebellar purkinje cells. Development 1995;121:1183–1193.

Okada Y, Shimizu T, Maeno E, Tanabe S, Wang X, Takahashi N: volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. J Membr Biol 2006;209:1–9.

Ordaz B, Tuz K, Ochoa LD, Lezama R, Peña-Segura C, Franco R: Osmolytes and mechanisms involved in regulatory volume decrease under conditions of sudden or gradual osmolarity decrease. Neurochem Res 2004a;29:65–72.

Ordaz B, Vaca L, Franco R, Pasantes-Morales H: Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K^+ channels. Am J Physiol 2004b;286:C1399–C1409.

Pal S, Hartnett KA, Nerbonne JM, Levitan ES, Aiznman E: Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. J Neurosci 2003;23:4798–4802.

Pal SK, Takimoto K, Aizenman E, Levitan ES: Apoptotic surface delivery of K^+ channels. Cell Death Differ 2006;13:661–667.

Pasantes-Morales H, Franco R: Astrocyte cellular swelling: mechanisms and relevance to brain edema; in Aschner M, Costa LG (eds): The Role of Glia in Neurotoxicity. ed 2, CRC Press, 2005, pp 173–190.

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

Rosen AS, Andrew RD: Osmotic effects upon excitability in rat neocortical slices. Neuroscience 1990;38:579–590.

Saly V, Andrew RD: CA3 neuron excitation and epileptiform discharge are sensitive to osmolality. J Neurophysiol 1993;69:2200–2208.

Saransaari P, Oja SS: Mechanisms of D-aspartate release under ischemic conditions in mouse hippocampal slices. Neurochem Res 1999;24:1009–1016.

Schwartzkroin PA, Baraban SC, Hochman DW: Osmolarity, ionic flux, and changes in brain excitability. Epilepsy Res 1998;32:275–285.

Small DL, Tauskela J, Xia Z: Role for chloride but not potassium channels in apoptosis in primary rat cortical cultures. Neurosci Lett 2002;334:95–98.

Staub F, Mackert B, Kempski O, Peters J, Baethmann A: Swelling and death of neuronal cells by lactic acid. J Neurol Sci 1993;19:79–84.

Szabo I, Lepple-Wienhues A, Kaba KN, Zoratti M, Gulbins E, Lang F: Tyrosine kinase-dependent activation of a chloride channel in CD95-induced apoptosis in T lymphocytes. Proc Natl Acad Sci USA 1998;95:6169–6174.

Takatani T, Takahashi K, Ouzumi Y, Shikata E, Yamamoto Y, Ito T, Matsuda T, Schaffer SW, Fujio Y, Azuma J: Taurine inhibits apoptosis by preventing formation of the Apaf-1/caspase-9 apoptosome. Am J Physiol Cell Physiol 2004;287:C949–C953.

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.

Tuz K, Pasantes-Morales H: Hyposmolarity evokes norepinephrine efflux from cynaptosomes by a depolarization- and Ca^{2+}-dependent exocytoic mechanism. Eur J Neurosci 2005;22:1636–1642.

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

Wei L, Xiao AY, Jin C, Yang A, Lu ZY, Yu SP: Effects of chloride and potassium channel blockers on apoptotic cell shrinkage and apoptosis in cortical neurons. Pflugers Arch 2004;448:325–334.

Yu SP: Regulation and critical role pf potassium homeostasis in apoptosis. Prog Neurol 2003;70: 363–386.

Yu SP, Farhangrazi ZS, Ying HS, Yeh CH, Choi DW: Enhancement of outward potassium current may participate in beta-amyloid peptide-induced cortical neuronal death. Neurobiol Dis 1998;5:81–88.

Yu SP, Yeh C-H, Grottron F, Wang X, Grabb MC, Choi DW: Role of the outward delayed rectifier K^+ current in ceramide-induced caspase activation and apoptosis in cultured cortical neurons. J Neurochem 1999;73:933–941.
Yu SP, Yeh C-H, Sensi SL, Gwag BJ, Canzoniero LMT, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, Choi DW: Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 1997;278:114–117.

Prof. Herminia Pasantes-Morales
Instituto de Fisiología Celular, UNAM
Apartado Postal 70–600
04510 Mexico D.F. (Mexico)
Tel. +52 55 5622 5588, Fax +52 55 5622 5607, E-Mail hpasante@ifc.unam.mx