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" MECANISMOS DE LIBERACIÓN DE OSMOLITOS DURANTE CAMBIOS GRADUALES Y CONTINUOS DE OSMOLARIDAD"

TESIS

QUE PARA OBTENER EL TITULO DE DOCTOR EN CIENCIAS
PRESENTA

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“Hasta el más grande de los viajes empieza dando un paso“
B. Franklin

"Los hombres inteligentes quieren aprender; los demás, enseñar."
Anton Chejov

"Lo más bello que podemos experimentar es el misterio de las cosas"
Albert Einstein

“Nunca consideres el estudio como una obligación, sino como una oportunidad para penetrar en el bello y maravilloso mundo del saber "
Albert Einstein

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RESUMEN

La respuesta adaptativa de las células ante una condición de hiposmolaridad implica la movilización de osmolitos, K^+, Cl^- y moléculas orgánicas, particularmente aminoácidos, para mantener un volumen constante frente a la entrada de agua generada por la condición hiposmótica. Los estudios hasta ahora se han realizado en su mayor parte, exponiendo a las células a decrementos drásticos y súbitos en la osmolaridad externa. Aún siendo útiles para amplificar las reacciones del proceso, estos modelos no reflejan lo que sucede en condiciones fisiológicas y patológicas, en las que los cambios en la osmolaridad externa son pequeños y graduales. La presente investigación en neuronas y astrocitos en cultivo se llevó cabo en un modelo experimental en el que se reproduce esta condición y los resultados más importantes son los siguientes:

1. La estimulación con cambios graduales en osmolaridad permite a las células nerviosas, neuronas y astrocitos, contrarrestar de manera más eficiente el cambio en volumen. El caso de las neuronas es más notable, ya que son capaces de mantener sin cambio su volumen a pesar de que la osmolaridad externa se redujo hasta en un 50%. Aunque este grado de eficiencia no se observó en las células gliales, sí puede decirse que en esta condición, el ajuste en el volumen fue más rápido y más eficiente que en condiciones de cambio súbito en la osmolaridad.
2. El cambio gradual en osmolaridad activa una corriente de Cl^-, con un umbral de osmolaridad muy bajo, con las características propias de los canales de Cl^- activados por volumen, tanto en su cinética como en su perfil farmacológico. En todas las condiciones examinadas, esta corriente de Cl^- fue independiente de Ca^{2+}.
3. Las corrientes de K^+ activadas por el volumen en las células de glioma C6 están mediadas por dos tipos de canales, dependiendo de la magnitud del estímulo hiposmótico. En condiciones de osmolaridad menores al 15%, el canal que participa es dependiente de Ca^{2+} y sensible a caribdotoxina, que posiblemente corresponde a un canal del tipo maxi K^+. Al ser mayor el decremento en la osmolaridad, participa un canal independiente de Ca^{2+}, insensible a caribdotoxina y a los inhibidores clásicos, pero inhibido por clofilio. Este canal podría pertenecer a la familia de los canales de K^+ 4TM2P. Este es el primer reporte en el que se describe la coexistencia de dos tipos de canales de K^+ asociados al cambio en volumen.
4. El umbral de activación de la salida de aminoácidos es notablemente más temprano en las neuronas que en las células gliales, permitiendo suponer que estas moléculas tienen un papel preponderante en la mayor eficiencia en el proceso observada en las neuronas.

ABSTRACT

The adaptive mechanism to hyposmotic swelling consistent in activation of osmolyte fluxes, has been extensively examined in a variety of cells, including brain cells. However, in most studies, cells are exposed to abrupt and large decrease in external osmolarity, that although being useful for the understanding of some basic features of the process, only rarely occurs even in pathological situations. In the present study carried out in cultured neurons and astrocytes, a paradigm was used in which cells were gradually exposed to small decreases in external osmolarity. Results are summarized as follows:

1. Neurons and glial cells exposed to gradual osmotic changes counteract swelling more efficiently that when the osmotic stimulus was large and abrupt. Neurons, in particular, were able to maintain a constant volume in face of osmolarity decreases up to 50%.
2. The gradual osmotic decrease activated a Cl^- current, with a low osmolarity threshold, showing an outwardly rectifying I-V relationship and a current inactivation at depolarizing voltages. Its pharmacological profile is that characteristic of the volume-activated Cl^- channels. This Cl^- current was consistenly Ca^{2+}-independent.
3. The K^+ currents activated by gradual osmotic changes seems mediated by two types of K^+ channels, depending upon the magnitud of the osmotic change. Small osmolarity reductions activate a Ca^{2+}-dependent, charyibdotoxin-sensitive K^+ channel, likely corresponding to a BK channel. Further decreases in osmolarity activate a Ca^{2+}-independent K^+ channel, insensitive to charybdotoxin and to the classical K^+-channel blockers but inhibited by clofilium. This channel might be a member of the 4TMP channels.
4. The osmolarity threshold of amino acid release is notably low in neurons as compared to glial cells, suggesting an important role for these osmolytes in the high efficiency of neurons for maintaining a constant volume in conditions of drastic reduction of osmolarity.

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INTRODUCCION

Regulación del volumen celular y su importancia.

La capacidad que tienen las células para mantener su volumen constante es una propiedad fundamental para su supervivencia y es uno de los mecanismos homeostáticos más importantes y probablemente uno de los más antiguos, siendo un distintivo a lo largo de las escala evolutiva (Chamberlin y Strange, 1989). Esta capacidad de adaptación se identificó inicialmente en las células de especies como las llamadas eurihalinas, que se encuentran expuestas a condiciones de osmolaridad externa variable. Sin embargo, esta respuesta adaptativa se ha encontrado consistentemente en prácticamente todas las células animales que siempre están rodeadas de fluidos de osmolaridad muy controlada, en valores de aproximadamente 285 mOsm/l, con sólo pequeñas fluctuaciones que no exceden de un 3% (Hoffman y Simonsen, 1989). Esta estabilidad puede alterarse en situaciones patológicas, que por distintos mecanismos, conducen a una situación de hiponatremia.

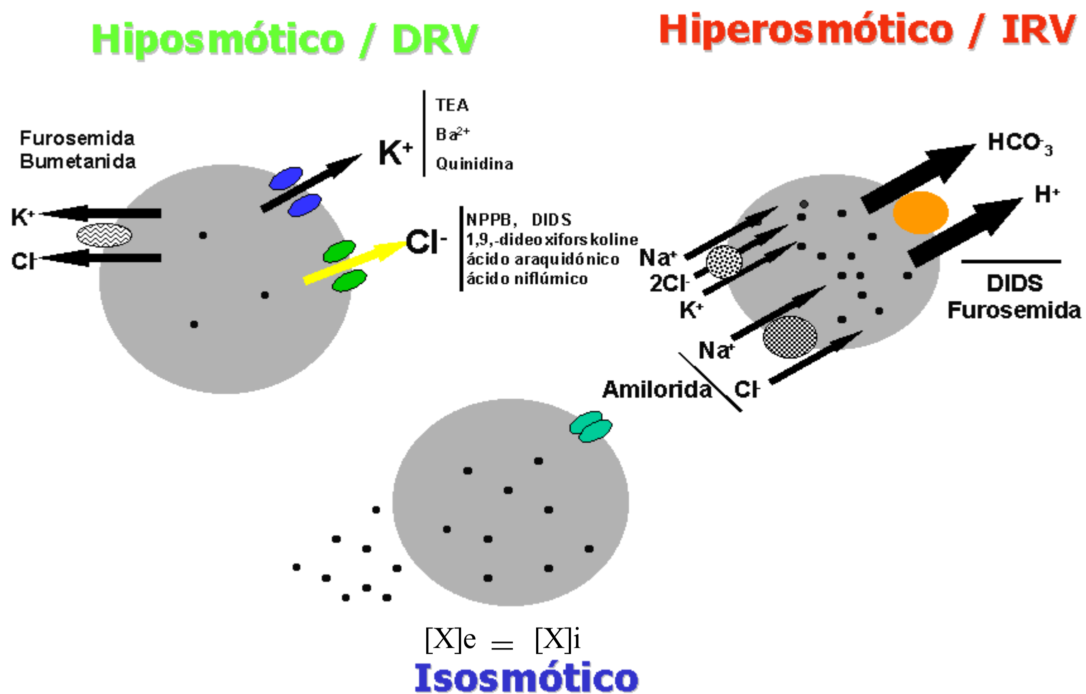
A nivel celular, cualquier desbalance osmótico que no sea corregido ocasiona alteraciones en el contenido de agua como resultado de la libre permeabilidad de la membrana plasmática a este fluido. Tales cambios en el contenido de agua modifican no sólo el volumen de las células sino también su forma y geometría, lo cual incide, en mayor o menor medida, sobre sus funciones metabólicas y el estado de hidratación de macromoléculas. Asimismo, el cambio en la concentración de moléculas que forma parte de la señalización intracelular y de la comunicación intercelular puede enviar mensajes equívocos para la función de células y órganos (Foskett, 1994; Hallows y Knauf, 1994). Se ha propuesto recientemente que el volumen celular también puede participar de manera directa en el control del metabolismo y expresión de genes, como señal de proliferación, muerte y migración celular, así como activar mecanismos que promuevan la inserción de proteínas transmembranles, canales, receptores y transportadores (Pasantes-Morales, 1996). Debido a su alta concentración intracelular, las proteínas normalmente se encuentran cercanas unas de otras, lo que facilita y modula su interacción física y enzimática, por lo que cambios en el volumen celular y por ende en el volumen citosólico, pueden alterar

dicha interacción así como las propiedades cinéticas de los diversos procesos enzimáticos. (Meijer, 2003).

Las células pueden hacer uso de varias estrategias para manejar el estrés osmótico: 1) los cambios osmóticos pueden ser ignorados por la presencia de una pared celular rígida como en las células vegetales, 2) pueden ser eludidos, como en el caso de ciertos moluscos al cerrar su concha ó 3) pueden ser contrarrestados, por la modificación de la concentración intracelular de solutos osmóticamente activos (osmolitos) y consecuentemente, por un cambio en el contenido de agua. Los organismos con pared celular, como las plantas y las bacterias, pueden ignorar cambios osmóticos dentro de cierto límite, ya que la rigidez de la pared previene cambios en el volumen celular. Las paredes de celulosa de las células vegetales llegan a tolerar turgencias internas tan altas como 50 atmósferas. En cambio, las membranas de las células animales son elásticas y no pueden soportar una diferencia significativa en la presión hidrostática, por lo que tienen que activar mecanismos de transporte de osmolitos, los que al ser traslocados, contrarrestan los cambios en el volumen celular en condiciones anisosmóticas (Chamberlin y Strange, 1989; Pasantes-Morales, 1990; Sarkadi y Parker, 1991; Lang 1998). La movilización de solutos en respuesta a los cambios en la osmolaridad del medio externo ocurre a través de canales y acarreadores, especializados en el transporte de especies moleculares diversas y en menor medida, a través de procesos de síntesis.

La regulación de volumen celular es un proceso complejo que implica varios eventos básicos: primero, la célula debe ser capaz de sensar los cambios en su volumen, posteriormente la célula debe reaccionar a los cambios de volumen que ha detectado, iniciando un proceso regulador que modifique el contenido intracelular de solutos en la dirección necesaria para corregir el contenido celular de agua. Finalmente, la célula durante el proceso regulador, debe "recordar" su volumen original e inactivar los mecanismos que se dispararon en el proceso regulador. En términos generales, cuando las células ganan agua, responden expulsando moléculas que se hallan en solución (osmolitos) hacia el exterior celular, para alcanzar el equilibrio osmótico con el medio externo. Este proceso se denomina decremento regulador de volumen (DRV). Cuando por el contrario, las células pierden agua, el mecanismo activado es opuesto, es decir, acumulan osmolitos en su

interior lo que induce una entrada de agua. Este proceso de conoce como incremento regulador de volumen (IRV) (Esquema 1). La recuperación del volumen celular después de un incremento producido por soluciones hiposmóticas, se ha descrito en una gran variedad de células animales, incluyendo células epiteliales (Davis y Finn, 1985; Kirk y Kirk, 1992; Welling, 1983), eritrocitos (Kregenow, 1981; Cala, 1983), células ascíticas de Ehrlich (Hoffmann, 1978), astrocitos en cultivo (Olson, 1986; Kimelberg, 1985; Pasantes-Morales, 1994 a,b) células granulares en cultivo (Pasantes-Morales, 1993a), linfocitos humanos (Grinstein, 1982), células MDCK (Roy y Sauve, 1987), células COS-7 (Ishii, 1996), glioma C6 (Strange y Morrison, 1992), neuroblastoma N1E115 (Falke y Misler, 1989), fibroblastos humanos (Bibby y McCulloch, 1994) y células IMCD (Tinel, 1994) entre otras.



Esquema 1. Control del volumen en condiciones anisomóticas. En condiciones isosmóticas la concentración de solutos en los compartimientos intra [X]i y extracelular[X]e es la misma, por lo que la presión osmótica en ambos lados es la misma. El proceso de ajuste en el volumen se lleva acabo a través de la modificación en la concentración de solutos intracelulares osmóticamente activos, que se reduce o aumenta, en soluciones hiposmóticas e hiperosmóticas.

Osmolitos

Los solutos osmóticamente activos u osmolitos, son aquellos que se encuentran en forma libre en solución en el citoplasma. En general, para que un soluto pueda participar en el DRV, debe encontrarse en concentraciones relativamente altas en el citoplasma, para que contribuya de manera significativa a corregir el desequilibrio osmótico impuesto por las situaciones anisosmóticas impuestas. Los osmolitos involucrados en la regulación de volumen son de dos tipos: Compuestos inorgánicos como K^+, Cl^- y el Na^+ (Grinstein, 1992, Roy y Sauve, 1987) y un grupo de moléculas orgánicas, como aminoácidos (Smith, 1987), aminas (Nakanishi, 1988), azucares y polialcoholes (Balaban, 1987, González, 1995). El K^+ está involucrado en el DRV, ya que es el ion más abundante en el interior de la célula. Los flujos de K^+ están acoplados a flujos de aniones (Cl^-, HCO^-_3) y de compuestos orgánicos cargados negativamente para mantener la electroneutralidad.

En general, durante la regulación de volumen las células de invertebrados y vertebrados de especies acuáticas movilizan preferentemente los osmolitos orgánicos, mientras que en las células de vertebrados terrestres, los iones parecen tener un papel más importante, a excepción tal vez, como se discutirá más adelante, en el caso de las células nerviosas. Del total de aminoácidos que existen en la naturaleza sólo algunos (alanina, glicina, taurina, ácido glutámico, ácido aspártico, β-alanina y prolina) son de importancia cuantitativa para la regulación del volumen celular. El uso consistente de uno o más de estos aminoácidos por muchos grupos de organismos, sugiere que tienen características compatibles con la función de la regulación del volumen celular (Lang, 1998). Entre ellos la taurina tiene un interés particular debido a que se encuentra en concentraciones muy elevadas en la mayoría de las células animales y a su relativa inercia metabólica. La taurina es un β-aminoácido sulfónico producto del metabolismo de la cisteína, que se encuentra presente en las células animales desde los grupos más sencillos hasta los más complejos. Se acumula intracelularmente y el gradiente de concentración se mantiene por un transportador dependiente de Na^+ (Banderali y Roy, 1992). La taurina cumple todos los requisitos de un osmolito ideal: es movilizada a través de la membrana en respuesta a cambios en la osmolaridad y en la composición iónica; debido a su naturaleza electroneutra puede acumularse en el citosol sin alterar el potencial de membrana ni otros procesos celulares;

metabólicamente es inerte, no forma parte de proteínas y el costo energético en su producción es muy bajo ya que es un producto final del metabolismo; su único papel bioquímico es la conjugación con el ácido cólico para formar el ácido taurocólico. Por el contrario, todos los otros aminoácidos usados como osmolitos tienen importancia en diversas funciones metabólicas y como transmisores sinápticos, por lo que su concentración y movilización está estrictamente regulada.

Una característica interesante de las moléculas orgánicas relacionadas con la función de la regulación osmótica es que, a diferencia de los iones inorgánicos, poseen lo que se conoce como propiedades osmoprotectoras, es decir, que además de funcionar como osmolitos pueden acumularse en concentraciones muy altas en el compartimiento intracelular sin alterar significativamente la estructura y función de las macromoléculas citosólicas, como enzimas o proteínas.

Mecanismos de movilización de osmolitos

El movimiento de los osmolitos es la consecuencia de un cambio en la permeabilidad de la membrana plasmática, ya sea por la activación de transportadores o de canales iónicos. Los mecanismos de liberación de osmolitos varían de una célula a otra. Las propiedades y el perfil farmacológico del DRV ha permitido establecer las bases para la identificación de los mecanismos de movilización de los osmolitos responsables de la corrección del volumen. Estas características y el perfil farmacológico del DRV son sugerentes de la participación de canales iónicos, más que de transportadores, en el mecanismo de movilización del K^+ y el Cl^-.

Las investigaciones en la última década han señalado que, efectivamente el mecanismo más generalizado para la movilización de estos iones es la apertura de canales específicos para cada ión, pero con cierta interdependencia. La coactivación de ambos tipos de canales es un mecanismo básico del DRV, que ha sido demostrado en una amplia variedad de tipos celulares, entre los que se encuentran las células tumorales de Erhlich (Hoffmann, 1989), células renales caninas (MDCK), linfocitos-T (Grinstein, 1992) y diversas líneas de células epiteliales (Lang, 1993) tubulo distal A6 (Nillius, 1995). En las células nerviosas, este

aspecto del DRV se conoce muy poco. Aunque estos dos iones son los más importantes para el DRV, existen otros iones involucrados en este proceso como el H^+ y el HCO_3-

Canales activados por volumen

Los canales de Cl^- activados por volumen tienen propiedades similares en la mayor parte de los tipos celulares estudiados. Son canales rectificadores salientes, con una conductancia unitaria de 40–50 pS a potenciales positivos y de 10-20 pS a potenciales negativos, con una inactivación dependiente de voltaje (generalmente mayor de +40 mV) son en general, independientes de Ca^{2+}, modulados por ATP y otros nucleótidos, y son sensibles a los bloqueadores generales de canales de Cl^- como el NPPB y el ácido niflúmico. Los canales de Cl^- sensibles a volumen son también permeables a una variedad de aniones incluyendo los de gran tamaño como benzoato, tiocianato y sulfato (Jackson 1993,1995, Pasantes-Morales 1994b, Nilius 1997). La identidad molecular del canal de Cl^- activado por volumen no se ha establecido. Varios tipos de canales se han propuesto como candidatos, sin que a la fecha, ninguno de ellos cumpla con todas las condiciones (Brandt y Jentsch, 1995,). Entre ellos puede señalarse el ClC2, canal de Cl^- de la familia de canales recientemente clonados (ClC0-7) (Grüder, 1992) la glicoproteína-P (Valverde, 1992), el ClC-2 y el fosfoleman (Moorman, 1995). Otra proteína que podría estar involucrada en la regulación del volumen es la pl_{Cln} (Paulmichl, 1992), ya que cuando es expresada en ovocitos de *Xenopus*, produce una prominente corriente de Cl^- con características semejantes a las corrientes aniónicas de cloro inducidas por hiposmolaridad observadas en células epiteliales.

En cuanto a los canales de K^+ involucrados en la regulación del volumen celular, se observan algunas diferencias notables con los canales de Cl^-. Así, a diferencia de lo que ocurre con el canal de Cl^-, cuyas características son esencialmente las mismas en los distintos tipos celulares, en el caso de los canales de K^+ que se activan por hiposmolaridad, se ha encontrado que participan canales con propiedades muy distintas. Así, en la mayor parte de las células epiteliales, los canales de K^+ activados por volumen son dependientes de Ca^{2+}. En los linfocitos, y en algunas neuronas, se ha encontrado que son dependientes de voltaje, mientras que en un gran número de otros tipos celulares, los canales de K^+ podrían

estar activados directamente por el cambio en volumen o en osmolaridad. Existen también variaciones en lo que se refiere a la conductancia de los canales activados por hiposmolaridad, encontrándose tanto del tipo de conductancia grande (100-200 pS), media (50-80pS) o pequeña (20-30pS) también dependiendo del tipo celular (ver revisión Pasantes-Morales 2000). Estas observaciones sugieren que, en algunos casos, los canales de K^+ presentes en la célula y que participan en otras diversas funciones celulares, se van a activar por fenómenos concurrentes con la hiposmolaridad y el cambio en volumen, como pueden ser un aumento en el Ca^{2+} intracelular o la despolarización iniciada por la activación temprana de canales de Cl^-. En otros casos, cuando no parece haber ningún otro factor asociado, podría pensarse en un canal de K^+ activado únicamente por hiposmolaridad o por volumen.

Los canales de K^+ dependientes de Ca^{2+}, que se activan por hiposmolaridad y que, como se mencionó, participan en la regulación del volumen en las células epiteliales, parecen estar activados por el incremento en Ca^{2+} citosólico que es característico del cambio en volumen hiposmótico. El incremento en la concentración de Ca^{2+} intracelular es un fenómeno característico del hinchamiento hiposmótico, que ocurre en casi todas las células. Estos canales de K^+ son del tipo BK, que se activan a concentraciones micromolares de Ca^{2+} y presentan una conductancia de 100-200 pS. Con base en esto, el hinchamiento en células epiteliales activa canales BK hiperpolarizando a la célula y posteriormente se activan los canales de Cl^-. En los demás tipos celulares, incluyendo astrocitos, la activación de la corriente de Cl^- precede a la de K^+ y en general ambas son independientes de Ca^{2+}.

Se ha sugerido que los canales BK son los que participan en la movilización de los flujos de K^+ activados por volumen. La participación de estos BK en las células epiteliales se apoya también en su perfil farmacológico, sensibilidad a iberiotoxina y caribdotoxina, que corresponde al de estos canales.

En muchos tipos celulares, incluyendo las células nerviosas, hay una salida de Cl^- producida por hinchamiento (Sánchez-Olea, 1996), que conduce a una despolarización, la cual podría activar a los canales Kv (canales de K^+ activados por voltaje). La participación de canales de K^+ sensibles a voltaje en la movilización de K^+ por hiposmolaridad, se ha sustentado en un estudio de Deutsch y Chen (1993) en linfocitos-T, en el que mostraron que

la transfección y subsecuente expresión de los canales Kv 1.3, confiere a los linfocitos la capacidad para regular su volumen, de la que carecían en ausencia de este canal. La participación de canales de K^+ sensibles a voltaje se ha propuesto también en interneuronas de hipocampo, con base en la observación de que la hiposmolaridad incrementa el volumen celular y activa una corriente de K^+ dependiente de voltaje, que es bloqueada por tetraetilamonio (TEA), un inhibidor típico de canales de K^+ dependientes de voltaje (Baraban, 1997).

En relación con un canal de K^+ exclusivamente activado por hiposmolaridad, la información disponible es todavía escasa. Estudios recientes en células tumorales ascíticas sugieren la participación de un miembro de la familia de canales de K^+ de dos poros, TASK-2 (Hougaard 2001). Las características de la corriente de K^+ activada por hiposmolaridad en estas células tiene una conductancia promedio de 5.5 pS, es independiente de calcio y de voltaje, y es sensible a cambios en el pH externo. No se inhibe por bloqueadores clásicos de canales de K^+, siendo sensible únicamente por clofilio. La participación de este canal en la regulación del volumen, y por tanto el sustento para identificarlo como un canal activado por hiposmolaridad, se sugiere en un estudio en el cual utilizando un tipo celular (HEK293) que no tiene la capacidad para regular el volumen en condiciones hiposmóticas y no expresa ningún miembro de la familia TASK, al ser transfectado el canal TASK2, las células adquieren la capacidad reguladora de volumen. La cinética, selectividad, modulación por pH externo, activación por osmolaridad y perfil farmacológico son similares a los presentados por el canal de K^+ activado por hiposmolaridad en células ascíticas (Niemeyer 2001).

Osmotransducción

Las vías de señalización que conectan el cambio en volumen con la activación de los efectores que conducen a la recuperación del volumen, es decir, la osmotransducción, está poco estudiada. Parte de la dificultad para establecer las cadenas de señalización involucradas en la salida adaptativa de los osmolitos es que el incremento en volumen y su recuperación subsecuente, son fenómenos muy complejos que involucran un gran número

de respuestas celulares, que no necesariamente se vinculan con la translocación de los osmolitos. Entre estos fenómenos concurrentes a los cambios en volumen pueden citarse, entre otros, las distintas reacciones de adhesión, el estrés y la reorganización del citoesqueleto. Cada uno de estos fenómenos tiene sus propias cadenas de señalización y sus mensajeros específicos, mismos que hay que separar de los propiamente involucrados en la osmotransducción.

Uno de los efectos primarios del incremento en el volumen celular observado en condiciones hiposmóticas en la mayor parte de las células es un incremento en los niveles de calcio libre $[Ca^{2+}]i$ (Rothstein, 1990). Esta observación ha llevado a postular que el Ca^{2+} puede estar participando en el DRV concretamente como señal activadora de los sistemas de transporte para la salida de los osmolitos. Sin embargo, la evidencia al respecto es controvertida ya que en general, los canales de Cl^- y las vías de movilización de los osmolitos orgánicos son independientes de Ca^{2+}. En el caso del K^+, el Ca^{2+} tiene un papel muy importante activando canales de K^+ dependientes de Ca^{2+} en las células epiteliales, pero no en las no epiteliales. Es posible, por lo tanto, que la función del Ca^{2+} como señal de osmotransducción sea distinta en los diferentes tipos celulares.

Otro grupo de elementos de señalización que se asocian con la osmotransducción es el de las cinasas de tirosina y algunas cinasas que son activadas por ellas. La evidencia se resume en el hecho de que los inhibidores de cinasas de tirosina en general, reducen la eficiencia del DRV y disminuyen la función de las vías de traslocación de los osmolitos, en tanto que los bloqueadores de las fosfatasas de tirosina tienen el efecto inverso. Asimismo, se cuenta con evidencia que muestra la activación de algunas de estas cinasas por hiposmolaridad. Entre ellas se encuentran las cinasas activadas por mitógenos, Erk-1 y Erk-2 (Tilly 1993, 1998; Crépel 1998; Schliess et al., 1995, 1996), la p38 y algunas cinasas de la familia c-Src. Sin embargo, como se mencionó, el hecho de que la hiposmolaridad y el incremento en volumen activen a estas enzimas, no lleva necesariamente a asociarlas con la activación de la salida de osmolitos. Un ejemplo ilustrativo de esto es el de las cinasas Erk-1 y Erk-2, que no están vinculadas con ninguna de las vías de movilización de osmolitos a pesar de que su activación por el incremento en el volumen se ha reportado en forma consistente. Algo

semejante sucede con la cinasa p38, cuya activación puede estar vinculada con una respuesta al estrés. La cinasa de fosfoinosítidos PI3K, que es una enzima blanco de las cinasas de tirosina, si parece tener una relación directa con la activación de la salida de los osmolitos, ya que además de que su actividad se incrementa en condiciones hiposmóticas, su inhibición siempre se asocia con una menor eficiencia de la liberación de osmolitos como la taurina y el Cl^-. Otras señales de transducción que están vinculadas con el cambio en el volumen celular son las cinasas PKC (Chou 1998) y PKA (Moran 2001) así como algunas fosfolipasas, en particular la fosfolipasa D (Tomassen 2004).

En relación con los mecanismos que permiten a las células detectar su volumen y los cambios inducidos por modificaciones externas o internas, y cómo la señal del volumen es amplificada y traducida al efector, es poco lo que se conoce. Recientemente se ha propuesto la posibilidad de que los receptores membranales con actividad intrínseca de tirosina-cinasas, que en general actúan como receptores a factores de crecimiento, puedan funcionar como intermediarios para detectar el cambio en la osmolaridad y/o en el volumen celular. Esta propuesta se basa en la observación de que el receptor al factor de crecimiento epidérmico en fibroblastos y el receptor a heregulinas en las neuronas granulares de cerebelo, se activan por hiposmolaridad y que las vías de señalización con las cuales están conectados estos receptores, establecen una cadena de información entre el receptor membranal y los canales a través de los cuales se movilizan los osmolitos (Franco 2004, Lezama 2005).

Cambios en el volumen cerebral

Si bien un aumento en el volumen celular es un inconveniente para cualquier órgano, es particularmente dramático en el caso del cerebro pues los limites a la expansión impuestos por el cráneo da márgenes limitados para el amortiguamiento de los cambios en el volumen intracraneal y a medida que la presión aumenta, el desplazamiento caudal del parénquima cerebral a través del *foramen magnum* lleva a la muerte por paro respiratorio y cardiaco. Si el edema cerebral no se controla, pueden originarse trastornos clínicos que ponen en peligro la vida ya que un incremento por arriba del 5% del volumen cerebral

origina morbilidad y mortalidad substancial. (Trachtman 1992). Durante el edema cerebral, el volumen del parénquima cerebral (espacio intra o extracelular) se incrementa en detrimento del volumen sanguíneo y del líquido cefalorraquídeo, lo que causa un desbalance entre el volumen intracraneal y la presión intracraneal. El edema cerebral puede ser vasogénico, en el que la entrada de agua y osmolitos ocurre como consecuencia del daño en la barrera hematoencefálica y el edema celular, que se presenta cuando ocurre un desequilibrio osmótico entre el plasma y el fluido extracelular cerebral, debido ya sea a una condición de hiponatremia, como en el edema anisosmótico, o a una redistribución de iones, como en el edema isosmótico, llamado también edema citotóxico. Aunque los mecanismos que generan el edema en cada caso son distintos, en condiciones patológicas es poco frecuente encontrar sólo un tipo de edema, ya que la aparición de uno de ellos generalmente evoluciona, con el tiempo, hasta producir la aparición del otro.

El edema anisosmótico se produce en las patologías que llevan asociada una condición de hiponatremia. La causa principal de edema hiposmótico es la hipotonicidad del plasma, la cual puede deberse a hiponatremia de distintos orígenes o a hipoglucemia. La hiponatremia es un desequilibrio en la distribución de los fluidos corporales y de los electrolitos disueltos en ellos, debido principalmente a la retención excesiva de agua y en ocasiones a una baja reabsorción de sodio. Se considera una condición hiponatrémica cuando la concentración de Na^+ en plasma alcanza valores iguales o menores a 130 mEq/l. La hiponatremia se desarrolla cuando el consumo de agua sobrepasa la capacidad del riñón de excretarla (intoxicación por agua), o cuando la pérdida de cationes monovalentes sobrepasa su consumo (Na^+ y K^+ fundamentalmente) (Fraser, 1997a,b). Esto trae como consecuencia la disminución de la concentración de Na^+ en el plasma y por tanto, una reducción en su osmolaridad. La hiponatremia se presenta como consecuencia en una amplia variedad de padecimientos crónicos, principalmente en las insuficiencias renales, debido a las críticas funciones de absorción y excreción de agua y electrolitos de ese sistema.

Otras causas de hiponatremia son la diabetes insípida, en donde la ingesta de agua puede ser muy elevada y está también asociada a condiciones que llevan a una intoxicación por agua como ocurre en ciertos estados psicóticos o por consumo de algunas drogas (Fraser y Arieff, 1990). Ocurre también durante el síndrome de secreción inapropiada de vasopresina, también conocida como hormona antidiurética (ADH), durante el cuál se descompensan los

mecanismos de regulación para la secreción de la hormona con lo que se presenta un cuadro de hiposmolaridad en el volumen intravascular y disminuciones en los niveles plasmáticos de Na^+, urea, ácido urico y creatinina (Fraser, 1997b). Una hiponatremia moderada es una condición normal en el embarazo y una hiponatremia crónica es también frecuente en la ancianidad.

El edema isosmótico o citotóxico es el incremento en el volumen de los elementos celulares del parénquima cerebral: inicialmente los elementos gliales y neuronales y después las células endoteliales. Se origina por alteraciones en el transporte activo de iones y moléculas con un cambio en su distribución intracelular, que conduce a la acumulación de agua. Este tipo de edema ocurre en patologías como epilepsia, encefalopatía hepática y traumatismo craneoencefálico A estas condiciones han de añadirse otras, como la anoxia, que puede presentarse como entidad aislada, o lo que es más frecuente, como parte de la isquemia, asociada a los accidentes vasculares cerebrales y aneurismas de causas diversas, así como a infecciones o tumores. En asociación con los cambios en la distribución iónica, originados por una falla energética, lactacidosis o acumulación de amonio, según el caso, que son inductores del edema citotóxico, se añaden otros efectos secundarios que lo agravan, tales como la sobrecarga iónica a través de las membranas afectadas por lipoperoxidación, o la acción citotóxica del glutamato liberado como consecuencia del edema, o durante la acumulación de sorbitol en animales hiperglicémicos (Goldberg y Choi, 1993). Bajo estas circunstancias, la muerte celular no se debe al hinchamiento per se, sino al incremento en los niveles de calcio citosólico asociado a estos fenómenos. Esto es seguido por un daño mayor y la extensión de este cuadro a zonas adyacentes, lo que excede las capacidades locales de compensación.

Además de estas situaciones extremas, cambios pequeños en el volumen celular pueden tener implicaciones importantes en la fisiología cerebral, dada la fina arquitectura neuronal, así como los mecanismos que gobiernan la actividad cerebral. Así, el aumento en el volumen celular puede promover hiperexcitabilidad, debido a la reducción del espacio extracelular como consecuencia del hinchamiento de los astrocitos y promoviendo la excitación neuronal a través de efectos de campo (Saly y Andrew, 1993), o a través del

incremento en la liberación de aminoácidos como el GABA y el glutamato que tienen la función dual de osmolitos y neurotransmisores.

Respuesta adaptativa del cerebro. Regulación del volumen.

Desde los primeros estudios sobre hiponatremia experimental, se advirtió que ante una reducción en la osmolaridad del plasma, el cerebro no reacciona como un osmómetro perfecto, sino que muestra una capacidad para recuperar el volumen expulsando el exceso de agua de las células. Simultáneamente se observa una disminución en el contenido de electrolitos y osmolitos orgánicos. El cambio en los electrolitos Na^+, K^+ y Cl^- es detectable entre 3 y 24 h después del inicio de la hiponatremia, (Melton , 1987) mientras que el descenso en los niveles celulares de osmolitos orgánicos es posterior (Thurston, 1980; Tratchman, 1991,1992; Lien, 1997; Verbalis y Gullans, 1991; Sterns, 1993). Los osmolitos orgánicos que predominantemente influyen en el proceso adaptativo son los aminoácidos taurina, glutamato, aspartato, glutamina y GABA, y otras moléculas como el myo-inositol, la creatina y el N-acetil aspartato. La disminución en los osmolitos orgánicos ocurre dentro de los dos días subsecuentes al inicio de la hiponatremia y su concentración permanece reducida durante el tiempo que persista la condición osmótica alterada. El porcentaje de disminución de los osmolitos orgánicos es de 50-80%, mientras que el de los electrolitos es de 10-30%, pero debido a que los electrolitos están más concentrados en el cerebro, su contribución a la regulación del volumen es mayor (60-70%) que la de los osmolitos orgánicos (30-40%) (Thurston, 1987; Lien, 1997). Estos cambios en la concentración de osmolitos y su curso temporal sugieren que la movilización de los electrolitos es un mecanismo rápido, de emergencia, para contrarrestar el edema, pero que debido a la participación de los iones en el mantenimiento de la excitabilidad neuronal, este mecanismo no puede mantenerse por mucho tiempo. Por el contrario, algunos de los osmolitos orgánicos, tales como la taurina y el mio-inositol, pueden contribuír a reducir el edema con menor riesgo para la homeostasis cerebral. En el caso del edema citotóxico, a diferencia del edema anisosmótico, no parece haber un mecanismo de corrección del volumen cerebral. Esto puede ser debido a que el edema citotóxico, en la mayor parte de los casos, se genera precisamente por la entrada de los iones Na^+ K^+ y Cl^-, los cuales, como se mencionó, son

elementos clave en el mecanismo que se activa para contrarrestar el edema anisosmótico. En este caso, pues, la estrategia para evitar el daño por edema debe dirigirse a la prevención, más que a la posible corrección una vez que se ha presentado el edema. En este trabajo se ha estudiado exclusivamente el incremento en volumen por condiciones hiposmóticas, por lo que tanto los antecedentes como los resultados se refieren solamente a esta condición de edema cerebral.

Decremento regulador del volumen en células nerviosas

El estudio de los mecanismos responsables de la respuesta adaptativa del cerebro para mantener el volumen celular constante se ha facilitado por la utilización de modelos como la exposición de las células nerviosas a medios de osmolaridad diferente de la normal, Las células se comportan inicialmente como osmómetros perfectos, es decir el volumen celular cambia en el sentido que tiende a disipar la diferencia de presión osmótica. Cuando las células se exponen a un medio hiposmótico incrementan su volumen de acuerdo al cambio en la osmolaridad externa, presentando el máximo de hinchamiento en general inmediatamente después del estimulo. A continuación, se activan los mecanismos de recuperación del volumen, la cuál se consigue en períodos de tiempo que varían de acuerdo al tipo celular. En las células nerviosas en cultivo, tanto neuronas como astrocitos, el proceso de recuperación del volumen tiene una fase rápida de cerca de 15-20 min, durante la cuál se recupera entre el 60 y el 80% del volumen inicial (Fig.1). Esta adaptación se consigue a través de cambios en el contenido intracelular de solutos en la dirección necesaria para contrarrestar el desequilibrio osmótico. Estos cambios requieren de la activación de mecanismos de transporte de iones y osmolitos orgánicos a través de la membrana.

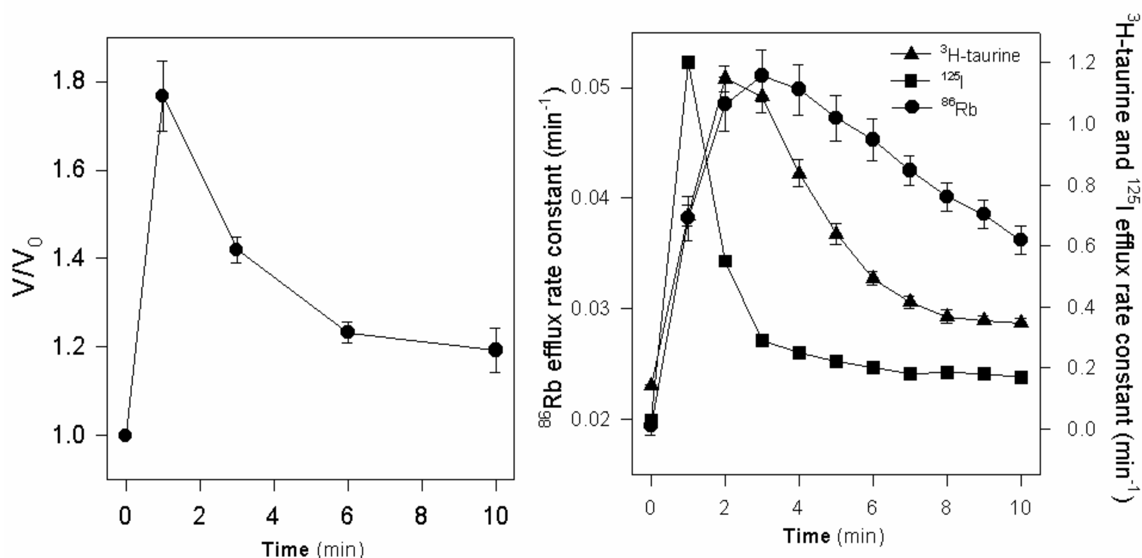


Figura .1- Curso temporal de la regulación de volumen celular en células nerviosas expuestas a cambios súbitos en la osmolaridad.

En el tejido nervioso, el DRV ha sido estudiado principalmente en células en cultivo, líneas celulares, neuronas y células gliales (Kimelberg y Ranson, 1986; Pasantes-Morales y Schousboe, 1988; Pasantes-Morales, 1993b, 1994a; Mountain, 1996; Pasantes-Morales, 1997). En astrocitos y neuronas en cultivo, se ha observado que el DRV es parcialmente dependiente de temperatura e independiente de la presencia de sodio en el medio; es insensible al bloqueo del sistema de cotransporte $Na^+/K^+/2Cl^-$ por compuestos como la furosemida o la bumetanida. Se inhibe por bloqueadores de los canales de Cl^- como el ácido 5-nitro-2(-3fenilpropilamino) benzoico (NPPB), la 1,9-dideoxiforskolina (DDF), el ácido niflúmico o el dipiridamol (Pasantes-Morales, 1994a). Es insensible a los bloqueadores generales de canales de K^+ como el tetraetilamonio (TEA), la 4-aminopiridina (4AP) o el bario, mientras que muestra sensibilidad a la quinidina y, notablemente, a los ácidos grasos poliinsaturados como el araquidónico, el linoléico y el linolénico (Pasantes-

Morales, 1994a; Sánchez-Olea, 1995; McManus, 1994). En cuanto a sus dependencias iónicas, si bien la presencia de bloqueadores de canales de Cl^- inhiben poderosamente el DRV, la omisión del Cl^- del medio no previene su expresión, lo cual sugiere una baja permeabilidad de las células nerviosas a este ión en condiciones isosmóticas, lo que mantendría una disponibilidad suficiente de Cl^- para sustentar el proceso regulador (Pasantes-Morales, 1993b; 1994a). En respuesta al hinchamiento celular, en contraste, la permeabilidad de la membrana al cloro aumenta notablemente, haciendo al movimiento de potasio el paso limitante del proceso. Lo anterior halla sustento en la observación de que en presencia de gramicidina (un ionóforo de cationes) y en ausencia de sodio, el DRV se ve notablemente acelerado, debido a la apertura de una vía alterna para la salida de potasio intracelular (Pasantes-Morales, 1993b; 1994a). Estas observaciones señalan al movimiento saliente de potasio como crítico en la expresión y desarrollo temporal de la respuesta regulatoria en las células nerviosas.

Vías de movilización de osmolitos en células nerviosas

La sensibilidad farmacológica del DRV descrita en la sección precedente, sugiere que los osmolitos involucrados en el DRV en las células nerviosas son los mismos que en la mayor parte de los tipos celulares estudiados, es decir, el K^+ el Cl^- y osmoltios orgánicos, incluyendo aminoácidos y mio-inositol. Los aminoácidos que participan en la regulación de volumen en el cerebro son el glutamato, el aspartato, la glutamina, el GABA y la taurina (Pasantes-Morales, 1993b, Kimelberg, 1990c).

La movilización de K^+ activada por medios hiposmóticos se ha estudiado en astrocitos en cultivo y se ha establecido que dicho movimiento es dirigido por el gradiente químico y constituye el paso limitante para que todo el proceso se lleve acabo. Esta salida se bloquea por bario a concentraciones altas pero no por TEA o 4AP. En estudios electrofisiológicos en astrocitos en cultivo se ha mostrado que la reducción en la osmolaridad produce un marcado aumento en la corriente total, junto con un desplazamiento del potencial de inversión de aproximadamente +40 mV, lo que indica que se incrementan las conductancias al K^+ y al Cl^- (Olson, 1997). En cuanto al canal de Cl^- un estudio en neuronas granulares del cerebelo describe la activación por hiposmolaridad de una corriente de Cl^- con las

características típicas del canal sensible a volumen. Es un rectificador saliente que se inactiva lentamente a potenciales despolarizantes. El canal muestra una conductancia de 36 pS, y es sensible a los bloqueadores generales de canales de Cl^- como NPPB, ácido niflúmico, SITS y DIDS. Consistentemente con las observaciones en otros tipos celulares, el canal requiere para su activación la presencia de ATP, aunque no su hidrólisis. En astrocitos en cultivo, el canal de Cl^- activado por hiposmolaridad es un rectificador saliente, sensible a NPPB y al ácido niflúmico (Olson, 1997; Parkerson and Sontheimer, 2004). Estos estudios señalan la similitud del canal de Cl^- sensible a hiposmolaridad en células nerviosas con los canales presentes en una gran variedad de tipos celulares y enfatizan la homogeneidad y persistencia de este canal a través de las especies y de los distintos órganos y tejidos animales.

Los mecanismos de movilización de los osmolitos orgánicos en el DRV aun no están totalmente identificados. Se ha propuesto una vía común para todos ellos aunque todavía no está claro si esto ocurre efectivamente así. La taurina es uno de los osmolitos más estudiados, debido a que su inercia metabólica sugiere que la taurina presente en las células tiene, en su mayor parte, una función como osmolito. La liberación de taurina en respuesta al cambio de volumen es independiente del Na^+ extracelular, lo que descarta la participación del transportador que es estrictamente dependiente de Na^+. El mecanismo de movilización parece ser una vía difusional a través de la cuál ocurre el movimiento de taurina en respuesta al aumento en el volumen, dirigido por su gradiente de concentración, que en condiciones fisiológicas es siempre del compartimiento intracelular al extracelular (Sánchez Olea, 1991). Un aspecto interesante en relación con la vía de movilización de la taurina es su sensibilidad a los bloqueadores de canales de Cl^- activados por hiposmolaridad, incluyendo una inhibición por ácidos grasos poliinsaturados. Esta característica que se comparte también por el myo-inositol, uno de los más importantes osmolitos orgánicos en el cerebro, ha sugerido una vía común para los osmolitos orgánicos y el Cl^- consistente en un canal poco selectivo. En apoyo a esta propuesta están los estudios realizados por Banderalli y Roy (1992) en células MDCK, en donde observaron corrientes de aminoácidos a través de canales aniónicos de rectificación saliente activados por hiposmolaridad. Los aminoácidos en efecto, permean a través el canal de Cl^- si se encuentran en forma aniónica, lo que si bien indica que el tamaño del poro es suficiente

para su traslocación, no asegura que los aminoácidos en condiciones de carga neta neutra, como se encuentran la mayor parte de ellos, puedan ser en efecto, traslocados por este canal.

Regulación Isovolumetrica

La información acerca de los fenómenos asociados a la regulación del volumen que se ha expuesto en las páginas anteriores, se refiere en todos los casos, a estudios llevados a cabo en células o preparaciones expuestas a cambios importantes y súbitos en la osmolaridad externa. Este abordaje experimental ha proporcionado información muy valiosa acerca de los mecanismos básicos de regulación del volumen, sin embargo, está muy alejado de las situaciones reales en las que se encuentran las células. Con excepción de algunos tipos de células renales y de células intestinales, que están expuestas efectivamente a cambios importantes en la osmolaridad externa, esto no ocurre para la mayor parte de las células animales, incluyendo las células nerviosas. Las condiciones de hiponatremia, aún la del tipo agudo, no se reflejan en cambios súbitos en osmolaridad, sino que estos ocurren en forma gradual y en general, las variaciones no van más allá de 4-5 mOsm. Por ello, se han diseñado modelos experimentales que tengan una similitud mayor con la situación del animal íntegro, consistentes en exponer a las células a un gradiente de osmolaridad, en el que los cambios no exceden de 2.5 mOsm/min. El primer estudio de este tipo se llevó a cabo por (Lohr y Grantham 1986) en células renales. Esta investigación demostró que las células expuestas a un gradiente osmótico de esta naturaleza, tenían la capacidad de mantener su volumen constante, aún cuando la osmolaridad externa se hubiera modificado hasta en un 50% por arriba y por debajo de la condición isosmótica, siempre que el cambio en la osmolaridad externa ocurriera de forma gradual. A este nuevo tipo de regulación de volumen se le llamó regulación isovolúmétrica (RIV) (Fig. 2). Se considera que la constancia en el volumen refleja, no la ausencia de cambio en el volumen sino una activación eficiente, constante y temprana, de mecanismos de regulación, de forma que se logra prevenir todo cambio neto en el volumen. Este supuesto se basa en la observación de que en el estudio de Lohr y Grantham, las células que mantienen su volumen constante durante la exposición al gradiente anisosmótico, al ser de nuevo expuestas a la condición

isosmótica, muestran cambios en el volumen consecuentes con el hecho de que hayan perdido solutos intracelulares cuando los cambios en la osmolaridad externa son decrecientes (el medio se hace hiposmótico), o ganado solutos intracelulares cuando los cambios son crecientes (el medio se hace hiperosmótico). En las investigaciones que llevamos a cabo en este proyecto, este supuesto quedó por primera vez, claramente fundamentado.

Mientras que los mecanismos de regulación en el DRV y en el IRV están bien caracterizados, en el caso de la RIV los mecanismos que la sustentan no están esclarecidos. A pesar de la importancia del tema, hay todavía muy pocos estudios con este enfoque. Además del trabajo mencionado en células renales, hay sólo dos reportes, uno en cardiomiocitos (Souza, 2000) y el otro en eritrocitos (Godart 1999). La regulación isovolumétrica en células nerviosas sólo se ha estudiado en la línea celular C6 (glioma de rata) ante condiciones hiperosmóticas (Mountian 1997). La importancia fisiológica de este mecanismo de regulación del volumen es el motivo por el cuál se llevó a cabo la presente investigación.

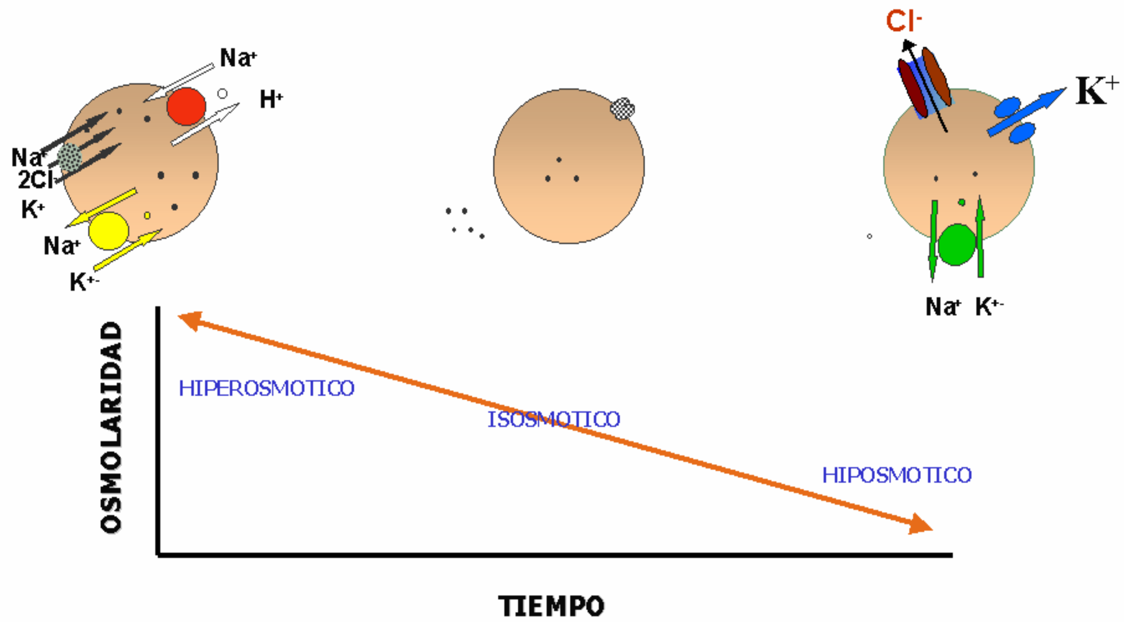


Figura 2. *Regulación isovolúmica*. Cambios graduales y continuos en la osmolaridad externa no afectan el volumen celular.

OBJETIVO GENERAL

Con base en estos antecedentes, el objetivo general de este proyecto es el de caracterizar los mecanismos involucrados en la regulación de volumen celular inducida por cambios graduales y continuos en la osmolaridad externa en células nerviosas. Particularmente a) el estudio de los mecanismos de liberación de osmolitos orgánicos e inorgánicos en distintos tipos celulares b) la caracterización de las corrientes iónicas en cambios graduales y continuos con énfasis en la vía de movilización de K^+ en estas condiciones. Para esto se establecieron los siguientes objetivos específicos:

1. Caracterización de los mecanismos de liberación de osmolitos que se activan en respuesta a cambios graduales y continuos en la osmolaridad y su contribución a la regulación del volumen celular. Se examinará la movilización de aminoácidos, K^+ y Cl^-.

2. Caracterización electrofisiológica de los canales iónicos que se activan en respuesta a cambios graduales y continuos en la osmolaridad y su contribución a la regulación del volumen celular.

Los experimentos que se llevaron a cabo en condiciones de cambios graduales y pequeños en osmolaridad, se compararon sistemáticamente con experimentos en paralelo efectuados en condiciones de exposición súbita a medios de osmolaridad reducida, con el propósito de comparar las características de la respuesta celular en los dos paradigmas experimentales. Se examinaron las respuestas en los siguientes tipos de células nerviosas: cultivos primarios de i) neuronas granulares de cerebelo ii) astrocitos de cerebelo y iii) células gliales de la línea C6.

RESULTADOS:

Los resultados de los experimentos se anexan en el formato de los artículos correspondientes ya publicados. La discusión se amplía al final de esta sección.

ARTICULO 1. Tuz K, Ordaz B, Vaca L, Quesada O, Pasantes-Morales H. Isovolumetric regulation mechanisms in cultured cerebellar granule neurons. J Neurochem. 2001 Oct;79(1):143-51.

ARTICULO 2.Ordaz B, Tuz K, Ochoa LD, Lezama R, Pena-Segura C, Franco R. Osmolytes and mechanisms involved in regulatory volume decrease under conditions of sudden or gradual osmolarity decrease. Neurochem Res. 2004 Jan;29(1):65-72.

ARTICULO 3. 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 Jun;286(6):C1399-409

DISCUSIÓN GENERAL

El presente trabajo se realizó con el propósito de investigar las características de la respuesta en cuanto al cambio en volumen en células nerviosas expuestas a reducciones pequeñas y graduales en la osmolaridad externa, una condición que, como se menciona en la introducción, es más cercana al entorno que pueden encontrar las células en condiciones tanto fisiológicas como patológicas. El estudio se llevó a cabo en neuronas y astrocitos de cerebelo en cultivo, así como en una línea celular de glia, cuyas características son similares a las de los astrocitos. Los resultados mostraron diferencias importantes en cuanto a la capacidad y eficiencia en la regulación del volumen entre las neuronas y las células gliales, que pueden ser interpretados a la luz de la importancia de los aminoácidos en el mecanismo de ajuste del volumen en estas condiciones de cambio gradual en la osmolaridad. Las neuronas granulares mostraron la capacidad de mantener su volumen sin cambio al ser expuestas a un medio en el que la osmolaridad externa se redujo a razón de 1.8 mosm/min. La constancia en el volumen en estas neuronas persiste, a pesar de que la osmolaridad externa disminuye hasta en un 50%. Estos resultados demuestran la presencia de regulación isovolúmica que se describió con detalle en la introducción, y señalan por primera vez la existencia de este fenómeno en células nerviosas. Hasta ahora este tipo de respuesta se ha encontrado únicamente en células renales, como se señala en la introducción, incluyendo túbulos proximales S_2 (Lohr y Grantham, 1986) y en la línea celular renal A6 (Van Driessche, 1997) y en rebanadas de hipocampo (Franco 2000).

Las células gliales, tanto los astrocitos del cultivo primario como la línea de glioma mostraron un comportamiento distinto al de las neuronas, en el sentido de que no fueron capaces de mantener su volumen sin cambio ante el decremento en la osmolaridad externa. Sin embargo, el aumento en volumen fue mucho menor al que se observa cuando las células son expuestas a cambios súbitos en la osmolaridad de igual magnitud, lo que indica que la regulación del volumen en la primera condición fue más eficiente que en la última. En los astrocitos, la exposición al cambio osmótico induce un incremento en volumen desde el inicio, lo que indica que estas células no mantienen un volumen constante como ocurre en las neuronas y en las células renales. Sin embargo, se puede decir que tiene lugar una regulación activa, ya que cuando las células son expuestas nuevamente a un medio

isosmótico, muestran un volumen menor que el inicial, lo que indica la salida de osmolitos intracelulares. Al ocurrir esta pérdida, el medio isosmótico es ahora hiperosmótico con respecto al medio intracelular lo que ocasiona que las células reduzcan su volumen y desencadenen inmediatamente después el incremento regulador de volumen. Una respuesta similar en el sentido de ausencia de regulación isovolúmica se observó en cardiomiocitos (Souza, 2000), aunque en este estudio no se hicieron las comparaciones con la respuesta ante el cambio súbito en osmolaridad, como hicimos en nuestro trabajo.

Mecanismos de regulación del volumen en condiciones de cambio gradual de osmolaridad.

Hasta antes de que se iniciaran nuestros estudios sobre el tema de la regulación isovolúmica, no se habían identificado los mecanismos responsables de esta regulación, incluyendo los osmolitos involucrados y su contribución al proceso. Nuestro trabajo sobre las neuronas granulares del cerebelo fue el primero en aportar datos en este sentido, aunque ya desde el estudio pionero de Lohr y Grantham (1986) se sabía que la regulación isovolúmica se acompaña de una disminución en el contenido intracelular de solutos osmóticamente activos. Los osmolitos que participan en la RIV son esencialmente los mismos que tienen a su cargo el DRV, es decir, los iones K^+ y Cl^- y moléculas orgánicas en particular los aminoácidos. En nuestro estudio en las neuronas encontramos que el Cl^- y los aminoácidos tienen un umbral de liberación muy temprano. En el caso de la taurina, por ejemplo, el umbral de liberación es de sólo H-2%. Su liberación fue de 4-10 veces más grande que la de glicina y glutamato y mostró la mayor disminución en el contenido celular. Estos resultados denotan un papel muy importante de este aminoácido en la RIV, principalmente como osmoefector en la fase temprana de regulación. El Cl^- muestra también un umbral de salida muy bajo, observándose su movilización casi inmediatamente después del inicio en la reducción de la osmolaridad. Por el contrario, la salida del K^+ sólo se observa más tarde, cuando la osmolaridad externa se ha reducido en 29%. La importancia del Cl^- y los aminoácidos como componentes esenciales de la RIV se pone de manifiesto por los resultados que muestran que en presencia del ácido niflúmico, un agente inhibidor de la movilización del Cl^- y de los aminoácidos, las células pierden la capacidad de regular su volumen.

En el caso de las células gliales, tanto astrocitos como células C6, la respuesta de los aminoácidos es claramente distinta de la de las neuronas, en el sentido de que su umbral de movilización en respuesta al cambio en osmolaridad es mucho mayor que en el caso de las neuronas. Así, para los astrocitos la liberación de aminoácidos ocurre solamente cuando la osmolaridad externa se ha reducido en 15%-20%. Sin embargo, al final del experimento, cuando la osmolaridad alcanzo un 50% de reducción, los astrocitos habían liberado 60-70% de la poza de aminoácidos, lo que es indicio de la participación de estos osmolitos en el control del volumen en estas células. En las células C6 se presenta una respuesta similar a la de los astrocitos, en el sentido de que hay una liberación tardía de los aminoácidos, con un umbral de activación de H-39% y H-33% para la taurina y el glutamato, respectivamente. En eritrocitos de salmón (*Oncorhynchus mykiss*) (Godart 1999) se muestra un tercer tipo de respuesta celular al decremento gradual de osmolaridad. En este caso, el cambio en volumen inducido por un decremento gradual en osmolaridad, es esencialmente el mismo que cuando el estímulo hiposmótico se administra en forma súbita. En estas células se examinó la movilización de taurina y de K^+ y se encontró que los dos osmolitos tienen un umbral de osmolaridad muy alto, ya que no es sino hasta que la osmolaridad se ha reducido en 30% que se inicia el mecanismo de liberación de estos osmolitos.

Considerando estos resultados, y como conclusión de esta primera parte del trabajo, se puede decir que existen tres tipos de respuesta celular al cambio gradual en osmolaridad en lo que se refiere a la regulación del volumen. Una, que se presenta en las células renales y en las neuronas, en la que las células son capaces de mantener constante el volumen a través de una gama muy amplia de mecanismos durante la reducción de osmolaridad. El segundo tipo de respuesta es aquélla en la que las células no mantienen su volumen constante, pero el incremento en volumen es menor que aquél que ocurre cuando el cambio en osmolaridad es súbito. Este tipo de respuesta se observa en células gliales, tanto astrocitos procedentes de cultivos primarios como en una línea celular de glioma. Finalmente, en los eritrocitos de salmón (*Oncorhynchus mykiss*) no hay diferencia en cuanto al cambio en volumen entre un decremento súbito o gradual de la osmolaridad

externa. Como resultado de nuestra investigación, puede señalarse una correlación interesante entre estos tres tipos de respuestas y la participación de los aminoácidos, en particular la taurina, en el mecanismo de mantenimiento del volumen en condiciones de cambio gradual en osmolaridad. En las células que mantienen un volumen constante, es decir, que presentan el fenómeno de RIV, el umbral de movilización de taurina es muy bajo, en tanto que en las células gliales que no tienen una respuesta tan eficiente ante el cambio gradual en osmolaridad, pero sí muestran una mejor regulación que ante el cambio súbito, el umbral de liberación de taurina es mayor. Finalmente, en los eritrocitos en los que no hay diferencia en la respuesta ante las dos formas de aplicar el estímulo hiposmótico, la taurina se moviliza sólo cuando el decremento en osmolaridad es muy pronunciado. Es necesario mencionar, sin embargo, que nuestros estudios son los únicos en los que se ha seguido el curso temporal y el umbral de activación de los aminoácidos frente al cambio en osmolaridad. En todos los trabajos previos, aunque en alguno de ellos (Souza 2000) se midió el contenido de taurina, no se siguió el proceso en forma dinámica. En tanto no se hagan más estudios esta salvedad debe tomarse en consideración con respecto a la correlación de la que se habla.

Las diferencias observadas entre neuronas y astrocitos, en particular la mayor sensibilidad observada en las neuronas, podría representar un mecanismo tendiente a proteger a las neuronas de los cambios en osmolaridad que pudieran presentarse tanto en condiciones fisiológicas como en especial en las numerosas patologías que conllevan una condición de hiponatremia. En este sentido pueden explicarse los resultados de un estudio llevado a cabo por Nagelhus (1993) en el que se observó que en el cerebelo de ratas expuestas a una condición hiponatrémica hay una redistribución de la taurina, que inicialmente se encuentra concentrada en las neuronas de Purkinje y no se encuentra en los astrocitos adyacentes. En respuesta al estímulo hiposmótico, la taurina sale de la neurona de Purkinje y es acumulada en los astrocitos, es decir, se mueve de acuerdo a su gradiente de concentración una vez que el poro de salida se ha abierto en respuesta al estímulo. Como resultado de esa movilización, los astrocitos muestran un incremento en volumen mientras que el de las neuronas permanece sin cambio.

Identificación de las vías de movilización de los osmolitos en respuesta a cambios graduales en osmolaridad.

Taurina, aminoácidos y otras moléculas orgánicas

En este trabajo se llevaron a cabo estudios tendientes a caracterizar las vías de movilización de los osmolitos responsables de la regulación del volumen en condiciones de cambio gradual en osmolaridad. El enfoque utilizado fue el farmacológico para la liberación de aminoácidos, Cl^- y K^+, y el electrofisiológico para un conocimiento más en detalle de las vías por las que permean los osmolitos iónicos.
La liberación de taurina en respuesta a un decremento en osmolaridad tiene lugar a través de una vía difusional, a través de la cuál el aminoácido se mueve en la dirección impuesta por el gradiente de concentración. A pesar de que esta característica de la vía de liberación de taurina se conoce desde hace varios años (Pasantes-Morales 1988, Schousboe 1990) su identidad molecular no se conoce. La salida osmosensible de taurina se inhibe por los bloqueadores de canales de Cl^-, una circunstancia que favoreció la propuesta de un canal aniónico no específico como la vía de movilización de la taurina, el Cl^- y posiblemente otros osmolitos orgánicos como el mio-inositol, que también es sensible a los inhibidores de canales de Cl^-. Esta propuesta, sin embargo, se ha visto cuestionada por estudios recientes con resultados que apuntan a la participación de vías distintas para la taurina y el Cl^-. Sin embargo, la inhibición de la liberación de taurina por los bloqueadores de canales de Cl^- es una observación muy consistente en todos los tipos celulares. Esta circunstancia hace suponer que si no se trata de la misma vía, al menos debe haber una conexión muy estrecha entre la vía de salida de la taurina y la activación y/o los niveles de Cl^- intracelulares, en forma tal que la inhibición del canal de Cl^- y la subsecuente salida del anión, impide la activación o la función óptima de la vía de traslocación de la taurina. Esta semejanza en la sensibilidad a los bloqueadores de Cl^- de dos de las vías más importantes de liberación de osmolitos hace que, como mostramos en el artículo correspondiente, la presencia de uno de estos bloqueadores, el ácido niflúmico, hace que se prevenga por completo la regulación isovolúmica en las neuronas del cerebelo.

Potasio y cloro

Corrientes totales evocadas por el cambio gradual en osmolaridad

Para el estudio de la movilización de K^+ y Cl^- se empleó un abordaje electrofisiológico y el estudio se llevó a cabo en los astrocitos en cultivo, y con mayor detalle en las células de glioma C6. Los iones K^+ y Cl^- son elementos claves en el decremento regulador de volumen en cambios súbitos de osmolaridad y estos mismos iones probablemente participen en la regulación que tiene lugar en respuesta a los cambios graduales en osmolaridad. En los astrocitos en cultivo se estudiaron las corrientes totales activadas por cambios graduales y continuos en la osmolaridad externa y en las células de glioma C6, se caracterizaron además, las corrientes iónicas de K^+ y de Cl^- en esa misma condición. . En los astrocitos las corrientes totales se activan cuando la osmolaridad se reduce un 12% (264 mOsm). Como consecuencia de esta activación de corrientes, las células muestran una marcada despolarización, ya que el potencial de reposo inicial cambia de -69 mV a –45 mV a los 20 min, y luego permanece sin cambio hasta el final del experimento. El curso temporal del cambio en el potencial de membrana sugiere que a decrementos pequeños en la osmolaridad se activa una corriente de Cl^-, seguida por la activación de una corriente catiónica (probablemente K^+), la cual previene una mayor despolarización. Estudios electrofisiológicos realizados por Kimelberg (1990 a,b) y Olson (1997) en astrocitos en cultivo en condiciones de cambio súbito en la osmolaridad, mostraron también una marcada despolarización, considerando un cambio en el potencial de membrana de –30 a -40 mV, lo que sugiere asimismo la activación inicial de una corriente de Cl^-.

En neuronas y células renales, los cambios osmóticos graduales (COG) provocan la liberación de Rb^{86} (como trazador de los movimientos de K^+) cuando la osmolaridad se reduce 27% y 20% respectivamente. En astrocitos en cultivo, no detectamos una movilización de Rb^{86} en respuesta a COG. Estos resultados son similares a los de Olson (1999), en los que los astrocitos expuestos a condiciones hiposmóticas muestran una disminución marcada y progresiva en el contenido de taurina mientras que el de K^+ no cambia. En rebanadas de hipocampo expuestas a condiciones de cambio gradual de osmolaridad, tampoco se pudo observar la liberación de K^+ (Rb^{86})(Franco 2000). Esta

diferencia notable entre los astrocitos por una parte y las neuronas y otros tipos celulares por otra, puede deberse a la capacidad especial de los astrocitos de acumular el K^+ extracelular, lo que podría contrarrestar la liberación activada por hiposmolaridad. Esta característica quizás contribuya a una regulación de volumen menos eficiente en los astrocitos en comparación con las neuronas.

Corrientes de K^+ y Cl^-

La caracterización de las corrientes de K^+ y Cl^- se llevó a cabo con detalle en las células C6 mediante el abordaje de sustitución iónica, y los resultados se muestran en el artículo correspondiente. Uno de los hallazgos más interesantes en este trabajo fue el de la activación de distintos canales de K^+ dependiendo de la magnitud del cambio osmótico. Haciendo estudios sobre la dependencia de Ca^{2+} de las corrientes iónicas, observamos que se activa una corriente de K^+ que fluye a través de un canal de K^+ dependiente de Ca^{2+} y sensible a uno de los inhibidores característicos de este tipo de canal, la caribdotoxina. En ausencia de Ca^{2+}, se activa una corriente de K^+ independiente de Ca^{2+} y que por su sensibilidad farmacológica correspondería a aquélla que ocurre a través de un canal activado esencialmente por volumen. Esta corriente es insensible a Ba^{2+} TEA, 4AP y en cambio se inhibe por clofilio, un agente que inhibe el canal de K^+ activado por volumen en varios tipos celulares. Nuestros resultados mostraron que la magnitud del cambio en osmolaridad es la que determina el tipo de canal que se activa por el incremento en el volumen. Los experimentos realizados haciendo cambios graduales en osmolaridad sugieren que este umbral se sitúa cerca de una reducción en osmolaridad del 15%, supuesto que fue comprobado por las características de las corrientes de K^+ que se activan cuando la osmolaridad se reduce de manera súbita 15% y 30%. Como demostramos en nuestro trabajo, en la primera condición, la corriente es dependiente de Ca^{2+} y sensible a caribdotoxina, mientras que en la segunda la corriente es independiente de Ca^{2+} y se inhibe por clofilio. La participación de estos canales se refleja en la eficiencia en la regulación del volumen, tanto en las condiciones de reducción gradual como en súbita, siendo menos eficiente en ausencia de Ca^{2+}. Por el contrario, no hay diferencia significativa en cuanto a la presencia de Ca^{2+} en relación con el volumen alcanzado cuando la osmolaridad externa es más allá del 15%. La corriente de Cl^- se activa muy tempranamente con un umbral de H-

3% y esta activación se encontró que es independiente de Ca^{2+}. El Cl^- entonces, puede estar acompañando a los dos tipos de canales de K^+ anteriormente descritos, ya que su funcionamiento no depende de la presencia de Ca^{2+}.

La identidad molecular de los canales a través de los cuales fluyen las corrientes estudiadas en este trabajo, no se ha establecido con precisión. En el caso del canal de Cl^-, como se señaló en la introducción, se han clonado varios tipos de canales, algunos de los cuales son activados por volumen. El ClC2 sería el candidato con más posibilidades, por su cinética de inactivación a voltajes más positivos, pero sin embargo, difiere en otros aspectos del canal que participa en la regulación del volumen. En cuanto a los canales de K^+, la evidencia disponible indica que el canal de K^+ dependiente de Ca^{2+} que participa en la regulación del volumen en condiciones de baja osmolaridad, podría ser del tipo de los maxi K, con base particularmente en su sensibilidad a la caribdotoxina. Este tipo de canales participan de manera prominente en la regulación del volumen en las células epiteliales, en las que la entrada de Ca^{2+} inducida por el decremento en la osmolaridad, activa estos canales de K^+, siendo ésta la respuesta inicial al aumento en el volumen, que es seguida por la activación de los canales de Cl^- sensibles al volumen. En congruencia con esta cadena de eventos, la regulación del volumen en células epiteliales es siempre dependiente de Ca^{2+}. Sin embargo, en células nerviosas, los reportes anteriores a nuestra publicación en las células C6, describían al proceso regulador en respuesta a un cambio súbito en osmolaridad como independiente de Ca^{2+} y a la corriente de Cl^- como el evento inicial. Es importante señalar que en la mayor parte de estos estudios previos, las condiciones de decremento en osmolaridad fueron siempre superiores al 15%, lo que a la luz de nuestros hallazgos sobre la diferente sensibilidad al Ca^{2+} de las corrientes de K^+, explica la independencia de Ca^{2+} previamente señalada. En cuanto al canal de K^+ independiente de Ca^{2+}, que participa en el ajuste en el volumen en condiciones de mayor decremento en osmolaridad, la evidencia reciente indica que se trata de un canal del tipo de los de la familia de canales de dominio de dos poros. Es en células ascíticas (Niemeyer, 2001) en las que se ha caracterizado este canal con mayor detalle en relación con su participación en los mecanismos de regulación del volumen, y la evidencia apunta hacia el canal TASK-2. No hay todavía evidencia, más

allá de lo reportado en este trabajo, que permita hacer la identificación molecular del canal de K^+ sensible a volumen e independiente de Ca^{2+} en células nerviosas.

En **conclusión**, los hallazgos más notables del trabajo de investigación que conforma esta tesis son los siguientes:

1. La estimulación con cambios graduales en osmolaridad permite a las células nerviosas, neuronas y astrocitos, contrarrestar de manera más eficiente el cambio en volumen que en cambios súbitos de osmolaridad. El caso de las neuronas que se estudiaron es el más notable, ya que son capaces de mantener sin cambio su volumen, a pesar de que la osmolaridad externa se redujo hasta en un 50%. Aunque este grado de eficiencia no se observó en las células gliales, sí puede decirse que en esta condición, el ajuste en el volumen fue más rápido y más eficiente que en condiciones de cambio súbito en la osmolaridad.
2. El cambio gradual en osmolaridad activa una corriente de Cl^-, con un umbral de osmolaridad muy bajo, con las características propias de los canales de Cl^- activadas por volumen, tanto en su cinética como en su perfil farmacológico. En todas las condiciones examinadas, esta corriente de Cl^- fue independiente de Ca^{2+}.
3. Las corrientes de K^+ activadas por el volumen en las células C6 están mediadas por dos tipos de canales, dependiendo de la magnitud del estímulo hiposmótico. En condiciones de osmolaridad menores al 15%, el canal que participa es dependiente de Ca^{2+} y sensible a caribdotoxina, que posiblemente corresponde a un canal del tipo maxi K^+. Al ser mayor el decremento en osmolaridad, participa un canal independiente de Ca^{2+}, insensible a caribdotoxina y a los inhibidores clásicos, pero inhibido por clofilio. Este canal podría pertenecer a la familia de los canales 4TM2P. Este es el primer reporte en el que se describe la coexistencia de dos tipos de canales de K^+ asociados al cambio en volumen.
4. El umbral de activación de la salida de aminoácidos es notablemente más temprano en las neuronas que en las células gliales, permitiendo suponer que estas moléculas tienen un papel preponderante en la mayor eficiencia en el proceso observada en las neuronas.

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Isovolumetric regulation mechanisms in cultured cerebellar granule neurons

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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.
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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

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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

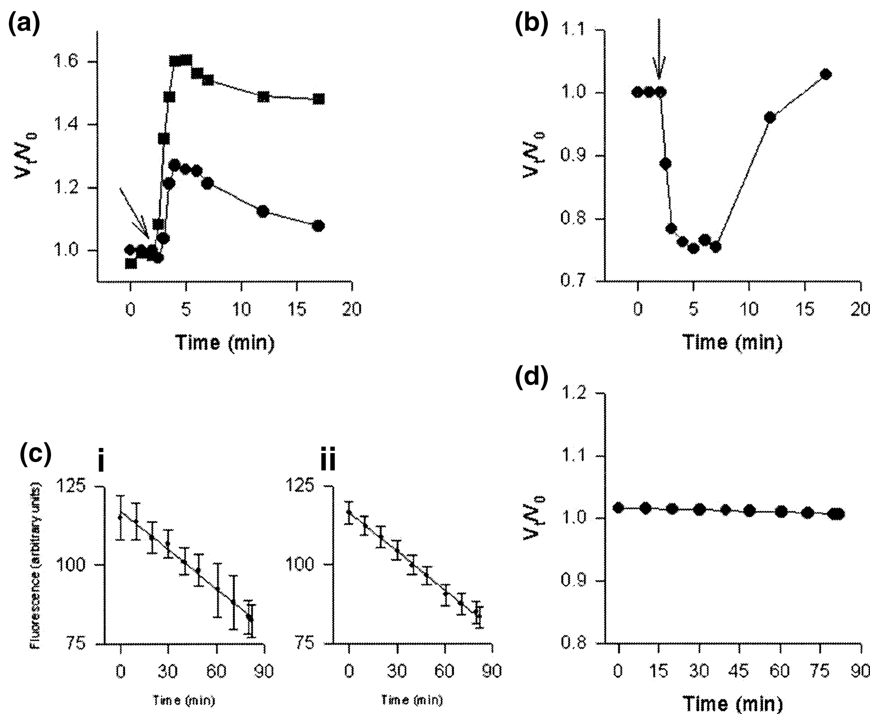

Fig. 1 Cell volume of cerebellar granule neurons exposed to sudden or gradual changes in external osmolarity. Cells were incubated with 1 μM calcein-AM, rinsed and superfused with iso-osmotic medium and subsequently (arrow) with hypo-osmotic or hyperosmotic solutions. Fluorescence measurements were transformed 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).

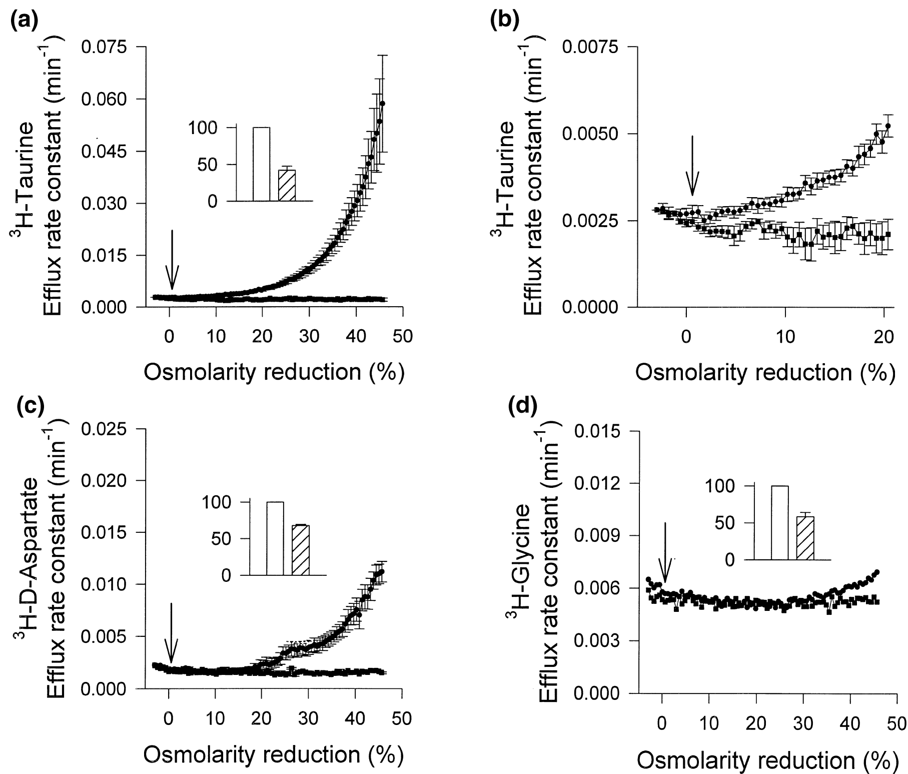

Fig. 2 Amino acid release from cerebellar granule neurons exposed to gradual reductions in external osmolarity. Cells were preloaded with [^{3}H]taurine (a and b), D-[^{3}H]aspartate (c), and [^{3}H]glycine (d), as described in Materials and methods. After loading, cells were washed and superfused (rate 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

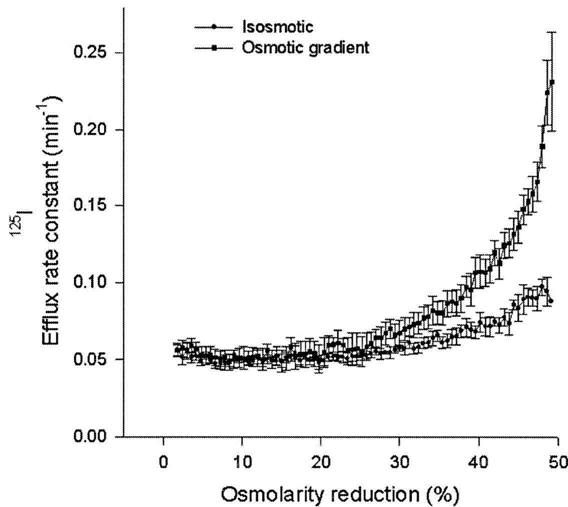

Fig. 3 ^{125}I efflux from cerebellar granule neurons in response to the osmotic gradient. Cells were preloaded with 5 μCi of ^{125}I, and then treated as in Fig. 2. (●) Controls in iso-osmotic medium. (■) Osmotic gradient of − 1.8 mOsm/min. Data are means of eight experiments ± SE.

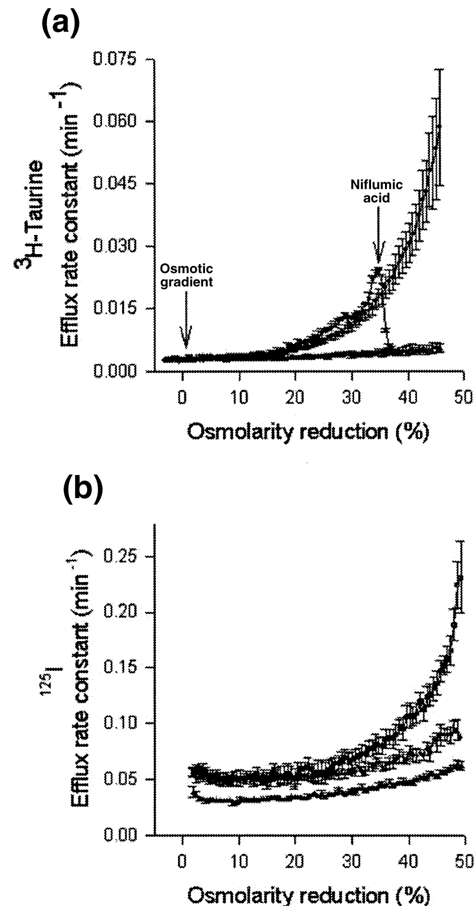

Fig. 4 Effects of niflumic acid on [^{3}H]taurine and ^{125}I efflux during IVR. Loading conditions and basal collection were as in Fig. 2. (a) [^{3}H]Taurine release elicited by the osmotic gradient (●); effect of 600 μM niflumic acid present throughout the experiment (▲) or added (arrow) at π_o (− 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

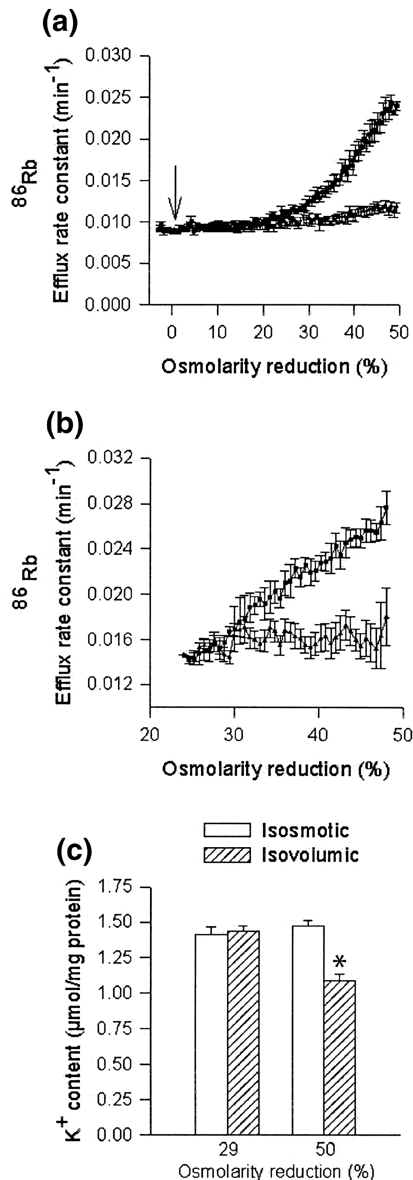

Fig. 5 ^{86}Rb release and changes in K^+ cell content in cerebellar granule neurons exposed to gradual or sudden decreases in osmolarity. (a) Cells preloaded with ^{86}Rb were superfused as in Fig. 2 with medium of gradually decreased osmolarity (■) or with iso-osmotic medium (●). At the end of the 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

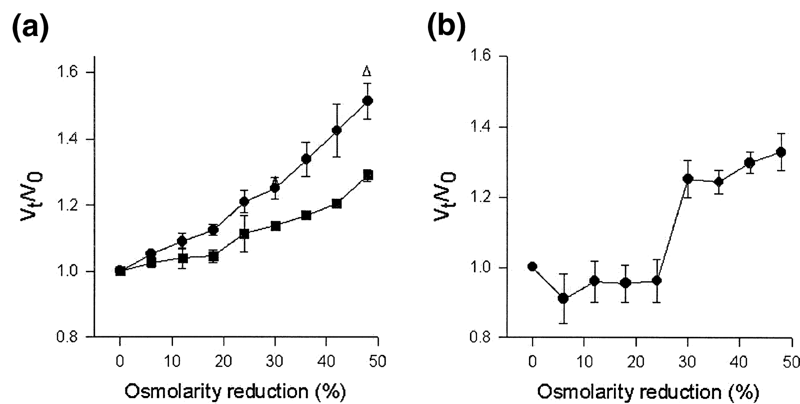

Fig. 6 Impairment of IVR by niflumic acid, Ba^{2+} and Cl^--free medium. Cell volume was measured as in Fig. 1. (a) Cell volume measured in cells exposed to the osmotic gradient in the presence of 600 µM niflumic acid (●), or Cl^--free medium (Cl^- replaced by gluconates) (■). The triangle in this figure (△) 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.

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

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

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

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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

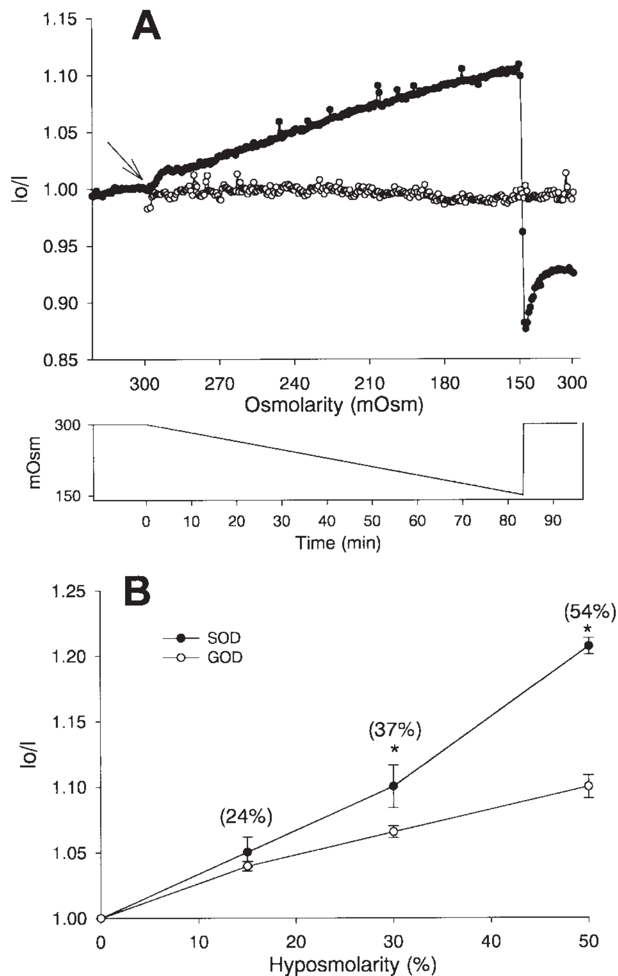

Fig. 1. Cell volume changes of cerebellar astrocytes exposed to gradual changes in external osmolarity. Estimation of relative cell volume was performed using large-angle light scattering as described by McManus et al., (35). Cells seeded on rectangular coverslips were placed on a superfusion chamber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

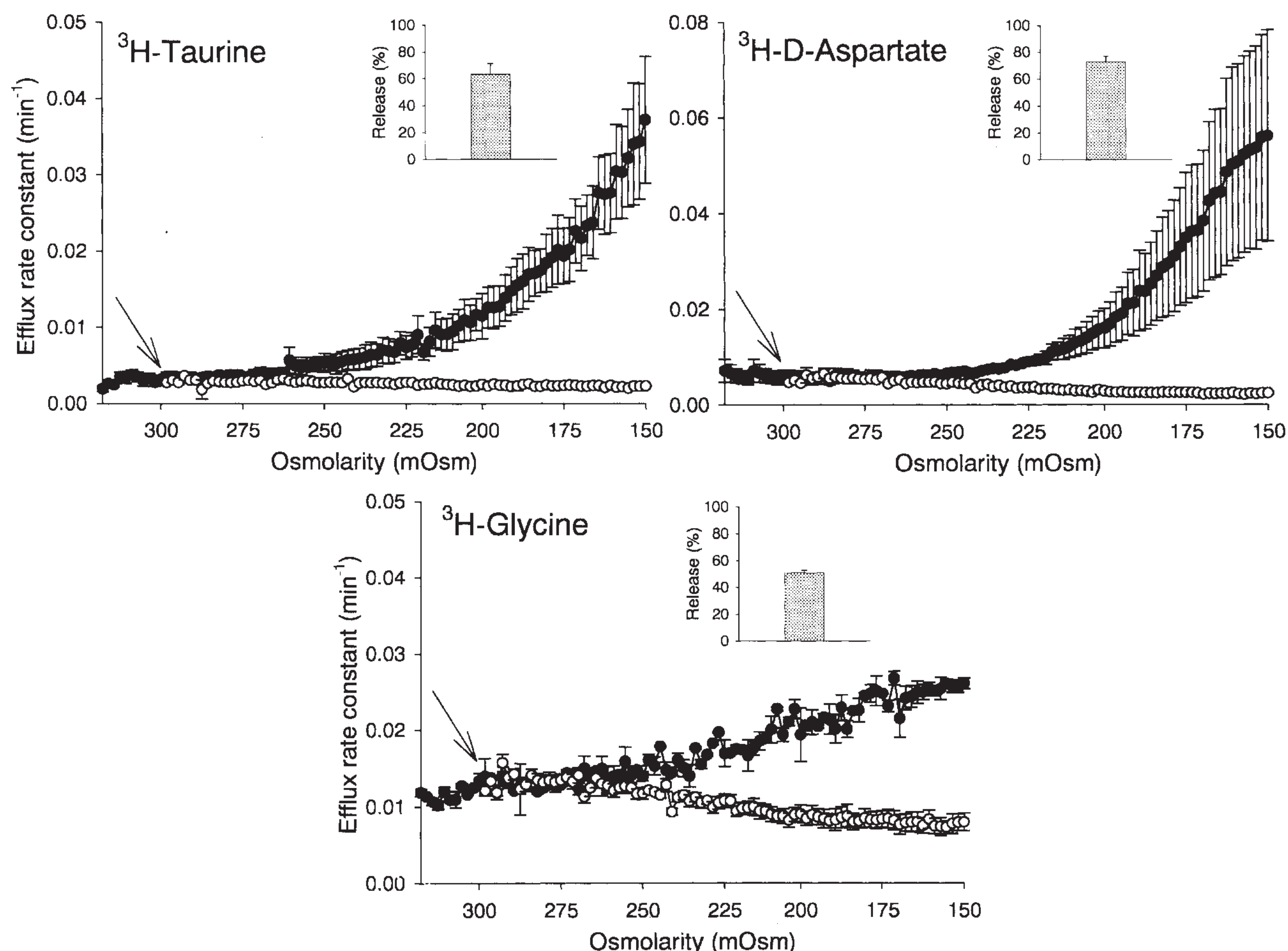

Fig. 2. Amino acid release from cerebellar astrocytes exposed to gradual reductions in external osmolarity. Cells were preloaded with ^{3}H-taurine, ^{3}H-D-aspartate, and ^{3}H-glycine as described in Tuz et al. (30) After the loading period, cells were washed and superfused (rate 2.0 ml/min) during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

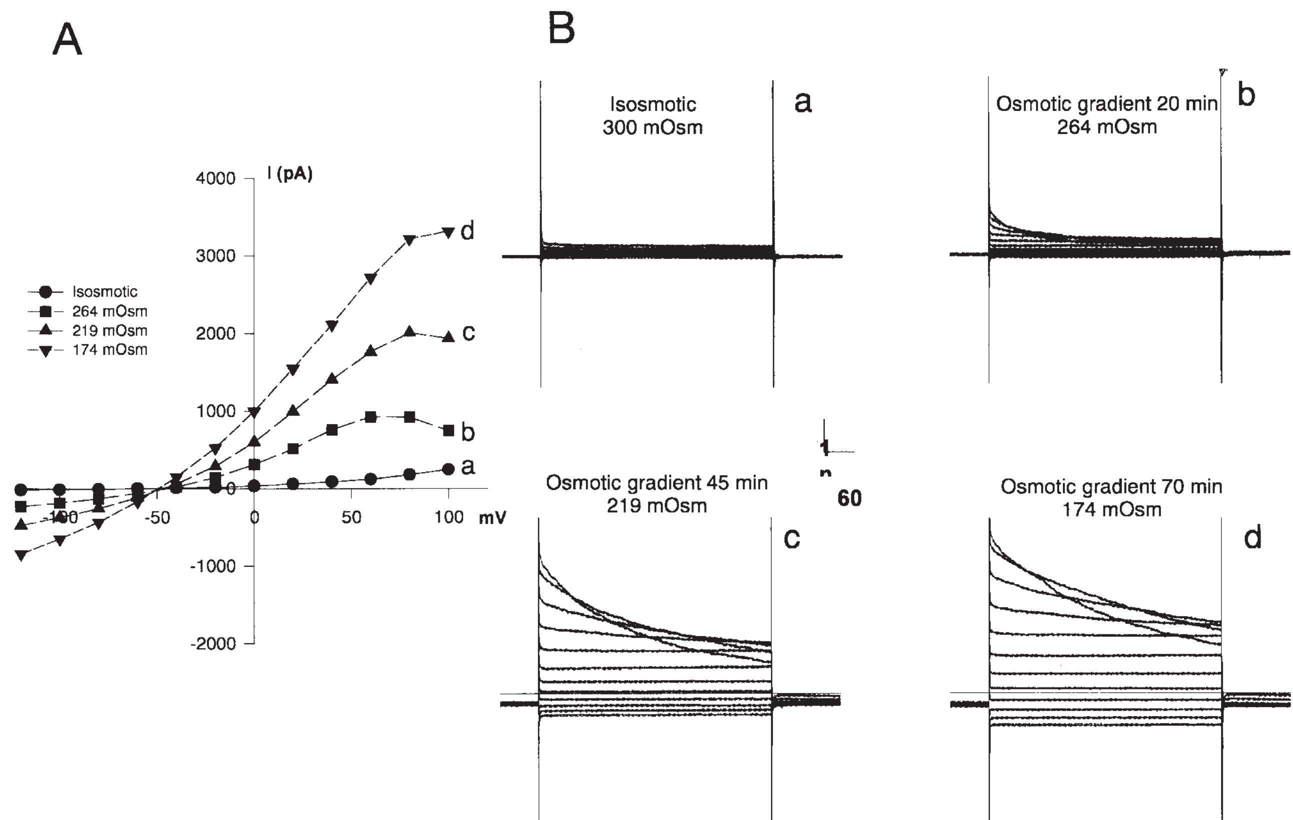

Fig. 3. Osmotic gradient–induced activation of whole-cell currents in cerebellar astrocytes. Astrocytes were seeded in 35-mm petri dishes at a low density of 0.3 mill/ml. Currents were monitored with Axopatch 200 patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell membrane currents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

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Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K^+ channels

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Ordaz, B., L. Vaca, R. Franco, and H. Pasantes-Morales. 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: C1399–C1409, 2004. First published January 21, 2004; 10.1152/ajpcell.00198.2003.—Volume changes and whole cell ionic currents activated by gradual osmolarity reductions (GOR) of 1.8 mosM/min were characterized in C6 glioma cells. Cells swell less in GOR than after sudden osmolarity reductions (SOR), the extent of swelling being partly Ca^{2+} dependent. In nominally Ca^{2+}-free conditions, GOR activated predominantly whole cell outward currents. Cells depolarized from the initial −79 mV to a steady state of −54 mV reached at 18% osmolarity reduction [hyposmolarity of −18% (H-18%)]. Recordings of Cl^- and K^+ currents showed activation at H-3% of an outwardly rectifying Cl^- current, with conductance of 1.6 nS, sensitive to niflumic acid and 5-nitro-2-(3-phenylpropylamino)benzoic acid, followed at H-18% by an outwardly rectifying K^+ current with conductance of 4.1 nS, inhibited by clofilium but insensitive to the typical K^+ channel blockers. With 200 nM Ca^{2+} in the patch pipette, whole cell currents activated at H-3% and at H-13% cells depolarized from −77 to −63 mV. A K^+ current activated at H-1%, showing a rapid increase in conductance, suppressed by charybdotoxin and insensitive to clofilium. These results show the operation of two different K^+ channels in response to GOR in the same cell type, activated by Ca^{2+} and osmolarity and with different osmolarity activation thresholds. Taurine and glutamate efflux, monitored by labeled tracers, showed delayed osmolarity thresholds of H-39 and H-33%, respectively. This observation clearly separates the Cl^- and amino acid osmosensitive pathways. The delayed amino acid efflux may contribute to counteract swelling at more stringent osmolarity reductions.

volume regulation; taurine; hyposmolarity; isovolumetric regulation; regulatory volume decrease

MOST CELLS RESPOND TO DECREASES in external osmolarity by rapid cell swelling followed by an active process of volume regulation accomplished by extrusion of intracellular osmotically active solutes (5). This process, known as regulatory volume decrease (RVD), has been examined extensively in a variety of cells, including brain cells (14). In most studies, however, cells are exposed to abrupt and large osmolarity reductions, which seldom occur in vivo, even in conditions of acute hyponatremia. An approach closer to the physiological situation is that devised by Lohr and Grantham (7), in which renal cells are exposed to small and gradual changes in external osmolarity. Under these conditions, cells are able to maintain a constant volume over a wide range of tonicities if the rate of change in osmolarity is <2.2 mosM/min. This response was named "isovolumetric regulation" (IVR), a term reflecting the active nature of this process, because the unchanged volume seems not to be due to the absence of swelling but to a continuous volume adjustment accomplished by the efflux of intracellular osmolytes. This notion is based on the shrinkage observed in cells returned to an isosmotic medium, which, because of the decrease of intracellular osmolytes, is now hyperosmotic with respect to the intracellular medium (6, 7). The ubiquity of IVR as a mechanism of cell volume control has not been extensively explored. IVR has been described so far in two types of renal cells (6, 7, 22), in cerebellar granule neurons (21), and in a subset of hippocampal neurons (24). Other cell types such as C6 cells and cultured myocytes show IVR only over very short ranges of osmolarity and/or when the change in osmolarity is very small (8, 18). It cannot be ruled out, however, that gradual exposure to hyposmolarity results in better mechanisms of cell volume control, i.e., less swelling or more rapid volume recuperation. The contribution of the different osmolytes and the nature of the translocation pathways activated in volume regulation in the gradual osmolarity reduction (GOR) condition or during IVR are not well known. K^+ and taurine efflux occurs during GOR (18, 21, 22), whereas in trout erythrocytes, in which GOR leads to continuous swelling, there is no evidence of rapid taurine or K^+ loss (1). All studies so far of the K^+ and Cl^- efflux on GOR have been carried out by monitoring ionic fluxes and final changes in ion intracellular content, but no electrophysiological studies have been carried out. In the present work we examined the changes in cell volume during GOR in glioma C6 cells and characterized the ionic currents activated under these conditions. Efflux of taurine and glutamate, monitored by the release of radioactive tracers, was also examined.

MATERIALS AND METHODS

Cells. The glioma C6 cell line (American Type Culture Collection, Rockville, MD) was grown in Eagle's medium (GIBCO Life Technologies) supplemented with 15% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The culture dishes were kept at 37°C in a humidified atmosphere (5% CO_2-95% O_2).

Solutions and gradually diluted solutions. The isosmotic medium contained (in mM) 135 NaCl, 1.0 $CaCl_2$, 1.17 $MgSO_4$, 4.7 KCl, 5 dextrose, and 10 HEPES (300 mosM, pH 7.4). The gradually diluted solutions (−1.8 mosM/min) were obtained by the procedure described

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by Lohr and Grantham (7) and by Van Driessche et al. (22). Briefly, the system consisted of two identical glass cylinders interconnected at their bases by a tube with an interrupting valve. The first container was filled with isosmotic medium and the second with the same volume of hyposmotic medium (50%). Media were kept at 39°C by placing the cylinders on a temperature-controlled hot plate and stirring. Superfusion medium was pulled from the first container with a polystaltic pump, allowing the hyposmotic medium to begin to enter this cylinder, mixing gradually and continuously with the isosmotic medium. In this way, a solution with linear dilution of −1.8 mosM/min was produced, which at the end of the experiment (82 min later) reached 150 mosM (50% hyposmotic). The linearity of the diluted solutions and the osmolarity of all solutions were verified in a freezing-point osmometer (Osmete A, Precision Systems). Reductions in osmolarity are indicated throughout the manuscript as hyposmolarity of negative percent change in each case. For instance, a 3% reduction is expressed as H-3%.

Estimation of changes in cell volume. Cell volume measurements were performed by estimating the changes in relative cell volume with a large-angle light-scattering system (12, 16). C6 cells were cultured on rectangular cover glasses (10 × 50 mm) at 90% confluence at the time of experiments. Cover glasses were placed at a 50° angle relative to the excitation light in a cuvette filled with isosmotic medium (300 mosM) in an Aminco-Bowman Series 2 luminescence spectrometer. Cells were excited at 585 nm with an argon arc lamp, and emission was detected at the same wavelength. Data are expressed as the inverse of the emission signal, because light intensity inversely correlates with cell volume. Cell volume changes were calculated according to the equation l_o/l_t, where l_o is isosmotic emission signal average and l_t is emission signal at time *t*.

Electrophysiological recordings. Currents were monitored with an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA). All recordings were performed at 35°C with a diluted solution-generating system. Whole cell membrane currents were measured by using ruptured patches. The time course of whole cell currents was obtained by following voltage protocols of holding potential of −70 mV to potentials ranging from −120 to +100 mV in 20-mV increments for 350 ms. Electrophysiological recording was carried out on cells seeded on 35-mm petri dishes, as described above. Once the whole cell configuration was obtained, cells were perfused for 5 min with isosmotic solution before the gradual dilution in osmolarity was initiated. The voltage protocol was carried out every 5 min for the duration of the experiment while the dilution gradient continued. This manipulation did not interfere with the whole cell recordings, because the entire whole cell voltage protocol lasted only 12 s. The voltage protocol was carried out fast enough to be concluded while the osmolarity reduction was only 0.36 mosM (H-0.2%) during the 12-s period. This protocol was repeated every 5 min for the duration of the experiments, as indicated. Patch electrodes were prepared from 1.5-mm OD, 1.5-mm ID borosilicate glass (World Precision Instru-

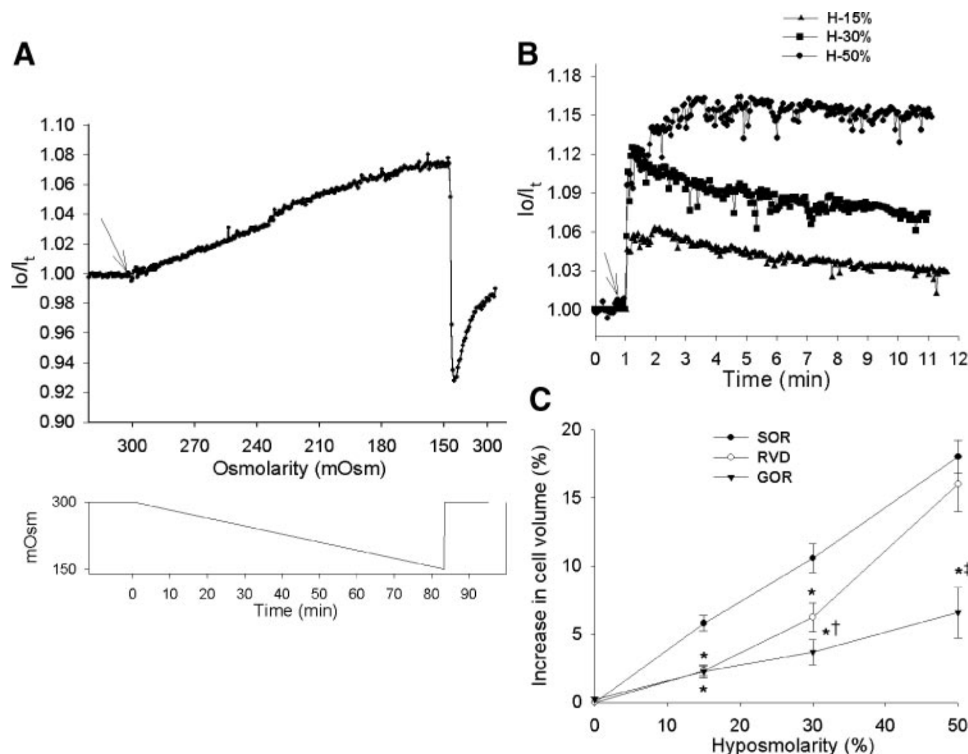

Fig. 1. Cell volume changes of C6 cells exposed to sudden (SOR) or gradual osmolarity reduction (GOR). Estimation of relative cell volume change (l_o/l_t, where l_o is isosmotic emission signal average and l_t is emission signal at time *t*) was performed with large-angle light scattering as described in materials and methods. Data are given as the inverse of the emission, because emitted light intensity inversely correlates with cell volume. *A*: *top*, a representative experiment of cell volume changes elicited by GOR. Cells were superfused (rate 2.0 ml/min at 37°C) for 10 min with isosmotic medium. At the indicated time (arrow), cells were exposed to the gradually diluted solution (−1.8 mosM/min) over 83 min (*bottom*), at which time the osmolarity had decreased 50% (150 mosM). At the end of the experiment, cells were returned to the isosmotic condition (300 mosM). *B*: representative traces of cell volume changes elicited by SOR. Cells were superfused with isosmotic medium, and then (at the arrow) sudden reduction in osmolarity was made by isosmotic medium dilution with distilled water until reaching −15% (H-15%), −30% (H-30%), and −50% (H-50%) hyposmotic medium. Cell volume changes were followed 10 min after hyposmotic stimulus. *C*: cell volume in C6 at H-15%, H-30%, and H-50% hyposmotic medium, reached in conditions of SOR (maximal swelling), after cell volume regulation in these conditions [regulatory volume decrease (RVD)], or when the same osmolarities were reached during exposure to GOR. Points are means ± SE; $n = 4$–10. *$P < 0.001$ with respect to SOR; †$P < 0.05$, ‡$P < 0.001$ with respect to RVD.

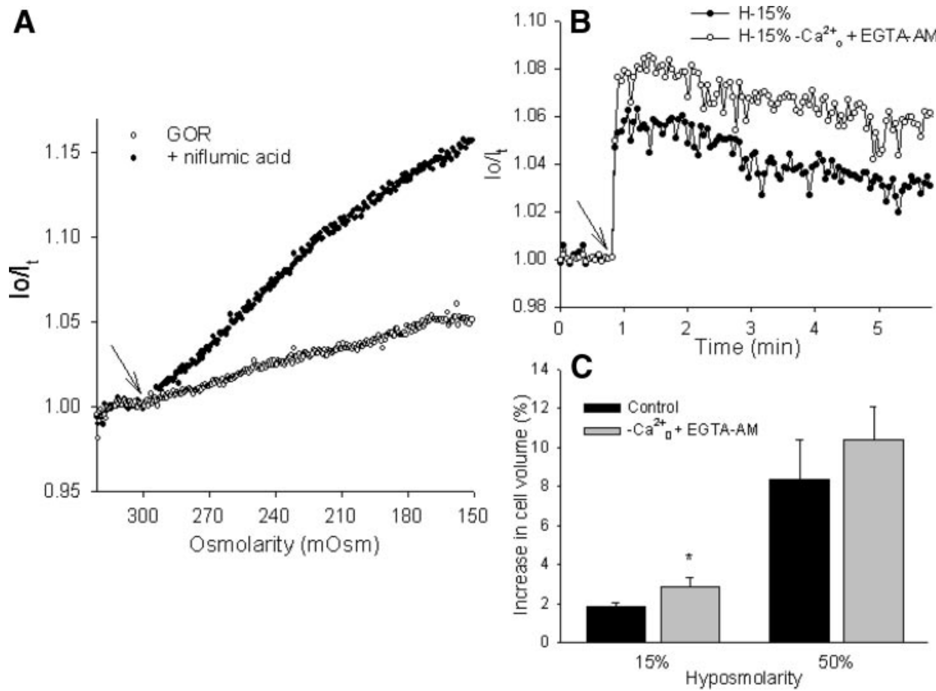

Fig. 2. Ca^{2+} dependence and effect of niflumic acid on cell volume changes of C6 cells exposed to SOR or GOR. Estimation and expression of relative cell volume change (l_o/l_t) as in Fig. 1. *A*: a representative experiment of cell volume changes elicited by GOR in the presence (●) or absence (○) of 600 μM niflumic acid. Niflumic acid was present throughout the experiment. *B*: representative traces of cell volume changes elicited by 15% SOR in the presence or absence of intracellular Ca^{2+}. Cells were superfused with isosmotic medium, and then (arrow) medium was made −15% hyposmotic (●) by addition of distilled water. Cell volume changes were followed 10 min after hyposmotic stimulus. ○, Cells were preloaded with 50 μM EGTA-AM 30 min before the experiment, and isosmotic medium was made nominally Ca^{2+} free by addition of 0.5 mM EGTA. *C*: effect of Ca^{2+}-free conditions (as described in *B*) on cell volume increase attained during GOR. Bars represent cell volume increase (% over isosmotic condition) reached at H-15% and H-50%. Solid bars, volume changes in the presence of Ca^{2+}; shaded bars, volume changes in cells in Ca^{2+}-free conditions as in *B*. Results are means ± SE; $n = 4$. $^*P < 0.05$ vs. control.

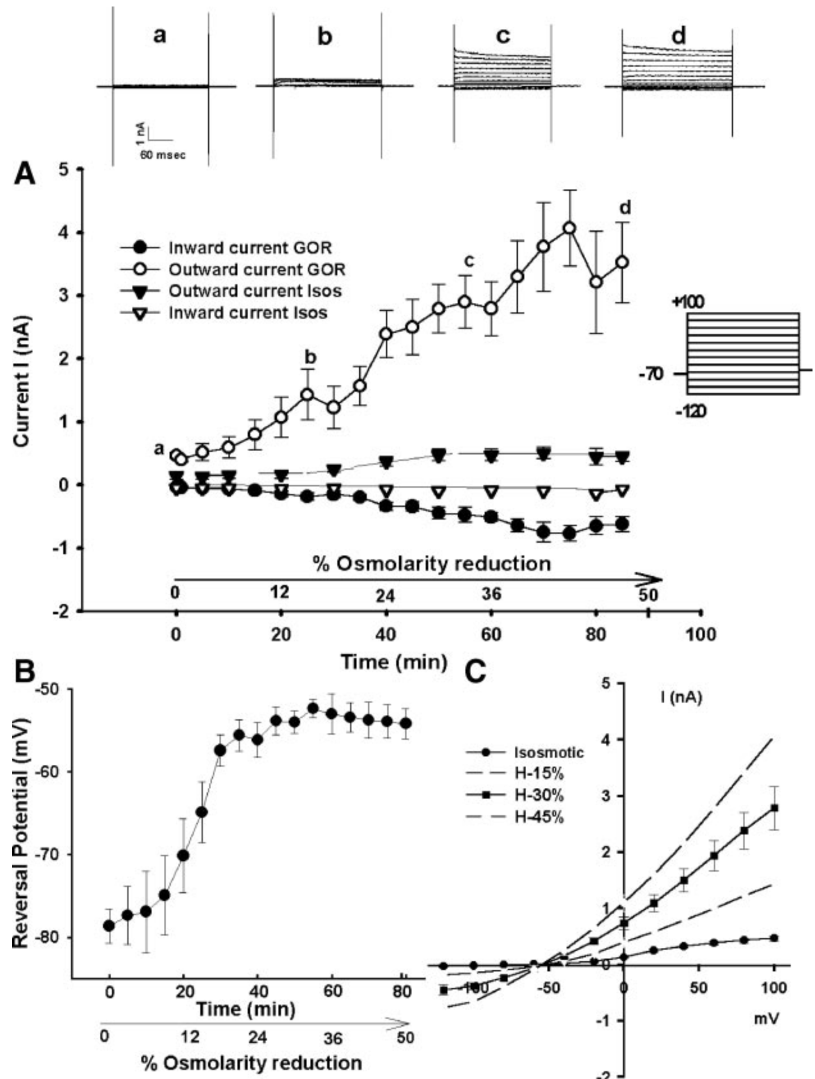

Fig. 3. Whole cell currents in C6 glioma cells induced by GOR in nominally intracellular Ca^{2+}-free solution. *A*: membrane currents (*I*) were measured in the whole cell recording mode of the patch-clamp technique with a holding potential of −70 mV. After GOR ($n = 17$): ●, inward currents; ○, outward currents. In isosmotic medium ($n = 4$): ▿, inward currents; ▾; outward currents. *Insets a–d* show the activation of currents at the time and osmolarity indicated by letters in the plot. *B*: change in the membrane potential (initial −79 ± 2 mV) through the effect of GOR. *C*: current-voltage (*I-V*) relationship in cells in isosmotic medium (●) and at the indicated osmolarity reductions.

ments), and the resistance was between 3 and 5 MΩ when filled with the pipette solution. The recorded signal was filtered at 10 kHz with a low-pass Bessel filter and transferred to a computer with the Digidata 1200 interface (Axon Instruments). Whole cell currents were analyzed with pCLAMP6 software (Axon Instruments).

The standard pipette solution contained (in mM) 110 K^+ aspartate, 30 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, and 5 Mg ATP, pH 7.4 adjusted with KOH (300 ± 3 mosM). In the anion-substitution experiments, K-aspartate and KCl were fully replaced by 140 mM CsCl. In experiments to isolate K^+ currents, aspartate was the major anion in the pipette and only 10 mM Cl^- was present in the intracellular solution.

Efflux of amino acids. Cells were incubated for 60 min in culture medium containing the labeled amino acids D-[^{3}H]aspartate (as a tracer for glutamate) and [^{3}H]taurine (0.5 μCi/ml). After the loading period, cultured dishes were superfused for 20 min with isosmotic medium, and the superfusion continued either with isosmotic medium or with the gradually diluted solutions (−1.8 mosM/min) during the time indicated. Samples were collected every minute, and radioactivity in samples and in cells after the experiment was quantified by scintillation. Labeled amino acid 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.

RESULTS

Cell volume changes after exposure to sudden decrease in osmolarity and GOR. C6 cells exposed to a sudden decrease in osmolarity (SOR) of H-15% and H-30% exhibited the typical RVD, as reported previously by Lohr and Yohe (8). As shown in Fig. 1*B*, the volume correction was more efficient at small osmolarity reductions. Cells exposed to GOR (−1.8 mosM/min) exhibited a progressive increase in cell volume, which continued over the time of the experiment, up to 83 min, when the osmolarity was decreased 50% (Fig. 1*A*). These results indicate the absence of IVR in the GOR model in C6 cells. However, cells swelled significantly less than those exposed to SOR at the same osmolarities (H-15%, H-30%, and H-50%), as shown in Fig. 1*C*. Moreover, at H-30%, cell volume in the GOR condition was still significantly lower than that in SOR after regulation, ~10 min later (Fig. 1*B*). At H-50%, no RVD was observed after SOR (Fig. 1*B*), but during GOR cells were notably less swollen (Fig. 1*C*). These results indicate a more rapid and efficient mechanism of volume regulation in GOR. Returning cells to isosmotic medium after GOR resulted in immediate decrease in cell volume to values lower than the

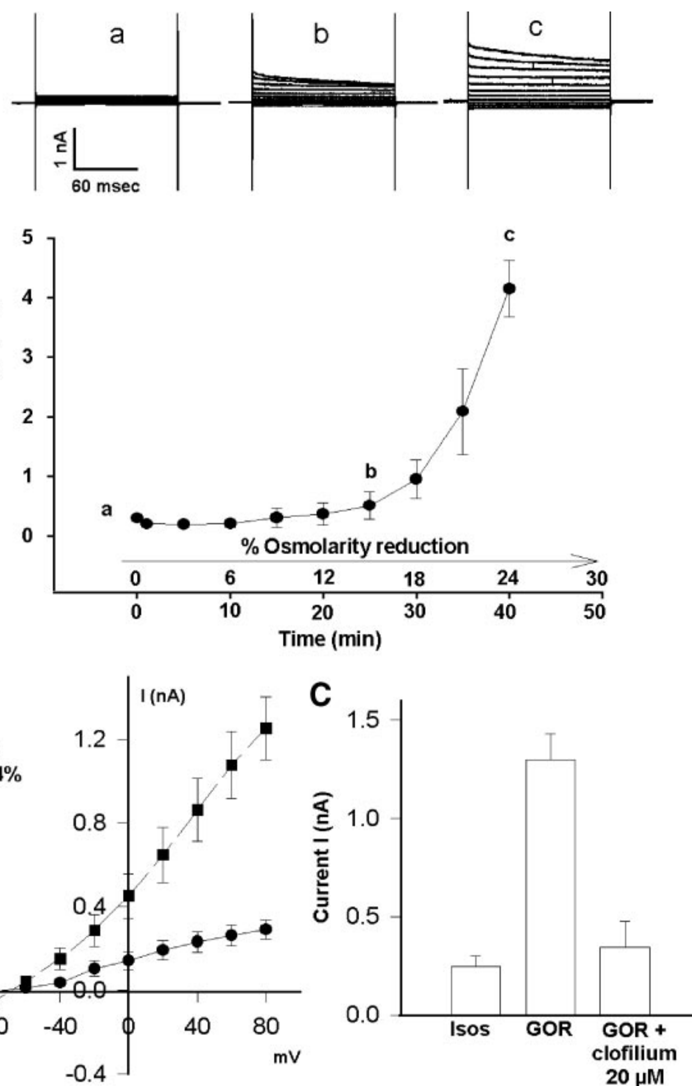

Fig. 4. K^+ conductance (*g*) elicited by GOR in intracellular Ca^{2+}-free conditions. The pipette was filled with 130 mM K-aspartate. The holding potential was −70 mV (*n* = 6). *A*: *insets a–c* show the currents obtained at the osmolarity indicated by letters in the plot. *B*: *I-V* relationships for K^+ currents (*n* = 6) under isosmotic conditions and at H-24%. *C*: effect of clofilium on the GOR-elicited K^+ current. Bars represent means ± SE (*n* = 5) of the current measured at +80 mV.

initial values, indicative of loss of intracellular osmolytes (Fig. 1*A*). Treatment with 600 μM niflumic acid increased cell volume to the same extent as in SOR at all osmolarities (Fig. 2*A*), further supporting the operation of an active mechanism of cell volume regulation. In cells incubated with EGTA-AM to reduce intracellular Ca^{2+}, a sudden osmolarity decrease of 15% resulted in higher cell swelling and less efficient RVD compared with cells not treated with EGTA-AM (Fig. 2*B*). In Ca^{2+}-free conditions, a significant increment in cell volume during GOR was observed at external osmolarity of H-15% but not at H-50% (Fig. 2*C*).

Studies in jejunal villus epithelial cells showed the operation of two mechanisms for cell volume regulation in response to small or large changes in cell volume, elicited either by nutrient uptake (9) or by hyposmolarity (10). The main differences were found in the volume regulatory response depending on the extent of cell swelling regarding Ca^{2+} dependence and sensitivity to charybdotoxin (10), thus suggesting the involvement of different K^+ channel types. To investigate a possible difference in whole cell currents depending on the extent of cell swelling and the influence of Ca^{2+}, experiments were carried out in either the presence or the absence of Ca^{2+} in the intracellular solution.

Whole cell currents activated during GOR in nominally Ca^{2+}-free intracellular solution. We first explored the currents activated by GOR in the patch-clamp whole cell configuration with 5 mM EGTA in the patch pipette. Figure 3*A* illustrates the activation and progressive increase with time of whole cell currents in this condition. As indicated in Fig. 3*A*, the outward current activated by changes in osmolarity was more prominent than the inward current, even though the time course was similar in both cases. No whole currents were activated when cells were maintained in isosmotic medium for the same time as those exposed to GOR (Fig. 3*A*). The membrane potential progressively depolarized within the first 30 min of the osmolarity decrease, then reached a steady-state level of about −54 mV at H-18% (Fig. 3*B*). Figure 3*C* illustrates the current-voltage (*I-V*) relationship for control (isosmotic) and 25, 50, and 75 min after GOR onset (H-15%, H-30%, and H-45%, respectively).

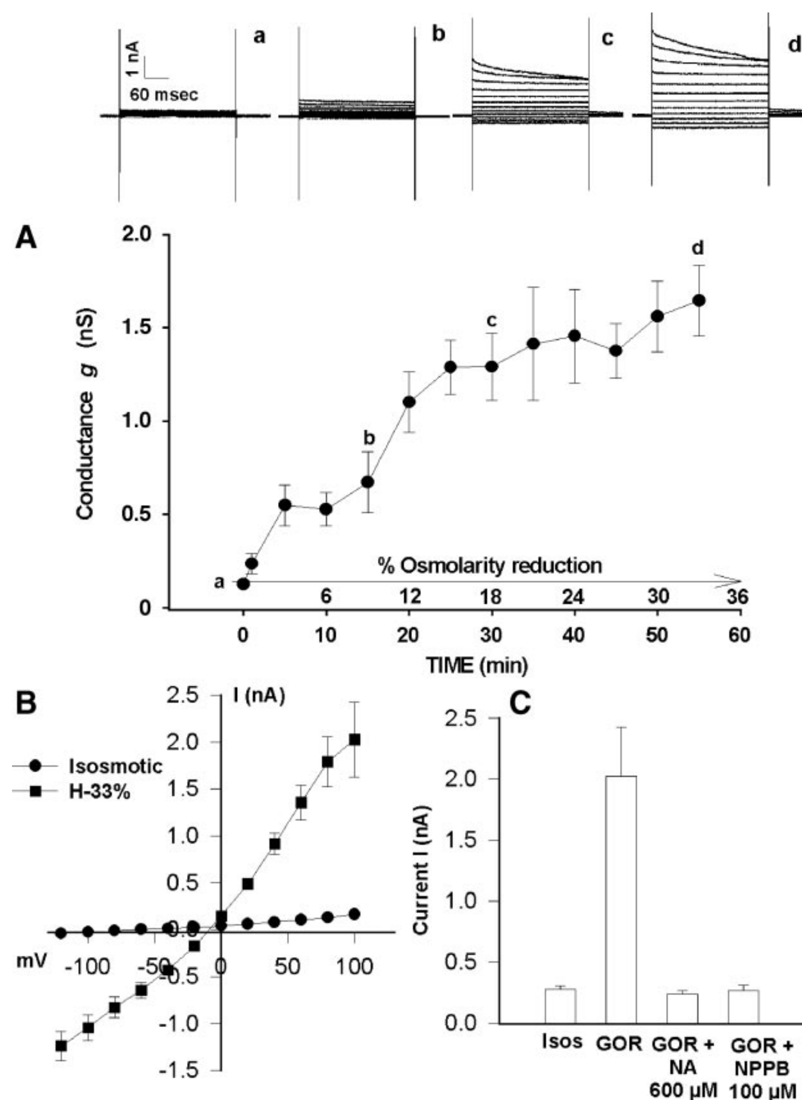

Fig. 5. *A*: Cl^- conductance elicited by GOR in intracellular Ca^{2+}-free conditions. The pipette was filled with 140 mM CsCl, the holding potential was −70 mV (n = 6). *Insets a–d* show the current activation at the osmolarity indicated by letters in the plot. *B*: *I-V* curves in cells in isosmotic medium and at H-33%. *C*: effect of the Cl^- channel blockers niflumic acid (NA) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on the GOR-elicited Cl^- current. The blockers were present in the solutions used to generate the media with gradually decreased osmolarities. Bars represent means ± SE (n = 4) of the current measured at +100 mV.

Using ionic replacements, we separated Cl^- and K^+ currents to study the relative contribution of each to the whole cell currents activated by GOR. K-current was examined by using 130 mM K-aspartate in the patch pipette to decrease the contribution of Cl^-. Figure 4*A* illustrates the time course of K^+ current activation after GOR. The activation of the K^+ conductance was delayed compared with the activation of the whole cell currents observed in Fig. 3*A*, because a significant increment in K^+ conductance was observed only after 30 min of GOR onset (H-18%). After this delay period, K^+ conductance activated completely within the next 15 min, to reach a maximum in the following 10 min (H-24%) (Fig. 4*A*). Figure 4*B* compares the *I-V* relationships in control conditions (isosmotic) and at the maximal current observed after GOR onset (H-24%). The cell membrane potential was not depolarized under these experimental conditions. The reversal potential observed at H-24% was −70 mV, a value close to the expected reversal potential for a K^+ electrode calculated from the Nernst equation (−84 mV). The K^+ current was inhibited in the presence of extracellular 20 μM clofilium (Fig. 4*C*) but was insensitive to 5 mM TEA, 2 mM barium, 1 mM 4-aminopyridine (4-AP), and 600 μM quinidine (not shown).

We next performed similar experiments but with solutions that minimize the contribution of K^+ channels to whole cell currents (140 mM CsCl in the patch pipette). Figure 5*A* illustrates the Cl^- conductance elicited by GOR. The Cl^- conductance activated early during the gradual hyposmotic reduction. Significant increments in conductance were detected as early as 2 min after GOR initiation (H-3%). The Cl^- conductance increased continuously with progressive osmolarity reduction.

Figure 5*B* illustrates the *I-V* relationship for the Cl^- current under isosmotic conditions (control) and at the maximal current observed after GOR onset (H-33%). In this case, the Cl^- current showed a reversal potential near 0 mV, a value expected when using symmetrical Cl^- concentrations. The Cl^- current was completely inhibited in the presence of extracellular 600 μM niflumic acid and 100 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Fig. 5*C*).

All of these results show that the initial increments of whole cell currents induced by GOR in the intracellular Ca^{2+}-free condition result from the activation of a Cl^- conductance, followed minutes later by the activation of a K^+-selective conductance. The maximal conductance values obtained were 1.6 ± 0.18 nS ($n = 7$) for Cl^- and 4.1 ± 0.47 nS ($n = 6$) for K^+.

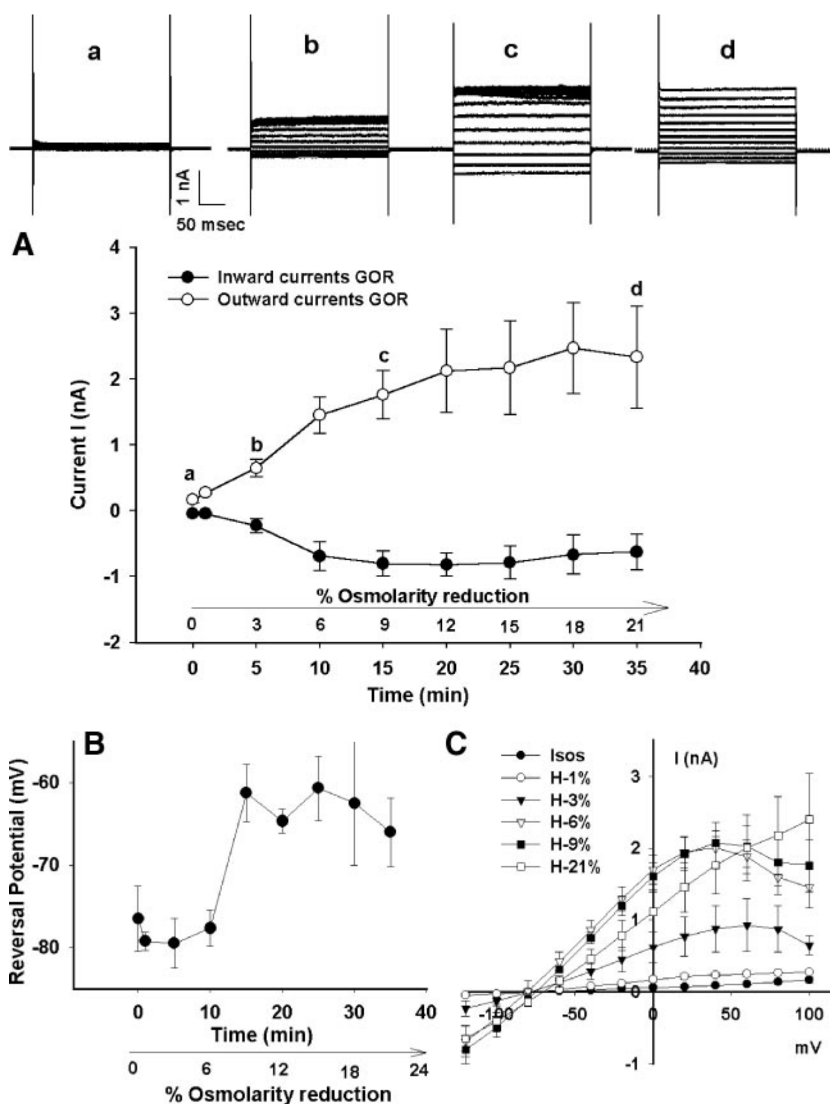

Fig. 6. Whole cell currents activated by GOR in the presence of intracellular Ca^{2+}. *A*: membrane currents measured as in Fig. 3 but with 200 nM Ca^{2+} in the patch pipette ($n = 6$). *Insets a–d* show the activation of currents at the time and osmolarity marked by letters in the plot. *B*: change in the membrane potential (initial −78 ± 3 mV) through the effect of GOR. *C*: *I-V* relationship in cells in isosmotic medium and at osmolarity reductions as indicated.

Whole cell currents activated by GOR in presence of intracellular Ca^{2+}. All of the above described recordings were performed in nominally intracellular Ca^{2+}-free conditions, and therefore all observed currents occurred through Ca^{2+}-independent channels. To investigate whether Ca^{2+}-dependent currents are elicited during the first minutes of osmolarity reduction and can contribute to cell volume control in cells showing small volume increase, whole cell currents were recorded in the presence of 200 nM Ca^{2+} in the patch pipette. As indicated in Fig. 6*A*, the change in osmolarity evoked inward and outward currents with similar time courses, the outward current being more prominent than the inward current at all osmolarities. Activation of these currents occurs at changes in osmolarity as low as H-3%. Figure 6*B* illustrates the change in membrane potential during osmolarity reductions. The potential remained hyperpolarized during the first 10 min (H-6%). After H-6%, the membrane potential depolarized to reach approximately −63 mV at H-9% and remained constant for the duration of the experiment. Figure 6*C* shows the *I-V* relationship for control (isosmotic) and at H-1%, H-3%, H-6%, H-9%, and H-21%. From H-6%, the maximal amount of current was reached. It is noteworthy that the total current at all points explored was lower in the Ca^{2+}-free condition than in the presence of Ca^{2+} (Figs. 3 and 6), indicating the superposition of Ca^{2+}-activated conductances.

We next examined the effect of GOR on K^+ currents in the presence of 200 nM Ca^{2+} in the patch pipette. K^+ conductance activated as early as H-1% and increased rapidly to reach a maximal value at H-3%, remaining at this value for the duration of the experiment (Fig. 7*A*). The *I-V* curves in Fig. 7*B* show a reversal potential of −76 mV, near the expected reversal potential for a K^+ electrode. From H-3% to H-12%, the K^+ current was reduced at potentials more positive than +60 mV. At larger osmolarity reductions (above H-15%) the *I-V* relationship was linear throughout the voltages explored. We did not explore further the reason for the current inactivation observed at very positive potentials.

These experiments showed in Ca^{2+}-free conditions an early activation of Cl^-, but not of K^+ currents, until osmolarity was reduced to H-18%, whereas in the presence of Ca^{2+} both Cl^- and K^+ currents activated as early as H-3%, thus suggesting a different osmolarity threshold for the K^+ current in the absence of Ca^{2+}. To confirm these differences, we examined K^+ and Cl^- currents in cells after sudden exposure to H-15%. In Ca^{2+}-free medium the osmolarity change activated only a Cl^- current, abolished by NPPB (Fig. 8*A*), whereas no K^+ current could be detected (Fig. 8*B*). In contrast, in the presence of 200 nM intracellular Ca^{2+}, H-15% evoked a K^+ current with an *I-V* curve similar to that observed at the same osmolarity reached by GOR, i.e., about the same maximal value and the same inactivation at positive voltages (Fig. 9*A*). This current was insensitive to clofilium (up to 100 μM) but was abolished by 100 nM charybdotoxin (Fig. 9*B*). At H-30% in Ca^{2+}-free conditions, a K^+ current activates that is abolished by clofilium and insensitive to charybdotoxin (not shown). These results clearly demonstrate the presence of two K^+ conductances with different pharmacological properties, showing distinguishable osmolarity thresholds for activation. The Ca^{2+}-activated K^+ channel contributes to cell volume regulation during small osmolarity reductions, whereas the clofilium-sensitive K^+ channel activates after more severe reductions in osmolarity. These two K^+ currents are accompanied by an early activated Cl^- channel.

Amino acid efflux during GOR. Figure 10 shows the efflux from C6 cells of [^{3}H]taurine and D-[^{3}H]aspartate (as tracer or glutamate) in response to GOR. The amino acid efflux in this condition was compared at each point with the release from cells bathed with isosmotic medium. The efflux of taurine and D-aspartate showed a delayed activation threshold at external osmolarity reductions of H-39% and H-33%, respectively. The

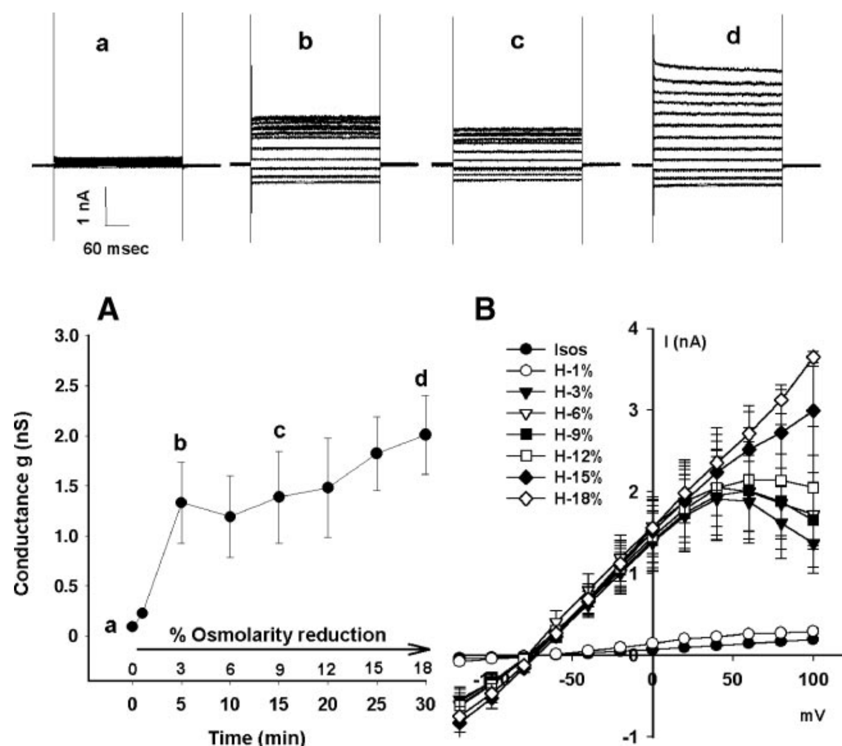

Fig. 7. *A*: K^+ conductance elicited by GOR in the presence of intracellular Ca^{2+}. The pipette was filled with 130 mM K-aspartate and contained 200 nM Ca^{2+}. The holding potential was −70 mV (n = 5). *Insets a–d* show the currents obtained at the osmolarity indicated by letters in the plot. *B*: *I-V* relationships for K^+ currents in cells in isosmotic medium and at osmolarity reductions as indicated (n = 5).

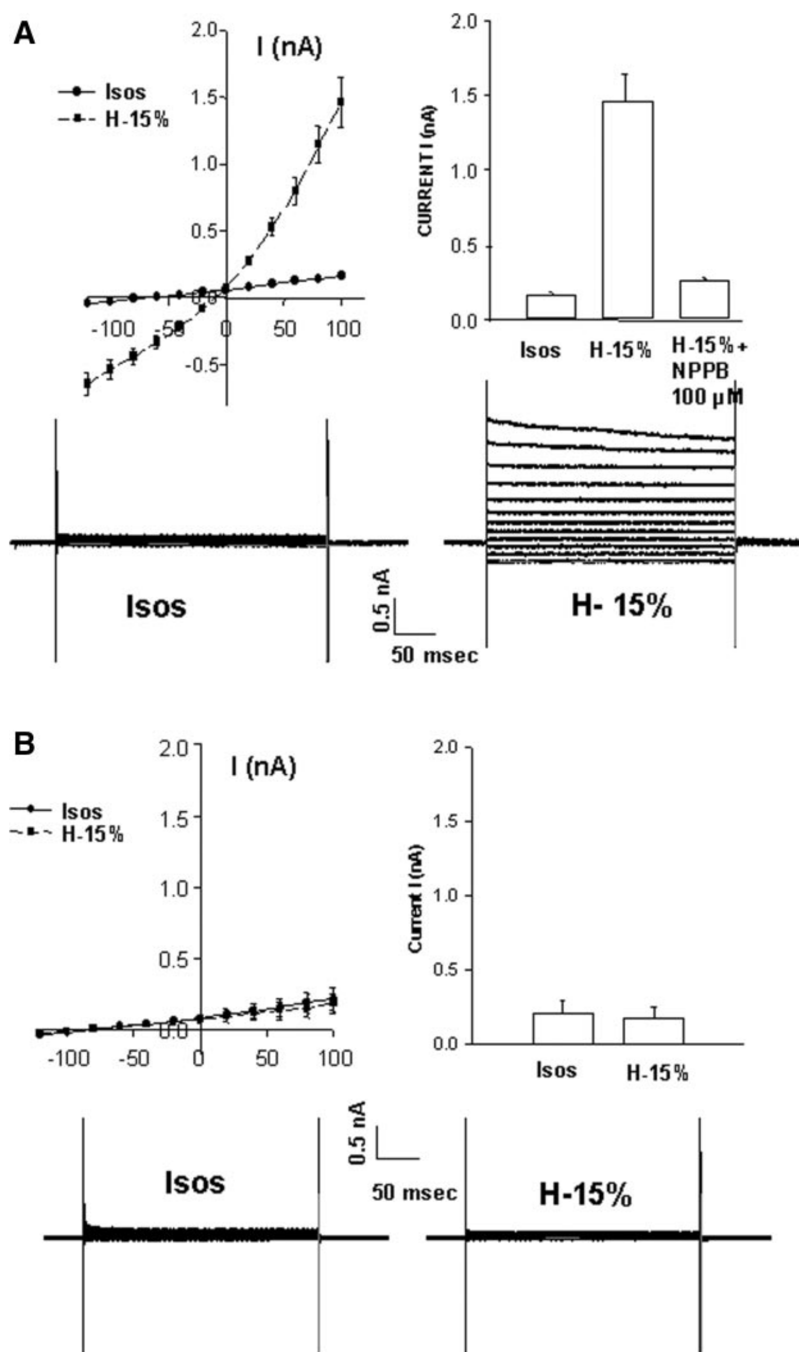

Fig. 8. K^+ and Cl^- currents activated by a sudden decrease in osmolarity of 15% in Ca^{2+}-free conditions. Cl^- (*A*) and K^+ (*B*) currents were measured as in Fig. 3, with ionic replacements and effect of Cl channel blockers as in Figs. 4 and 5; $n = 6$.

amino acid efflux in isosmotic and GOR conditions was followed from *minutes 1* to *82*, but because no difference was observed during the first 50 min, only the time course of efflux from *minute 40* to the end is shown (Fig. 10).

DISCUSSION

In the present study we compared the changes in cell volume evoked by sudden or gradual reductions in osmolarity in C6 cells. We found that cell swelling after GOR was consistently lower than that attained after SOR at the same osmolarities. Moreover, at H-15%, cell volume after GOR was still significantly lower than after 10 min of RVD subsequent to a sudden hyposmotic stimulus. Furthermore, at H-50%, when cells do not exhibit RVD after SOR, cell volume is notably lower. Thus the mechanisms activated by GOR in our conditions, although not sufficient to fully prevent swelling, can substantially reduce it. According to the study of Lohr and Yohe (8) in C6 cells, swelling is prevented only when the osmolarity decrease is of 0.3–0.4 mosM/min and the osmolarity reductions do not exceed 20%. Therefore, C6 cells possess mechanisms to counteract hyposmotic swelling, which, however, appear less efficient than those present in renal cells, A6 cells, and some neurons, which are able to maintain constant volume in the face of osmolarity reductions similar to those used in the present study (7, 21, 24). This may be due to the contribution of amino acids and possibly other organic osmolytes, as discussed below.

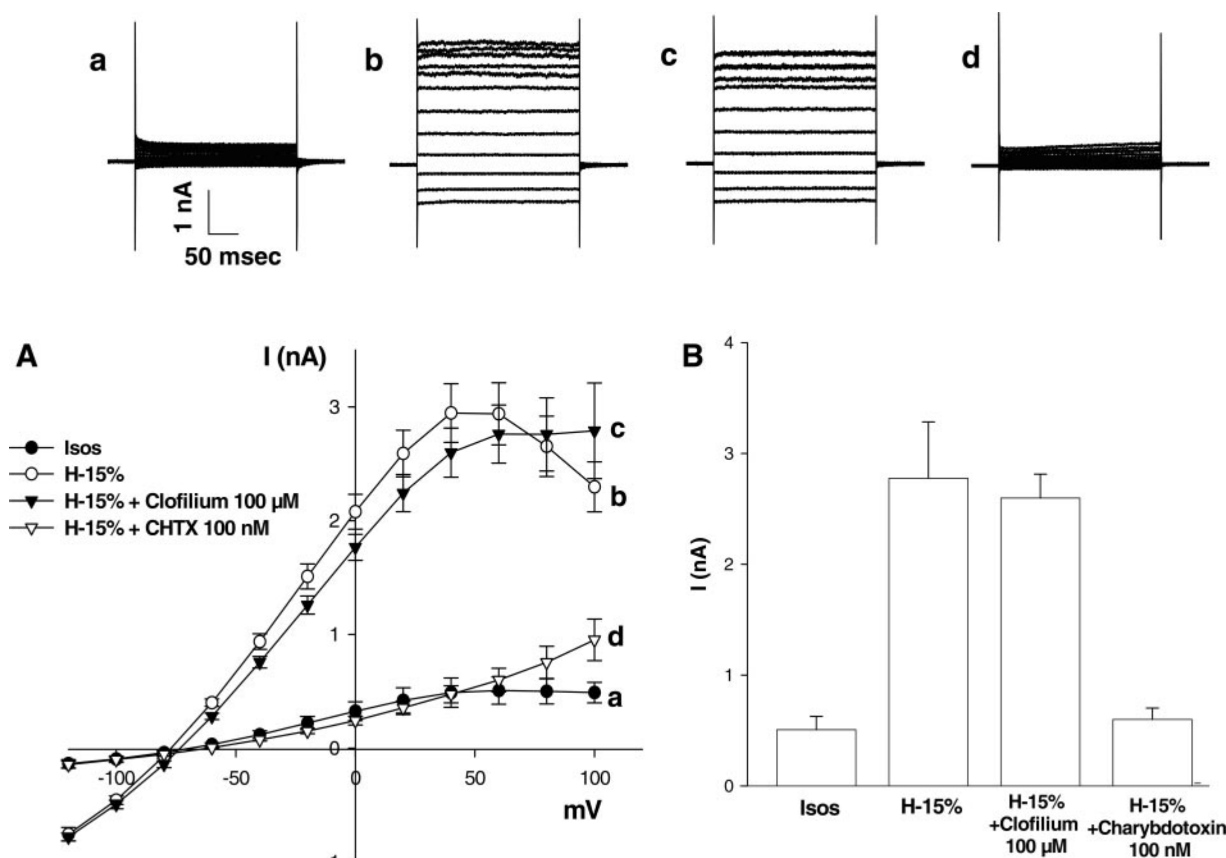

Fig. 9. K^+ current activated by SOR of H-15% in the presence of intracellular Ca^{2+}. The pipette was filled with 130 mM K-aspartate and contained 200 nM Ca^{2+}. The holding potential was −70 mV. *A*: *I-V* relationships for K^+ currents in cells in isosmotic medium and at H-15%, +100 μM clofilium, and 100 nM charybdotoxin (CHTX). *Insets a–d* show the currents obtained at isosmolarity and osmolarity of H-15% indicated by letters in the plot. *B*: effect of clofilium and charybdotoxin on the SOR-elicited K^+ current. Bars represent means ± SE (n = 5) of the current measured at +60 mV.

In both the sudden and the gradual model, at small reductions in osmolarity and less swelling cell volume control was more efficient in the presence of Ca^{2+}, suggesting a Ca^{2+}-dependent element in the volume-regulatory mechanism in this condition. The swelling-activated Cl^- channels, as well as the osmosensitive amino acid fluxes, are largely Ca^{2+} independent (15), thus making the K^+ efflux pathway the likely candidate to be influenced by Ca^{2+}. Volume-activated K^+ channels show marked differences with respect to Ca^{2+} dependence, depending on the cell type (reviewed in Ref. 15). In epithelial cells hyposmolarity activates Ca^{2+}-dependent K^+ channels, whereas in most nonepithelial cells the volume-sensitive K^+ channels are Ca^{2+} independent (15). Interestingly, in the present work we found the operation of both Ca^{2+}-dependent and Ca^{2+}-independent K^+ channels in the same cell type, which activate at different times and by different signals. For the Ca^{2+}-dependent channel, the activation signal may not primarily be the change in osmolarity but the increase in intracellular Ca^{2+} concurrent with swelling, known to occur in most cells (11), whereas the Ca^{2+}-independent channel may respond to the osmolarity reduction or the magnitude of the volume change. The two types of K^+ channels also differ in their pharmacological profile. The Ca^{2+}-dependent channel is sensitive to charybdotoxin and insensitive to clofilium, whereas the opposite is found for the Ca^{2+}-independent channel. This coincidence of two different types of K^+ channels activated by swelling in the same cell has not been previously reported, to our knowledge, but was first suggested by an interesting study in jejunal villus epithelial cells showing that volume regulation at small volume increases is Ca^{2+}- and charybdotoxin sensitive, although it turns to be Ca^{2+} independent and charybdotoxin insensitive at larger cell volume increases (10).

The relative contribution of the Ca^{2+}-dependent and the Ca^{2+}-independent K^+ channel to the total currents evoked by GOR can be estimated by the current magnitude as well as by the changes in membrane potential observed in the presence or absence of intracellular Ca^{2+}. In the Ca^{2+}-free condition, the first event elicited by GOR is an early activation (H-5%) of a Cl^- current, which increases as the external osmolarity drops. In the absence of a significant accompanying K^+ current, cells markedly depolarize from the resting potential of −79 ± 2 mV to −54 mV. Activation of a K^+ current at H-19% prevents further depolarization and stabilizes the membrane potential. A different response is observed in the presence of Ca^{2+}. During the first 10 min (up to H-6%), no significant change in membrane potential is observed, suggesting that Cl^- and K^+ currents already activated at that time stabilize the membrane potential. After this time, cells slightly depolarize, reaching −63 mV at H-9%, and this value remains unchanged for the duration of the experiment, suggestive of a predominant Cl^-

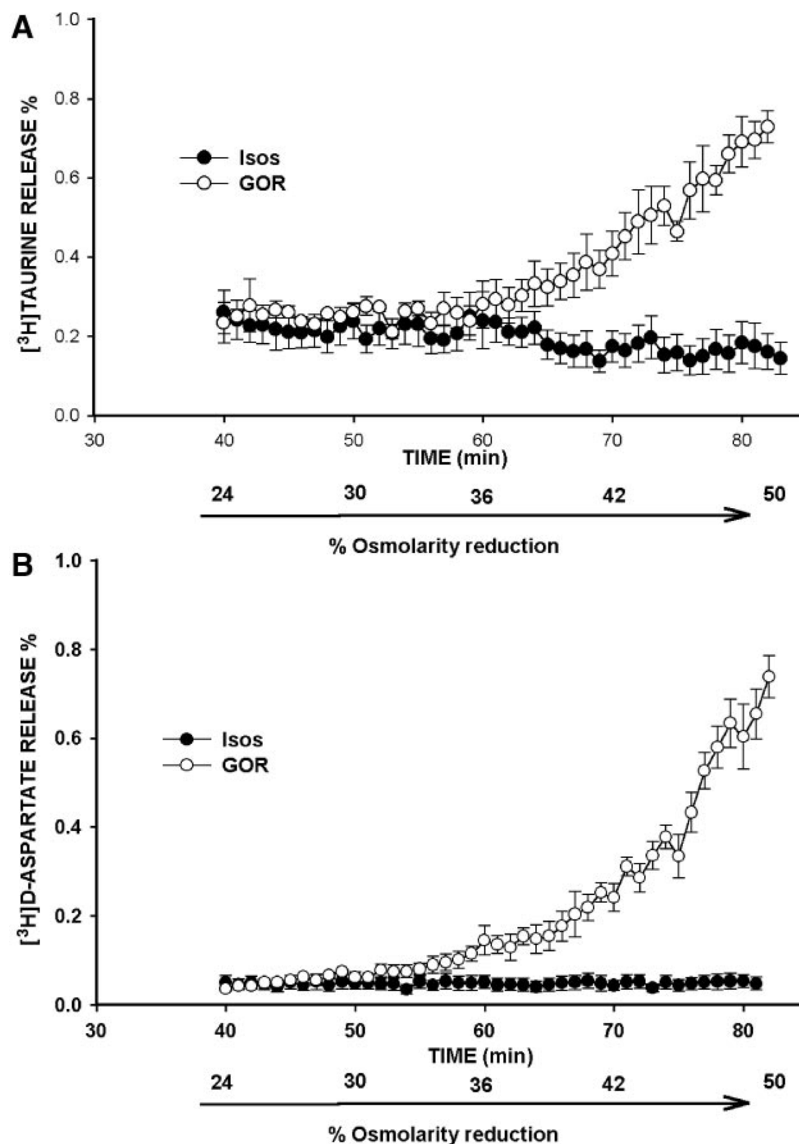

Fig. 10. Amino acid release from C6 glioma cells elicited by GOR. Cells were preloaded with [^{3}H]taurine (*A*) or D-[^{3}H]aspartate (*B*) as described in MATERIALS AND METHODS. After a basal collection period of 6 min with isosmotic medium, the external osmolarity was decreased at a rate of 1.8 mosM/min and superfusion continued until −50% reduction in osmolarity was achieved (final osmolarity 150 mosM). Controls were continuously superfused with isosmotic medium. Fractions were collected every minute from *minutes 1* to *82*, but because no difference was observed during the first 50 min, the points show only the time course of efflux from *minute 40* to the end. Data are expressed as efflux rate constants (min^{-1}) and are means ± SE of 6 experiments.

current. The maximal value of total currents is consistently higher in the presence of Ca^{2+} than in the Ca^{2+}-free condition at the same osmolarities, most likely reflecting the contribution of the Ca^{2+}-activated K^+ current. However, larger differences are observed at small osmolarity reductions, suggesting a more important contribution of the Ca^{2+}-dependent channel in this condition, in line with the observation in jejunal villus cells on Ca^{2+}-dependent RVD at small cell volume changes (10). The influence of the two types of K^+ channels with respect to Ca^{2+} dependence is also reflected in the cell volume regulation. At H-15%, volume regulation after SOR is less efficient and swelling is higher after GOR in the Ca^{2+}-free condition, whereas Ca^{2+} has no significant influence at H-30%. These results, similar to those reported in jejunal epithelium cells (10), stress the fact that volume regulatory mechanisms in conditions of physiological cell volume changes, such as those elicited by nutrient uptake or ionic gradients, differ from those observed at large cell volume changes, which may not occur even in pathological conditions. The type of K^+ channel activated appears to be determinant in the mechanisms operating in the two different situations. The fact that the same differences in the type of K^+ channel involved in volume regulation at small or large cell volume changes were observed in conditions of hyposmolarity (Ref. 10 and present results) or isosmolarity-evoked swelling (9) supports the notion that it is the change in cell volume rather than in osmolarity that determines the type of K^+ channel activation and the consequent mechanism of cell volume adjustment.

The Cl^- and K^+ efflux pathways during GOR may or may not be identical to those operating for the RVD after SOR. In A6 cells, the anion selectivity for the Cl^- efflux pathway in GOR and SOR is different, suggesting different mechanisms for Cl^- efflux (22). In C6 cells, the electrophysiological and kinetic properties of the Cl^- current activated by GOR, including an outwardly rectifying *I-V* relationship and current inactivation at hyperpolarizing voltages, as well as its pharmacological sensitivity, are similar to those of the Cl^- conductance activated in SOR described by Jackson and Strange (3). As to the K^+ channels, the sensitivity to charybdotoxin of RVD in jejunal epithelium cells (10) and of the K^+ current in C6 cells

(present results) suggests that the Ca^{2+}-dependent, swelling-activated K^+ channel is a maxi-K^+ channel, which is distinctively sensitive to this blocker. This type of channel appears similar to that involved in RVD in most epithelial cells (15). The Ca^{2+}-independent channel, which is apparently also voltage independent, activates only by osmolarity and shows a pharmacological profile characterized by insensitivity to typical K^+ channel blockers such as TEA, quinidine, 4-AP, and Ba^{2+} but sensitivity to clofilium. This feature relates this channel to the volume-activated K^+ channel recently described in Ehrlich ascites cells (2, 13). Putative candidates for this channel include members of the 4M2P family of channels (13).

The gradual decrease in osmolarity elicited the release of taurine and glutamate in C6 cells. However, the amino acid efflux was a delayed cell response, the fluxes being activated at very low osmolarities. This is in marked contrast with the early activation of a Cl^- current. This result strongly supports the notion of different permeability pathways for the two osmolytes (4, 20) and provides further evidence against a common pathway for amino acids and Cl^- efflux, which was based mainly on similarities in their pharmacological profile (17, 19). The sensitivity to Cl^- channel blockers, however, is suggestive of some interdependence of the two osmolyte pathways.

The contribution of amino acids, and possibly other organic osmolytes permeating through the same pathway, may be crucial for the efficacy of cell volume regulation elicited by small cell volume changes. According to their behavior in the face of GOR, three types of cell responses have been observed so far. In cells responding by IVR such as tubule renal cells, A6 cells, a subset of hippocampal neurons, and cerebellar granule neurons (7, 21, 22, 24), amino acid efflux activates early after the osmotic stimulus, as documented in brain cells (21). Other cells such as cultured myocytes (18) and C6 cells in the present study in conditions of GOR do not exhibit IVR but show a higher efficiency for volume regulation (evidenced as less swelling) in conditions of large cell volume increase, concomitant with a delayed activation of amino acid fluxes. Finally, in trout erythrocytes (1), cell swelling is the same in both SOR and GOR, with no efflux of taurine observed until very late. The importance of taurine, amino acids, and other organic osmolytes in preventing cell swelling in brain cells may operate also in vivo. The long-term swelling prevention in brain during chronic hyponatremia relies not on electrolytes but on the sustained decrease in the brain content of organic osmolytes, which may be as large as 90% in the case of taurine (23). The superior ability of neurons compared with C6 cells to resist to changes in external osmolarity, which seems to be based primarily on the contribution of organic osmolytes, may represent a protective mechanism to spare neurons from the deleterious consequences of swelling.

In summary, a main conclusion of the present study is that the mechanisms operating in response to small or large changes in cell volume differ essentially in the type of K^+ channel involved as well as in the sensitivity of amino acid efflux pathways.

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