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"Participación del calcio y tirosina cinasa en la regulación del volumen y la salida de osmolitos en condiciones hiposmóticas en células nerviosas de rata en cultivo"

TESIS

QUE PARA OBTENER EL GRADO DE:

DOCTOR EN CIENCIAS BIOMEDICAS

P R E S E N T A :

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RESUMEN

Las neuronas granulares y astrocitos de cerebelo de rata tienen la capacidad de regular su volumen en condiciones hiposmóticas, mediante la activación de mecanismos de transporte transmembranal para la salida de osmolitos. En este trabajo se estudio la participación del calcio y de tirosina y serina/treonina cinasas como señales de transducción en la regulación del volumen y la salida de ^{3}H-taurina, ^{125}I, y ^{86}Rb. Se observó un incremento en la concentración de calcio citosólico asociado con el aumento en volumen. Esto es resultado tanto de la liberación de calcio de almacenes intracelulares como de la entrada de calcio extracelular. Sin embargo, estos eventos no son necesarios para la regulación del volumen ni para la liberación de ^{3}H-taurina y ^{125}I en neuronas y astrocitos. Los flujos de ^{86}Rb en neuronas son independientes de calcio; mientras que en astrocitos, la liberación de éste osmolito se incrementa en ausencia de calcio extracelular y se inhibe parcialmente en ausencia de calcio intracelular.

En neuronas, la liberación de ^{3}H-taurina se inhibe en presencia de bloqueadores de cinasas de tirosina ó se incrementa inhibiendo fosfatasas de tirosina, indicando la participación de estas enzimas en la movilización de este osmolito. El estrés hiposmótico induce un incremento en la actividad de Erk1/Erk2 en estas neuronas. No obstante, la inhibición de éstas, no modificó la liberación de ^{3}H-taurina. La inhibición de la fosfatidil inositol-3 cinasa disminuye la liberación de ^{3}H-taurina, sugiriendo la participación de ésta enzima en la activación de los flujos de este osmolito en neuronas granulares.

ABSTRACT

Cerebellar astrocytes and granule neurons exhibit regulatory volume decrease (RVD) subsequent to swelling in hyposmotic solutions. This is accomplished by extrusion of intracellular osmolytes, mainly K^+ and Cl^-, as well as amino acids. In this work the role of Ca^{2+}, tyrosine or serine/threonine kinases as a transduction signals on RVD and osmolyte fluxes were examined. Hyposmotic swelling induced an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). Extracellular Ca^{2+} influx as well as release from internal stores contributed to this osmosensitive $[Ca^{2+}]i$ elevation. However, RVD, ^{3}H-taurine or $^{125}I^-$ fluxes were not affected by extracellular Ca^{2+} omission neither by endogenous Ca^{2+} release blockers or some maneuvers that deplete Ca^{2+} stores. ^{86}Rb efflux was Ca^{2+}-independent in granule neurons, whereas in astrocytes, ^{86}Rb release is largely Ca^{2+}-independent; the Ca^{2+}-dependent fraction is sustained essentially by Ca^{2+} released from endoplasmic reticulum in a mechanism mediated by Ca^{2+}/calmodulin. In cerebellar granule neurons the osmosensitive ^{3}H-taurine release was markedly inhibited by blockers of tyrosine kinases or potentiated by blockers of tyrosine phosphatases. By other hand activation of Erk1/Erk2 was induced by hyposmotic stimulus, but blockade of this kinases only produced a marginally decreased in osmosensitive ^{3}H-taurine release, suggesting that the Erk1/Erk2 is an epiphenomenon not directly involved in ^{3}H-taurine release. Finally, the inhibition of PI-3 kinase essentially abolished the osmosensitive ^{3}H-taurine efflux in granule neurons suggesting a role for PI-3 kinase in the mechanism that activates ^{3}H-taurine efflux.

INTRODUCCION

IMPORTANCIA DEL VOLUMEN CELULAR

El control y mantenimiento del volumen celular es una propiedad que poseen la mayoría de las células. Las alteraciones en la osmolaridad externa, así como la presencia de hormonas, nutrientes y estrés oxidativo, ocasionan cambios en el volumen celular. Estos cambios son amortiguados por la activación de mecanismos de transporte transmembranal de agua y solutos que permiten a las células, corregir los cambios en su volumen.

La capacidad de las células para responder de manera rápida y eficiente a variaciones en la osmolaridad externa mediante mecanismos de regulación del volumen celular, es un mecanismo homeostático que se ha conservado a lo largo de la evolución (Chamberlin y Strange, 1989). Cualquier desbalance osmótico que no sea corregido podría ocasionar alteraciones en el volumen celular como resultado de la libre permeabilidad de la membrana plasmática al agua. La disminución o incremento en el volumen celular traerían como consecuencia modificaciones en la morfología celular, en el estado metabólico, en el contenido de agua y por lo tanto, en el estado de hidratación de macromoléculas, así como en la concentración de moléculas que forman parte de los mecanismos de señalización celular y comunicación intercelular (Foskett, 1994; Hallows y Knauf, 1994; Alvarez-Leefmmans y Reuss, 1996).

El volumen celular tiene un papel importante como señal para el crecimiento y proliferación celular; en la expresión genética, en procesos de transporte de sustancias nutritivas como azúcares y aminoácidos, en funciones de secreción, en el movimiento de organelos intracelulares, y como una señal en mecanismos de inserción de

proteínas membranales, canales, receptores y transportadores (McManus y Churchwell, 1994). En todas estas situaciones se generan microgradientes osmóticos que dan lugar a cambios locales y transitorios en el volumen celular.

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 regulados, por la modificación de la concentración intracelular de iones inorgánicos, compuestos orgánicos y el contenido de agua. Los organismos con paredes celulares, 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 frágiles y no pueden soportar una diferencia significativa en la presión hidrostática, por lo que, tienen que activar mecanismos de transporte de solutos intracelulares osmóticamente activos, que ayudan a contrarrestar los cambios en el volumen celular ocasionados por alteraciones en la osmolaridad del medio externo (Chamberlin y Strange, 1989; Pasantes-Morales *et al.*, 1990; Sarkadi y Parker, 1991).

En la clínica, el estudio de los procesos que intervienen en la regulación del volumen contribuye a la comprensión y mejor manejo de estados patológicos, que conllevan alteraciones en el contenido plasmático de osmolitos: Por ejemplo, severas diarreas, deficiencias renales, diabetes mellitus e insipidus, diuresis osmótica con deshidratación, deprivación de agua y ciertos envenenamientos (como por etanol). La hipotonicidad del plasma, usualmente debido a hiponatremia, es clínicamente mas frecuente que la hipertonicidad. La hiponatremia se presenta en circunstancias de

malnutrición y cirrosis hepática. En el cerebro, el aumento en el volumen celular, particularmente de células gliales está asociado a numerosas neuropatologías, como son epilepsia, isquemia, traumatismos craneanos y encefalopatía hepática. El edema cerebral es una patología muy frecuente si se considera el número de patologías asociadas causalmente con él. El edema cerebral se presenta como complicación de 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 son la diabetes insípida, en donde la ingesta de agua puede ser muy elevada y las insuficiencias hepática y cardíaca; esta última al propiciar entre otros, episodios de hipoglucemia e hipoxia de documentadas propiedades edematogénicas. A estas causas de edema cerebral han de añadirse otras, que él mismo agrava y retroalimenta. Entre ellas está principalmente la anoxia, que puede presentarse como entidad aislada (p.e. en la asfixia), o lo que es más frecuente, como parte de la isquemia, como en las embolias o los accidentes vasculares cerebrales y aneurismas de causas diversas (revisado en Quesada y Morales-Mulia, en preparación).

Otros grupos de entidades clínicas con un componente edematogénico cerebral incluyen los traumatismos y diversas causas de muerte celular, como las infecciones y los tumores. Dada la restricción que impone la caja del cráneo a la expansión del tejido cerebral, el edema que resulta de esta alteración en el volumen celular es en muchos casos, una complicación aún más grave que la propia patología que le dio origen. La causa directa de gravedad clínica es el incremento del volumen cerebral, lo cual, provoca un incremento casi inmediato de la presión interna. La compresión del tejido cerebral compromete a su vez la eficiencia de la irrigación sanguínea, dando lugar entre otros a episodios de isquemia, luego muerte neuronal y daño permanente. Si el edema es generalizado y/o persistente puede sobrevenir la herniación del cerebro a través del *foramen magnum*, y la muerte por paro respiratorio ó cardíaco, por

compresión y falla de los correspondientes centros autonómicos en el tallo cerebral (revisado en Quesada y Morales-Mulia, en preparación).

En el cerebro, el aumento del volumen celular puede representar un importante componente en la hiperexcitabilidad debido a la reducción del espacio extracelular después del hinchamiento de los astrocitos causado por la actividad neuronal, promoviendo la excitación así a través de efectos de campo (Saly y Andrew, 1993).

También en el cerebro, bajo condiciones isosmóticas, las células pueden incrementar o disminuir su volumen por situaciones que modifiquen la concentración intracelular o extracelular de solutos. El primer caso, se da por ejemplo como consecuencia de la acumulación de K^+ en los astrocitos o de iones inorgánicos a través de las membranas afectadas por lipoperoxidación en neuronas y en otros tipos celulares, o durante la acumulación de sorbitol en animales hiperglicémicos. El hinchamiento en tipos celulares como neuronas y glia, durante la isquemia ocurre como consecuencia de la acción citotóxica del glutamato, lo cual conlleva a la acumulación de Na^+, Cl^- y agua (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 al efecto citotóxico del glutamato. La reducción en el volumen celular resulta de cuadros de deshidratación, y de modificaciones de los niveles de hormona antidiurética en sangre, lo cual puede provocar cuadros clínicos de hipo o hipernatremia.

Entonces, un mejor conocimiento de los mecanismos responsables del mantenimiento del volumen celular del cerebro, puede llevar a un manejo más racional de este tipo de secuelas e idealmente, al diseño de fármacos y procedimientos para prevenir dichas alteraciones en el volumen celular (Foskett, 1994).

MANTENIMIENTO Y REGULACION DEL VOLUMEN CELULAR

Regulación crónica del volumen celular.

El mantenimiento de un equilibrio dinámico en el volumen celular es crítico para la sobrevivencia y funcionamiento de todas las células ya que la dilución de moléculas importantes en el metabolismo y mecanismos de señalización celular puede llevar a alteraciones en su homeostasis. Las dos principales clases de solutos en una célula incluyen: 1) compuestos orgánicos (proteínas solubles) generalmente sintetizados dentro de la célula y que constituyen la mayor parte de las moléculas impermeables; y 2) pequeños solutos, orgánicos e inorgánicos, que pueden potencialmente permear a través de la membrana plasmática.

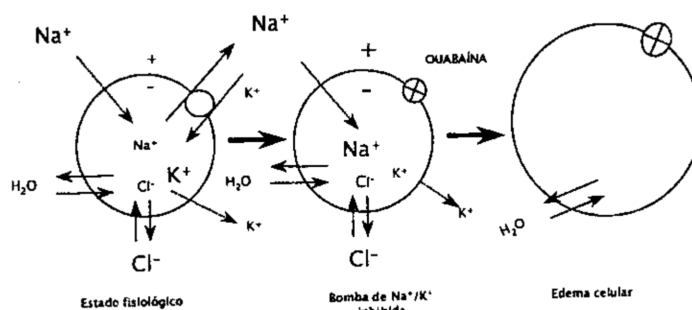
Dado que el pH intracelular se mantiene a niveles ligeramente alcalinos (7.2–7.4) con respecto al punto isoeléctrico de la mayoría de las proteínas, éstas se encuentran como aniones polivalentes. Esto trae como consecuencia que la presión coloidosmótica intracelular sea mayor que la del líquido extracelular y como la membrana es permeable al agua y a pequeños solutos tales como Na^+, K^+, Cl^-, si los iones y el agua se distribuyesen tal y como lo predice la teoría de Gibbs-Donnan, las células se hincharían en condiciones isosmóticas. De acuerdo a esta teoría, en el estado de equilibrio, la concentración total de solutos intracelulares debería ser mayor que la de los extracelulares, lo cual resultaría en una menor concentración de agua (o más exactamente en una disminución de su potencial químico) en el interior, con respecto al exterior celular. Este gradiente de concentración (o de potencial químico) del agua causaría un flujo osmótico neto de este solvente hacia el interior celular.

Como la membrana de las células animales es distensible, cede ante pequeñas presiones hidrostáticas, por lo que el flujo osmótico neto de agua causaría un aumento de volumen y eventual lisis celular. Sin embargo, normalmente este aumento de

volumen celular coloidosmótico no ocurre y las células mantienen niveles constantes de agua y solutos. Esto indica que aunque las células animales contienen aniones orgánicos impermeables, los iones inorgánicos permeables no están distríbuidos de acuerdo con la teoría de Gibbs-Donnan. Si hubiese equilibrio de Gibbs-Donnan a través de la membrana celular, la osmolaridad de la fase acuosa intracelular excedería a la de la fase acuosa extracelular por 25 a 30 mosmoles/l, lo cual equivale a una diferencia de presión hidrostática a través de la membrana de 6587 a 7904 mm de H_2O a 37°C (revisado en Alvarez-Leefmans y Reuss, 1996).

Los eritrocitos se utilizan frecuentemente como modelo para el estudio de las propiedades osmóticas de las células animales. En ellos, se ha demostrado que la presión hidrostática es sólo 2 ó 3 mm de H_2O más alta en el interior con respecto al exterior celular (Rand y Burton, 1964). Esta diferencia de presión hidrostática a través de la membrana corresponde a una concentración total de solutos intracelulares de aproximadamente 0.01 mosmoles/l, más alta que la del medio extracelular. En conclusión, no existe gradiente de presión hidrostática a través de la membrana. Esto indica que los eritrocitos, al igual que otras células, no se encuentran en equilibrio de Gibbs-Donnan. Si esto es así ¿qué es lo que evita el edema y la lisis celular en condiciones isosmóticas?. Una respuesta a esta pregunta, basada en evidencia experimental, es que las células animales mantienen su volumen constante a través de mecanismos de transporte activo primario, principalmente mediante la operación de la bomba de Na^+/K^+. De acuerdo con esta hipótesis, el volumen celular estaría determinado por un balance entre el eflujo neto de iones mediado por sistemas de transporte activo primario y el influjo neto de iones a través de canales y sistemas de transporte activo secundario. Debido a la acción de la bomba de sodio, el Na^+ que entra a la célula por electrodifusión (canales de Na^+) o por sistemas de transporte secundario (cotransportadores de Na^+ /H^+ y de Na^+ /Ca^{2+}, el cotransportador de Na^+–

K^+-Cl^-, o los cotransportadores de Na^+ y aminoácidos) es transportado hacia el exterior, de tal manera que la membrana se comporta como si fuese impermeable al Na^+ (y al K^+), por lo menos en el estado de reposo. Esta noción se conoce como la "hipótesis de bombeo y fuga" (the pump-leak hypothesis). El corolario de esta hipótesis es que las células animales en equilibrio osmótico se comportan como si fuesen impermeables al Na^+. La hipótesis predice que si se inhibe la bomba de Na^+, por ejemplo con el glucósido cardiaco ouabaina (estrofantina G) o por agotamiento del ATP celular a consecuencia de anoxia, las células se cargarán de Na^+ y de Cl^- con la consecuente entrada de agua. Dado que el gradiente electroquímico del Na^+ esta dirigido hacia el interior celular, este catión se "movilizará" hacia el citosol a través de canales y sistemas de transporte activo secundario (transportadores y cotransportadores). Como el sistema que expele activamente al Na^+ está inhibido, éste se acumulará en el interior celular. Paralelamente a la ganancia de Na^+ se producirá un influjo neto de Cl^-, debido a la despolarización de la membrana. La entrada de ambos iones se acompañará de la entrada osmótica de agua, lo cual resultará en un aumento del volumen celular (esquema I).



Esquema I. Distribución asimétrica de electrolitos entre el medio interno y externo de una célula animal. La concentración de Na^+ y Cl^- en el espacio extracelular es mucho mayor que la de K^+ y aniones proteicos, los cuales presentan una distribución inversa. Esto genera una diferencia de potencial eléctrico en la membrana que es negativa en el interior. El Na^+ tiende a fluir hacia el interior celular favorecido por su gradiente electroquímico. El potencial químico del K^+ tiende a desplazarlo hacia el exterior pero el gradiente eléctrico favorece su entrada. Esta distribución se mantiene en el reposo gracias a procesos activos como la ATPasa de Na^+/K^+.

En algunos tipos celulares se ha demostrado que el bloqueo de la bomba de Na^+ con uabaina resulta en un incremento del volumen celular, como lo predice la hipótesis de la bomba. Sin embargo, existen células en las que no se ha podido detectar este fenómeno. Más aún, hay casos en los que la inhibición de la bomba de Na^+ produce disminución del volumen celular (revisado en Alvarez-Leefmans y Reuss, 1996).

Estas observaciones sugieren que los mecanismos de mantenimiento del volumen celular varían de célula a célula, o que involucran sistemas de transporte de salida adicionales a los considerados originalmente dentro de la hipótesis de bomba y fuga. En apoyo a esta última idea hay observaciones que muestran que algunas células, en presencia de ouabaina, aumentan inicialmente de volumen pero subsecuentemente lo disminuyen. Este decremento del volumen, implica la activación de sistemas de transporte de agua y solutos distintos de la bomba de Na^+. Lo que parece suceder en estos casos es que la inhibición de la bomba de Na^+ resulta no sólo en la acumulación intracelular de Na^+, sino también en un aumento en la concentración de calcio intracelular ($[Ca^{2+}]i$) que produce la activación de canales de K^+ con el consiguiente eflujo de éste acompañado de aniones. Si el eflujo de solutos inducido por el Ca^{2+} es mayor que el influjo neto de solutos que queda sin ser contrarrestado al inhibir a la bomba de Na^+, el resultado final será una disminución del volumen celular. Si inicialmente el influjo neto sobrepasa al eflujo neto de solutos, pero tiempo después esta tendencia se invierte, el patrón de cambios en el volumen celular será bifásico, es decir, habrá un incremento transitorio del volumen celular seguido de una disminución del mismo. Si al inhibir la bomba de Na^+ el influjo neto de solutos es igual al eflujo neto de los mismos, el volumen celular no cambiará, como también se ha observado experimentalmente (revisado en Alvarez-Leefmans y Reuss, 1996).

Regulación aguda del volumen celular.

La membrana plasmática es muy permeable al agua, lo que permite que siempre exista una diferencia en la concentración de solutos intracelulares con el exterior, es decir, un gradiente osmótico entre el exterior y el interior de la célula. Cuando dos soluciones presentan una misma presión osmótica se hace referencia a soluciones isosmóticas. Por el contrario, si las presiones osmóticas de las soluciones son diferentes, se hace referencia a soluciones anisosmóticas. Cuando la presión osmótica del medio extracelular aumenta debido al incremento en la concentración de soluto (medio hiperosmótico), el agua tiende a desplazarse por ósmosis hacia el exterior y la célula se encoje. Si por el contrario la presión osmótica disminuye en el medio extracelular (medio hiposmótico), el agua tiende a desplazarse hacia el interior de la célula y ésta se hicha.

Además de poseer la capacidad de mantener su volumen constante, las células animales están dotadas de mecanismos que les permiten ajustar su volumen en medios anisosmóticos. Esto se debe a la activación de mecanismos de transporte de solutos intracelulares osmóticamente activos, que al movilizarse van acompañados de agua. La activación de estos mecanismos trae como consecuencia, la corrección del volumen celular. En el caso particular en el que una célula animal es expuesta a una solución hiposmótica, inicialmente el volumen celular aumenta debido a la entrada de agua. Después de este aumento en el volumen, que frecuentemente corresponde al volumen predicho para una respuesta osmométrica, el volumen celular disminuye; aún cuando la osmolaridad extracelular se mantenga por debajo de la inicial. Este proceso compensatorio del volumen, que generalmente ocurre en un período de varios minutos, se conoce como Decremento Regulador del Volumen (DRV). En la mayoría de los casos, las células no retornan totalmente a su volumen inicial, sino que alcanzan un nivel estable ligeramente superior. Análogamente, cuando las células se exponen

súbitamente a un medio hiperosmótico, hay una disminución inicial rápida del volumen celular debida a la diferencia de presión osmótica. El volumen celular se recupera lentamente hasta alcanzar un nivel cercano al inicial, aun cuando persista la condición hiperosmótica mediante el proceso conocido como Incremento Regulador del Volumen (IRV). Este proceso regulador es el resultado de la activación de transportadores e intercambiadores iónicos, pero no de canales iónicos como en el caso del DRV.

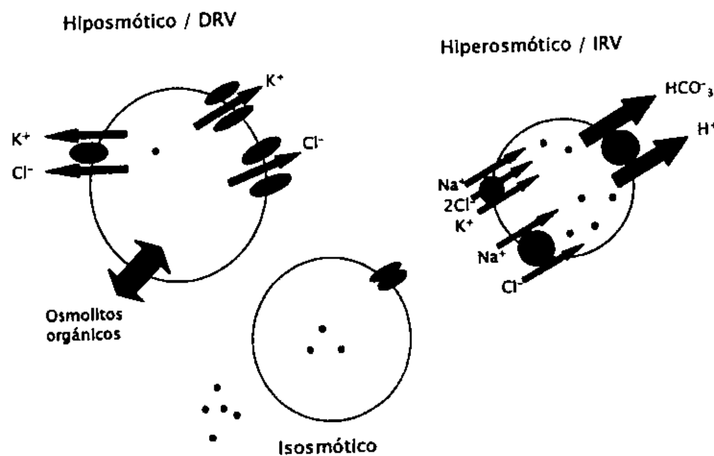
MECANISMOS DE TRANSPORTE EN LA REGULACION DEL VOLUMEN

Decremento Regulador del Volumen (DRV).

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, 1987; Welling *et al.*, 1983), eritrocitos (Kregenow, 1981; Cala, 1983), células ascíticas de Ehrlich (Hoffmann *et al.*, 1978), astrocitos en cultivo (Olson y Holtzman, 1986; Kimelberg, 1985; Pasantes-Morales, *et al.*, 1994 a y b), células granulares en cultivo (Pasantes-Morales *et al.*, 1993), linfocitos humanos (Grinstein *et al.*, 1982), células MDCK (Roy y Sauve, 1982), células COS-7 (Ishii *et al.*, 1996), glioma C6 (Strange y Morrison, 1992), neuroblastoma N1E115 (Falke y Misler, 1989), fibroblastos humanos (Bibby y McCulloch, 1994) y células IMCD (Tinel *et al.*, 1994) entre otras.

Las diversas investigaciones indican que el DRV esta mediado por la activación de mecanismos de transporte de iones como el K^+ y el Cl^- (Grinstein *et al.*, 1982; Eveloff y Warnock, 1987), que normalmente (en condiciones isosmóticas) están inactivos y/o a la liberación de compuestos orgánicos osmóticamente activos como

polialcoholes, azúcares, aminas y aminoácidos como la taurina (Pierce y Greenberg, 1972; Fugelli y Thoroed, 1986; Fincham *et al.*, 1987). El movimiento de estos solutos es la consecuencia de un cambio en la permeabilidad de la membrana plasmática, ya sea por la activación de transportadores o por la activación de canales. Se sabe que el mecanismo más generalizado para el movimiento de K^+ y Cl^- ocurre a través de vías electrogénicas y que el flujo de los osmolitos orgánicos es mediante un proceso difusional, dirigido solo por el gradiente de concentración (esquema II).



Esquema II. La mayoría de las células animales poseen la capacidad de recuperar su volumen inicial después de que éste se ha incrementado, ó disminuido, por exposición a condiciones hiposmóticas ó hiperosmóticas, respectivamente. El proceso de ajuste de volumen se lleva a cabo a través de la modificación en la concentración de solutos intracelulares osmóticamente activos. En el esquema se ilustran los diferentes mecanismos de transporte utilizados para la movilización de estos solutos.

Osmolitos inorgánicos.

El mecanismo de salida para los iones K^+ y Cl^- bajo condiciones hiposmóticas ya sea mediante la activación de canales o de transportadores depende del tipo celular de que se trate. Existen también otros aniones que intervienen en este proceso, como el

H^+ y el HCO_3^-. Un sistema de cotransporte K^+/Cl^- activado por un incremento de volumen participa en la expulsión de estos iones en eritrocitos de distintas especies, incluyendo el pato (Mayer, 1985), el borrego (Lauf, 1985) y algunos peces (Bourne y Cossins, 1984; Borgese *et al.*, 1987). La activación acoplada de los intercambiadores K^+/H^+ y $Cl^-/HCO3^-$ parece mediar la salida de Cl^- y K^+ en eritrocitos de *Amphiuma* (Kregenow, 1981; Siebens, 1985; Cala, 1985 a y b). En túbulos proximales de riñón de conejo y de ratón la salida de K^+ durante la regulación de volumen se encuentra asociada con la salida de HCO_3^- y no de Cl^- (Volkl y Lang, 1988).

A pesar de la existencia de estos sistemas de cotransporte electroneutro para la salida de Cl^- y K^+, el mecanismo más generalizado para expulsar estos iones es la activación de vías electrogénicas constituídas por canales iónicos presentes en la membrana celular. El mecanismo utilizado para disminuir los niveles intracelulares de Cl^- y K^+ determina el curso temporal de la recuperación del volumen (esquema II). Cuando se encuentran involucrados los sistemas de cotransporte K^+/Cl^- y de intercambio $Cl^-/HCO3^-$ y K^+/H^+, el proceso es muy lento y tarda alrededor de 2 a 4 horas y si se activan los canales de Cl^- y K^+, el volumen se recupera entre 10 y 30 min dependiendo del tipo celular (Lauf, 1985).

Se ha postulado que la salida de Cl^- y K^+ ocurre a través de canales separados, pero interdependientes. En las células de Ehrlich la evidencia experimental en apoyo a esta suposición esta basada en las siguientes observaciones: 1) al inhibirse la salida de K^+ con quinidina se inhibe la recuperación del volumen, sin embargo, si se provee una vía alterna para la salida de K^+ con valinomicina (Hoffmann *et al.*, 1984) o con gramicidina en un medio sin Na^+ (Hofmann *et al*, 1986) el volumen disminuye normalmente, señalando que el canal de Cl^- se encuentra activo, además de indicar que la salida de K^+ es el paso limitante en la recuperación del volumen; 2) el curso temporal de la inactivación del canal de Cl^- es diferente al del canal de K^+: el primero se activa abruptamente con el aumento de volumen y se inactiva en aproximadamente

10 min, mientras que el canal de K^+ permanece activado por más tiempo (Sarkadi *et al.*, 1984); 3) la despolarización (de aproximadamente 10 mV) observada durante la fase reguladora de volumen, es consistente con un mayor aumento en la permeabilidad de la membrana para el Cl^- que para el K^+. Esta despolarización aumenta la fuerza electromotriz para la salida de K^+ y puede explicar al menos parte de esta salida (Lang *et al.*, 1987).

Osmolitos orgánicos.

Los principales osmolitos orgánicos pueden clasificarse en tres grupos: 1) azúcares como la glucosa y la sacarosa; 2) polialcoholes como el manitol, sorbitol y el mio-inositol; 3) aminoácidos como la taurina, el glutamato, la prolina, la glicina, la serina y la alanina; 4) metilaminas como la betaína y la glicerofosforilcolina; 5) otros compuestos como la urea, la creatina y la fosfocreatina.

El uso de un osmolito en particular puede estar determinado por diferentes condiciones. Por ejemplo, los animales que tienen una dieta alta en nitrógeno, como los elasmobranquios, utilizan urea y metilaminas como osmolitos. Las células renales en condiciones hiperosmóticas acumulan inositol y glicerofosforilcolina, pero no sorbitol, mientras que la taurina es el principal osmolito en células nerviosas en cultivo cuando son expuestas a soluciones hiposmóticas.

Los osmolitos orgánicos son específicamente importantes para la regulación del volumen celular en la medula renal, ya que el fluido renal puede incrementarse hasta 3800 mosmol/l durante la antidiuresis y disminuir hasta 170 mosmol/l durante la diuresis (Graf, 1993). También son importantes en el cerebro, donde las alteraciones en el volumen se traducen en las complicaciones antes descritas y donde los cambios en la composición iónica podrían afectar la excitabilidad neuronal.

Existen distintos mecanismos que contribuyen a la acumulación de osmolitos orgánicos durante el estrés hiperosmótico: 1) disminución en su degradación (glicerofosfoforilcolina), 2) incremento en su síntesis (inducción de la aldosa reductasa) e 3) incremento en la recaptura mediado por los transportadores acoplados a Na^+ (mio-inositol, betaína y taurina). El incremento en la síntesis de sorbitol a partir de glucosa por la aldosa reductasa bajo estas condiciones involucra un incremento en la expresión de la enzima debido a la activación hiperosmótica del gen codificante. De igual manera la expresión de los transportadores de inositol y betaína es inducida por hiperosmolaridad (revisado en Häussinger, 1996).

Entre los aminoácidos libres con función osmorreguladora la taurina es el más importante en la mayoría de las células, debido a que se encuentra presente en concentraciones muy elevadas constituyendo hasta el 70 % de la poza total de aminoácidos libres (80 mM en la retina, 28 mM en linfocitos y 60 mM en músculo esquelético y cardiaco). La taurina es un aminoácido inerte desde el punto de vista fisiológico, no participa en la estructura primaria de proteínas ni en ninguna reacción del metabolismo primario de la célula (Jacobsen y Smith, 1968; Pasantes-Morales *et al.*, 1991). Esto permite que los niveles celulares de la taurina puedan modificarse, con el fin de ajustar la presión osmótica interna sin que las variaciones en la concentración intra o extracelular alteren otras funciones celulares.

IDENTIDAD MOLECULAR DE LAS VIAS DE MOVILIZACION DE LOS OSMOLITOS

La identidad molecular de las proteínas responsables del movimiento de osmolitos asociados a la regulación del volumen celular en condiciones hiposmóticas no es del todo conocida. Los canales de K^+ activados por volumen se encuentran en muchos tipos celulares. Estos canales son muy específicos para el K^+ ya que no son permeables a otros cationes como el Na^+. Sin embargo, este tipo de canales se ha estudiado poco y se desconocen sus características moleculares. El estudio más concluyente acerca de la participación de un tipo particular de canales de K^+ se hizo en una línea celular de linfocitos que no tienen la capacidad de regular su volumen y no muestran la activación de corrientes de K^+. Cuando estas células son transfectadas con el canal Kv1.3 adquieren la capacidad de regular su volumen al mismo tiempo que presentan una corriente de K^+ activada por el hinchamiento (Sackin, 1994).

Los canales de Cl^- sensibles a volumen se han estudiado con mayor detalle y se sabe que son poco selectivos, ya que permiten el paso de un gran número de aniones. Hasta el momento se han clonado seis canales de Cl^- entre los cuales se encuentran la glicoproteína-P (Valverde *et al.*, 1992); el CLC-2 (Grüder *et al.*, 1992); el pI_{Cln} (Paulmich *et al.*, 1992); un intercambiador aniónico parecido a la banda 3 en eritrocitos (Goldstein y Brill, 1991); las porinas de la mitocondria (Reymann et al., 1995) y el fosfoleman una proteína muy pequeña de tan sólo 72 aminoácidos que es muy permeable a la taurina (Moorman, *et al.*, 1995).

Los mecanismos de liberación de los osmolitos orgánicos están aún menos caracterizados. Se ha demostrado que la liberación de taurina en respuesta al cambio de volumen, es independiente de la presencia de Na^+ en el medio extracelular y de la

temperatura, lo cual descarta la participación del transportador del aminoácido dependiente de sodio durante su liberación inducida por hiposmolaridad. En cambio, el movimiento de taurina en respuesta al aumento en el volumen celular, se da a través de vías difusionales en los que el movimiento de este aminoácido está dirigido por su gradiente de concentración (Sánchez-Olea *et al.*, 1991). Una característica de los mecanismos de movilización de la taurina asociada a cambios en el volumen celular es que se inhibe por bloqueadores de canales de Cl^- como el DIDS, el dipiridamol, el NPPB, la DDF, los ácidos grasos poliinsaturados y el ácido niflúmico (Sánchez-Olea *et al.*, 1991; Pasantes-Morales *et al.*, 1994). Estas observaciones han llevado a suponer que el transporte de este osmolito se realiza a través de un canal aniónico con características tales que permitan el paso de osmolitos zwiteriones como son los aminoácidos (Strange et al., 1996).

SEÑALES QUE ACTIVAN LA LIBERACION DE OSMOLITOS

El cuestionamiento acerca de cómo una célula percibe su volumen y cómo la señal del volumen es amplificada y transducida al efector (mecanismos para la movilización de osmolitos), es un requisito necesario e importante para entender el fenómeno de regulación. El hecho de que las células regulen su volumen en soluciones anisosmóticas, o de que lo mantengan constante en condiciones isosmóticas, supone que deben estar dotadas de "sensores" que les permiten detectar cambios en su propio volumen. Los mecanismos mediante los cuales una célula sensa cambios en su propio volumen y, traduce la señal detectada en una activación de los sistemas de transporte de los solutos responsables de la respuesta reguladora, constituyen uno de los temas más estudiados en la actualidad.

En relación con el mecanismo sensor o detector de cambios de volumen celular, se han postulado dos grupos de teorías que incluyen mecanismos químicos y físicos. De acuerdo con la teoría química, un cambio del volumen celular conduciría a un cambio en la actividad química del agua intracelular, lo que a su vez llevaría a una modificación en la concentración de uno o varios "factores citosólicos" (iones o moléculas), cuya naturaleza aún no ha sido determinada. La dilución o la concentración de estos "factores", llevaría a la activación de los mecanismos de transporte de solutos osmóticamente activos a través de la membrana, dando lugar a la liberación de osmolitos. Existen dos variantes de la teoría química: la hipótesis que podría llamarse del "factor químico específico" y la del "apiñamiento de macromoléculas" (macromolecular crowding). La primera hipótesis postula que los cambios en la concentración del Mg^{2+} libre intracelular, constituyen la señal detonante de los mecanismos efectores de la respuesta reguladora. Esta hipótesis carece de evidencia experimental sólida y actualmente es controvertida, ya que no se ha demostrado que los cambios en el volumen celular se correlacionen causalmente con modificaciones en la concentración de Mg^{2+} y que las respuestas efectoras sean moduladas por el Mg^{2+}.

En la hipótesis del apiñamiento de macromoléculas intracelulares, se postula que la concentración de macromoléculas citoplásmicas es la señal intracelular que inicia el proceso regulador, esto es, que un cambio en la concentración de macromoléculas modifica la actividad funcional de los transportadores efectores. El argumento se basa en el hecho de que pequeños cambios en la concentración total de macromoléculas intracelulares produce grandes cambios en la reactividad (o en el coeficiente de actividad) de otras macromoléculas. El efecto se caracteriza por tener un poder amplificador de 10 a 1000 veces. Los cambios en la actividad funcional de los transportadores de soluto serían consecuencia de la reactividad de solutos

citoplásmicos, por ejemplo, enzimas que modulan la fosforilación de proteínas reguladoras o de los mismos transportadores. La hipótesis se derivó inicialmente de estudios realizados en fantasmas de eritrocitos en los que se demostró que la regulación del volumen celular no depende del valor absoluto del volumen, sino de la concentración de proteínas intracelulares. El efecto es inespecífico puesto que puede reproducirse con albúmina o hemoglobina. Aunque muy interesante, la posibilidad de que el fenómeno de apiñamiento macromolecular juegue un papel determinante en la regulación de volumen celular carece de evidencia definitiva (Alvarez-Leefmans y Reuss, 1996). El tema ha sido estudiado y tratado por Sarkadi y Parker (1991) y por Allen Minton (1994).

La teoría física del sensor de volumen comprende la hipótesis mecánica con sus variantes. De acuerdo con dicha hipótesis, el sensor del volumen estaría localizado en la membrana plasmática o en el citoesqueleto adyacente a la misma y su activación pondría en marcha los mecanismos de transporte de solutos que subyacen a las respuestas reguladoras. Existe evidencia en favor de que algunos canales mecanosensibles conocidos como "cation-nonspecific stretch-activated channels" (SACS), presentes en la membrana plasmática, constituyen la parte central del sensor de volumen, y en algunos casos de los mecanismos efectores. La evidencia proviene de experimentos realizados en células en las que se ha logrado detectar, con técnicas electrofisiológicas, un aumento en la probabilidad de apertura de estos canales en respuesta a la distensión de la membrana cuando la célula aumenta de volumen.

Existen por lo menos dos propuestas basadas en datos experimentales, que implican a este tipo de canales en el mecanismo sensor de volumen. En el primer modelo, postulado por Christensen (1987), la distensión de la membrana como consecuencia del aumento en el volumen celular, activa canales catiónicos no

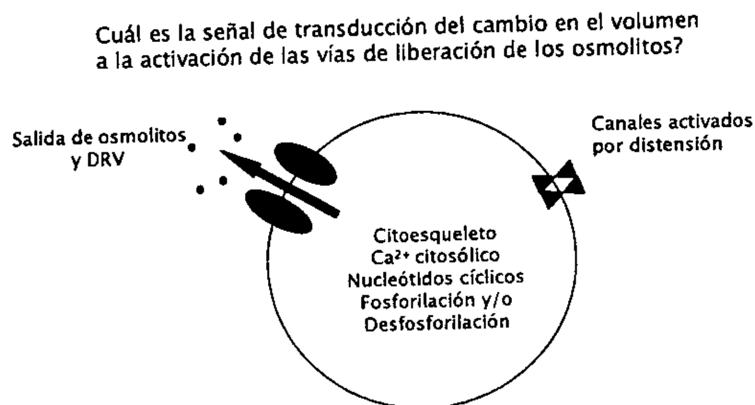
selectivos permeables a cationes monovalentes (K^+ y Na^+) y divalentes (Ca^{2+}). La entrada de Ca^{2+} produce un aumento en la $[Ca^{2+}]i$, lo cual causa la activación de canales de K^+ y/o Cl^- con la consecuente pérdida de agua osmóticamente obligada, produciéndose el DRV. En el segundo modelo, representado por los trabajos de Falke y Misler (1989), así como los de Grinstein y colaboradores (1990 y 1992), el DRV puede producirse en ausencia de Ca^{2+} extracelular, por lo que se ha postulado que el estiramiento de la membrana abre canales catiónicos no selectivos que dan como resultado una despolarización de la membrana y causa la apertura de canales de K^+ sensibles a voltaje y canales permeables a aniones. La activación de este conjunto de canales permite que la célula recupere su volumen.

También se ha propuesto que el citoesqueleto puede ser otro de los sensores del cambio en el volumen celular, ya que las alteraciones en la forma celular esta relacionada con cambios en la actividad de diversas proteínas asociadas al citoesqueleto. Además, los canales iónicos y otras proteínas de membrana como transportadores e intercambiadores (entre los cuales se encuentran algunos que participan en la regulación del volumen, p.ej. la banda 3), estan relacionados estrechamente con el citoesqueleto (Goodman and Zagon, 1986).

A pesar de que hasta el momento no se conocen con exactitud las señales intracelulares responsables del DRV, diversos estudios han identificado algunos posibles candidatos dentro de los cuales se encuentran: el Ca^{2+} y la activación de proteínas cinasas, que al parecer son importantes para la activación de las vías de liberación de los osmolitos (esquema III). Es ampliamente documentado que uno de los fenómenos primarios asociados con el aumento en el volumen celular producido por soluciones hiposmóticas, es un incremento en la $[Ca^{2+}]i$; por lo que se ha propuesto que el Ca^{2+} puede estar participando como señal transductora del cambio de volumen en la activación de las vías de permeabilidad de los osmolitos. Las acciones biológicas

del calcio son variadas y pueden estar mediadas por vías bioquímicas, como la activación de proteínas cinasas y fosfatasas, que a su vez pueden activar o desactivar canales iónicos y elementos del citoesqueleto.

La participación de proteínas cinasas como son la cinasa dependiente de Ca^{2+} y calmodulina (Ca^{2+}/CAM-c), la proteína cinasa C (PKC), la cinasa dependiente de AMPc (PKA); así como de la fosfolipasa-C (PLC) y la fosfolipasa-A_2 citosólica (PLA_2c), ha sido establecida en diferentes tipos celulares. Otras vías de señalización que implican la fosforilación en residuos de tirosina también se han identificado (revisado en Nilius et al., 1997) (esquema III). Varias observaciones puntuales relacionan la fosforilación en tirosina y la activación de flujos de Cl^- sensibles a volumen. Coincidente con un aumento en el volumen celular en diferentes estirpes celulares, se ha demostrado un incremento en la fosforilación de proteínas cinasas como la cinasa de adhesiones focales (FAK), la fosfatidilinositol-3 cinasa (PI-3c), así como de tres de las cinasas de la familia de proteínas activadas por mitógenos (MAP): Erk1/Erk2, p38, y JNK.



Esquema III. Aún no se conocen con exactitud las señales intracelulares responsables de la activación de los mecanismos que permiten la liberación de los osmolitos durante el DRV. En el esquema se enlistan posibles señales y mensajeros que pueden estar participando en el proceso de regulación.

ANTECEDENTES

Pocas son las investigaciones en las que se ha realizado un estudio paralelo del incremento en la $[Ca^{2+}]i$ y el DRV en la misma preparación y bajo las mismas condiciones experimentales, por lo que existe una gran controversia con respecto a la participación de este catión como una señal de transducción en el proceso regulador.

En un primer trabajo realizado por Wong y Chase (1986) se reportó que un incremento en la $[Ca^{2+}]i$ es una característica constante del hinchamiento celular inducido por soluciones hiposmóticas. Este incremento en la $[Ca^{2+}]i$ es una respuesta bifásica, muy similar entre los diferentes tipos celulares; esta respuesta consiste en un incremento inmediato en la $[Ca^{2+}]i$ debido a la liberación de Ca^{2+} a partir de almacenes intracelulares, seguido de una fase sostenida que representa la entrada de Ca^{2+} extracelular. En la mayoría de las células, la omisión de Ca^{2+} externo no afecta significativamente la primera fase de la respuesta, mientras que la fase sostenida es marcadamente reducida. En otras, ambas fases son reducidas, pero solo en pocos casos esta condición abole el incremento en Ca^{2+}. Es claro que tanto la entrada de Ca^{2+} como la liberación de éste de almacenes intracelulares, contribuyen al incremento en la $[Ca^{2+}]i$ inducido por hiposmolaridad pero la relativa contribución de estos elementos varia entre células.

CALCIO EXTRACELULAR

McCarthy y O´Neil (1992) en una primera revisión sobre el tema, sugirieron que canales de Ca^{2+} sensibles a voltaje, canales tipo SACS y canales cationicos no específicos (corriente capacitativa), son las rutas principales para la entrada de Ca^{2+} durante el hinchamiento celular. El efecto de bloqueadores de estos tres tipos de

canales sugiere que todos estos mecanismos pueden estar implicados en la entrada de Ca^{2+}, con diferencias en su contribución dependiendo del tipo celular.

La inhibición de la entrada de Ca^{2+} por bloqueadores de canales sensibles a voltaje se ha encontrado en células de pituitaria de rata GH3 (Chen *et al.*, 1996), en células de osteosarcoma UMR-106 (Yamaguchi *et al.*, 1989), en células del túbulo proximal de conejo (McCarty y O'Neil, 1991a y b; O'Neil y Leng, 1997), en células renales IMCD (Mooren y Kinne, 1994), en células de vejiga de sapo (Wong *et al.*, 1990), en músculo liso de aorta (Lang *et al.*, 1995), en fibroblastos humanos (Bibby y McCulloch, 1994), en MTAL (Montrose-Rafizadeh y Guggino, 1991), en cardiomiocitos de rata (Taouil *et al.*, 1998) y astrocitos corticales de rata (Bender and Norenberg, 1994). En otros tipos celulares parece que estos canales no estan involucrados en la entrada de Ca^{2+} como es el caso de las células MDCK (Rothstein and Mack, 1992), en el epitelio de vejiga de sapo (Wong y Chase, 1986), de las células del túbulo proximal de conejo (Suzuki *et al.*, 1990) y de células epiteliales de mamífero (Sudlow y Burgoyne, 1997).

Como ya se mencionó el hinchamiento hiposmótico activa canales tipo SACS en el epitelio del plexo coroideo (Christensen y Hoffmann, 1992), el neuroblastoma N1E115 (Falke y Misler, 1989) y células GH3 (Chen *et al.*, 1996). La reducción del incremento en $[Ca^{2+}]i$ por bloqueadores de estos canales con Gd^{3+} o La^{3+} ha sido reportada en fibroblastos (Bibby y McCulloch, 1994), células de vejiga de sapo (Wong *et al.*, 1990), células UMR-106 (Yamaguchi *et al.*, 1989), GH3 (Chen *et al.*, 1996), COS-7 (Ishii *et al.*, 1996), en linfocitos T (Schlichter y Sakellaropoulos, 1994) y en células epiteliales de mamífero (Sudlow y Burgoyne, 1997). La entrada de Ca^{2+} puede ocurrir también por un mecanismo conocido como entrada capacitativa, el cual esta regulado por el estado de relleno de las pozas intracelulares (Putney, 1986). La liberación de Ca^{2+} proveniente de éstas durante el hinchamiento puede iniciar la entrada de Ca^{2+} a

través de canales catiónicos no específicos de la membrana plasmática, mediante dicha vía capacitativa. En células IMCD, la liberación de Ca^{2+} de pozas intracelulares precede la entrada de Ca^{2+} y ésta es requerida para que el Ca^{2+} entre, lo cual sustenta esta propuesta (Tinel *et al.*, 1994). De todos estos resultados se desprende la idea de que no es uno, sino varios los mecanismos que están mediando la entrada de Ca^{2+} durante el aumento del volumen por hiposmolaridad, y que la contribución relativa de cada uno es probablemente específica de cada tipo celular.

CALCIO INTRACELULAR

Cuando el incremento en la $[Ca^{2+}]i$ fue cuantificado utilizando colorantes fluorescentes, se determinó la persistencia de un componente transitorio de la respuesta en Ca^{2+} aún en un medio libre de éste, lo cual enfatiza la intervención de pozas endógenas de Ca^{2+}. Sin embargo, existe cierta discrepancia entre los estudios que proponen el mecanismo mediante el cual el Ca^{2+} es liberado a partir de dichos almacenes. El retículo endoplásmico (RE), ha sido reportado como el mayor contribuidor, ya que su vaciamiento con tapsigargina y ácido ciclopiazónico previene el incremento en $[Ca^{2+}]i$ (Fisher *et al.*, 1997; Jena *et al.*, 1997; Wu *et al.*, 1997; Tinel *et al.*, 1994). Se ha reportado la activación del receptor a 1,4,5-trifosfato (IP_3) en respuesta a hiposmolaridad, indicando que el hinchamiento aumenta los niveles de IP_3 en células del túbulo proximal de conejo (Suzuki *et al.*, 1990), en células COS-7 (Ishii *et al.*, 1996) y en epitelio pulmonar (Felix *et al.*, 1996) pero en otras células tales cambios no ocurren o son solo marginales, o tienen un curso temporal que no se relaciona con el incremento en la $[Ca^{2+}]i$ (Bender *et al.*, 1992; Tinel *et al.*, 1997). Otras evidencias que no sustentan una relación causal entre la generación de IP_3 y el $[Ca^{2+}]i$ vienen de estudios que demuestran que la inhibición de la PLC con el bloqueador U-73122 no tiene efecto alguno sobre el incremento en la $[Ca^{2+}]i$ (Fisher *et al.*, 1997). Además, en

células ascíticas de Ehrlich, hay un incremento en la formación de IP_3 sin que se presente un incremento en $[Ca^{2+}]i$ (Svane *et al.*, 1990).

La liberación de Ca^{2+} por receptores a ryanodina solo se ha documentado en un trabajo realizado en células epiteliales transformadas (Wu *et al.*, 1997). Un estudio en células IMCD implica directamente al ácido araquidónico en el mecanismo de liberación de Ca^{2+} de pozas intracelulares, basado sobre los efectos que tiene el ácido araquídonico liberando Ca^{2+} en soluciones isosmóticas y la supresión de la liberación de éste en soluciones hiposmóticas al utilizar ETYA, un análogo no metabolizable del ácido araquidónico (Tinel *et al.*, 1997).

Finalmente, se ha propuesto que la reducción en el hinchamiento hiposmótico induce la movilización de Ca^{2+} de compartimentos intracelulares no mitocondriales a través de vías de fuga pasiva, una propiedad inherente de estos organelos. Es decir, que posiblemente el retículo endoplásmico esté funcionando como un osmosensor capaz de detectar la dilución del citosol cuando las células se encuentran en un medio hiposmótico. Entonces, el Ca^{2+} es liberado de estos compartimentos mediante canales que normalmente dejan fugar Ca^{2+}, pero que además son sensibles a la distensión de su membrana (Jena *et al.*, 1997; Missiaen *et al.*, 1996).

IMPORTANCIA DEL Ca^{2+} EN EL DRV Y LIBERACION DE OSMOLITOS

En general, parece que la mayoría de las células en las que el DRV es dependiente de Ca^{2+} son de origen epitelial (revisado en Pasantes-Morales y Morales-Mulia en prensa). Los hepatocitos son una excepción ya que el DRV no depende de Ca^{2+}. A diferencia de las células epiteliales, en otros tipos celulares el DRV es esencialmente independiente de Ca^{2+}. En células sanguíneas incluyendo plaquetas

(Margalit et al., 1993), linfocitos (Grinstein et al., 1982) y leucocitos HL60 (Gallin et al., 1994; Hallows y Knauf, 1994), el DRV es independiente de Ca^{2+}. En astrocitos existe cierta controversia pues se ha reportado el DRV como dependiente (Vitarella et al., 1994), pero también como independiente (Medrano y Gruenstein, 1993) de Ca^{2+}. En neuronas simpáticas y líneas de neuroblastoma, el DRV es completamente independiente de Ca^{2+} (Altamirano et al., 1998; Falke and Misler, 1989; Leaney et al., 1997).

En general, la liberación de Cl^- activada por hiposmolaridad es característicamente independiente de Ca^{2+} en la mayoría de las células, aunque existen algunas excepciones. En algunos tipos celulares de origen epitelial la corriente de Cl^- sensible a volumen es dependiente de Ca^{2+} extracelular. En células de túbulo proximal (McCarty y O'Neil, 1992), túbulos distales de riñón (Rubera *et al.*, 1997), células pancreáticas (Verdon *et al.*, 1995) células acinares lacrimales (Kotera y Brown, 1993) y la línea celular de tráquea 9HTEo⁻ (Galietta *et al.*, 1997) la corriente de Cl^- es activada por la entrada de Ca^{2+} extracelular.

Con respecto a la liberación de osmolitos orgánicos, solamente en el neuroblastoma CHP-100 y en la línea celular de tráquea 9HTEo⁻ (Galietta *et al.*, 1997), la liberación de taurina se reduce en ausencia de Ca^{2+} extracelular. La liberación de mio-inositol es dependiente de Ca^{2+} en células MDCK (Bagnasco *et al.*, 1993) e independiente en el glioma C6 (Strange *et al.*, 1993) y en células renales papilares (Kinne et al., 1996). Los flujos de sorbitol son dependientes de Ca^{2+} en células IMCD (Bevan *et al.*, 1990) mientras que cierta confusión existe en células renales papilares ya que este osmolito se reporta como dependiente (Kinne *et al.*, 1996) o independiente (Siebens y Spring, 1989; Timothy *et al.*, 1991) de Ca^{2+}.

En varios reportes se establece una coincidencia entre la dependencia de Ca^{2+} del DRV y la dependencia de Ca^{2+} de canales de K^+ sensibles a volumen. Esta coincidencia se presenta principalmente en células epiteliales, mientras que la independencia de Ca^{2+} de ambos, DRV y canales de K^+, caracteriza a células no epiteliales (Pasantes-Morales y Morales-Mulia, en prensa).

FOSFORILACIONES EN RESIDUOS DE TIROSINA

Además del Ca^{2+}, existe evidencia experimental de la participación de cinasas de tirosina en los mecanismos que subyacen a la regulación del volumen en condiciones hiposmóticas. La liberación de Cl^- y osmolitos orgánicos es inhibida ó potenciada por bloqueadores de cinasas ó de fosfatasas de tirosina, respectivamente. También se ha demostrado que la exposición a un medio hiposmótico, incrementa la actividad de diferentes cinasas de tirosina. En linfocitos T, el hinchamiento activa una cinasa conocida como $p56^{lck}$ lo cual a su vez, es indispensable para la activación de la corriente de Cl^- en estas células (Lépple-Wienhues *et al.*, 1998); en la línea celular 407, la activación de la corriente de Cl^- correlaciona con un incremento en la fosforilación de PI-3 cinasa y FAK (Tilly *et al.*, 1996a); en una línea celular de hepatoma (Schliess *et al.*, 1995), en el glioma C6 (Sinning *et al.*, 1997), en células intestinales 407 (Tilly *et al.*, 1993) y en astrocitos de corteza de rata (Schliess *et al.*, 1996; Crépel *et al*, 1998) la hiposmolaridad incrementa la actividad de las MAPs cinasas: Erk1/Erk2.

Erk1/Erk2 pertenecen a la familia de las proteínas cinasas activadas por mitógenos y son elementos clave en eventos de señalización durante el crecimiento celular (Seger y Krebs, 1995; Marais y Marshall, 1996). Estas cinasas fosforilan múltiples substratos como son las proteínas MAP-2 y Tau asociadas a los microtúbulos, a la proteína cinasa S6 y a factores de transcripción como c-Jun (Davis,

1993). La vía que conduce a la activación de Erk1/Erk2 estudiada con más detalle es la cascada de señalización iniciada por los receptores a factores de crecimiento como son el EGF y PDGF, cuya activación involucra a la GTPasa $p21^{ras}$ (Ras). La conversión de Ras-GDP hacia el estado activo Ras-GTP ocurre vía el acoplamiento del complejo Grb2-Sos con el receptor y el reclutamiento de la cinasa Raf-1 a la membrana plasmática. Una vez que Raf-1 ha sido activada fosforila a MEK (la cinasa de las MAP cinasas), y ésta a su vez activa a Erk1/Erk2 (Leefers *et al.*, 1994; Kyriakis *et al.*, 1993; Ahn *et al.*, 1991). Además de los receptores a factores de crecimiento, algunas proteínas G acopladas a receptores de siete segmentos transmembranales, así como receptores de la familia de las integrinas, se han identificado como activadores de Erk1/Erk2 (Coob y Goldsmith, 1995; Zhu y Assoian, 1995).

Se ha sugerido que la activación de la vía Ras/Erk por estos receptores es mediada por la interacción de PI-3 cinasa (receptores acoplados a proteínas G), o por la cinasa FAK (integrinas), con el complejo Grb2-Sos (Lopez-Ilasaca *et al.*, 1997; Schlaepfer y Hunter, 1996). Finalmente se ha reportado la activación de Erk1/Erk2 independientemente de Ras y la cual involucra un incremento en la $[Ca^{2+}]i$ y a la PKC (De Vries-Smits *et al.*, 1992; Burgering *et al.*, 1993).

Es claro que frecuentemente existe una correlación entre cambios en la $[Ca^{2+}]i$ y el aumento en el volumen celular en diversos tipos celulares; no obstante, en la mayoría de los estudios la implicación del Ca^{2+} como una señal de transducción subyacente al DRV no ha sido establecida conclusivamente. Parte del problema es que la$[Ca^{2+}]i$ y el DRV (incluyendo la asociada liberación de osmolitos) no siempre se han estudiado en la misma preparación o bajo condiciones similares: células en suspención ó en monocapa, concentración y tiempo de exposición a bloqueadores y seguimiento del curso temporal de los eventos estudiados. De lo anterior se desprende la necesidad

de realizar un estudio detallado sobre la influencia del incremento en la $[Ca^{2+}]i$ en el proceso de regulación, tratando de establecer condiciones experimentales similares.

Por otra parte, es necesario explorar otras posibles vías de señalización ya que los antecedentes muestran claramente que tanto la dependencia del Ca^{2+} como la participación de cinasas de tirosina, parecen ser eventos específicos dependiendo del tipo celular estudiado; resulta interesante suponer que cada estirpe celular utiliza mecanismos y entidades particulares (enzimas, canales, moléculas mensajeras, etc.) que son la base del mantenimiento de su homeostasis.

En el sistema nervioso se ha estudiado poco sobre las vías de señalización involucradas en el DRV, no obstante la implícita importancia del volumen celular tanto en condiciones fisiológicas como en situaciones patológicas. Esta investigación se propone definir el papel del Ca^{2+} en la regulación del volumen en células nerviosas de cerebelo de rata, así como explorar la participación de otras vías de transducción que incluyen la participación de tirosinas cinasas, de la PI-3 cinasa y de las cinasas Erk1/Erk2, pues existe evidencia de la activación de estas cinasas durante el estrés hiposmótico y que se sustenta por el hecho de que estas proteínas se encuentran asociadas con diversos elementos del citoesqueleto.

OBJETIVO GENERAL

El presente trabajo tuvo como objetivo principal estudiar las vías de señalización que participan en la transducción del cambio en el volumen celular a la activación de las vías de movilización de los osmolitos responsables de la regulación del volumen en células nerviosas de rata en cultivo, examinando los siguientes aspectos: 1) el papel del Ca^{2+} en los flujos asociados al DRV en astrocitos y neuronas granulares de cerebelo, 2) la participación de Erk1/Erkr2 en la vía de señalización que conlleva a la activación de la vía para la liberación de taurina bajo condiciones hiposmóticas en neuronas granulares y 3) la participación de cinasas de tirosina en la activación de los flujos de taurina en neuronas granulares.

OBJETIVOS ESPECIFICOS

Con tal finalidad se establecieron como objetivos específicos los siguientes:

1. Determinar si se presenta un incremento en la $[Ca^{2+}]i$ asociado al aumento en el volumen celular bajo condiciones hiposmóticas en células granulares y astrocitos de cerebelo de rata en cultivo.

2. Investigar si el incremento en la $[Ca^{2+}]i$ inducido por hiposmolaridad en estas células nerviosas se debe a la entrada de Ca^{2+} extracelular y/o a la liberación de Ca^{2+}de almacenes intracelulares.

3. Determinar si el DRV y la salida de I^{125} (como trazador de Cl^{-}), de ^{3}H-taurina (como representante de los aminoácidos) y de ^{86}Rb (como trazador de K^{+}) en neuronas granulares de rata en cultivo bajo condiciones hiposmóticas, dependen de la entrada

de Ca^{2+} y/o la liberación de Ca^{2+} de almacenes intracelulares asociadas al aumento en el volumen celular.

4. Determinar si el DRV y la liberación de ^{86}Rb en respuesta a hiposmolaridad dependen de la entrada de Ca^{2+} y/o la liberación de Ca^{2+}de almacenes intracelulares asociadas al aumento en el volumen celular, en astrocitos de cerebelo de rata en cultivo.

5. Determinar si la exposición de neuronas granulares a un medio hiposmótico 30%, induce un aumento en la actividad de las MAPs cinasas Erk1/Erk2 cuantificando el grado de fosforilación de la proteína básica de mielina como substrato de estas cinasas.

6. Examinar el efecto de la inhibición de MEK sobre la actividad de Erk1/Erk2 asociada al estrés hiposmótico y sobre la liberación de taurina en neuronas granulares de cerebelo de rata en cultivo.

7. Examinar el efecto de inhibidores de cinasas de tirosina sobre el curso temporal de la liberación de taurina sensible a volumen en neuronas granulares de cerebelo de rata en cultivo.

8. Examinar la participación de la PI-3 cinasa sobre el curso temporal de la liberación de taurina estimulada por hiposmolaridad en neuronas granulares de cerebelo de rata en cultivo.

La metodología y resultados de esta investigación indicados en la discusión se encuentran descritos en los artículos y anexo que se encuentran al final del escrito.

DISCUSION

Como muchas otras células, las neuronas granulares y astrocitos de cerebelo de rata en cultivo poseen la capacidad de regular su volumen cuando han sido expuestas a un medio hiposmótico. El Decremento regulador del volumen de las células nerviosas es un proceso activo que implica la activación de mecanismos de transporte de solutos intracelulares osmóticamente activos. La activación de estos mecanismos correctores trae como consecuencia, el restablecimiento del volumen celular (Pasantes-Morales, *et al.*, 1993; 1994).

Uno de los aspectos importantes relacionados al estudio de los mecanismos que subyacen a la regulación del volumen celular ha sido el conocimiento de cómo la célula detecta que ha ocurrido un cambio en su volumen y que señal (o señales) emplea para activar las vías mediante las cuales se movilizan los osmolitos.

INCREMENTO EN LA [Ca^{2+}]i EN CONDICIONES HIPOSMÓTICAS

Participación del Ca^{2+} extracelular.

En esta investigación se demostró que en las células granulares (artículo I, fig.4; artículo III, fig. 4A) y astrocitos (artículo II, fig. 1A) de cerebelo de rata en cultivo, como en muchos otros tipos celulares, también se presenta un incremento en la [Ca^{2+}]i. La [Ca^{2+}]i promedio en neuronas granulares mantenidas en un medio isosmótico fue de 90 $\pm$ 7.7 nM (n=12). La dilución del medio al 50% ocasionó un incremento gradual en la [Ca^{2+}]i a un máximo de 148 $\pm$ 9.8 nM, la cual disminuyó lentamente hasta alcanzar un valor de 100 $\pm$ 8.7 nM que permaneció estable hasta 400 seg después del estímulo. En el caso de los astrocitos, el valor basal en la [Ca^{2+}]i fue de 117 $\pm$ 12.4 nM e incremento hasta 386 $\pm$ 37 nM durante la dilución del medio al 50%. La señal se

estabilizó a 201 ± 21 nM a los 400 seg. Tanto en neuronas granulares como en astrocitos, el incremento de la $[Ca^{2+}]i$ inducido por hiposmolaridad disminuyó notablemente en soluciones libres de Ca^{2+} y suplementadas con 0.5 mM de EGTA (artículo II, fig. 1B; artículo III, fig. 4B). Bajo estas condiciones la $[Ca^{2+}]i$ en neuronas antes del estímulo hiposmótico, fue de 89 ± 8.0 nM con un incremento máximo de 130 ± 12 nM, mientras que en astrocitos la $[Ca^{2+}]i$ en un medio isosmótico fue de 81.1 ± 19 nM, incrementándose hasta 190.7 ± 26 nM durante la dilución. Estos datos indican que la entrada de Ca^{2+} contribuye de manera predominante en la respuesta total del incremento en la $[Ca^{2+}]i$ bajo condiciones hiposmóticas.

Las vías que permiten la entrada de Ca^{2+} a la célula durante el hinchamiento aún se desconocen; se ha propuesto que el Ca^{2+} puede permear al interior celular por los canales tipo SACS, o bien que la hiposmolaridad genera la despolarización de la membrana celular activando canales de Ca^{2+} sensibles a voltaje, como se ha observado en células ascíticas de Ehrlich (Lambert, 1989; Lang, 1987), células MDCK (Paulmichl, 1993), células renales (Ubl, 1988), linfocitos (Grinstein, 1982; Sarkadi, 1984), en astrocitos en cultivo (Kimerlberg, 1988) y en la línea celular de neuroblastoma N1E115 (Falke y Misler, 1989). Sin embargo, no en todas las células se presenta una despolarización por el aumento en volumen sino por el contrario, se ha registrado que la membrana celular de hepatocitos se hiperpolariza en condiciones hiposmóticas (Graf, 1988), mientras que en otros tipos celulares se presenta una hiperpolarización temporal seguida de una despolarización más sostenida cuando las células se exponen a soluciones de baja osmolaridad (Hazama y Okada, 1988 y 1990).

Durante la realización del presente estudio no fue posible analizar el efecto de cationes como Gd^{3+}, La^{3+} y Cd^{2+} sobre el incremento en la $[Ca^{2+}]i$ ya que al ser adicionados al medio experimental, estos cationes afectaron la señal fluorescente a

340 y 380 nm por lo que la relación 340/380 también se vio alterada (anexo I, fig. 1). Esto indica que estos cationes pueden estar permeando al interior celular interfiriendo con la señal de Ca^{2+}.

El bloqueo de canales de Ca^{2+} sensibles a voltaje tipo L con verapamil y nitrendipina, no modificó la respuesta de Ca^{2+} de astrocitos (artículo IV, fig. 5B, 5C) ni de neuronas (anexo I, fig. 2); lo cual descarta que este tipo de canales sea la vía de entrada al Ca^{2+} durante el hinchamiento.

O´Neil y Leng (1997), en un trabajo realizado con células renales del túbulo proximal de conejo, determinaron que el hinchamiento aparentemente activa dos vías de entrada para el Ca^{2+}, una sensible y otra insensible a la dihidropiridina nifedipina. La activación de estas vías esta regulada tanto por la PLC como por la PKC ya que al inhibir estas proteínas, la fase que corresponde a la entrada de Ca^{2+}, desapareció completamente. El hecho de que en este mismo tipo celular no se haya identificado la presencia de canales de Ca^{2+} tipo L, llevó a los autores a suponer que la entrada de Ca^{2+} a la célula esté mediada por un canal tipo L modificado, en el que la sensibilidad al voltaje y a las dihidropiridinas se encuentra alterada; o bien, que sea el resultado de la activación de una entrada "capacitativa" de Ca^{2+} la cual es regulada por el estado de llenado del retículo endoplásmico (Putney, 1986). En este sentido es necesario contemplar la posibilidad de que un mecanismo similar sea el responsable de la entrada de Ca^{2+} durante hiposmolaridad en el caso de los astrocitos de cerebelo (presente trabajo), ya que cuando se agrega Ca^{2+} después de que el incremento en la $[Ca^{2+}]i$ regresa a niveles basales se observa un nuevo incremento en la $[Ca^{2+}]i$, lo cual se debe a la entrada de Ca^{2+}. Este incremento se presenta en condiciones en las que los astrocitos se encuentran en un medio con ó sin Ca^{2+}. Cuando se añade Ca^{2+} a las células mantenidas en un medio isosmótico no se observa ningún cambio en la $[Ca^{2+}]i$.

Estos resultados muestran que la vía de entrada para el Ca^{2+} permanece abierta mientras las células se mantienen en condiciones hiposmóticas (anexo I, fig. 3).

Liberación de Ca^{2+} de almacenes intracelulares.

La persistencia de la respuesta de elevación de $[Ca^{2+}]i$ tanto en astrocitos como en neuronas aún en ausencia de Ca^{2+} extracelular, sugiere la contribución de Ca^{2+} proveniente de almacenes intracelulares. Con el propósito de determinar la fuente endógena de aporte de Ca^{2+} a la respuesta total asociada al aumento en el volumen celular, se utilizaron estrategias para depletar de Ca^{2+} los almacenes intracelulares: las células se trataron con ionomicina ó tapsigargina en un medio sin Ca^{2+} (0.5 mM EGTA). El estímulo hiposmótico no provocó incremento alguno en la $[Ca^{2+}]i$ después de cualquiera de estos tratamientos en ambos tipos celulares (artículo II, fig. 2; artículo III, fig. 5A, 5B), lo cual indica que una parte del Ca^{2+} liberado durante el hinchamiento proviene del retículo endoplásmico.

Han sido varias las propuestas acerca de cual es el mecanismo mediante el cual el Ca^{2+} es liberado desde las pozas endógenas; aunque todas ellas resultan algo contradictorias. Por una parte se propone que la liberación de Ca^{2+} es el resultado de la activación del receptor a inositol 1,4,5-trifosfato (IP_3) en el retículo endoplásmico, esta evidencia es sustentada por el hecho de que en células del túbulo contorneado de conejo (Suzuki, 1990), células COS-7 (Ishii, 1996), y en astrocitos de corteza en cultivo (Bender, 1994) se presenta un incremento en la hidrólisis de fosfatidilinositol 4,5-bifosfato (PIP_2) bajo condiciones hiposmóticas, generando la acumulación de IP_3. Sin embargo, este incremento en los niveles de IP_3 es marginal y se presenta retardado con respecto al estímulo hiposmótico en astrocitos de corteza (Bender et al., 1992). Por otra parte, Schliess y colaboradores (1996) reportaron que en estas células, la inhibición de la PLC no modifica el incremento en la $[Ca^{2+}]i$ inducido por

hiposmolaridad. En células hepáticas de rata, se genera un aumento en los niveles de IP_3 previo al incremento en la $[Ca^{2+}]i$ en condiciones hiposmóticas. Teóricamente se esperaba que este incremento en la $[Ca^{2+}]i$ pudiera estar activando canales de K^+ dependientes de Ca^{2+} involucrados en el DRV. Estos eventos no pudieron ser relacionados, ya que la acumulación de IP_3 sólo se presenta cuando las células se hinchan por la reducción de la osmolaridad a partir de un medio isosmótico, y no cuando la osmolaridad se reduce a partir de un medio hiperosmótico, es decir, cuando las células se encuentran previamente encogidas. En ambas situaciones, los flujos de K^+ que median la regulación del volumen celular son similares, por lo cual se presume que es necesaria una distensión importante de la membrana plasmática para que se estimule la hidrólisis de los PIP_2 y que este evento es sólo un epifenómeno asociado al hinchamiento (vom Dahl, *et al.,* 1991).

La participación del receptor a ryanodina se ha descrito mediando la liberación de Ca^{2+} asociada al hinchamiento en células transformadas de epitelio este evento a su vez, ocasiona la activación de la entrada Capacitativa de Ca^{2+} (Wu et al., 1997). En estas células, tanto el DRV como el incremento en la $[Ca^{2+}]i$ son inhibidos por ryanodina (100 μM) y por la ausencia de Ca^{2+} extracelular.

En neuronas y astrocitos también se pudo descartar la participación tanto del receptor a IP_3 como del receptor a ryanodina como proveedores de Ca^{2+} durante el hinchamiento, ya que la inhibición de la PLC (anexo I, fig. 4A, 4B) y concentraciones altas de ryanodina (50-100 μM), no modificaron el incremento en la $[Ca^{2+}]i$. La falta de activación de estos receptores lleva a especular la intervención de otra (s) vías a través de las cuales el Ca^{2+} sea liberado durante el hinchamiento; en este sentido existe la posibilidad de que la mera dilución del citosol por la entrada de agua bajo condiciones hiposmóticas sea detectada por los almacenes de Ca^{2+}, que a su vez, actuando como

osmómetros, también aumenten su volumen y esto a su vez lleve a que la tensión de su membrana abra una vía permeable al Ca^{2+}. Se sabe que existe una asociación entre el retículo endoplásmico con elementos del citoesqueleto que lo interconectan con el citoesqueleto basal de membrana, de tal forma que a su vez la distensión de la membrana plasmática puede ser detectada por la membrana del RE. Estos eventos pueden ocasionar la apertura de canales catiónicos (que se sabe estan presentes en este organelo) con la subsecuente liberación de Ca^{2+}.

En apoyo a estas suposiciones existen dos trabajos en células de endotelio permeabilizadas, en los que se demuestra que la liberación de $^{45}Ca^{2+}$ inducida por hinchamiento de almacenes intracelulares sensibles a tapsigargina, proviene de un compartimiento distinto al de los receptores a IP_3 y ryanodina, ya que la previa estimulación con agonistas para ambos receptores no impidió la liberación de $^{45}Ca^{2+}$ por hiposmolaridad (Missiaen, *et al.*, 1996 y Jena, *et al.*, 1997). Estos resultados conducen a la posibilidad de que solo fuerzas físicas (distención de la membrana) actuando sobre los compartimentos intracelulares los que puedan liberar Ca^{2+} sin el requerimiento de intermediarios bioquímicos.

INFLUENCIA DEL Ca^{2+} SOBRE EL DRV Y LA LIBERACION DE ^{125}I Y ^{3}H-TAURINA

En el caso de las neuronas granulares, la omisión de Ca^{2+} extracelular y la inhibición de canales tipo SACS ó de canales sensibles a voltaje, no tuvo ningún efecto sobre el DRV y los flujos asociados de ^{3}H-taurina y ^{125}I (artículo I, fig. 1; tablas 1 y 2). Estos resultados contrastan con los reportados por Bassavappa en el neuroblastoma CHP-100, en donde tanto el incremento de la $[Ca^{2+}]i$ como la salida de ^{125}I inducidos por hiposmolaridad se inhiben en ausencia de Ca^{2+} extracelular o bien, cuando la entrada de Ca^{2+} es bloqueada con ω-ctx-MVIIC, un bloqueador de canales de Ca^{2+} tipo

Q y R. Es de hacer notar, sin embargo, que estos resultados se observaron bajo condiciones innecesariamente extremas, tales como el uso de EGTA 5 mM.

El DRV y los flujos asociados de ^{3}H-taurina y ^{125}I en neuronas granulares no se modificaron por agentes que bloquean la liberación de Ca^{2+} mediada por el receptor a ryanodina (dantroleno) ó por el receptor a IP_3 (TMB-8). Sólo el tratamiento de las células con BAPTA-AM tuvo un ligero efecto sobre la liberación de taurina (artículo I, tabla III).

Con respecto a los astrocitos, previamente se demostró que la liberación de ^{3}H-taurina y ^{125}I inducida por un medio hiposmótico no se afecta por la omisión de Ca^{2+} extracelular ni por bloqueadores de la entrada de Ca^{2+} a la célula (Sánchez-Olea, et al., 1995). En este trabajo la ausencia de Ca^{2+} extracelular no inhibe el DRV; por el contrario, bajo estas condiciones se observó que la regulación del volumen se hace más eficiente durante los últimos cinco minutos (artículo II, fig. 1C), lo cual puede estar reflejando un aumento en la permeabilidad al K^{+} como se discute más adelante. A excepción del La^{3+}, otros cationes y el verapamil no afectaron de manera significativa el proceso regulador (artículo II, tabla1).

Tanto en astrocitos (Sánchez-Olea, et al., 1995) como en neuronas (presente trabajo), el efecto inhibidor de las dihidropiridinas sobre el DRV (artículo I, tabla 1) y los flujos de ^{125}I y ^{3}H-taurina (artículo I, fig. 2 y 3) no pudo ser atribuido al bloqueo de canales de Ca^{2+} tipo L ya que este efecto persistió aún en ausencia de Ca^{2+} extracelular, lo cual sugiere que podría tratarse de un efecto *per se* inespecífico de estos fármacos. En células granulares de cerebelo de ratón en cultivo, las dihidropiridinas disminuyen las corrientes de K^{+}; efecto que se atribuye a posibles similitudes en las propiedades de unión electrostática hacia las dihidropiridinas entre los canales de Ca^{2+} y canales de K^{+} (Fagni, 1994).

La depleción de Ca^{2+} de los almacenes intracelulares y la incubación de las células con BAPTA-AM, condición bajo la cual se previenen cualquier incremento en los niveles de Ca^{2+} citosólico, no afectaron ni el curso temporal ni la eficiencia de la regulación del volumen ni en astrocitos (artículo II, fig. 3A, fig.4, fig. 6) ni en neuronas (artículo I, tabla 3, anexo I, fig. 5).

INFLUENCIA DEL Ca^{2+} SOBRE LOS FLUJOS DE K^+

En las neuronas granulares, la cantidad de K^+ liberado medido como flujo de ^{86}Rb en respuesta a hiposmolaridad, es proporcional a la disminución en la osmolaridad (artículo III, fig.1). Estos flujos de ^{86}Rb fueron insensibles a la mayoría de los bloqueadores de canales de K^+, y sólo fueron afectados por el Ba^{2+}, Cs^+ y quinidina (artículo III, tabla 1). Además, los resultados señalan que en estas células los flujos de ^{86}Rb asociados al volumen son también independientes del Ca^{2+} extracelular. En un medio libre de Ca^{2+} la liberación no se inhibe, sino por el contrario, se incrementa. Bloqueadores de la entrada de Ca^{2+} y bloqueadores de canales de K^+ activados por Ca^{2+} no afectan los flujos de ^{86}Rb activados por hinchamiento (artículo III, tabla 1; fig. 6A).

La liberación de ^{86}Rb también fue insensible a la depleción de Ca^{2+} de los almacenes intracelulares y al tratamiento de las células con BAPTA-AM (artículo III, fig. 7A, 7B). La adición de ionomicina en un medio isosmótico ó hiposmótico no modificó la liberación de ^{86}Rb, no obstante que la $[Ca^{2+}]i$ incrementó hasta alcanzar niveles cercanos a 1 μM (artículo III, fig.5D). Esto descarta cualquier participación de canales activados por Ca^{2+} o bien, algún efecto modulador como el observado en los astrocitos (ver abajo).

Como se mencionó, en el caso particular de los astrocitos de rata en cultivo, las evidencias acerca de la dependencia de Ca^{2+} sobre la liberación de K^+ bajo condiciones hiposmóticas, han sido particularmente contradictorias. En astrocitos corticales O´Connor y Kimelberg (1993) reportaron un bloqueo de los flujos de ^{86}Rb activados por hiposmolaridad en un medio sin Ca^{2+} extracelular. En un posterior estudio Vitarella y colaboradores (1994), reportaron un incremento en la liberación de ^{86}Rb en ausencia de Ca^{2+} extracelular, no obstante al hecho de que el tratamiento con BAPTA-AM (en el mismo trabajo), sólo inhibió parcialmente la salida de este osmolito. Bender y Norenberg (1994) también observaron un aumento en la liberación de ^{86}Rb cuando el Ca^{2+} extracelular es omitido; sin embargo, concluyeron que la activación de los flujos de ^{86}Rb activados por hiposmolaridad es dependiente de Ca^{2+}.

Por lo anterior, en este trabajo examinamos muy detalladamente la influencia del Ca^{2+} sobre los movimientos de ^{86}Rb activados por un medio hiposmótico, en neuronas y astrocitos de cerebelo de rata en cultivo. Uno de los resultados más importantes de este estudio fué la descripción y caracterización de la activación de un flujo de ^{86}Rb cuando el Ca^{2+} extracelular es removido. Bajo condiciones hiposmóticas, la ausencia de Ca^{2+} extracelular provocó un aumento en la liberación de ^{86}Rb con respecto a su liberación en un medio con Ca^{2+} (artículo IV, fig. 2B). El mecanismo por el cual la omisión de Ca^{2+} activa los flujos de ^{86}Rb no es del todo claro, aunque en células epiteliales se ha descrito la activación de una conductancia de K^+ con características similares a las observadas en este trabajo. En ambos casos el Ba^{2+}, el verapamil, las dihidropiridinas y un decremento en el pH inhiben de manera importante esta movilización de K^+ activada por la omisión de Ca^{2+} extracelular (artículo IV, fig. 3A, 3B).

La activación del flujo de ^{86}Rb por ausencia de Ca^{2+} extracelular podría explicar la paradójica aceleración del DRV bajo estas condiciones (artículo II, fig. 1C) pues como se mencionó en la introducción la liberación de K^+ es el paso limitante en la regulación del volumen en células nerviosas. La activación de este flujo de ^{86}Rb podría explicar por qué en astrocitos corticales, la inhibición con BAPTA-AM de la salida de ^{86}Rb en condiciones hiposmóticas es revertida en ausencia de Ca^{2+} extracelular (Bender y Norenberg, 1994). Además, es importante subrayar que este flujo de ^{86}Rb representa un obstáculo para determinar si el Ca^{2+} extracelular es necesario para la activación de la liberación de ^{86}Rb activada por hinchamiento. En el presente trabajo, el tratamiento de los astrocitos con EGTA-AM, condición bajo la cual cualquier incremento en la $[Ca^{2+}]i$ es prevenido, sólo disminuyó un 30% la salida de ^{86}Rb bajo condiciones hiposmóticas (artículo IV, fig. 4A, 4B). En presencia de Gd^{3+}, Cd^{2+}, La^{3+} y diltiazem, los flujos de ^{86}Rb no se modificaron (artículo IV, página 353). La inhibición observada con verapamil y nitrendipina no puede ser atribuída al bloqueo de la entrada de Ca^{2+} ya que la inhibición persiste aún en ausencia de Ca^{2+} extracelular (artículo IV, fig. 5). En este sentido es importante señalar que ambos compuestos también bloquean los flujos de K^+ activados por la omisión de Ca^{2+} y que se ha reportado que las dihidropiridinas bloquean canales de K^+ (Fagni et al., 1994).

En astrocitos corticales, la inhibición de la PLC disminuye marcadamente la salida de ^{86}Rb en un medio hiposmótico, resultado que sugiere la participación del receptor a IP_3 (Bender et al., 1993), contrastando con lo observado en el presente trabajo, en donde la inhibición de la PLC no modificó de manera significativa la liberación de ^{86}Rb en astrocitos de cerebelo (artículo IV, página 353). Sin embargo, el tratamiento de las células con W-7 y KN-93 (inhibidores de calmodulina y la cinasa dependiente Ca^{2+}/CAM), disminuyó la salida de ^{86}Rb de forma similar a lo observado con EGTA-AM (artículo IV, fig. 6). Estos resultados sustentan la participación del

sistema Ca^{2+}/CAM en la activación de una fracción de las vías para K^+ durante el DRV en astrocitos de cerebelo de rata en cultivo.

Otro aspecto importante del presente estudio fue la observación de que la liberación de ^{86}Rb en astrocitos es modulada por el incremento en la $[Ca^{2+}]i$. Si el estímulo hiposmótico es aplicado en la presencia de ionomicina cuando la concentración extracelular de Ca^{2+} es de 1-2 mM, el incremento en la $[Ca^{2+}]i$ alcanza niveles superiores al 1 µM. Cuando esto sucede se observa una potenciación de la liberación de ^{86}Rb (artículo IV, fig. 7), lo cual puede ser concecuencia de la contribución de vías adicionales activadas por este umbral de Ca^{2+}. En células epiteliales, la corriente de K^+ activada por hiposmolaridad es dependiente de Ca^{2+} y en algunos casos se ha identificado tanto farmacológica como electrofisiológicamente, que este tipo de canales corresponde a los llamados maxi-K los cuales requieren de concentraciones µM de Ca^{2+} y de una despolarización para activarse (Sarkadi and Parker, 1991).

Es difícil determinar si los componentes Ca^{2+}-dependiente y Ca^{2+}-independiente en la liberación de ^{86}Rb en astrocitos corresponden a vías separadas o bien, si se trata de una sola entidad con dependencia parcial del Ca^{2+}. De crucial importancia resulta el hecho de investigar no sólo para el caso de las células nerviosas sino para cualquier tipo celular, si en realidad existe un canal de K^+ sensible al aumento en el volumen, o si la salida de este ion ocurre a través de canales de K^+ activados por otros eventos que suceden paralelamente al hichamiento. Si el hichamiento por hiposmolaridad incrementa la $[Ca^{2+}]i$ y produce depolarización de la membrana, es posible que ambos sucesos conlleven a la activación de canales de K^+ activados por voltaje y canales de K^+ dependientes de Ca^{2+}.

Baraban y colaboradores (1997) realizaron un trabajo con técnicas electrofisiológicas en rebanadas de hipocampo de rata, reportando que el estrés hiposmótico activa una corriente de K^+ en interneuronas de las capas lacuosum/moleculare, lo cual no ocurrió en neuronas de la región CA1 del hipocampo. Los autores discuten acerca del papel modulador de estas interneuronas sobre la región CA1 ya que la función de estas interneuronas es la de inhibir a las neuronas piramidales de la región CA1 y el hecho de que el hinchamiento celular active una corriente de K^+ en las interneuronas, eventualmente conducirá a una disminución de la inhibición de la actividad de las neuronas piramidales, lo que a su vez puede contribuir a la hiperexitabilidad observada bajo episodios de hipoxia-isquemia y epilepsia. Estas evidencias ponen de relieve la importancia del manejo del K^+ como osmolito en células nerviosas.

LIBERACION DE TAURINA, (Ca^{2+}/CAM-c), PKC Y PKA

La inhibición de Ca^{2+}/CAM-c con KN-93 no modificó la liberación de taurina en neuronas granulares (artículo V, fig. 2A). Este resultado concuerda con la independencia de Ca^{2+} de los mecanismos correctores del volumen discutido anteriormente. Sin embargo, es importante destacar que el W-7, un inhibidor de la calmodulina, potenció marcadamente la liberación de taurina (artículo V, fig. 2A). Este efecto puede deberse a una acción inespecífica del fármaco o bien, puede ser el resultado de un incremento en los niveles de ácido araquídonico (Wolf y Gross, 1996), y que este a su vez induzca la activación de la vía para la movilización de taurina como se ha reportado en células de neuroblastoma (Basavappa et al., 1998).

La liberación de taurina en neuronas granulares no se ve afectada por la inhibición (artículo V, fig. 2B) o activación (artículo V, fig. 2C) de la PKC, lo cual descarta la participación de esta cinasa en la liberación de taurina sensible a volumen.

La disminución observada en la liberación de este osmolito cuando la PKC es regulada a la baja después del tratamiento crónico con un éster de forbol (trabajo V, fig. 2C), resulta algo contradictoria pero podría explicarse si se piensa que este tratamiento pudiera estar afectando a isoformas de PKC independientes de Ca^{2+} y sensibles a ésteres de forbol, pertenecientes a las no convencionales, que posiblemente esten participando cascada abajo en la vía de señalización del DRV. Existe también la posibilidad de que el PMA esté modificando la arquitectura del citoesqueleto como se ha reportado previamente (Nobes y Hall, 1994) y, que cuando las células reciben el estímulo hiposmótico no sean capaces de detectar el cambio en su volumen. Sin embargo, no puede excluirse la posibilidad de algún efecto inespecífico como en el caso del W-7.

En células cardíacas la cleritina un bloqueador de la PKC inhibe la liberación de taurina (Song et al., 1998). En corteza cerebral de rata la cleritina disminuye 28% la liberación de éste osmolito. Con respecto a la corriene de Cl^- asociada al volumen, la influencia de PKC depende del tipo celular. En algunos casos la activación o regulación a la baja conllevan a una reducción ó estimulación, respectivamente, de la corriente de Cl^-; en otros casos la activación de esta enzima inhibe completamente el movimiento de Cl^- (revisado en Okada, 1997). Los presentes resultados sugieren que la PKC no tiene una participación determinante en la cascada de señalización que subyace a la liberación de taurina en neuronas granulares.

Se sabe que la PKA y su activador el AMPc, tienen efectos moduladores sobre la corriente de Cl^- sensible a volumen en algunos tipos celulares (Okada, 1997) aunque su vía de señalización no es del todo clara. En neuronas granulares la participación de esta cinasa queda excluída pues la activación de la adenilato ciclasa con fosrkolina y el aumento en los niveles de AMPc no tuvieron efectos importantes sobre la liberación de

taurina (artículo V, fig. 2D); asímismo los bloqueadores H-7 y H-8 que tienen efecto sobre la PKA, no modificaron esta respuesta (trabajo V, fig. 2B).

LIBERACIÓN DE TAURINA Y ERK1/ERK2

Como se menciona en la Introducción se ha propuesto que puede ocurrir el transporte osmosensible de taurina y Cl^- a través de un canal aniónico inespecífico; sin embargo, aún se conoce poco acerca de la identidad molecular de este canal. En este sentido y ya que en muchos tipos celulares el Ca^{2+} no tiene participación como señal en el DRV ha sido necesario explorar otras posibles señales que puedan estar regulando la liberación de los osmolitos.

Los resultados del presente estudio muestran claramente la participación de cinasas de tirosina en la cascada de señalización que conduce a la liberación de taurina en neuronas granulares. La liberación de taurina es inhibida casi por completo en la presencia de tirfostina, un inhibidor de cinasas de tirosina y por el contrario, la inhbición de fosfatasas de tirosina potenció de manera importante esta liberación (artículo V, fig. 3). En astrocitos corticales (Crépel et al., 1998) y en células de intestino 407 (Tilly et al., 1993), la inhbición de cinasas de tirosina también impidieron la activación de la corriente de Cl^- y la liberación de ^{125}I respectivamente.

La exposición de las neuronas granulares a un medio hiposmótico indujo un incremento en la actividad de Erk1/Erk2 (artículo V, fig. 4A), lo cual también se ha observado en otras estirpes celulares. La inhibición de MEK con PD 98059 disminuyó significativamente el incremento en la actividad de Erk1/Erk2 (artículo V, fig. 4A). Sin embargo, esta condición no afectó la liberación de taurina en estas neuronas (artículo V, fig. 4B). En astrocitos corticales de rata se ha reportado que la inhibición de MEK

disminuye la actividad de Erk1/Erk2 e impide la activación de la corriente de Cl^- sensible a hichamiento (Crépel et al., 1998); esto marca una clara diferencia entre ambos tipos de células nerviosas y que probablemente este en relación con su función específica como parte del sistema nervioso. En la línea celular de intestino 407 se reportaron resultados similares y a diferencia de lo que ocurre en los astrocitos, en el intestino la hipotonicidad induce un aumento en la actividad de Erk1/Erk2 pero tal evento no esta relacionado con la vía de liberación de Cl^- ya que la inhibición de MEK no modificó la liberación de ^{125}I (Van der Wijk et al., 1998).

La activación de Erk1/Erk2 en respuesta a hinchamiento osmótico parece ser un fenómeno generalizado pues ha sido observado en hapatocitos y células de hepatoma (Schliess et al., 1995; Wiese et al., 1998), en astrocitos (Schliess et al., 1997; Crépel et al., 1998), en glioma C6 (Sinning et al., 1997), células intestinales (van der Wijk et al., 1998) y miocitos cardiacos (Sadoshima et al., 1996). Sin embargo, existen diferencias entre los diferentes tipos celulares respecto a la vía de señalización corriente arriba que conduce a la activación de Erk1/Erk2: la activación de estas cinasas en astrocitos requiere la entrada de Ca^{2+} extracelular, mientras que en hepatocitos, hepatoma y miocitos cardíacos la activación de Erk1/Erk2 es independiente de Ca^{2+} pero sensible a toxina pertusis, lo cual sugiere la intervención de proteínas G. En células intestinales la activación osmótica de Erk1/Erk2 ocurre vía Ras/Raf pero independiente de PI-3 cinasa, PKC y $p21^{rho}$.

Es escasa la información acerca del papel fisiológico de la activación de Erk1/Erk2 durante el hichamiento osmótico. Las MAP-cinasas representan puntos centrales de integración en las vías de señalización inducidas por factores de crecimiento, citocinas y diferentes formas de estrés ambiental. La activación de Erk1/Erk2 por hiposmolaridad resulta relevante en la regulación de la función celular. Se ha reportado que el estrés hiposmótico induce un incremento en la expresión de

genes de expresión inmediata como c-*fos* y c-*jun* posterior al aumento en la actividad de Erk1/Erk2 en células de hepatoma (Schliess et al., 1995) y en células cardiacas (Sadoshima et al., 1996), lo cual sugiere un papel para Erk1/Erk2 en la regulación de la transcripción y/o la sobrevivencia a largo plazo. La activación de la cascada de señalización Ras/Erk ha sido ampliamente reconocida como evento clave en la proliferación celular. Además la traslocación al núcleo de Erk1/Erk2 es un prerrequisito para la transición de G_0-S y para la división celular (Davis, 1993). En el hígado, la activación de Erk1/Erk2 tiene importantes implicaciones sobre el metabolismo celular y la expresión genética; el hichamiento celular actúa como una señal anabólica estimulando la síntesis de proteínas y glicógeno, inhibiendo simultáneamente la glicogenólisis y la proteolisis entre otros procesos (revisado en Häussinger y Schliess, 1999).

LIBERACIÓN DE TAURINA Y PI-3 CINASA

El tratamiento de las neuronas granulares con wortmannina (desde 100 nM hasta 1 μM), un conocido inhibidor de la PI-3 cinasa disminuyó potentemente (85%) la liberación de taurina (artículo V, fig. 5A). Sin embargo el LY294002, otro inhbidor de esta cinasa no tuvo ningún efecto sobre la liberación de este osmolito (artículo V, fig. 5B). La activación de PI-3 cinasa asociada al hichamiento ha sido reportada en glioma C6 y células intestinales (Sinning et al., 1997; Tilly et al., 1996a), en estas últimas la presencia de wortmannina previno la liberación de ^{125}I. La wortmannina y el LY294002 también bloquearon el DRV y la corriente de Cl^- en células de hepatoma (Feranchak et al., 1998). La discrepancia observada entre ambos inhibidores en el presente trabajo podría ser explicada por diferencias en la sensibilidad de las isoformas de esta cinasa estos fármacos, como sugiere un estudio realizado en neuronas sensoriales y simpáticas, donde el LY294002 resultó ser 100 veces menos potente que la

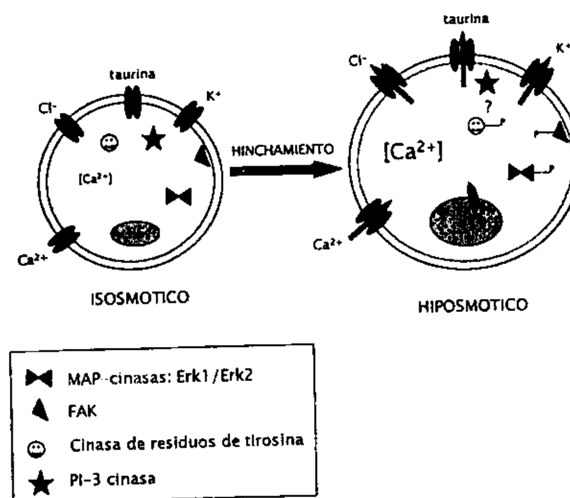
wortmannina para inhibir el transporte retrógrado del receptor a NGF (Reynolds et al., 1998; Bartlett et al., 1999).

Otra posible explicación de esta diferencia entre los dos bloqueadores de la PI-3 cinasa sobre la liberación de taurina en neuronas granulares, puede ser que la wortmannina no esté bloqueando a esta cinasa sino que su efecto se deba a la inhibición de alguna fosfolipasa. Un estudio realizado en fibroblastos mostró que la wortmannina es capaz de inhibir la actividad de la PLC, la PLD y la PLA_2 con una IC_{50} similar a la reportada para inhibir a la PI-3 cinasa (Cross et al., 1995). Estos resultados son relevantes, ya que existe evidencia que sugiere la participación de la PLA_2 en el mecanismo de liberación de taurina y Cl^- asociados a volumen en diferentes tipos celulares (Margalit et al., 1993; Toroed et al., 1997; Basavappa et al., 1998).

En células de neuroblastoma la inhibición de PLA_2 disminuye la liberación de Cl^-, taurina y ácido araquidónico inducida por hiposmolaridad (Basavappa et al., 1998). Por lo tanto, es necesario realizar un estudio más detallado sobre el papel de la PI-3 cinasa en la vía de liberación de taurina en neuronas granulares. Algunas estrategias pueden ser: 1) cuantificar la actividad de la enzima utilizando un substrato, 2) cuantificar el incremento de su producto fosforilado (fosfatidilinositol 3,4,5 trifosfato), 3) determinar la participación de otras proteínas que se sabe están relacionadas con la activación de esta enzima, como son FAK, Rho y src. Actualmente en el laboratorio se están realizando este tipo de estudios. Una perspectiva interesante resulta el hecho de que cambios en la organización del citoesqueleto sean la señal para la activación de los mecanismos correctores del volumen celular. Se ha propuesto que la PI-3 cinasa puede estar funcionando como sensor del hichamiento osmótico detectando la distensión de la membrana plasmática (Low et al., 1997).

Los resultados de la presente investigación indican que en células nerviosas de cerebelo de rata en cultivo, la respuesta en Ca^{2+} al medio hiposmótico no esta

relacionada con la activación de los mecanismos correctores del volumen celular y que éste evento podría ser simplemente un epifenómeno, como resultado de una mayor permeabilidad de las membranas celulares al Ca^{2+} ocasionada por el hichamiento. Además, se puede especular que la activación de Erk1/Erk2 por soluciones hiposmóticas sea una respuesta al estrés asociado al hichamiento y/o este relacionada a procesos metabólicos y transcripcionales relacionados con el mantenimiento de la homeostasis celular y no necesariamente con la activación de las vías para la liberación de osmolitos que participan en el DRV (esquema IV). En cuanto a la participación de la PI-3 cinasa, los resultados no son del todo concluyentes y no es posible hasta el momento, determinar qué papel este desepeñando esta cinasa en la vía de señalización involucrada en el proceso regulador (esquema IV).



Esquema IV. El hichamiento osmótico induce un incremento en la $[Ca^{2+}]i$, así como en la actividad de Erk1/Erk2. Sin embargo, ningúno de estos eventos está relacionado con la activación de los mecanismos correctores del volumen celular. Por otra parte, es evidente la implicación de alguna (as) cinasa (s) de tirosina en la liberación de taurina de neuronas granulares y es probable que FAK y PI-3 cinasa también participen en la vía de transducción subyacente al proceso de regulación del volumen en estas células.

CONCLUSIONES

- El aumento del volumen celular de astrocitos y neuronas granulares de cerebelo de rata en cultivo, inducido por un medio 30% y 50% hiposmótico, está asociado a un incremento en la $[Ca^{2+}]i$, el cual presenta dos fases: una respuesta inicial máxima transitoria seguida de una fase sostenida.

- El incremento de la $[Ca^{2+}]i$ inducido por hiposmolaridad, en astrocitos y neuronas granulares de rata en cultivo, es la consecuencia tanto de la liberación de Ca^{2+} de almacenes intracelulares como de la entrada de Ca^{2+} desde el medio extracelular.

- El Decremento Regulador del Volumen y la salida de taurina, Cl^- y K^+ que lo acompañan, no se modificó por la ausencia de Ca^{2+} extracelular.

- Las dihidropiridinas tienen un efecto inhibidor sobre la regulación del volumen y los flujos asociados de taurina, Cl^- y K^+. Este efecto sin embargo, no está relacionado con la acción de estos fármacos como bloqueadores de canales de Ca^{2+} sensibles a voltaje tipo L.

- La liberación de Ca^{2+} de pozas endógenas inducida por hiposmolaridad, no es un evento necesario para que se activen los flujos de taurina, Cl^- y K^+ durante la regulación del volumen de las células nerviosas de cerebelo de rata en cultivo.

- El incremento en la $[Ca^{2+}]i$ inducido por un medio hiposmótico, en células nerviosas de cerebelo de rata en cultivo, es sólo un epifenómeno asociado al aumento en el

volumen y no participa en la activación de los mecanismos que subyacen a la regulación del volumen celular.

- El estrés hiposmótico (30%) induce un incremento en la actividad de Erk1/Erk2, en neuronas granulares, este incremento se previene cuando MEK es inhibida con PD 98059.

- El incremento en la actividad de Erk1/Erk2 asociada al hichamiento no esta relacionada con la activación de la vía para la liberación de taurina en neuronas granulares.

- La wortmannina pero no el LY194002 inhibió la liberación de taurina en neuronas granulares. Es necesario realizar un estudio más detallado para determinar la participación de la PI-3 cinasa en la vía de señalización implicada en la liberación de este osmolito.

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ARTICULO 1

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Regulatory Volume Decrease and Associated Osmolyte Fluxes in Cerebellar Granule Neurons Are Calcium Independent

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To investigate a possible role for Ca as a transduction signal for regulatory volume decrease (RVD), the effects of external Ca removal, Ca channel blockers (Cd, Co, La, Gd, verapamil, diltiazem, dihydropyridines) and inhibitors of endoplasmic reticulum Ca release (dantrolene, ryanodine, TMB-8) were examined on RVD and on the swelling-activated efflux of two main osmolytes: Cl (traced by ^{125}I) and [^{3}H]taurine. Omission of Ca plus EGTA did not affect RVD or osmolyte release but when BAPTA was the chelator, RVD decreased 20%, ^{125}I fluxes were unaffected and taurine stimulated efflux decreased (20%) while the basal efflux slightly increased (<10%). Verapamil, diltiazem, Co, Cd, La and Gd did not affect RVD or osmolyte fluxes. Nimodipine and nitrendipine (25–50 μM) markedly decreased RVD and osmolyte fluxes (>90%) through a mechanism independent of extracellular Ca. Swelling elicited an increase in cytosolic Ca measured by fura-2, which was notably variable ranging 50–350 nM. However, RVD and osmolyte fluxes were not affected by the blockers of endogenous Ca release dantrolene, ryanodine and TMB-8 or by the permeable Ca chelator BAPTA-AM, even when the cytosolic Ca increase was abolished by the chelator. These results indicate that 1) RVD and osmolyte fluxes are independent of extracellular Ca 2) RVD, osmolyte release and cytosolic Ca raise are only coincident events. Consequently, Ca is unlikely to be a transducing signal for RVD in neurons. J. Neurosci. Res.47:144–154, 1997.
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Key words: intracellular Ca; taurine; swelling; Cl

INTRODUCTION

Cerebellar granule neurons exhibit regulatory volume decrease (RVD) subsequent to swelling in hyposmotic solutions (Pasantes-Morales et al., 1993). As in many other cell types, RVD in neurons is an active process accomplished by the extrusion of intracellular osmotic solutes, i.e., K, Cl, and a number of organic molecules (Pasantes-Morales et al., 1994). Outcoming evidence indicates that in neurons, the efflux of both organic and inorganic osmolytes occurs through channel-like molecules (Schousboe et al., 1991). Less is known about the signals and messengers that link the change in cell volume with the activation of the osmolyte efflux pathways. Calcium (Ca) has been repeatedly suggested as a likely candidate to play this role in numerous cell types, including brain astrocytes (McCarthy and O'Neil, 1992). Evidence on this respect is somewhat confusing, as RVD has been reported as completely independent or dependent on extracellular Ca, or dependent only on changes in intracellular Ca which in turn may or may not be dependent of extracellular Ca. The same apparent variability exists about the Ca dependence of the osmolytes involved in RVD (Bender and Norenberg, 1994; O'Connor and Kimelberg, 1993; Olson et al., 1990; Vitarella et al., 1994). Part of the difficulty in solving these questions is that the Ca dependence of the various components of cell volume regulation such as cell volume changes, efflux of osmolytes, changes in cell Ca levels, etc., has not always been examined in the same cell type. This is important because, in contrast to the similarities in the basic features of RVD such as time course, handled osmolytes and nature of the efflux pathways observed in various cell types, the transduction mechanisms appear different, and therefore the response of one cell type in this respect cannot be inferred from observations made in other cells.

In neurons, evidence relating Ca to RVD is scarce and controverted. In cultured cortical and cerebellar neurons and in neuroblastoma NIE115 cells, swelling elicits an increase in intracellular Ca which is dependent of

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external Ca whereas RVD has been found independent of external Ca in NIE115 cells and in cerebellar granule neurons (Falke and Misler, 1989; Sánchez-Olea et al., 1993). In order to clarify the influence of Ca during RVD in neurons, in the present study we examined in the same type of cells, namely cultured cerebellar granule neurons: 1) the change in intracellular Ca elicited by swelling in individual neurons, 2) the influence of removal of external Ca on RVD and on the efflux of the osmolytes taurine and ^{125}I (as tracer for Cl), and 3) the effect of conditions and drugs that potentially modify extracellular Ca fluxes and intracellular Ca levels on RVD and osmolyte fluxes.

MATERIALS AND METHODS

Materials

Fetal calf serum and penicillin/streptomycin were obtained from GIBCO (Grand Island, NY). Poly-L-lysine (molecular weight >300,000), trypsin, soybean trypsin inhibitor, DNAse, diltiazem, dantrolene, TMB-8 and verapamil were obtained from Sigma Chemical Corp. (St. Louis, MO). Nitrendipine and nimodipine were purchased from RBI (Natick, MD), BAPTA-AM, A23187 and ryanodine from Calbiochem (San Diego, CA) and ∝-Conotoxin MVIIC from Peptide Int. Inc. (Louisville, KY). Fura-2 AM and fura-2 pentapotassium salt were purchased from Molecular Probes (Eugene, OR). All other chemicals were of the purest grade available from regular commercial sources.

Cell Cultures

Cultures of cerebellar granule neurons were prepared as previously described by Morán and Patel (1988). Briefly, the dissociated cell suspensions from the cerebellum of 8-day-old rats were plated at a density of 265×10^3 cells/cm^2 in plastic dishes previously coated with poly-L-lysine (5 μg/ml). The culture medium contained basal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The culture dishes were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. When cultures were maintained for more than 2 days in vitro (DIV), they were added with both cytosine arabinoside (10 μM) at about 20 hr after seeding and 150 μM NMDA to increase cell survival (Balàzs et al., 1988). The enrichment of cultures with neurons was assessed by counting cells immunostained with polyclonal antibodies against neuron-specific enolase (Dakopatts, Carpinteria, CA). Cells stained with antibodies against glial fibrillar acidic protein (Dakopatts) were identified as glial cells. Our cultures contained about 95% neurons, from which 90% correspond to glutamatergic granule cells and the remaining 5% neurons represent GABAergic non granule cells, most likely stellate and basket neurons (Gallo et al., 1986).

Cell Volume

Cell volume measurements were made as described in Pasantes-Morales et al. (1993). Briefly, neurons cultured for 1–2 days were detached by treatment for 2–3 min with phosphate-buffered saline without calcium and containing 1 mM EDTA and 0.01% trypsin. Detached cells were mixed with the same volume of serum-containing medium, and then centrifuged and resuspended in isosmotic Krebs-HEPES medium. Then, an aliquot of the cell suspension was diluted approximately 100-fold with media. Exactly 1 min later, cell volume was measured at the indicated times using a Coulter cell counter (model ZF) associated to a Coulter channelizer (model 256). Cell volumes were measured in picoliters but results were routinely expressed as relative volume, i.e. the change with time over the initial volume recorded in isosmotic medium. HEPES-buffered isosmotic medium contained (in mM) NaCl, 135; KCl, 5; $MgSO_4$, 0.6; $CaCl_2$, 1; KH_2PO_4, 1.7; glucose, 10; HEPES, 10; pH 7.4. The osmolarity calculated was 320 mOsm/kg medium and was verified in a freezing point osmometer. Hyposmotic solutions were always prepared by reducing the concentration of NaCl.

Release

For osmolyte release experiments (Pasantes-Morales et al., 1993; Sánchez-Olea et al., 1996), cultured cells were loaded by incubation at 37°C with either [^3H]-taurine (20–40 Ci/mmol; 2.5 μCi/ml) for 1 hr or ^{125}I (17 Ci/mg; 1 μCi/ml) for 15 min in the culture medium. After loading, cells were washed three times (5 min each) with isosmotic medium. For [^3H]-taurine release experiments, cells were first incubated for 5 min in isosmotic medium and then for 5 min in 50% hyposmotic medium. The efflux of ^{125}I was measured by collecting fractions every 20 sec during 2.5 min in isosmotic medium and 2.5 min in hyposmotic media. At the end of the experiment, radioactivity in the collected fractions and that remaining in cells was measured by scintillation spectrometry. Results are expressed as the percent of radioactivity released at each incubation period of the total radioactivity accumulated by cells during loading, excluding the washing period. In some experiments on ^{125}I release, results are expressed as efflux rate coefficients as previously described (Venglarik et al., 1990; Sánchez-Olea et al., 1996). In all parallel experiments control cells were treated with the vehicle used to prepare solutions containing the tested compounds.

Intracellular Ca Measurements

Methods are described in detail elsewhere (Hernández-Cruz et al., 1995). Briefly, a glass coverslip con-

taining cultured cerebellar neurons was transferred to a recording chamber (Warner Instruments, Mod. RC-25, Hamden, CT) on an inverted microscope (Nikon Diaphot TMD, Nikon Corp., Japan). Cells were loaded with fura-2 by incubation with the acetoxymethyl (AM) ester form of the dye fura-2/AM (Molecular Probes), at a final concentration of 1 μM, with no dispersing agents added. Cells were allowed to load for 30 min at room temperature and then rinsed for another 15 min before the beginning of the experiments. Ca levels were determined by recording pairs of images using alternating illumination with 340 nm and 380 nm excitation. Dual wavelength excitation was provided by two nitrogen pulsed lasers (3 ns pulse duration), one emitting at its natural wavelength of 337 nm and the second one dye-tuned at 380 nm. These lasers were triggered alternatively at frequencies ranging from 2 to 15 Hz under computer control. (Biolase Imaging System, Newton, MA). Background images at 340 and 380 nm illumination were obtained from an area of the coverslip free of cells. These images were stored separately and used for on-line background subtraction. We found that a correction for cell autofluorescence was not essential for calibration purposes. The key elements of the fluorescence Ca imaging system were a high numerical aperture UV objective (Nikon UV-F 100×, 1.3 N.A.), an intensified charge coupled device camera (c2400–87, Hamamatsu, Bridgewater, NJ) and the Biolase Imaging System running under their FL-2 software. The system allows real-time simultaneous acquisition of fluorescence measurements from multiple areas of interest placed on individual cells. Ca concentrations were calculated from fluorescence measurements at 340 and 380 nm excitation wavelengths using the formula:

$$[Ca] = KD\ (Ff/Fb)\ (R - Rmin)/(Rmax - R),$$

where the calculated dissociation constant (KD) for fura-2 is 300 nM, Ff/Fb is the ratio of fluorescence values for Ca-free/Ca-bound indicator at 380 nm excitation, R is the ratio of fluorescence at 340/380 nm for the unknown [Ca], and Rmin and Rmax are the ratio of fura-2 fluorescences at 340/380 nm of Ca-free and Ca-bound fura-2. The values of Ff/Fb, Rmin, and Rmax were determined by measuring under identical conditions the fluorescence of a glass capillary 100 μm in diameter containing calibration solutions with 100 μM fura-2 pentapotassium salt (Molecular Probes), and known Ca concentrations in the range from 10 nM to 40 μM. Although in situ calibrations were attempted in initial experiments, we found very difficult to manipulate [Ca] over the required range. Our Ca measurements, based exclusively in the in vitro calibrations, could be underestimated to some extent because of effects of viscosity and dye binding to cytoplasmic constituents (Konishi et al., 1988). Cells were continuously superfused with a recording solution containing (in mM) NaCl 130, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 1, NaH_2PO_4 0.5, HEPES-Na 5, and glucose 5 (pH 7.4). Hyposmotic test solutions were pressure-applied (10 psi) via a puffer pipette located within 100 μm from the cells under examination, or by superfusing the whole recording chamber. Puffer application of the hyposmotic solution was controlled by the solenoid valve of a Picospritzer II device (General Valve, Fairfield, NJ). Control experiments showed that with this procedure, the external medium surrounding the cell(s) was replaced within less than 150 msec. Fura-2 experiments were carried out at 22–23°C.

RESULTS

Effect of Changes of External Ca

Regulatory volume decrease. Figure 1A shows the time course of RVD in cerebellar neurons exposed to 50% hyposmotic medium which is similar to that previously described (Pasantes-Morales et al., 1994). At the time of the experiment, volume recovery is not complete and the efficiency of the regulatory process is about 60%. When RVD was measured in Ca-free medium containing 0.5 mM EGTA (preincubated during 10 min), the extent and time course of RVD was not significantly changed (Fig. 1A). No difference was observed when cells were preincubated up to 20 min with the EGTA, Ca-free media (not shown). When cells were preincubated with 0.5 mM BAPTA and RVD measured in the presence of the chelator, there was an inhibition of 20%–25% in the volume recovery at 10–16 min as compared to control (Fig. 1A). The same reduction in RVD efficiency was observed at lower concentrations of BAPTA (0.1–0.2 mM). This inhibitory effect of BAPTA was not observed when the medium contained 0.2 mM BAPTA and 1.5 mM Ca (not shown) excluding a direct pharmacological effect of BAPTA. RVD was unaffected by the addition of divalent cations Co and Cd (0.5 mM) or by the trivalent cations Gd (30 μM) and La (0.5 mM), which are potent antagonists of voltage-gated transmembrane Ca transport (Table I). The DHPs nitrendipine and nimodipine, specific blockers of the voltage-dependent L-type Ca channels, exhibited an inhibitory effect on RVD (Table I). This effect, however, was independent of the presence of extracellular Ca, as the observed inhibition of RVD was the same in nominally Ca-free medium containing 0.5 mM EGTA (not shown). Other blockers of L-type Ca channels, verapamil and diltiazem did not affect RVD (Table I).

^{125}I release. Cerebellar granule neurons responded to cell swelling elicited by 50% hyposmotic solutions with an increase in ^{125}I efflux which is rapid in onset and offset. During the activation of ^{125}I fluxes

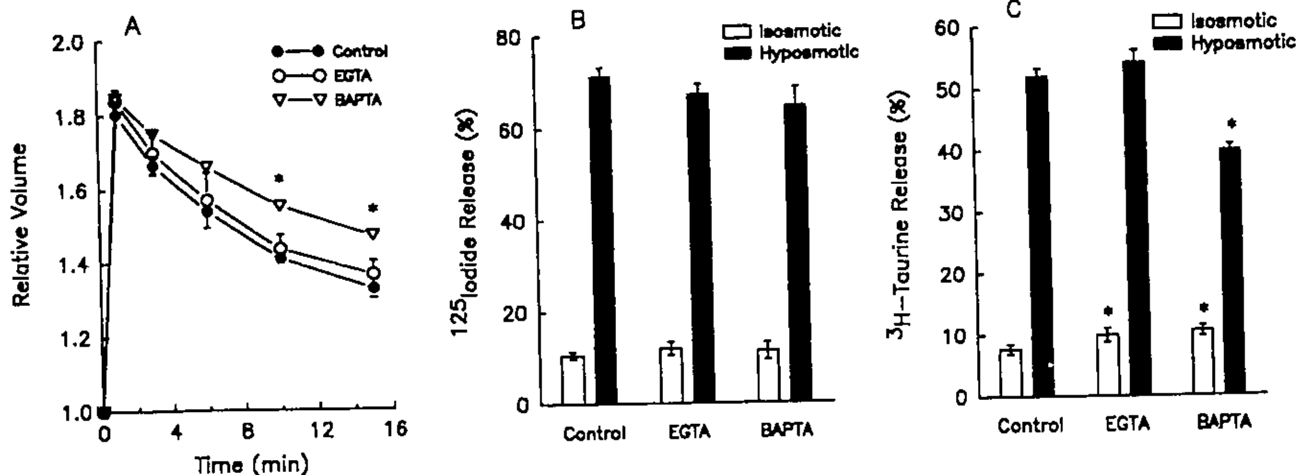

Fig. 1. Effect of calcium chelators on RVD (A) and the release of ^{125}I (B) and [^{3}H]-taurine (C) induced by hyposmolarity in cultured neurons. Cell volume was measured in cerebellar granule neurons after 1–2 days in culture, detached by treatment with trypsin. Cells were preincubated during 5–10 min with 0.5 mM EGTA or BAPTA before cell volume assay. Results are expressed as relative volume considering cell volume in isosmotic conditions as 1. For release experiments cells loaded with [^{3}H]-taurine were incubated for 5 min with isosmotic medium and 5 min with 50% hyposmotic medium. ^{125}I release was measured by collecting fractions every 20 sec during 2.5 min in isosmotic and 50% hyposmotic media. Results are expressed as radioactivity released under isosmotic and hyposmotic conditions as the percent of total radioactivity in cells after washing. Results are means of 6–18 experiments with SEM values shown as vertical bars if they extend beyond the symbols. Asterisk indicates statistically significant difference from control ($P<0.001$) using Student's *t*-test.

TABLE I. Effect of Ca^{2+} Channel Blockers on Hyposmolarity Stimulated ^{125}I Release and RVD in Cultured Cerebellar Granule Cells

			^{125}I Efflux (%)	
Inhibitor	μM	RVD (%)	Basal	Stimulated
None		52.7 ± 2.2	7.5 ± 0.4	78.9 ± 1.6
Verapamil	100	51.4 ± 1.6	8.5 ± 1.4	77.8 ± 0.0
Diltiazem	50	52.9 ± 2.5	8.6 ± 0.1	77.6 ± 0.1
ω-ctx-MVIIC	0.5	—	8.3 ± 1.2	74.6 ± 0.1
La^{3+}	500	—	8.0 ± 1.0	76.8 ± 3.0
Cd^{2+}	500	41.6 ± 0.8*	8.3 ± 0.5	78.4 ± 0.3
Co^{2+}	500	50.7 ± 1.1	6.7 ± 0.7	79.1 ± 0.4
Gd^{3+}	30	49.3 ± 1.5	7.3 ± 0.2	71.4 ± 3.7
Nitrendipine	10	33.5 ± 3.0*		
	25	20.4 ± 4.0*		
Nimodipine	10	44.6 ± 3.5*		
	25	31.9 ± 2.5*		

Cells were loaded with ^{125}I and preincubated 10 min with the indicated concentrations of drugs. Cells were washed and release was measured. Cells were exposed to isosmotic and then to 50% hyposmotic medium, and five fractions of each medium were collected at 20 sec intervals during 5 min. For RVD cells were resuspended in isosmotic medium and then the volume regulation was measured in 50% hyposmotic media containing the indicated concentrations of the drugs. Results are expressed as percentage of RVD, i.e., the percentage of recovery of cell volume between min 1 (maximal volume) and min 15. Values are means ± SE of 6–10 experiments. Asterisk indicates statistically significant difference from control ($P<0.001$) using Student's *t*-test.

evoked by cell swelling (50% hyposmotic medium), about 80% of the total ^{125}I accumulated during loading was released (Fig. 1B). This release pattern of ^{125}I is essentially similar to that previously described (Sánchez-Olea et al., 1996). Removal of external Ca, using the chelators EGTA (0.5 mM) and BAPTA (0.5 mM), did not significantly modify the amount of ^{125}I release, although a tendency to decrease ^{125}I efflux in the presence of BAPTA was observed (Fig. 1B). The efflux of ^{125}I elicited by 15% and 30% hyposmotic solutions was similarly unaffected by removal of extracellular Ca (not shown). Table I shows that Co, Cd, Gd and La did not affect significantly the ^{125}I efflux associated to cell volume regulation. Diltiazem and verapamil were also ineffective (Table I). None of these compounds affected the basal release of ^{125}I in isosmotic conditions. The DHPs nitrendipine and nimodipine exerted a significant inhibitory effect of ^{125}I efflux activated by hyposmolarity. At 50 μM ^{125}I release was inhibited by more than 90% (Fig. 2). The inhibitory effect of DHP was essentially identical in Ca-free media containing 0.5 mM EGTA (Fig. 2).

[^{3}H]Taurine release. In accordance with previous observations (Pasantes-Morales et al., 1993), cerebellar granule neurons preloaded with [^{3}H]taurine and then exposed to 50% hyposmotic solutions responded with a rapid and marked release of the amino acid following the time course of RVD. About 54% of the taurine pool was released during the first 5 min of exposure to the hyposmotic medium and up to 80% was lost at the end of the volume regulatory process (Fig. 1C). Removal of external Ca by addition of the chelator EGTA (0.5 mM) did

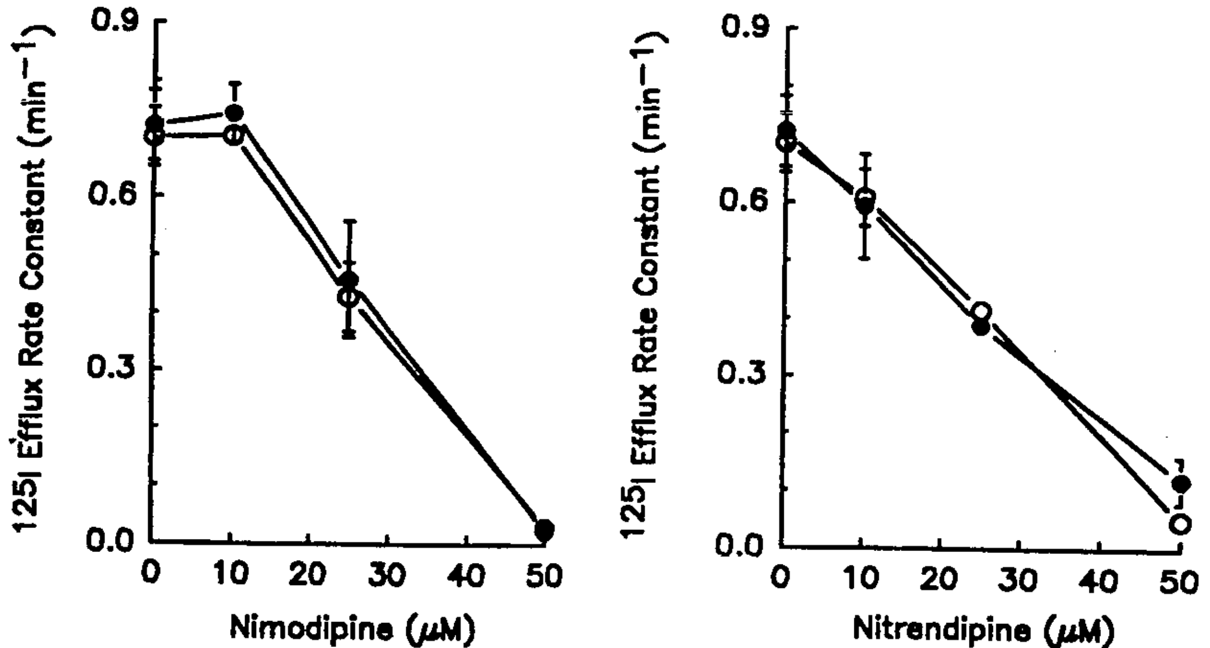

Fig. 2. Effect of nimodipine and nitrendipine on the release of ^{125}I stimulated by hyposmolarity in cultured neurons. Cells loaded with ^{125}I for 15 min were preincubated with nimodipine or nitrendipine for 5 min at the indicated concentrations. After washing cells, fractions were collected every 20 sec during 2.5 min in isosmotic and 50% hyposmotic media containing the same drug concentration. Results are expressed as efflux rate coefficients as described in Materials and Methods. Results are means of six experiments with SEM values shown as vertical bars if they extend beyond the symbols. ●, solutions with 1 mM Ca; ○, solutions with no Ca plus 0.5 mM EGTA.

not significantly affect [^{3}H]taurine release (Fig. 1C). Similarly, no effects were observed when the cells were preincubated in the presence of the chelator in Ca free media during 10–30 min (not shown). Both BAPTA and EGTA induced a small increase in the basal (isosmotic) release of [^{3}H]taurine (Fig. 1C) and BAPTA, but not EGTA, inhibited by 20% the efflux of [^{3}H]taurine (Fig. 1C). As for RVD and ^{125}I efflux, Co, Cd (0.5 mM) and Gd (30 µM) did not reduce [^{3}H]taurine release associated to cell swelling (Table II). Lanthanum at a concentration of 0.1 mM was ineffective and at 0.5 mM reduced [^{3}H]taurine release by 30% (Table II). Diltiazem and verapamil had no effect on [^{3}H]taurine release (Table II). DHPs exhibited a marked concentration-dependent inhibition of the volume sensitive [^{3}H]taurine release, with maximal inhibition (>95%) at 50 µM (Fig. 3). At any given concentration, DHPs affected the release of [^{3}H]taurine in isosmotic conditions (Fig. 3). In media with no Ca added and containing 0.5 mM EGTA, the effect of DHPs was essentially the same as in media with Ca (Fig. 3).

All the above results were obtained from neurons of 1–2 days in culture. No difference regarding the effect of the examined conditions and drugs was observed in neurons of 7–8 days in culture (not shown).

Changes in Intracellular Ca

The effect of an hyposmotic solution on cell swelling and intracellular Ca levels of granular neurons was examined using the fluorescent probe fura-2 (Figs. 4 and 5). Typically, fluorescence measurements at 340 and 380 nm excitation were collected simultaneously at intervals ranging from 0.2 to 0.5 sec from several cells selected by drawing areas of interest on the phase contrast image. The selected cells were first challenged with a 1 sec puff of a high K+ depolarizing solution (140 mM KCl, 10 mM HEPES and 2 mM $CaCl_2$, pH 7.4) delivered from a nearby pipette. Figure 4C shows the simultaneous recording of [Ca]i changes in 12 individual cells morphologically identified as granular neurons. All cells responded to the application of a high K+ solution, suggesting that they possess functional voltage-gated Ca channels, and therefore are likely to be cerebellar neurons. These changes were absent in cells morphologically identified as glial cells. They were also absent in nerve cells if Ca was omitted from the depolarizing solution (data not shown). Spontaneous fluctuations of [Ca]i were not observed in these cultures. As shown in Figure 4A, when a 50% hyposmotic solution was applied with a second puffer pipette, a moderate cell swelling was produced (judged by the decrease in fluorescence

TABLE II. Effect of Ca^{2+} Channel Blockers on the Hyposmolarity Stimulated [^{3}H]-Taurine Release in Cultured Granule Cells

		[^{3}H]-Taurine efflux (%)			
		With Ca^{2+} external		Without Ca^{2+} external	
Inhibitor	μM	Basal	Stimulated	Basal	Stimulated
None		7.7 ± 0.1	54.1 ± 0.4	10.3 ± 0.2	53.8 ± 0.2
Verapamil	100	8.5 ± 1.5	63.0 ± 2.0	10.3 ± 1.1	58.7 ± 2.2
	250	8.0 ± 1.0	59.0 ± 1.8	9.4 ± 1.0	59.8 ± 1.5
Diltiazem	50	8.7 ± 0.5	54.6 ± 2.0	10.6 ± 0.7	53.3 ± 1.4
ω-ctx-MVIIC	0.5	6.7 ± 0.3	58.2 ± 1.0		
La^{3+}	500	5.6 ± 0.6	38.1 ± 2.0*		
Cd^{2+}	500	6.0 ± 0.7	61.6 ± 1.5		
Co^{2+}	500	6.5 ± 0.7	63.4 ± 1.7		
Gd^{3+}	30	8.3 ± 0.6	54.9 ± 1.4		

Cells were preloaded with [^{3}H]-taurine and then preincubated during 10 min with the indicated concentrations of the drugs and subsequently a 5 min period in isosmotic or 50% hyposmotic medium was collected; results are expressed as % [^{3}H]-taurine released. Values are averages ± SE of 6–10 different experiments. Asterisk indicates statistically significant difference from control (P <0.001) using Student's t-test.

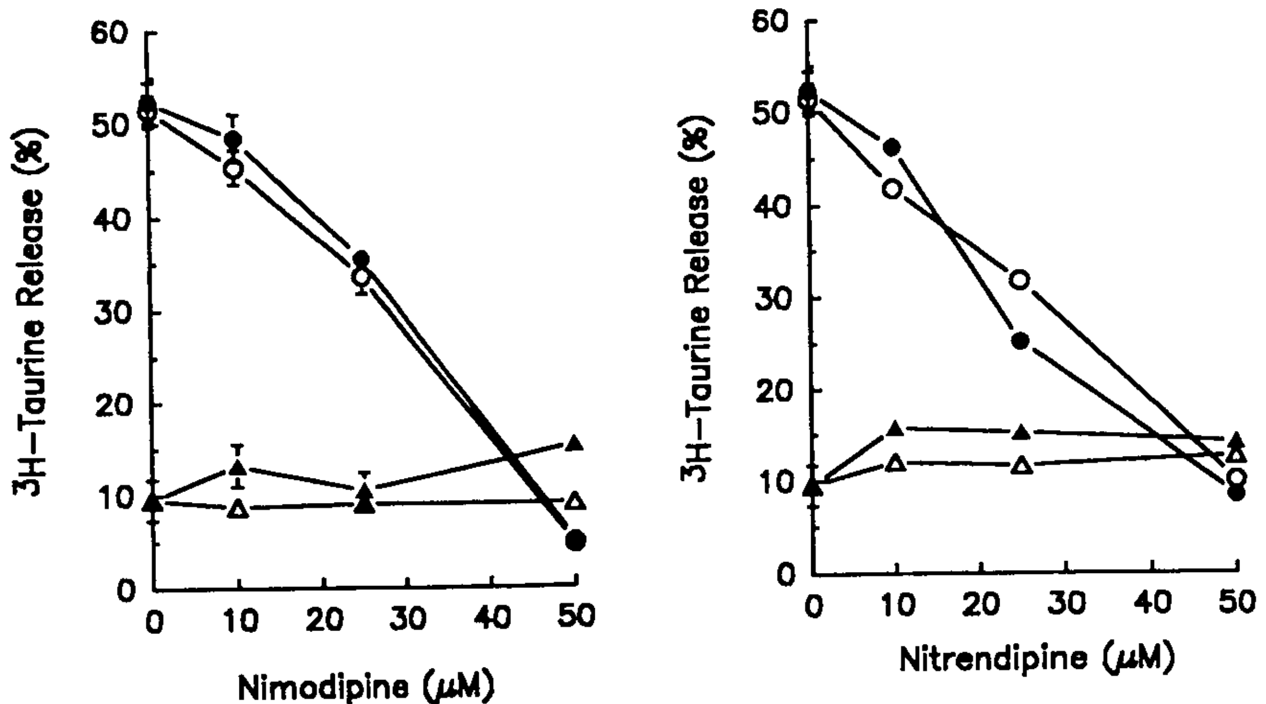

Fig. 3. Effect of nimodipine and nitrendipine on the release of [^{3}H]-taurine stimulated by hyposmolarity in cultured neurons. Cells loaded with [^{3}H]-taurine for 1 hr were preincubated during 5 min with nimodipine or nitrendipine at the indicated concentrations. After washing with isosmotic medium cells were incubated for 5 min with isosmotic medium and 5 min with 50% hyposmotic medium in the presence of the same concentrations of the drugs. Results are expressed as radioactivity released under isosmotic (triangles) and hyposmotic (circles) conditions as the percent of total radioactivity in cells after the washing period. Results are means of eight experiments with SEM values shown as vertical bars if they extend beyond the symbols. ●, hyposmotic, 1 mM Ca; ○, hyposmotic, no Ca plus 0.5 mM EGTA; ▲, isosmotic, 1 mM Ca; △, isosmotic, no Ca plus 0.5 mM EGTA.

recorded at the 380 nm excitation), but no detectable changes in [Ca]i were observed. To ensure a complete replacement of the external medium with the hyposmotic solution, the entire recording chamber was then rapidly superfused (flow rate, 3 ml/min) with the same hyposmotic solution. This produced a more severe cell swell-

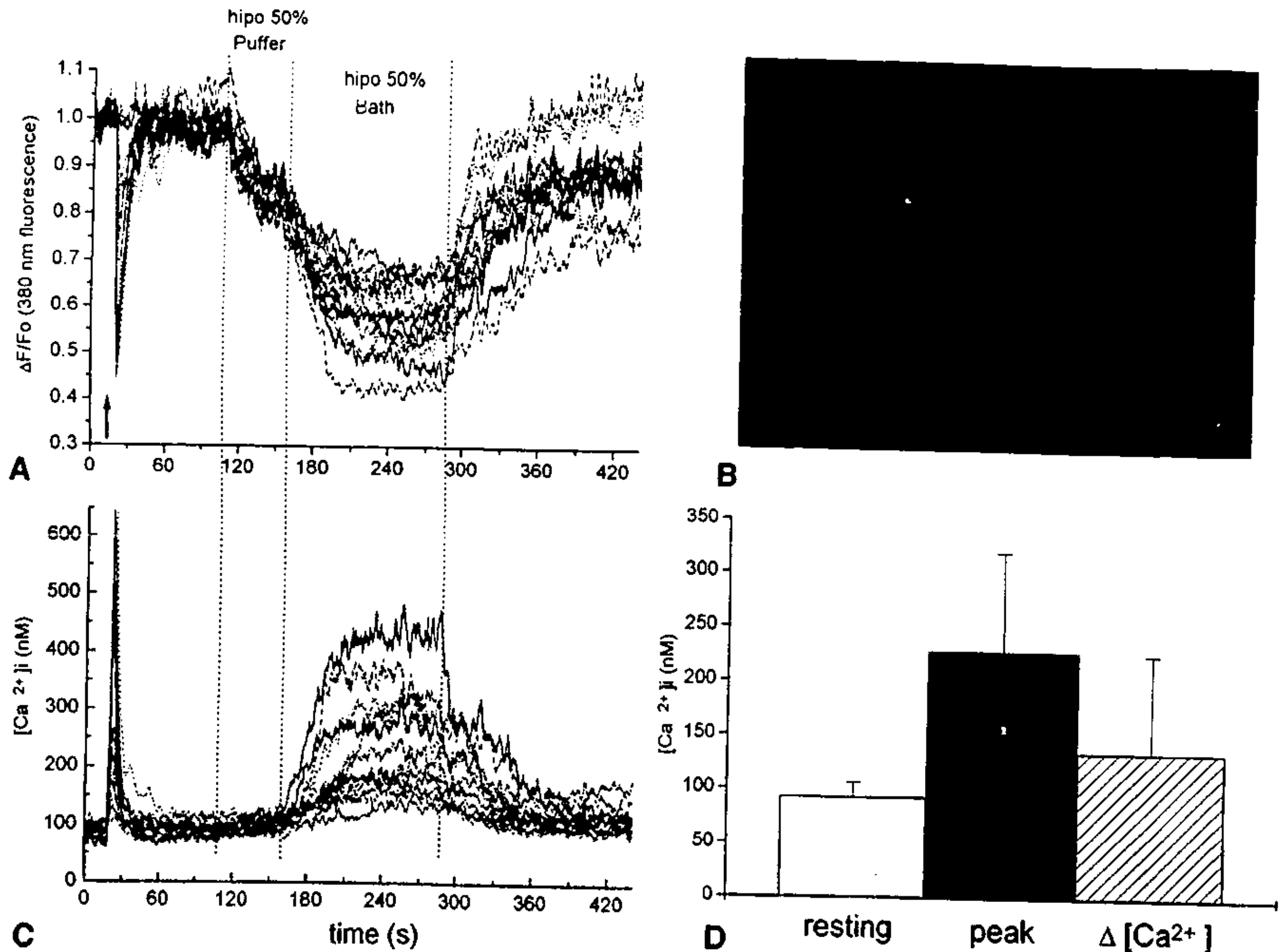

Fig. 4. Hyposmotic-induced changes in intracellular Ca concentration in cultured cerebellar granule neurons. Fluorescence changes (340/380 nm excitation) were simultaneously recorded in 12 individual cerebellar granule cells chosen from the field whose micrograph is shown in **B. A:** Normalized fluorescence changes (380 nm excitation) obtained from areas of interest centered on individual cells. **C:** Ca concentration changes occurring at each individual cell, calculated from the 340/380 nm fluorescence ratio (see Materials and Methods). The cells were first challenged with a 1 sec puff of a high K solution delivered by pressure from a nearby micropipette (arrow). Later, a puff of 50% hyposmotic saline was delivered with a second pipette an then the entire recording chamber was perfused with hyposmotic solution. This produced the changes in Ca concentration shown in C. **D:** Mean Ca concentration values obtained from granular cells at rest and at the peak of the swelling-induced Ca rise (n = 22).

ing, followed by [Ca]i increases in most, but not all neurons (Fig. 4C). The increase in intracellular Ca ranged from 20 nM to about 350 nM. In all cases the increase in intracellular Ca levels induced by hyposmolarity was abolished when cells were preincubated 10 min with 10 μM BAPTA-AM (Fig. 5). The following experiments were carried out to determine if the increase in [Ca]i observed in granular neurons upon cell swelling is important for osmotic regulation.

Effect of drugs affecting intracellular Ca on RVD and ^{125}I and [^{3}H]taurine fluxes. The effects of compounds known to modify intracellular Ca pools on RVD and osmolyte fluxes were examined. The compounds used were the permeable Ca chelator BAPTA-AM (30 μM) and the blockers of Ca release from endoplasmic reticulum dantrolene (30 μM), TMB-8 (100 μM), and ryanodine (1 μM). RVD was not significantly affected by BAPTA-AM (Table III), which completely blocks intracellular Ca increases induced by hyposmolarity (Fig. 5) without affecting cell swelling (Fig. 5D). This chelator marginally (11%) reduced the swelling-stimulated efflux of taurine without affecting the basal release (Table III). BAPTA-AM also inhibited by 16% the volume-activated efflux of ^{125}I and increased about twofold the basal efflux (Table III). None of the blockers of endogenous Ca release, dantrolene, TMB-8, and ry-

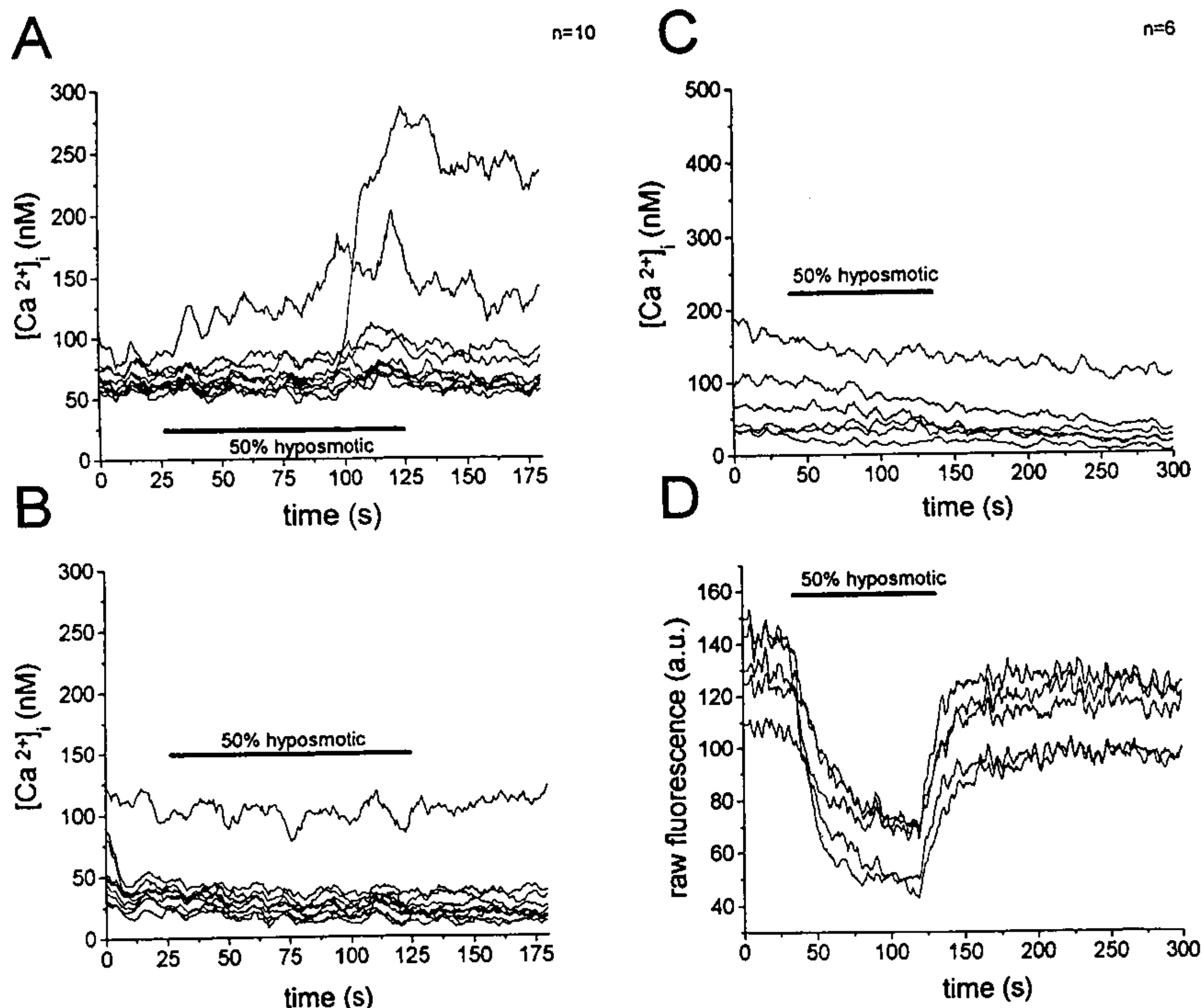

Fig. 5. Effect of BAPTA-AM on the swelling-induced Ca rises in cultured cerebellar neurons. **A:** Ca concentration changes elicited by a 90 sec exposure to an hyposmotic solution were recorded from 10 individual granule neurons. The responses were small and became more apparent towards the end of the pulse. **B:** Responses from the same group of cells after 10 min incubation with 10 μM BAPTA-AM. Notice that except for one cell that responded with a Ca increase in A, all remaining cells reduced their resting Ca concentration. In addition, swelling-induced Ca rises were completely abolished by the Ca chelator. **C:** In a different group of six cells from the same dish (after BAPTA-AM incubation), the complete absence of Ca rises upon exposure to the hyposmotic challenge, in spite of the significant volume changes recorded as fluorescence decreases (380 nm excitation) due to fura-2 dilution (**D**).

anodine, significantly affected RVD or osmolyte fluxes (Table III).

The effect of the Ca ionophore A23187 on taurine and ^{125}I release in isosmotic conditions was examined to investigate whether an increase in intracellular Ca by itself was able to activate the efflux of osmolytes associated to RVD. The ionophore elicited a small increase in taurine efflux, from 0.2% to 0.35%. Nevertheless, this efflux was negligible as compared to that observed during cell swelling induced by hyposmotic conditions (9.5% at the peak release). ^{125}I efflux was unaffected by the ionophore (results not shown).

DISCUSSION

RVD in cerebellar granule neurons from primary cultures was found independent of extracellular Ca as the time course and efficiency of volume regulation remained essentially unchanged in Ca-free medium containing EGTA as chelator as a nonsignificant decrease of

TABLE III. Effect of Drugs Influencing Cytosolic Ca^{2+} on Hyposmolarity Induced [^{3}H]-Taurine and ^{125}I Efflux and RVD, Using Cultured Granule Cells

Condition	μM	[^{3}H]-Taurine efflux (%)		^{125}I Efflux (%)		RVD(%)
		Basal	Stimulated	Basal	Stimulated	
None		6.9 ± 0.5	53.5 ± 0.4	7.2 ± 0.8	77.0 ± 2.1	58.4 ± 2.3
BAPTA-AM	30	6.4 ± 0.2	47.9 ± 1.2*	16.0 ± 1.0*	73.9 ± 0.5	58.2 ± 1.8
Dantrolene	50	6.6 ± 0.8	49.0 ± 4.5	4.9 ± 0.1	82.0 ± 0.3	50.2 ± 1.4
TMB-8	100	7.9 ± 0.1	55.2 ± 1.6	14.9 ± 0.3*	74.7 ± 3.7	51.8 ± 2.5
Ryanodine	1	6.6 ± 0.5	54.5 ± 0.7	6.8 ± 0.6	67.3 ± 3.5	60.9 ± 1.8

Cells were loaded with ^{125}I or [^{3}H]-taurine and preincubated 10 min with the indicated concentrations of drugs. Cells were washed and release was measured. Cells were exposed to isosmotic and then to 50% hyposmotic medium, and five fractions of each medium were collected at 20 sec intervals during 5 min for ^{125}I and a 5 min period in isosmotic or hyposmotic medium for [^{3}H]-taurine; results are expressed as % tracer released. Values are means ± SE of 6–16 experiments. Asterisk indicates statistically significant difference from control ($P < 0.001$) using Student's *t*-test.

no more than 7–8% on RVD efficiency was observed. However, this decrease was higher (25%) when BAPTA was used as chelator. These effects of the chelators may be related to possible actions on surface charges in the membrane conducing to ion leakage leading to cell swelling which would counteract the cell volume decrease. In fact, small increases in the basal efflux of osmolytes were also observed under these conditions. The lack of effect on RVD or osmolyte fluxes of divalent cations, which are general antagonists of transmembranal Ca transport, further supports the independence of these processes of extracellular Ca. Drugs such as verapamil and diltiazem which block the L-type Ca channels were also without effect on RVD. Only the DHP exhibited a marked, dose-dependent inhibitory effect on RVD. However, this inhibitory action is most likely unrelated to Ca entry via the L-type channels, since 1) the inhibitory concentrations are larger than those required for Ca channel inhibition, 2) as mentioned above, other L-type Ca channel blockers such as verapamil and diltiazem were ineffective, and 3) the DHP inhibition occurs even in the absence of extracellular Ca. Other reports addressed to the Ca dependence of RVD in neurons are that of Falke and Misler (1989) in N1E115 neuroblastoma cells and ours in cerebellar granule neurons in which the volume regulatory process was found unaffected by removal of extracellular Ca, in agreement with the present results. In accordance with this Ca independence of RVD, the associated fluxes of ^{125}I and taurine in these cells were also essentially unaffected by all maneuvers directed to decrease extracellular Ca availability for volume regulation, with the exception of the DHP which also exhibited a marked inhibitory effect on osmolyte fluxes, independent of extracellular Ca, in total agreement with results on RVD. A report by Bassavappa et al. (1995) on CHP-100 neuroblastoma cell line describes inhibition of ^{125}I fluxes in the presence of a very high concentration (2.5 mM) of EGTA. However, no inhibition by blockers of Ca channels such as Ni, diltiazem, and the o-conotoxin GVIA could be observed. An indirect evidence of the Ca-independence of osmolyte release in cerebellar granule neurons is the lack of effect of increasing cytosolic Ca by the ionophore A23187, which failed to elicit any efflux of [^{3}H]taurine and ^{125}I in isosmotic conditions. This result contrasts with that of Hoffmann et al. (1986) in Ehrlich ascites cells, in which the ionophore markedly activates the efflux of taurine in isosmotic medium, with properties similar to those of the efflux activated by hyposmolarity.

In most cell types, swelling consistently elicits a rise in intracellular Ca levels with increases reported between 35 to 400 nM (McCarthy and O'Neil, 1992). A correlation between RVD and this increase in cytosolic Ca has not been conclusively established, mainly because the features of the two events have not been examined in parallel. This has been done in the present study in cerebellar granule neurons, in which the effects of conditions and drugs affecting changes in intracellular Ca elicited by swelling, have been examined also on the time course and efficiency of RVD, and on the osmolyte fluxes which subserve it. In agreement with our previous work using fluo-3 to measure Ca changes in a population of neurons in monolayer (Sánchez-Olea, et al., 1993), an increase in intracellular Ca associated to cell swelling was observed in the present study, measuring Ca levels with fura-2 in individual neurons. This single-cell measurement approach revealed a marked variability in the response among the different cells, which was not clearly related to the extent and rate of cell swelling but makes difficult accurate quantitative studies on the effect of Ca channel blockers. The origin of this cytosolic Ca increased upon swelling in cerebellar granule neurons is unclear. It may come either from an increased permeability to cytosolic Ca through the membranes stretched by swelling, or from endogenous store sites activated by volume or/and voltage changes associated to cell swell-

ing. In our previous work in cerebellar granule neurons (Sánchez-Olea et al., 1993) it was shown that the increase in intracellular Ca was largely dependent of extracellular Ca, suggesting that swelling induces a change in cell membrane permeability to Ca. The nature of this Ca influx is still not well characterized since it is unaffected by blockers of voltage-dependent Ca channels such as La and verapamil. In neuroblastoma cells CHP-100, the P- and Q-type Ca channel blocker o-conotoxin MVIIC was found to prevent the increase in intracellular Ca raised by swelling (Basavappa et al., 1995) but the high concentration used (10 μM), which is many times higher than that required to block these channels, makes it difficult to explain its mechanism with basis on the inhibition of Ca-channels.

In any event, and whatever the source and mechanism of the increased cytosolic Ca might be, it seems not to be a requirement for RVD as revealed by the insensitivity of cell volume regulation and osmolyte fluxes to the numerous drugs tested in the present work directed to block both Ca influx and the raise in intracellular Ca. Moreover, the failure of the permeable Ca chelator BAPTA-AM to modify RVD or osmolyte fluxes, even when it fully prevented the rise in cytosolic Ca, is an indication that this event in neurons is unrelated to the activation of the volume corrective mechanisms and is simply an epiphenomenon, resulting from a passive change in Ca permeability of cell membranes. Therefore, it is unlikely that in cerebellar granule neurons Ca is a signal implicated in the mechanisms transducing the change in cell volume and the activation of the osmolyte efflux pathways by which RVD is accomplished.

An increase in intracellular Ca coincident with Ca independence of RVD has been also reported in cultured rabbit medullary cells (Perry and O'Neill, 1994) and in rat salivary gland acinar cells (Tilly et al., 1994) whereas in other cell types, the two events appear associated. It seems therefore that whereas in most cell types the basic features of RVD such as extent, time course, osmolytes and nature of the corrective fluxes are remarkably similar, the signals and messengers which link the change in cell volume and the activation of osmolyte extrusion may be different in the different cell types.

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ARTICULO 2

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Osmotic Swelling-Induced Changes in Cytosolic Calcium Do Not Affect Regulatory Volume Decrease in Rat Cultured Suspended Cerebellar Astrocytes

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Abstract: Hyposmotic swelling-induced changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and their influence on regulatory volume decrease (RVD) were examined in rat cultured suspended cerebellar astrocytes. Hyposmotic media (50 or 30%) evoked an immediate rise in $[Ca^{2+}]_i$ from 117 n*M* to a mean peak increase of 386 (50%) and 220 n*M* (30%), followed by a maintained plateau phase. Ca^{2+} influx through the plasmalemma as well as release from internal stores contributed to this osmosensitive $[Ca^{2+}]_i$ elevation. Omission of external Ca^{2+} or addition of Cd^{2+}, Mn^{2+}, or Gd^{3+} did not reduce RVD, although it was decreased by La^{3+} (0.1–1 m*M*). Verapamil did not affect either the swelling-evoked $[Ca^{2+}]_i$ or RVD. Maneuvers that deplete endoplasmic reticulum (ER) Ca^{2+} stores, such as treatment (in Ca^{2+}-free medium) with 0.2 μM thapsigargin (Tg), 10 μM 2,5-di-*tert*-butylhydroquinone, 1 μM ionomycin, or 100 μM ATP abolished the increase in $[Ca^{2+}]_i$ but did not affect RVD. However, prolonged exposure to 1 μM Tg blocked RVD regardless of ER Ca^{2+} content or cytosolic Ca^{2+} levels. Ryanodine (up to 100 μM) and caffeine (10 m*M*) did not modify $[Ca^{2+}]_i$ or RVD. BAPTA-acetoxymethyl ester (20 μM) abolished $[Ca^{2+}]_i$ elevation without affecting RVD, but at higher concentrations BAPTA prevented cell swelling and blocked RVD. We conclude that the osmosensitive $[Ca^{2+}]_i$ rise occurs as a consequence of increased Ca^{2+} permeability of plasma and organelle membranes, but it appears not relevant as a transduction signal for RVD in rat cultured cerebellar astrocytes. **Key Words:** Regulatory volume decrease—Thapsigargin—BAPTA acetoxymethyl ester—Glial cells.
J. Neurochem. **71,** 2330–2338 (1998).

Control of cell volume in astrocytes is of particular interest because of their prominent role in generating brain cytotoxic edema in various clinical situations, including hyposmolar states (Kimelberg and Ransom, 1986). A widely used approach to this issue has been the study of the response of cultured astrocytes to swelling induced by hyposmotic solutions. After a rapid phase of volume increase, astrocytes display an active regulatory volume decrease (RVD) accomplished by extrusion of intracellular osmolytes, mainly K^+ and Cl^-, as well as amino acids and other organic molecules (Kimelberg et al., 1990; Pasantes-Morales et al., 1993; Sánchez-Olea et al., 1993; Bender and Norenberg, 1994; Kirk, 1997). The swelling-activated efflux of osmolytes in astrocytes occurs primarily by separate channels for K^+ and Cl^- (Jackson and Strange, 1993; Pasantes-Morales et al., 1994*a,b*) and by a leak pathway for amino acids, which shares with the anion channel many physiological and pharmacological features (Strange and Jackson, 1995; Pasantes-Morales, 1996; Okada, 1997).

The signal responsible for transducing the change in cell volume into activation of the osmolyte efflux pathways has not been conclusively identified in astrocytes or in many other cell types. Ca^{2+} has been considered as a likely candidate because in most cells, swelling leads to an immediate rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) originating from both external and internal sources (McCarty and O'Neil, 1992; Foskett, 1994). However, the link between $[Ca^{2+}]_i$ elevation and RVD has not been conclusively established because volume regulation in many cells is Ca^{2+}-independent (Foskett, 1994). In cultured astrocytes the involvement of Ca^{2+} in RVD is poorly understood owing to several controversial results, briefly summarized here: (a) RVD in cortical and cerebellar astrocytes in suspension was found to be independent of external Ca^{2+} (Sánchez-Olea et al., 1995), whereas in cortical astrocytes grown in monolayer, RVD was reported as strictly (O'Connor and Kimelberg, 1993) or partly (Vitarella et al., 1994) dependent

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Abbreviations used: AM, acetoxymethyl ester; BHQ, 2,5-di-*tert*-butylhydroquinone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; RVD, regulatory volume decrease; Tg, thapsigargin.

on extracellular Ca^{2+}. In C6 glioma cells in suspension, excessive swelling and RVD blockade were observed after treatment with Ca^{2+}-free medium supplemented with 2 m*M* EGTA, but a swelling-induced rise in $[Ca^{2+}]_i$ was not observed (Lohr and Yohe, 1994). (b) RVD was markedly reduced by dihydropyridines but not by other voltage-activated Ca^{2+} channel blockers, such as verapamil or diltiazem. The effect of dihydropyridines seems unrelated to Ca^{2+} because it persists in Ca^{2+}-free media (Sánchez-Olea et al., 1995). (c) Cl^- efflux activated by swelling, which has a critical influence on RVD, has been reported as dependent (O'Connor and Kimelberg, 1993) or independent (Sánchez-Olea et al., 1995) of extracellular Ca^{2+}. (d) K^+ efflux (traced by $^{86}Rb^+$) evoked by osmotic swelling has been reported to decrease (O'Connor and Kimelberg, 1993) or increase (Sánchez-Olea et al., 1993; Bender and Norenberg, 1994; Vitarella et al., 1994) in the absence of external Ca^{2+}. Controversial results also exist regarding the influence on RVD of Ca^{2+} release from internal stores, as there is evidence in support of (Bender et al., 1993) or against (Schliess et al., 1996) the involvement of inositol 1,4,5-trisphosphate (IP_3) on the change in $[Ca^{2+}]_i$ associated with hyposmotic swelling. In a recent report, Fischer et al. (1997), after carefully characterizing the Ca^{2+} response to swelling in cortical astrocytes, found drugs and conditions affecting it, but volume changes were not investigated.

To establish a direct correlation between Ca^{2+} responses and RVD, in the present work we examined the change in $[Ca^{2+}]_i$ elicited by cell swelling, and in parallel experiments we tested the effect on RVD of drugs or conditions that influence $[Ca^{2+}]_i$ homeostasis. The study was carried out in the same cell preparation, i.e., cultured cerebellar astrocytes in suspension. Our results about the origin and changes in $[Ca^{2+}]_i$ elicited by swelling were remarkably similar to those observed in attached cortical astrocytes (Fischer et al., 1997).

MATERIALS AND METHODS

Cell cultures

Primary cultures of cerebellar astrocytes were obtained as previously described (Sánchez-Olea et al., 1995). In brief, the dissociated cell suspensions from cerebella of 8-day-old rats were plated at a density of 2.1×10^4 cells/cm^2 in plastic dishes. The culture medium contained basal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 m*M* glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The culture dishes were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. The enrichment of cultures in astrocytes and the proportion of other cells were assessed by immunocytochemical techniques as previously described (Sánchez-Olea et al., 1995).

Volume measurements

Volume changes were followed by electronic sizing as previously described (Sánchez-Olea et al., 1995). Cells cultured for 2–3 weeks were detached by treatment for 3 min with phosphate-buffered Ca^{2+}-free saline containing 1 m*M* EDTA and 0.01% trypsin. The detached cells were mixed with the same volume of serum-containing medium and then centrifuged and resuspended in isosmotic medium. A sample of the cell suspension was diluted ~100-fold with the experimental medium, and exactly 1 min later, cell volume was measured at the indicated times using a Coulter Counter (model ZF) linked to a Coulter Channelyzer (model 256). Cell volumes were expressed as relative volume, i.e., the percent increase with time after swelling over the initial volume recorded in isosmotic medium. HEPES-buffered isosmotic medium contained 135 m*M* NaCl, 5 m*M* KCl, 0.6 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 10 m*M* glucose, and 10 m*M* HEPES, pH 7.4. The osmolarity calculated was 320 mOsmol/kg of medium and was verified with a freezing point osmometer. Hyposmotic solutions were always prepared by reducing the concentration of NaCl to 60 m*M* (150 mOsmol; 50%). Control cells were always treated in parallel with the vehicle used to prepare solutions containing tested drugs.

Intracellular Ca^{2+}

For measurements of change in $[Ca^{2+}]_i$, cells were detached as above described and loaded at 37°C during 1 h in basal Eagle's medium containing 5 μ*M* fura-2 acetoxymethyl ester (AM). After loading, cells were centrifuged, resuspended in an equal volume of medium, and incubated for an additional 30 min. The cells were then washed, and fluorescence was measured in an Aminco-Bowman series 2 luminescence spectrometer. The excitation wavelength alternated between 340 and 380 nm, and fluorescent intensity was monitored at an emission wavelength of 510 nm. Each experiment was individually calibrated to obtain the maximal fluorescence after disrupting the cells with 0.1% Triton X-100, and the minimal fluorescence obtained after buffering the calcium in the solution with 20 m*M* EGTA. The values obtained with this procedure were used to calculate the $[Ca^{2+}]_i$ according to previously published equations (Grynkiewicz et al., 1985).

Statistical analysis

All data are presented as mean ± SE values. Statistical differences between means were assessed with a one-way ANOVA and Tukey's test, modified by Spjøtvoll y Stoline (Daniel, 1994), for multiple comparisons. Student's *t* test for unpaired data was applied as appropriate. Differences in means were considered significantly different when $p < 0.05$.

RESULTS

Swelling-induced changes in $[Ca^{2+}]_i$ in astrocytes stimulated with 50% hyposmotic solutions: effects of removal of external Ca^{2+}

The mean ± SE $[Ca^{2+}]_i$ in astrocytes suspended in isosmotic medium was 117 ± 12.4 n*M*. Exposure to 50% hyposmotic medium caused a rapid, transient increase in $[Ca^{2+}]_i$ reaching at the peak 386 ± 36 n*M*, followed by a slower second phase lasting several minutes in which Ca^{2+} levels attained 201 ± 21 n*M*. After 5 min in the hyposmotic medium $[Ca^{2+}]_i$ was still above the baseline (Fig. 1A). When swelling occurs in medium without Ca^{2+} and supplemented with 0.5 m*M* EGTA, the baseline decreased to 87.1 n*M*, and the change in $[Ca^{2+}]_i$ induced by the hyposmotic medium was substantially decreased to 190.7 ± 26 n*M*

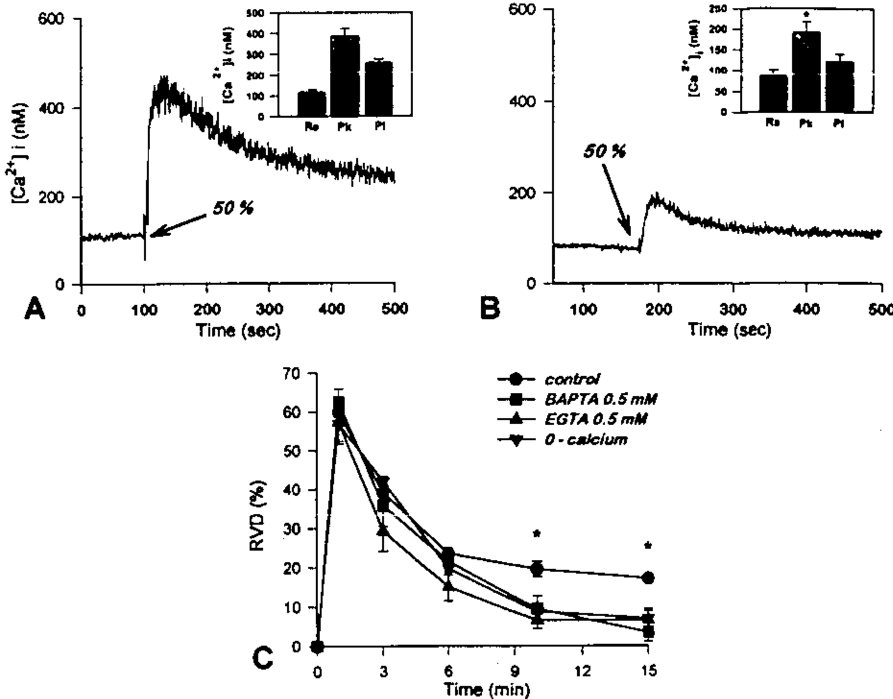

FIG. 1. Changes in $[Ca^{2+}]_i$ and RVD evoked by hypotonic swelling in the presence or absence of external Ca^{2+}. Cultured cerebellar astrocytes in suspension were loaded with fura-2/AM as described in Materials and Methods. **A:** The basal trace corresponds to $[Ca^{2+}]_i$ in astrocytes in isosmotic medium. At the time indicated by the arrow, the osmolarity in the medium was reduced to 150 mOsmol (50%) by addition of diluent medium without NaCl. **Inset:** Rs, resting; Pk, peak; Pl, plateau. **B:** $[Ca^{2+}]_i$ changes in Ca^{2+}-free medium supplemented with 0.5 m*M* EGTA. Traces are representative experiments. **Inset:** Mean ± SE (bars) values of seven to 16 experiments. **C:** RVD after hyposmotic swelling in media with or without Ca^{2+}, in the presence or absence of the Ca^{2+} chelator EGTA or BAPTA. Results are expressed as relative volume and represent the percent increase over cell volume in isosmotic medium at the indicated times after exposure to 50% hyposmotic medium. Data are mean ± SE (bars) values of six experiments. $^*p < 0.05$, significant differences from control (Student's *t* test and ANOVA, Tukey's test).

(Fig. 1B). This effect was similar in Ca^{2+}-free medium not supplemented with EGTA (data not shown). When Ca^{2+} was added to the medium up to 300 s after the hyposmotic shock, $[Ca^{2+}]_i$ levels still increased, indicating that the influx pathway remains open at that time. Verapamil (100 μ*M*) did not affect the magnitude of the $[Ca^{2+}]_i$ change (Table 1) or its time course. Effects of the Ca^{2+} antagonist Cd^{2+} (300 μ*M*) and the stretch-activated channel blocker Gd^{3+} (30 μ*M*) could not be examined on $[Ca^{2+}]_i$. These cations affected both 340 and 380 nm wavelengths, resulting in a very noisy 340/380 nm ratio. This was observed in the basal condition and a few seconds after addition of the cations. The further increment in noise of the 340/380 nm ratio in hyposmotic conditions suggests that the cations are entering the cell and interfering with fura-2 fluorescence.

The influence of external Ca^{2+} removal and of blockers of Ca^{2+} influx was examined on RVD. In 50% hyposmotic solutions, omission of external Ca^{2+},

TABLE 1. *Effects of Ca^{2+} channel blockers on $[Ca^{2+}]_i$ and RVD evoked by osmotic swelling*

	$[Ca^{2+}]_i$ (n*M*)			
Blocker (m*M*)	Baseline	Peak	Plateau	RVD %
No additions	117 ± 12.4	386 ± 37	201 ± 21	68.3 ± 2.9 (n = 30)
Verapamil (0.1)	123 ± 5.9	345 ± 17	216 ± 3	67.8 ± 4.6 (n = 6)
Cd^{2+} (1)				66.9 ± 5.6 (n = 6)
Co^{2+} (5)				65.9 ± 6.1 (n = 4)
Mg^{2+} (10)				67.2 ± 5.8 (n = 4)
La^{3+} (0.1)				36.6 ± 2.4 (n = 4)[a]
La^{3+} (1)				9.5 ± 0.7 (n = 4)[a]

Cultured cerebellar astrocytes were detached, and $[Ca^{2+}]_i$ and RVD were measured as described in Materials and Methods. Peak corresponds to the maximal value in $[Ca^{2+}]_i$ irrespective of the baseline value. Plateau is the $[Ca^{2+}]_i$ level measured at 300 s after the hyposmotic (50%) stimulus. RVD corresponds to the volume recovery efficiency after 15 min of exposure to 50% hyposmotic medium. Results are mean ± SE values.

[a] Significantly different from controls (Student's *t* test, $p < 0.01$).

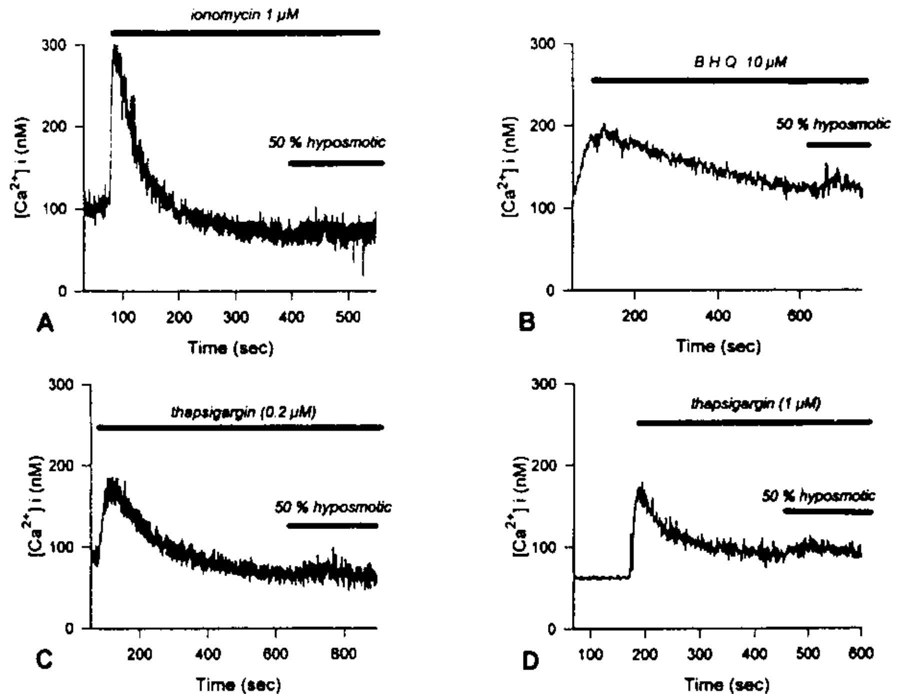

FIG. 2. Effect of Tg, BHQ, and ionomycin on $[Ca^{2+}]_i$ in cultured cerebellar astrocytes in isosmotic and hyposmotic conditions. Astrocytes in suspension were loaded with fura-2, and $[Ca^{2+}]_i$ was measured as described in Materials and Methods. After 50 s to determine the basal $[Ca^{2+}]_i$, cells were treated with (**A**) 1 μM ionomycin, (**B**) 10 μM BHQ, (**C**) 0.2 μM Tg, or (**D**) 1 μM Tg in Ca^{2+}-free (0.5 mM EGTA) medium. At the time indicated by the bar the osmolarity was reduced to 150 mOsmol. Traces are representative of at least four experiments.

in either the presence or absence of Ca^{2+} chelators, did not decrease the efficiency of RVD, which was instead increased by ~20% (Fig. 1C). The divalent cations Co^{2+}, Mg^{2+}, and Mn^{2+} did not affect RVD, and of the trivalent cations, La^{3+} but not Gd^{3+} decreased it significantly (Table 1). As previously reported, verapamil and diltiazem did not modify RVD (Sánchez-Olea et al., 1995).

Origin and contribution of Ca^{2+} released from intracellular stores on $[Ca^{2+}]_i$ and RVD

To examine the origin and possible mechanisms of the $[Ca^{2+}]_i$ rise observed in conditions of low external Ca^{2+} concentration, the endogenous Ca^{2+} stores were depleted by exposing the astrocytes to (a) ionomycin, a Ca^{2+} ionophore that in the absence of external Ca^{2+} collapses the Ca^{2+} gradient and depletes most intracellular Ca^{2+} stores, (b) thapsigargin (Tg) and 2,5-di-*tert*-butylhydroquinone (BHQ), inhibitors of the endoplasmic reticulum (ER) Ca^{2+}-ATPase, preventing the Ca^{2+} replenishment of ER, eventually leading to depletion, (c) ATP, which activates Ca^{2+} release from ER mediated by IP_3 receptors and could compete with the Ca^{2+} pool available for RVD, and (d) ryanodine (100 μM) or caffeine (10 mM), which inhibits Ca^{2+} movements from ER via the ryanodine receptor.

Figure 2 shows the effect of 1 μM ionomycin, 10 μM BHQ, and 0.2 and 1 μM Tg, tested in nominally Ca^{2+}-free medium. All these drugs exhibited essentially the same effect. Ionomycin (1 μM) transiently increased $[Ca^{2+}]_i$ to a peak after a few seconds, followed by a decline to basal levels (Fig. 2A). BHQ induced a rise in $[Ca^{2+}]_i$ that was sustained for a short time and then slowly returned to baseline. Similar responses were evoked by 0.2 and 1 μM Tg (Fig. 2C and D). After treatment with these drugs, osmotic swelling in 50% hyposmotic solutions did not elicit any further increase in $[Ca^{2+}]_i$ (Fig. 2). Conversely, after the swelling-induced $[Ca^{2+}]_i$ elevation, treatment with ionomycin, BHQ, and Tg did not evoke any further rise in $[Ca^{2+}]_i$ (data not shown).

Figure 3 shows the effect on RVD of treatment with ionomycin and ATPase blockers in nominally Ca^{2+}-free conditions. Astrocytes were exposed to the drugs during 10 min in isosmotic medium to deplete the internal Ca^{2+} stores before stimulation with the hyposmotic medium. Astrocyte volume was not significantly changed by this treatment. After this Ca^{2+}-depletion period, cells were transferred to hyposmotic medium also containing the drugs. Under these conditions, ionomycin, BHQ, and 0.2 μM Tg did not influence RVD (Fig. 3A and B), but 1 μM Tg blocked it completely (Fig. 3B).

The following experiments were devised to investigate whether RVD inhibition resulted from a deleterious action of Tg at this concentration when present in the hyposmotic medium. First, cells were exposed to 1 μM Tg only during the preincubation period of 10 min in isosmotic medium, to deplete ER Ca^{2+} stores, and then RVD was measured in the absence of the drug. As shown in Fig. 3B, 1 μM Tg did not inhibit RVD. Second, 1 μM Tg was applied without preincubation, only in the hyposmotic medium, in the presence or absence of external Ca^{2+}. In both conditions $[Ca^{2+}]_i$ levels were raised, in the first case transiently, by the

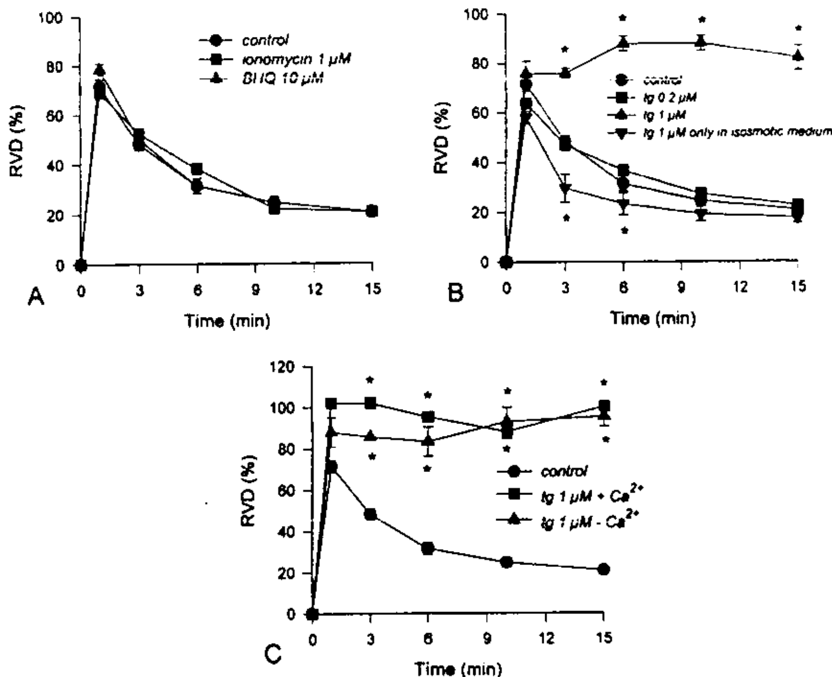

FIG. 3. Effect of Tg, BHQ, and ionomycin on RVD in cultured cerebellar astrocytes. **A:** Cells were preincubated with 1 μM ionomycin (■) or 10 μM BHQ (▲) for 10 min in isosmotic, Ca^{2+}-free (0.5 mM EGTA) medium before exposure to 50% hyposmotic medium that was also Ca^{2+}-free and contained the drugs. **B:** Cells were preincubated during 10 min with 0.2 (■) or 1 μM (▲) Tg, in Ca^{2+}-free medium and then exposed to 50% hyposmotic medium containing the drug. In (▼) 1 μM Tg was only present in the isosmotic medium (preincubation) but not in the hyposmotic medium. **C:** Cells were not preincubated with Tg, but the drug (1 μM) was present in the hyposmotic medium, with (■) or without (▲) Ca^{2+}. Results are expressed as described in Fig. 1. Data are mean ± SE (bars) values of four experiments. *$p < 0.05$, significant differences (ANOVA, Tukey's test).

effect of Tg on the ER Ca^{2+} pool, and in the second case, in addition, by the Ca^{2+} entry subsequent to swelling. A blockade of RVD since its early phase was always observed when 1 μM Tg was present (Fig. 3C). Therefore, 1 μM Tg (but not 0.2 μM Tg) in the hyposmotic medium prevented RVD, regardless of $[Ca^{2+}]_i$ levels.

Application of ryanodine (1–100 μM) and caffeine (10 mM) did not modify the resting $[Ca^{2+}]_i$ or the amplitude or rate of $[Ca^{2+}]_i$ increase activated by hyposmolarity. Similarly, ryanodine and caffeine did not affect RVD (data not shown).

Stimulation of astrocytes with ATP (5–100 μM) in Ca^{2+}-free medium caused an immediate increase in $[Ca^{2+}]_i$ to 108 ± 26 nM at the peak of the response. This rise was followed by a decline, and after 5 min $[Ca^{2+}]_i$ returned to basal levels. Subsequent stimulation with 50% hyposmotic solution still evoked a modest increase (52 ± 10 nM) in $[Ca^{2+}]_i$. This may be due to the weak response to ATP of cerebellar astrocytes (108 nM), which contrasts with the much higher (837 nM) reported for cortical astrocytes (Fischer et al., 1997). Incubating astrocytes for 5 min with ATP before 50% hyposmotic stimulus did not decrease but rather increased RVD by ~30% (data not shown).

The effect of BAPTA-AM could not be tested accurately, owing to some complex effects of the chelator. Incubating astrocytes with 30 μM BAPTA-AM for 10–30 min markedly decreased astrocyte swelling. When the concentration was decreased to 20 μM, cells still swelled less, but RVD was unaffected (Fig. 4A). In all these conditions, BAPTA-AM abolished the $[Ca^{2+}]_i$ elevation induced by swelling. Even shorter times (5 min), as shown in Fig. 4B, were sufficient to suppress the rise in $[Ca^{2+}]_i$. In all these experiments, astrocytes were treated with BAPTA-AM only in the isosmotic medium. If the chelator was present in the hyposmotic medium as well, RVD was blocked. To investigate whether BAPTA-AM was per se producing some effect on RVD under these conditions, the chelator was added at different times to the hyposmotic medium during the volume recovery response. It was observed that RVD was immediately arrested by BAPTA-AM, regardless of the time of application and the time course of $[Ca^{2+}]_i$ rise (Fig. 4A). This effect of BAPTA-AM impairing RVD was observed even when astrocytes were depleted of $[Ca^{2+}]_i$ by prior exposure to Ca^{2+}-free medium and treatment with ionomycin (Fig. 4C).

$[Ca^{2+}]_i$ and RVD in astrocytes stimulated by 30% hyposmotic solutions

Differences in the osmosignaling systems have been observed according to the extent of the hyposmotic shock (Tilly et al., 1993). Therefore, some experiments were carried out in astrocytes stimulated by 30% hyposmotic solutions. These conditions still evoked a marked $[Ca^{2+}]_i$ elevation, also exhibiting a transient and a sustained phase (Fig. 5A). In Ca^{2+}-free (0.5 mM EGTA) solutions, these two components were substantially decreased. At variance with results in 50% hyposmotic solutions, Ca^{2+} basal levels were attained within 5 min after the stimulus (Fig. 6A). Depletion of intracellular Ca^{2+} stores with ionomycin and BHQ abolished any $[Ca^{2+}]_i$ elevation in response to the hyposmotic stimulus (Fig. 5B and C). A marginal rise in $[Ca^{2+}]_i$ was elicited by the hyposmotic stimulus in astrocytes previously exposed to 100 μM

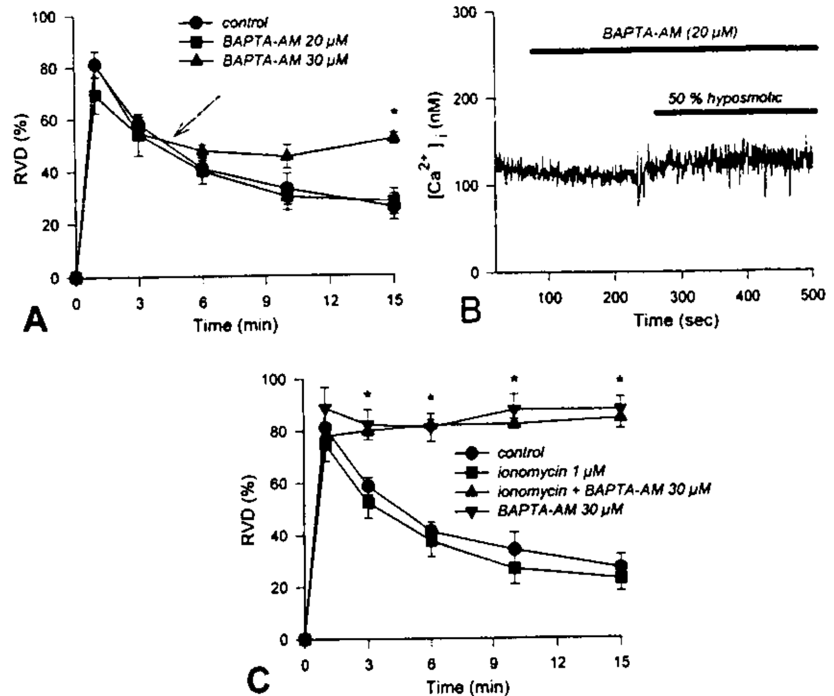

FIG. 4. Effect of BAPTA-AM on $[Ca^{2+}]_i$ and RVD in cultured cerebellar astrocytes. **A:** Cells were incubated for 20 min in isosmotic, Ca^{2+}-free medium (0.5 m*M* EGTA) containing 20 μM BAPTA-AM (■) and then exposed to 50% hyposmotic medium, or 30 μM BAPTA-AM was added at 4 min (arrow; ▲). **B:** Cells were incubated with 20 μM BAPTA-AM for 5 min in Ca^{2+}-free medium. At the time indicated by the bar, the osmolarity was reduced to 50%. **C:** Astrocytes were preincubated with 1 μM ionomycin for 1 min in Ca^{2+}-free isosmotic medium and then exposed to Ca^{2+}-free, 50% hyposmotic medium with (▲) or without (■) 30 μM BAPTA-AM. For comparison, 30 μM BAPTA-AM alone (▼) was used. Data are mean ± SE (bars) values of four experiments. $^*p < 0.05$, significant differences (ANOVA, Tukey's test).

ATP (data not shown). It is noteworthy that in cerebellar astrocytes markedly less Ca^{2+} is released by ATP than in cortical astrocytes (Fischer et al., 1997). This may possibly explain why in cerebellar astrocytes the hyposmotic stimulus still evoked some $[Ca^{2+}]_i$ rise, of a magnitude related to the decrease in osmolarity, which is not seen in cortical astrocytes (Fischer et al., 1997).

When exposed to 30% hyposmotic solutions, astrocytes swelled to a peak volume of 48% above the control (isosmotic) value. This was followed by RVD leading to 75% cell volume recovery (Fig. 6A). Essentially the same time course and efficiency of the volume regulatory process were observed in Ca^{2+}-free solutions (Fig. 6A). Depletion of intracellular Ca^{2+} stores by preincubation for 5–10 min with 1 μM iono-

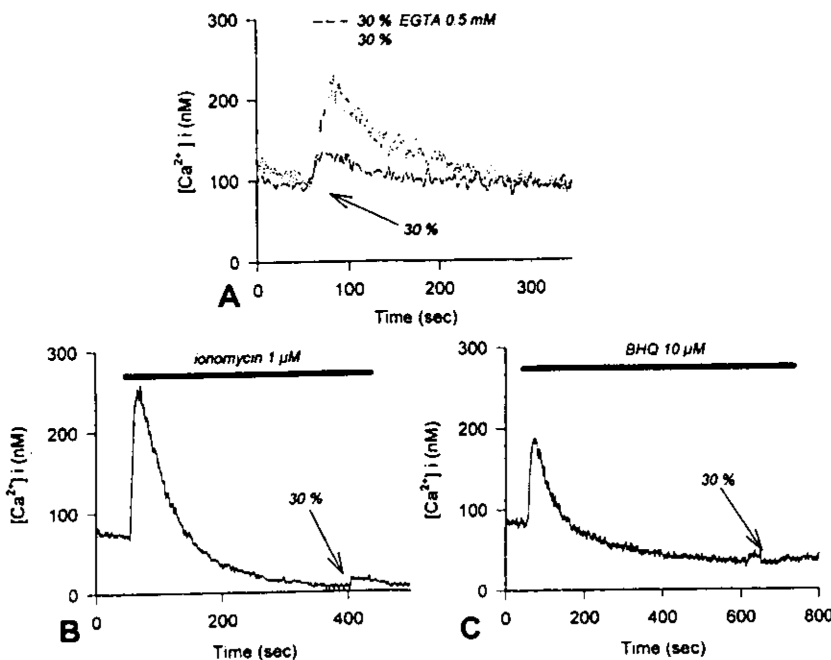

FIG. 5. Changes in $[Ca^{2+}]_i$ evoked by 30% hyposmotic solutions. **A:** The basal trace corresponds to $[Ca^{2+}]_i$ in astrocytes in isosmotic medium. At the time indicated by the arrow, the osmolarity was reduced to 210 mOsmol by addition of diluent medium without NaCl, in the presence (· · ·) or absence (- - -) of external Ca^{2+}. **B** and **C:** After 50 s to determine the basal $[Ca^{2+}]_i$, cells were exposed to 1 μM ionomycin (B) or 10 μM BHQ (C), in Ca^{2+}-free (0.5 m*M* EGTA) medium. At the time indicated by the arrow the osmolarity was reduced to 210 mOsmol. Results are representative of four separate experiments.

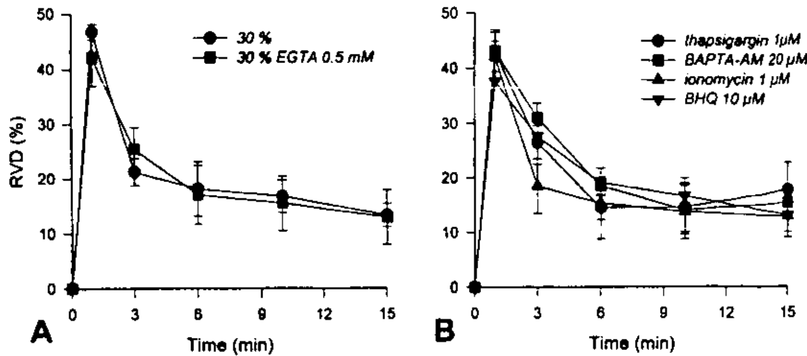

FIG. 6. RVD in cultured astrocytes exposed to a 30% hyposmotic solution. **A:** RVD after hyposmotic swelling in medium with (●) or without (■) external Ca^{2+}. **B:** Effect of BHQ, ionomycin, Tg, and BAPTA-AM. Cells were preincubated for 10 min in isosmotic, Ca^{2+}-free (0.5 m*M* EGTA) medium containing 1 μ*M* ionomycin (▲), 10 μ*M* BHQ (▼), 1 μ*M* Tg (●), or 20 μ*M* BAPTA-AM (■) and then exposed to 30% hyposmotic Ca^{2+}-free medium. Hyposmotic medium was prepared reducing the NaCl concentration from 135 to 90 m*M* (210 mOsmol; 30%). Data are mean ± SE (bars) values of four experiments.

mycin, 1 μ*M* Tg, and 10 μ*M* BHQ did not affect RVD. Similarly, treatment with 20 μ*M* BAPTA-AM for 10 min before the hyposmotic stimulus did not modify astrocyte volume or RVD (Fig. 6B).

DISCUSSION

An increase in $[Ca^{2+}]_i$ triggered by osmotic swelling has been consistently observed in a large variety of cells in culture. Only a few cell types do not exhibit this feature (Foskett, 1994). The time course and the contribution of external and internal Ca^{2+} sources to this increase in $[Ca^{2+}]_i$ are also similar in different cells. Despite this general response, a correlation between changes in $[Ca^{2+}]_i$ and RVD has not been always observed (Foskett, 1994), and therefore the implication of Ca^{2+} as a transduction signal underlying RVD has not been conclusively established. Part of the problem is that $[Ca^{2+}]_i$ and RVD have not always been measured in the same cell preparation or under identical conditions with respect to concentration and time of exposure to blockers (McCarty and O'Neil, 1992; Foskett, 1994).

Cerebellar astrocytes from primary cultures exhibited the typical response of increased $[Ca^{2+}]_i$ immediately after osmotic swelling elicited by 50 or 30% hyposmotic solutions. It is noteworthy that our results on changes in $[Ca^{2+}]_i$ in suspended cerebellar astrocytes were remarkably similar to those recently reported in cortical astrocytes attached to coverslips (Fischer et al., 1997), showing no differences with respect to the astrocyte origin or the preparation used (cells attached or in suspension). Also, no differences in RVD have been observed between cortical and cerebellar astrocytes in suspension or in monolayer measured by radiochemical (Bender and Norenberg, 1994) or electrical impedance (O'Connor and Kimelberg, 1993) procedures.

As the present work showed, omission of external Ca^{2+}, while importantly contributing to the increase in $[Ca^{2+}]_i$ evoked by swelling, did not decrease RVD but rather increased it in 50% hyposmotic medium. Available information about the effect on RVD of external Ca^{2+} removal in astrocytes is scarce. Besides our present and previous results (Sánchez-Olea et al., 1995) showing complete independence of RVD on external Ca^{2+}, only two studies in cortical astrocytes have examined this question with notably different results (O'Connor and Kimelberg, 1993; Vitarella et al., 1994). In C6 glioma cells, although RVD was found to be Ca^{2+}-dependent, $[Ca^{2+}]_i$ was not raised by swelling (Lohr and Yohe, 1994). The route for Ca^{2+} entry on swelling is unclear. Suggested pathways are voltage-dependent Ca^{2+} channels, stretch-activated channels (Sackin, 1994), or capacitative nonspecific cationic channels (McCarty and O'Neil, 1991; Fischer et al., 1997). Some of these channels, in addition, could be modulated in hyposmotic conditions by the dilution of intracellular macromolecules as proposed by the "macromolecular crowding" hypothesis (Minton, 1994; Summers et al., 1997). Our evidence on the lack of effect of voltage-dependent and stretch-activated channel blockers on RVD seems to discount these two pathways. The apparent membrane permeability to cations such as Gd^{3+} and Cd^{2+} on swelling, together with the low selectivity of the Ca^{2+} influx pathway reported in cortical astrocytes, rather favors the capacitive channel as the mechanism of Ca^{2+} influx during swelling. Such interpretation could explain the insensitivity of RVD to external Ca^{2+}, as the purpose of this entry might only be the replenishment of endogenous stores depleted after cell swelling. On the same line, the Ca^{2+} influx pathway did not inactivate over several minutes after the hyposmotic stimulus, when membrane distension is decreasing but the Ca^{2+} internal stores are still not replenished. The capacitive Ca^{2+} entry has been also implicated in the mechanism for Ca^{2+} influx during swelling in rabbit cultured proximal tubule cells (O'Neil and Leng, 1997). La^{3+} exhibited a striking inhibitory effect on RVD, but the concentration required was largely in excess of that necessary to block Ca^{2+} entry, suggesting another action of this cation. Screening of surface charges, influencing in turn some of the osmolyte pathways involved in RVD, may be responsible for the observed effect (Elinder and Århem, 1994). Although astrocytes were viable, a potentially cytotoxic effect of La^{3+} at these high concentrations cannot be totally excluded.

Consistent with findings in cortical astrocytes (Fischer et al., 1997), the present results showed a substantial contribution of endogenous stores, essentially the ER, to the osmosensitive $[Ca^{2+}]_i$ elevation. The mechanism of its release, though, remains unclear (McCarty and O'Neil, 1992; Foskett, 1994). The ryanodine receptor, which seems to participate in other cell types (Wu et al., 1997), appears not to be involved in astrocytes, because concentrations that saturate the receptor in these cells did not affect $[Ca^{2+}]_i$ nor RVD. IP_3-sensitive receptors have been implicated by evidence showing phosphoinositide hydrolysis associated with swelling in cortical astrocytes (Bender et al., 1993). However, blockade of this avenue by U-73122 has no effect on the swelling-induced $[Ca^{2+}]_i$ increase in astrocytes (Fischer et al., 1997). Osmosensitive Ca^{2+} release via IP_3 may also result from a direct interaction between ankyrin and IP_3 receptors as a consequence of swelling. Such an interaction has been demonstrated in brain (Joseph and Samanta, 1993) and in lymphoma cells (Bourguignon and Jin, 1995), and it is suggested to play a role in the regulation of IP_3 receptor-mediated Ca^{2+} release during lymphocyte activation. An alternate mechanism for swelling-induced Ca^{2+} release from intracellular stores not involving IP_3 hydrolysis has been recently proposed, implicating arachidonic acid release at the end of a chain of reactions initiated by a G protein, possibly activated by a mechanoreceptor. In rat inner medullary collecting duct cells, blockade of arachidonic acid metabolism reduced the Ca^{2+} response to hyposmotic stress (Tinel et al., 1997). Finally, an option supported by recent evidence from different groups proposes ER as an osmosensor liberating Ca^{2+} either by mechanical opening of Ca^{2+} channels or by voltage-dependent channels, sensing a voltage change after swelling, between ER and cytosol (McCarty and O'Neil, 1991; Missiaen et al., 1996; Jena et al., 1997).

Although the astrocyte volume change clearly evoked Ca^{2+} release from endogenous stores, the difference among the effects on RVD of ionomycin and BHQ (unaffected), Tg (blocked), and ATP (increased), despite their similar action of depleting Ca^{2+} stores, raises serious doubts about the contribution of this Ca^{2+} pool to RVD. A notion that might be considered regarding these differences is that residual $[Ca^{2+}]_i$ levels, at required sites within microcellular domains, could be depleted only by high concentrations of Tg or by the combined treatment with BAPTA-AM and ionomycin but not by ionomycin alone. A minimal $[Ca^{2+}]_i$ concentration of ~50 nM (which has been termed the "permissive" Ca^{2+} concentration) seems essential for full and sustained activation of Cl^- channels during swelling in endothelial cells, but an increase in $[Ca^{2+}]_i$ above this level has no effect on the Cl^- current (Szücs et al., 1996). The possibility of decreasing $[Ca^{2+}]_i$ below this threshold by some but not all treatments used in the present study cannot be totally excluded. However, an action of Tg on RVD unrelated to Ca^{2+} seems clearly demonstrated. The use of BAPTA-AM as a tool to evaluate the contribution of $[Ca^{2+}]_i$ to RVD was restricted by a secondary action of the drug preventing astrocyte swelling in this preparation as well as by some apparently direct effects of the chelator on RVD.

Even considering the notion of a "permissive" Ca^{2+} concentration, a main conclusion of the present work is that $[Ca^{2+}]_i$ elevation evoked by swelling occurs as a consequence of an increased permeability to Ca^{2+} of plasma and organelle membranes by mechanisms still not fully understood, but the present study challenges the actual importance of this increase in $[Ca^{2+}]_i$ as a transduction signal for RVD in astrocytes.

In any event, a fact to be considered in terms of the physiology of astrocytes is that the depletion of intracellular Ca^{2+} stores by swelling, reported in the present study and in that of Fischer et al. (1997), may compromise those functions involving changes in $[Ca^{2+}]_i$ as part of the signaling mechanisms of agonists such as ATP, hormones, and other factors.

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ARTICULO 3

Ca^{2+} Changes and ^{86}Rb Efflux Activated by Hyposmolarity in Cerebellar Granule Neurons

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Hyposmotic swelling increased ^{86}Rb release in cultured cerebellar granule neurons (1 day in vitro [DIV]) with a magnitude related to the change in osmolarity. ^{86}Rb release was partially blocked by quinidine, Ba^{2+}, and Cs^{+} but not by TEA, 4-AP, or Gd^{3+}. ^{86}Rb efflux decreased in Cl^{-}-depleted cells or cells treated with DDF or DIDS, suggesting an interconnection between Cl^{-} and K^{+} fluxes. Swelling induced a substantial increase in $[Ca^{2+}]_i$ to which both external and internal sources contribute. However, ^{86}Rb efflux was independent of $[Ca^{2+}]_o$, unaffected by depleting the endoplasmic reticulum (ER) by ionomycin or thapsigargin and insensitive to charybdotoxin, iberiotoxin, and apamin. Swelling-activated ^{86}Rb efflux in differentiated granule neurons after 8 DIV, which express Ca^{2+}-sensitive K^{+} channels, was not different from that in 1 DIV neurons, nor in time course, net release, Ca^{2+}-dependence, or pharmacological sensitivity. We conclude that the swelling-activated K^{+} efflux in cerebellar granule neurons is not mediated by Ca^{2+}-sensitive large conductance K^{+} channels (BK) as in many cell types but resembles that in lymphocytes where it is possibly carried by voltage-gated K^{+} channels. J. Neurosci. Res. 53:626–635, 1998. © 1998 Wiley-Liss, Inc.

Key words: volume regulation; RVD; swelling; K^{+} channels

INTRODUCTION

Active volume regulation after swelling due to a reduction in external osmolarity appears to be a general feature of animal cells (Hoffmann and Simonsen, 1989). Brain cells, astrocytes and neurons also exhibit this property (Kimelberg and Ransom, 1986; Pasantes-Morales et al., 1993). This process of cell volume recovery has been examined preferentially in astrocytes, which are those showing prominent swelling during the numerous pathologies conveying cellular brain edema (Kimelberg and Ransom, 1986). However, it is unclear whether neurons do not exhibit swelling because water permeability is restricted or if the mechanisms of cell volume control are more efficient than in astrocytes. In most cells the regulatory volume decrease occurs by activation of efflux pathways for intracellular osmolytes including K^{+}, Cl^{-}, and a number of organic molecules (Hoffmann and Simonsen, 1989). The swelling-activated release of K^{+} to the microenvironment of synaptic conexions may have a profound influence on the functioning of neuronal networks. In fact, there is evidence showing that changes in osmolarity result in marked alteration of brain excitability (Andrew, 1991; Roper et al., 1992; Rosen and Andrew, 1990; Hochman et al., 1995). It is therefore of interest to characterize the volume-activated K^{+} fluxes in brain cells, particularly when the pivotal role of astrocytes clearing K^{+} is also disturbed by swelling.

Studies on cell volume regulation in neurons including activation of K^{+} fluxes are scarce. The pharmacology of regulatory volume decrease (RVD) and the activation by swelling of stretch-sensitive cationic channels and voltage-gated K^{+} currents have been reported in a neuroblastoma cell line (Falke and Misler, 1989). A study in rat sympathetic neurons (Leaney et al., 1997) describes the activation of Cl^{-} currents, but K^{+} currents were not examined. Stretch-activated K^{+} channels have been described in hypothalamic neurons (Baraban et al., 1997; Kim et al., 1995), and these K^{+} channels respond to hyposmolarity in the interneurons, but not in pyramidal cell of hippocampal slices. This suggests that the volume-sensitive efflux of K^{+} may not be a general response of neurons. We have characterized RVD in cerebellar granule neurons in culture after 1–2 days in vitro (DIV) and showed that the corrective fluxes of Cl^{-} and K^{+} appear to occur through separate conductive channels (Pasantes-Morales et al., 1994). It was also shown that the anion channel is poorly selective in contrast to the cation pathway, which is essentially restricted to K^{+} and ^{86}Rb (Pasantes-Morales et al., 1994). The properties of the

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anion fluxes have been characterized (Sánchez-Olea et al., 1996), but there is no information about those of K^+, despite the fact that K^+ efflux is the rate limiting step for RVD in these cells (Pasantes-Morales et al., 1994). It is unclear at present whether the channels carrying K^+ and Cl^- fluxes may function in a separate way or if they are somewhat interdependent (this question was addressed in the present work for cerebellar granule neurons). The changes in $[Ca^{2+}]_i$ evoked by swelling and its possible influence on K^+ efflux were also investigated. In a variety of cell types, K^+ fluxes elicited by swelling are carried by Ca^{2+}-dependent BK channels, likely activated by an increase in $[Ca^{2+}]_i$—which also occurs as consequence of swelling. To our knowledge, the influence of swelling in the Ca^{2+} cell content has not been examined in neurons.

MATERIALS AND METHODS

Cell Culture

Primary cultures of cerebellar granule neurons were prepared as previously described (Pasantes-Morales et al., 1994). Briefly, the dissociated cell suspensions from cerebellum from 8-day-old rats were plated at a density of 265×10^3 cells/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) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The culture dishes were incubated at 37°C in a humidified atmosphere (5% CO_2/95% air). When cultures were maintained for more than 2 days in vitro (DIV), 20 hr after seeding they were added with cytosine arabinoside (10 μM) and 150 μM NMDA to increase cell survival (Balàz et al., 1988). The enrichment of cultures with neurons was assessed by counting immunostained cells marked with polyclonal antibodies against neuron-specific enolase (Dakopatts, Carpinteria, CA). Dishes stained with antiglial fibrillar acidic protein antibodies (Dakopatts) showed the presence of less than 5% glial cells. These cultures contained about 95% glutamatergic granule cells while the remaining 5% represent GABAergic neurons, most likely stellate and basket neurons (Gallo et al., 1986).

^{86}Rb Release Measurements

Cultured cells were incubated in culture medium containing ^{86}Rb (2.5 μCi/ml) for 60 min. After incubation, the medium was replaced with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline containing (in mM): 135 NaCl, 1.0 $CaCl_2$, 1.17 $MgCl_2$, 1.7 KH_2PO_4, 5 KCl, 10 HEPES, and 5 glucose, pH 7.4. The medium osmolarity, determined with a freezing-point osmometer (Mod. 5002, Precision Instruments, MA), was 295 ± 6 mosm/lt. After 15 min of successive washes with this medium, cells were superfused at a rate of 1 ml/min for 5 min, after which a stable ^{86}Rb efflux baseline was attained. Then, the superfusion medium was replaced by one of reduced osmolarity (150 mosm; 50% hyposmotic), and samples collected for 10 min more. Hyposmotic media were prepared by reducing the NaCl concentration to 60 mM. Control dishes were added with the vehicle used to prepare solutions containing the drugs tested. At the end of the experiments, radioactivity in media and that remaining in the cells was measured by in a liquid scintillation counter. Results are expressed as the radioactivity released in each collected sample as percent of the total radioactivity present in the cells at that time.

Intracellular-Free Calcium Measurement

For Ca^{2+} measurements, cells were detached from dishes by treatment with phosphate-buffered Ca^{2+}-free saline containing 1 mM EDTA and 0.01% trypsin for 2 min, and loaded with 5 μM Fura-2/AM at 37°C during 1 hr in basal Eagle's medium. After loading, cells were centrifuged, resuspended in an equal volume of medium, and incubated for additional 30 min. The cells were then washed and fluorescence was measured in an Aminco-Bowman luminescence spectrometer (series 2), SLM-AMINCO® (Rochester, NY). Excitation wavelength was alternated between 340 and 380 nm and fluorescence intensity was monitored at 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the cells with 0.1% triton X-100, and the minimum fluorescence obtained after buffering the calcium in the solution with 20 mM EGTA. The values obtained through this procedure were used to calculate the intracellular calcium concentration according to previously published equations (Grynkiewicz, et al., 1985).

RESULTS

Swelling-Activated K^+ (^{86}Rb) Efflux

Under isosmotic conditions, the efflux of ^{86}Rb from cultured cerebellar granule neurons was about 1.1% × min^{-1}. Replacement of the isosmotic solution by a 50% hyposmotic solution increased the efflux to a peak release of 4.5% at about 2 min after the stimulus. Then release slowly inactivated almost attaining the initial rates (Fig. 1). Mild hyposmotic solutions (30%) still increased ^{86}Rb efflux clearly above the baseline. Low (15%) hyposmotic solutions elicited only a marginal increase in ^{86}Rb release (Fig. 1).

Effects of K^+ Channel Blockers

TEA (1–20 mM) and 4-AP (1–5 mM) did not modify the ^{86}Rb efflux activated by hyposmolarity in

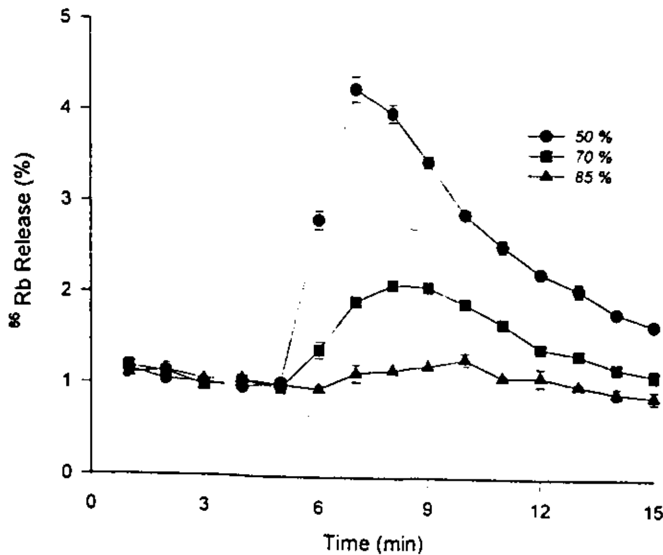

Fig. 1. Time course of swelling-induced ^{86}Rb release from cerebellar granule neurons (1 DIV). After 1 hr loading period with ^{86}Rb (2.5 μCi/ml), cells were washed for 20 min with isosmotic medium and fractions collected every min for the last 5 min (basal efflux). Afterwards, a hyposmotic medium (50%, 75%, or 85%) was introduced, and fractions collected for 10 min more. Data are expressed as percentage of ^{86}Rb released/min of the total accumulated as described in Materials and Methods. Results are means ± SE of 4–12 experiments: SE are represented by vertical bars when they exceeded size of symbols.

TABLE I. Effect of K^+ and Ca^{2+} Channel Blockers on ^{86}Rb Release From Cerebellar Granule Neurons Exposed to 50% Hyposmotic Media

Condition	Concentration	^{86}Rb release (%)
Control	—	27.13 ± 0.24
Quinidine	600 μM	19.11 ± 0.59*
Barium	1 mM	22.67 ± 0.71*
	5 mM	19.29 ± 0.39*
Cesium	5 mM	23.89 ± 1.56**
4-AP	1 mM	24.53 ± 1.47
	5 mM	31.95 ± 2.75
TEA	1 mM	27.48 ± 1.30
	20 mM	27.00 ± 1.71
Gadolinium	50 μM	25.61 ± 1.83
Cadmium	300 μM	29.14 ± 0.23
Verapamil	100 μM	30.00 ± 0.87
Iberiotoxin	10 nM	26.40 ± 1.14
Apamin	200 nM	25.09 ± 1.96
Charybdotoxin	10 nM	26.37 ± 2.00
	20 nM	23.92 ± 2.24

*After 15-min washing period with isosmotic medium, culture dishes previously loaded with ^{86}Rb were exposed sequentially to isosmotic medium (5 min) and to 50% hyposmotic medium (10 min). Results are mean ± SE of the percentile fraction of ^{86}Rb released during the hyposmotic stress. n = 79 for control and 4–12 for the experimental conditions. Significantly different from control with $P < 0.01$ (*), or $P < 0.05$ (**).

cerebellar granule neurons. Barium (1–5 mM) decreased ^{86}Rb efflux by 16% and 29%, respectively; quinidine (1 mM) decreased it by 29%, and Cs (5 mM) by 12% (Table I). The trivalent cation Gd^{3+}, which is blocker of stretch-activated channels, did not significantly influence ^{86}Rb efflux (Table I).

Effects of Cl^--Free Media and of Cl^--Channel Blockers

To investigate a possible interconnection between the volume-activated K^+ and Cl^- fluxes, Cl^- was replaced by the impermeant anion gluconate. Replacement of Cl^-, in the hyposmotic solution only, accelerated the onset and increased the net ^{86}Rb efflux. The inactivation phase was also accelerated (Fig. 2). When cells were exposed to the Cl^--free medium during 10 min prior to the hyposmotic stimulus, thus decreasing the internal Cl^- pool, ^{86}Rb efflux was markedly reduced (Fig. 2). To further investigate the interdependence of ^{86}Rb and Cl^- fluxes, we examined the effect on ^{86}Rb efflux of the Cl^- channel blockers 5-nitro-2(-3-phenylpropylamino)benzoic acid (NPPB), niflumic acid, 1,9,-dideoxyforskoline (DDF), and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), all of which block swelling-activated Cl^- efflux (Pasantes-Morales et al., 1994). When present in the hyposmotic medium, DDF (100 μM) and DIDS (400 μM) reduced (30–40%) the release of ^{86}Rb (Fig. 3A), but niflumic acid and NPPB lead to an unexpected increase in ^{86}Rb efflux activated by hyposmolarity (Fig. 3B). This increase is likely due to an effect of these blockers increasing, per se, ^{86}Rb efflux, since they elicited a marked release in isosmotic conditions as shown in Figure 3C. It has been reported that NPPB and niflumic acid, but not DIDS nor DDF, deplete the cell ATP content (Ballatori et al., 1994), and by this mechanism impair the Cl^- efflux pathway which requires ATP for activation. This may have an opposite effect stimulating ATP-sensitive K^+ fluxes. To examine this possibility, ^{86}Rb efflux was measured in cerebellar granule neurons treated with the metabolic blockers iodoacetate and dinitrophenol to impair ATP synthesis. Figure 3D shows that ^{86}Rb release in isosmotic solutions was unchanged, and the time course of of ^{86}Rb release in hyposmotic medium was similar to that in Cl^--depleted cells illustrated in Figure 2.

Changes in Cytosolic Ca^{2+} Evoked by Swelling in Cerebellar Granule Neurons

The mean basal $[Ca^{2+}]_i$ in cerebellar granule neurons in isosmotic medium was about 90 nM. The hypotonic solution (50%) gradually increased $[Ca^{2+}]_i$ to a maximal of 148 nM, reached after about 70 s. This initial phase was followed by a sustained phase, which lasts up to 300 s (Fig. 4A). In Ca^{2+}-free (0.5 mM EGTA) medium,

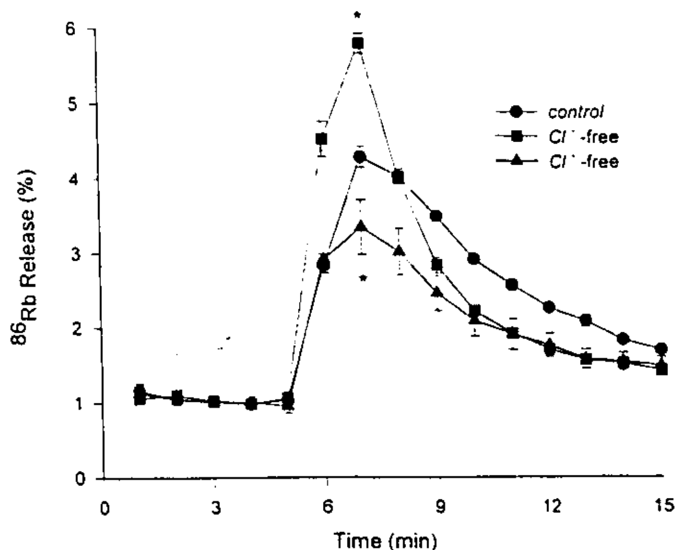

Fig. 2. Effect of Cl^--free medium on swelling-induced ^{86}Rb release from cultured cerebellar granule neurons. ^{86}Rb efflux was measured in cells exposed to hyposmotic Cl^--containing (●), Cl^--free medium (■), or in cells incubated during 10 min in isosmotic Cl^--free medium prior to the perfusion with isosmotic and hyposmotic Cl^--free solutions (▲). Cl^- was replaced with gluconate in all cases. Data are expressed as in Figure 1. Results are means ± SE of 4–12 experiments. *Significantly different from controls, $P < 0.05$.

the rise in $[Ca^{2+}]_i$ was only partly reduced (Fig. 4B), suggesting a contribution of Ca^{2+} from internal sources. ER depletion obtained incubating cells with 1 μM ionomycin or thapsigargin in the absence of external Ca^{2+} and prevented any further increase in $[Ca^{2+}]_i$ by the hyposmotic solution (Fig. 5A,B). Incubating neurons with 50 μM BAPTA-AM during 30 min prior to the hyposmotic stimulus markedly decreased the swelling-induced $[Ca^{2+}]_i$ elevation (Fig. 5C). When cells were treated with 1 μM ionomycin in media containing external Ca^{2+}, $[Ca^{2+}]_i$ was notably increased, as expected (Fig. 5D).

Influence of Ca^{2+} on the Swelling-Activated ^{86}Rb Efflux

In a variety of cells, Ca^{2+}-gated K^+ channels, essentially of the BK type, activate upon swelling; and consequently, K^+ efflux and RVD are dependent of external Ca^{2+} (Sarkadi and Parker, 1991). In cerebellar granule neurons, ^{86}Rb efflux activated by swelling was not decreased in the absence of Ca_o^{2+}, but rather an increase of about 15% was observed under this condition (Fig. 6A). Blockers of Ca^{2+} influx or Ca^{2+} antagonists such as Cd^{2+} (300 μM) or verapamil (100 μM) did not modify the time course nor the magnitude of ^{86}Rb efflux (Table I). Nimodipine (50 μM) markedly reduced the initial peak release and abolished the sustained phase (Fig. 6B). This effect was identical in the presence or absence of external Ca^{2+} (Fig. 6B). ^{86}Rb efflux was insensitive to charybdotoxin, apamin, and iberiotoxin, blockers of the Ca^{2+}-sensitive K^+ channels of large (BK), small (SK), and medium conductance (Table I). ^{86}Rb release in cerebellar granule neurons was also independent of $[Ca^{2+}]_i$. In Ca^{2+}-depleted cells, by treatment with 1 μM ionomycin or thapsigargin in Ca^{2+}-free media, the efflux of ^{86}Rb activated by swelling was unchanged (Fig. 7A). Treatment with BAPTA-AM, 50 μM during 30 min, decreased ^{86}Rb efflux by 15% (Fig. 7B). ^{86}Rb efflux was unaffected in cells treated with ionomycin, in media containing Ca^{2+} (results not shown) despite the remarkable elevation of $[Ca^{2+}]_i$ induced by the ionophore shown in Fig. 5D. All these experiments were carried out in cells after 1 day in culture which might not have expressed yet Ca^{2+}-activated K^+ channels (Carignani et al., 1991; Watkins and Mathie, 1994). Therefore, ^{86}Rb release was examined in fully differentiated cerebellar granule neurons after 8 days in culture (8 DIV). The time course of ^{86}Rb release in these cells was not different from that in immature cells (Fig. 8). Similarly, ^{86}Rb efflux was independent of $[Ca^{2+}]_o$ and was not blocked by charybdotoxin nor decreased by treatments with ionomycin or thapsigargin in Ca^{2+}-free medium (Fig. 8).

DISCUSSION

The present work showed that K^+ efflux is part of the response of cerebellar granule neurons to swelling evoked by hyposmotic solutions. The onset and the amount of released K^+ (traced with ^{86}Rb) were graded responses dependent on the magnitude of cell swelling; but in comparison to other osmolytes, particularly Cl^- and taurine (Sánchez-Olea et al., 1996), the activation threshold to is higher and the onset is delayed. It is then not surprising that K^+ efflux is rate-limiting for RVD (Pasantes-Morales et al., 1994).

The swelling-activated ^{86}Rb efflux in cerebellar granule neurons was insensitive to most of the known blockers of K^+ channels. Only Ba^{2+}, Cs^+, and quinidine significantly affected these fluxes. This contrasts with results in N1E115 neuroblastoma cells (Lippmann et al., 1995) and in other cell types in which RVD is also blocked by 20 mM TEA, suggesting the involvement of TEA-sensitive K^+ channels in cell volume recovery. A study in mesencephalic and hypothalamic neurons reported stretch-activated K^+ channels blocked by Ba^{2+} but not by TEA, 4-AP, or Gd^{3+} (Kim et al., 1995)—but increased after lowering pH, thus showing some similarities with the ^{86}Rb efflux in cerebellar granule neurons.

The present results suggest that the swelling-activated K^+ and Cl^- efflux pathways are interconnected in cerebellar granule neurons. ^{86}Rb efflux was reduced in cells exposed to Cl^--free solutions prior to the hypos-

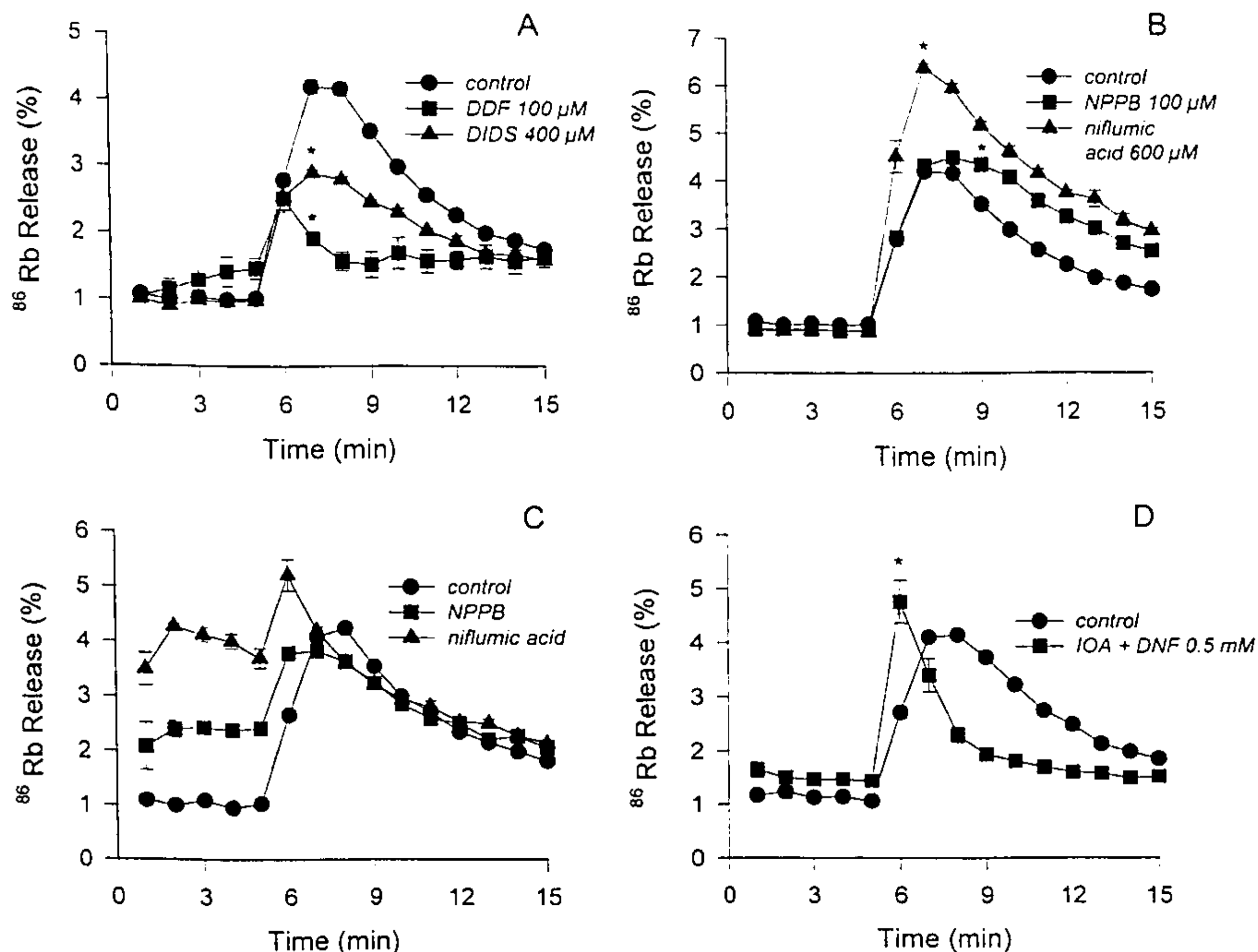

Fig. 3. Effects of Cl⁻ channels blockers on swelling-induced ^{86}Rb release from cultured cerebellar granule neurons. A and B: Cells were exposed to the tested drugs at the concentrations indicated during the hyposmotic stimulus only. C: Cells were preincubated with NPPB or niflumic acid during 10 min before and during the hyposmotic stimulus. D: cells were treated with 1 mM iodoacetate (IOA) and 0.5 mM dinitrophenol (DNF). Data are expressed as in Figure 1. Results are means ± SE of 4–12 experiments. *Significantly different from controls. $P < 0.05$.

motic stimulus, a maneuver directed to deplete intracellular Cl⁻, and also by the Cl⁻ channel blockers DIDS and DDF. The effect of these conditions was observed both on the initial and the sustained phases of ^{86}Rb efflux. The release of Cl⁻ in cerebellar granule neurons occurs immediately after the hyposmotic stimulus, and this may result in cell membrane depolarization which could in turn activate voltage-gated K⁺ channels. In fact, the peak release of ^{86}Rb is delayed with respect to the ^{125}I peak (Sánchez-Olea et al., 1996). In N1E115 neuroblastoma cells, an early depolarization evoked by swelling preceeds the opening of K⁺ channels (Falke and Misler, 1989). Therefore, Cl⁻ efflux reduction in Cl⁻-depleted cells or in cells exposed to Cl⁻ channel blockers may influence a depolarizing-dependent component of K⁺ fluxes and could account for the decrease in ^{86}Rb efflux observed under these conditions. Further supporting a close interconnection between the swelling-activated Cl⁻ and K⁺ fluxes in cerebellar granule neurons is the increase and acceleration of ^{86}Rb efflux observed in cells only exposed to Cl⁻-free medium at the time of the hyposmotic stimulus, without prior Cl⁻ depletion: a condition likely increasing the Cl⁻ exit driving force. The effect of Cl⁻ channels blockers on ^{86}Rb release was examined to further investigate this interdependence. However, not all of them exerted the same action on ^{86}Rb release from cerebellar granule neurons. Whereas DIDS and DDF decreased the volume-sensitive ^{86}Rb efflux, NPPB and niflumic acid increased it. It was observed, though, that NPPB and niflumic acid evoked a marked release of ^{86}Rb fluxes in isosmotic conditions. We initially considered this effect as mediated by the activation of ATP-sensitive K⁺ channels, since these drugs decrease the ATP cell content (Ballatori et al., 1994). This was not

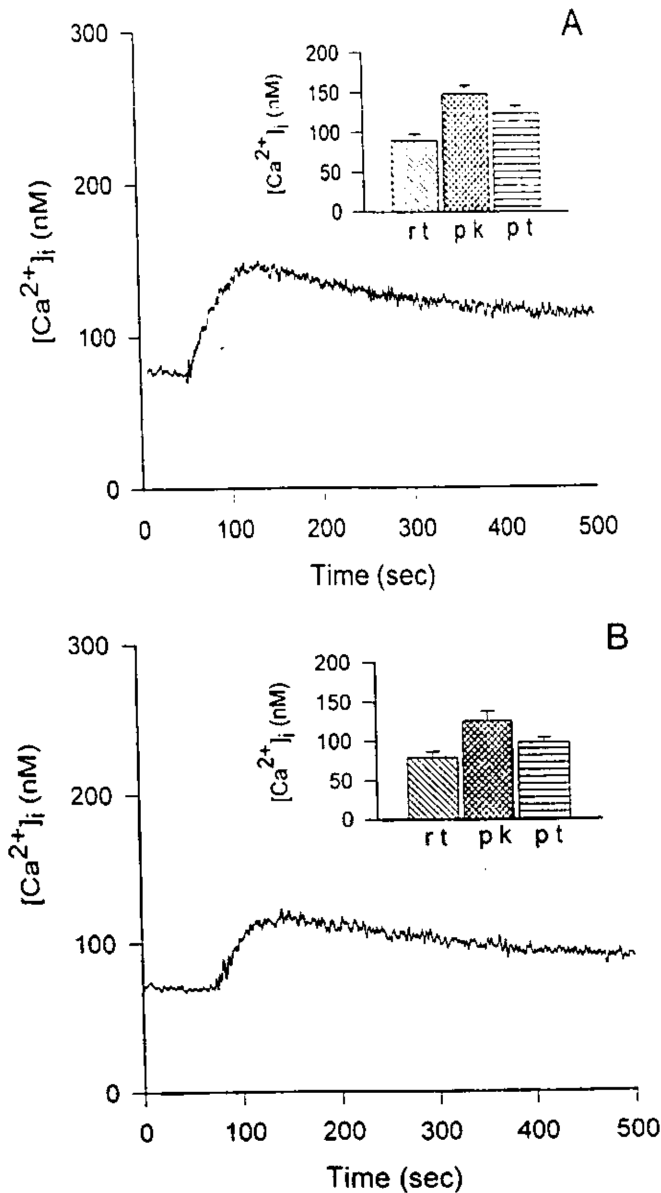

Fig. 4. Changes in $[Ca^{2+}]_i$ evoked by hyposmotic medium in the presence or absence of external Ca^{2+}. Suspended cultured cerebellar granule neurons were loaded with fura-2/AM and the $[Ca^{2+}]_i$ measured as described in Materials and Methods. Traces are from representative experiments. **A:** The initial part of the trace corresponds to the $[Ca^{2+}]_i$ in isosmotic medium. At sec 50 (or 75 in B) medium was changed by a 50% hyposmotic medium. **B:** Same as in A, but in the absence of external Ca^{2+}, plus 0.5 mM EGTA. Insets represent the $[Ca^{2+}]_i$ at different moments of the response: at rest (rt), at the peak (pk), and once a plateau was reached (pt). Bars represents the mean ± SE of 4–10 experiments.

the case, however, since the effect of the Cl^- channel blockers could not be reproduced by depletion of cellular ATP with iodoacetate and dinitrophenol. This treatment decreased the swelling-activated ^{86}Rb efflux with a pattern similar to that of DDF and DIDS, likely reflecting a reduction of Cl^- efflux, which is known to be highly sensitive to cellular ATP levels (Jackson et al., 1994).

In a variety of cell types, particularly in epithelial cells, RVD is markedly Ca^{2+}-dependent (Foskett, 1994). In these cells, Ca^{2+}-sensitive K^+ channels—particularly the BK channels—have been proposed as the channels carrying K^+ fluxes involved in RVD, subsequent to the rise in $[Ca^{2+}]_i$ elicited by swelling (Sarkadi and Parker, 1991). In other cell types (including lymphocytes, Ehrlich ascitic cells, platelets, and hepatocytes), RVD is Ca^{2+}-independent (Foskett, 1994; McCarty and O'Neil, 1992). Results of the present work show that the volume-associated ^{86}Rb efflux in cerebellar granule neurons is independent on external Ca^{2+}. The following evidence supports this notion: 1) ^{86}Rb release was enhanced rather than decreased in Ca^{2+}-free medium; 2) blockers of Ca^{2+} channels or Ca^{2+} antagonists such as verapamil and Cd^{2+} did not affect ^{86}Rb efflux; 3) nimodipine blocked ^{86}Rb efflux (though this is an effect unrelated to Ca^{2+}, since it occurs in the absence of external Ca^{2+}); 4) Gd^{3+}, a blocker of stretch-activated cation channels leading to Ca^{2+} entry which supposedly acts as trigger for K^+ efflux, did not affect ^{86}Rb release; 5) ^{86}Rb fluxes were insensitive to TEA and to iberiotoxin, charybdotoxin, and apamin (blockers of the Ca^{2+}-activated BK, SK, and MK channels); and 6) ^{86}Rb efflux in isosmotic or hyposmotic conditions was unchanged after increasing $[Ca^{2+}]_i$ by ionomycin. ^{86}Rb efflux was also insensitive to Ca^{2+} released from internal stores since it was not affected when the internal reservoirs were depleted (thapsigargin or ionomycin in Ca^{2+}-free medium) or intracellular Ca^{2+}-chelated (BAPTA-AM). Altogether, these results do not associate BK nor other Ca^{2+}-sensitive channels to ^{86}Rb fluxes activated by swelling in cerebellar granule neurons and exclude Ca^{2+} as the transducing signal for the swelling-sensitive K^+ efflux in these cells. Since cerebellar granule neurons used in this study are immature neurons, and even though showing a robust RVD may not have full expression of Ca^{2+}-sensitive K^+ channels (Carignani et al., 1991; Leaney et al., 1997), the Ca^{2+}-dependence of ^{86}Rb efflux was examined in neurons differentiated after 8 days in culture. The insensitivity to both external and internal Ca^{2+} observed in these cells confirms that volume-activated K^+ efflux is Ca^{2+}-independent, regardless of the state of differentiation. Anyway, it is known that even in differentiated granule neurons, after several days in culture, the density of Ca^{2+}-activated K^+ channels is not very high (Cull-Candy et al., 1989; Olesen et al., 1994).

The volume-sensitive fluxes of Cl^- and taurine in cerebellar granule neurons are also Ca^{2+}-independent (Morán et al., 1997), and in accordance with those and the present results, RVD is insensitive to omission of external

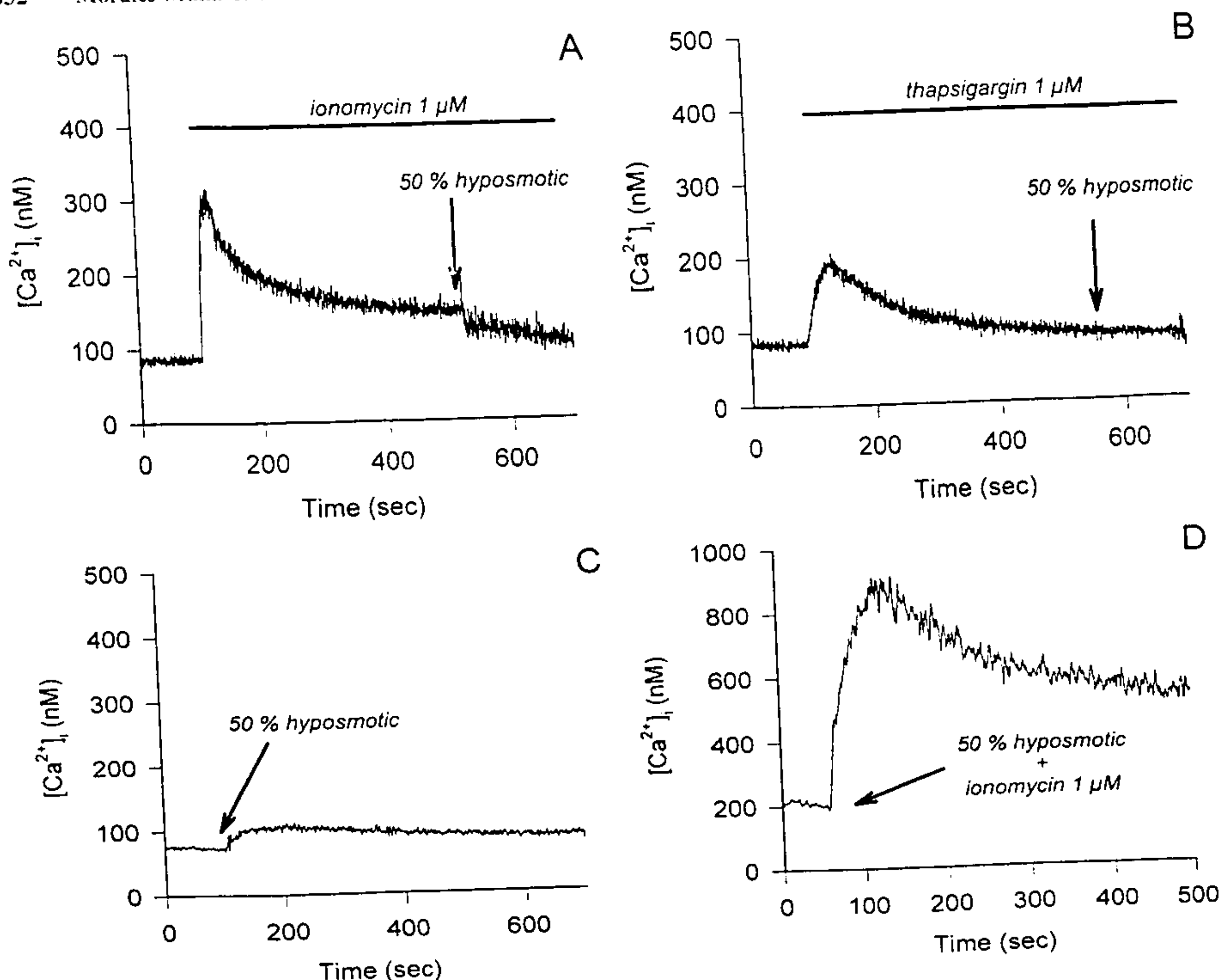

Fig. 5. Effect of ionomycin, thapsigargin, and BAPTA-AM on $[Ca^{2+}]_i$ in cultured cerebellar granule neurons. After 100-sec recording of basal $[Ca^{2+}]_i$, cells were exposed to 1 μM ionomycin (**A**) or 1 μM thapsigargin (**B**), in Ca^{2+}-free medium plus EGTA 0.5 mM; 450 sec later (arrow), the osmolarity was reduced to 150 mOsmol. **C:** Cells were preincubated with 50 μM BAPTA-AM during 30 min before the $[Ca^{2+}]_i$ recording. At the time indicated by the arrow, the osmolarity was by reduced to 150 mOsmol. **D:** The basal trace corresponds to $[Ca^{2+}]_i$ in cells in isosmotic medium. At the time indicated by the arrow, the osmolarity was reduced to 150 mOsmol, supplemented with 1 μM ionomycin. Traces are representative experiments of three separate determinations.

Ca^{2+} or to depletion of internal Ca^{2+} stores. By showing the Ca^{2+}-independence of ^{86}Rb fluxes activated by swelling, this work contributes to a more complete picture of the influence of Ca^{2+} on the efflux pathways for the main osmolytes responsible for RVD in cerebellar granule neurons. Despite this apparent Ca^{2+} independence, in cerebellar granule neurons (as in many other cell types), the hyposmotic stimulus elicited an increase in $[Ca^{2+}]_i$, largely due to Ca^{2+} influx from the external medium but with some contribution of Ca^{2+} released from internal stores (Foskett, 1994; McCarty and O'Neil, 1992). This $[Ca^{2+}]_i$ elevation may be consequence of an increase in cell and organelle membrane permeability to Ca^{2+} by mechanisms still not fully understood (Madhumita et al., 1997; Missiaen et al., 1996), but clearly it seems not linked to the osmolyte fluxes which accomplish RVD.

For those cells, including cerebellar granule neurons (in which K^+ efflux does not occur via Ca^{2+}-sensitive channels), the question remains open of whether some of the K^+ channels observed in the resting state are those modulated by swelling or by factors associated to the change in cell volume, or if there is a specific channel gated only by swelling. Swelling results in a large variety of cell changes including $[Ca^{2+}]_i$ rise, changes in the cytoskeleton organization, phosphorylation of several proteins, and expression of early genes (Ninning et al.,

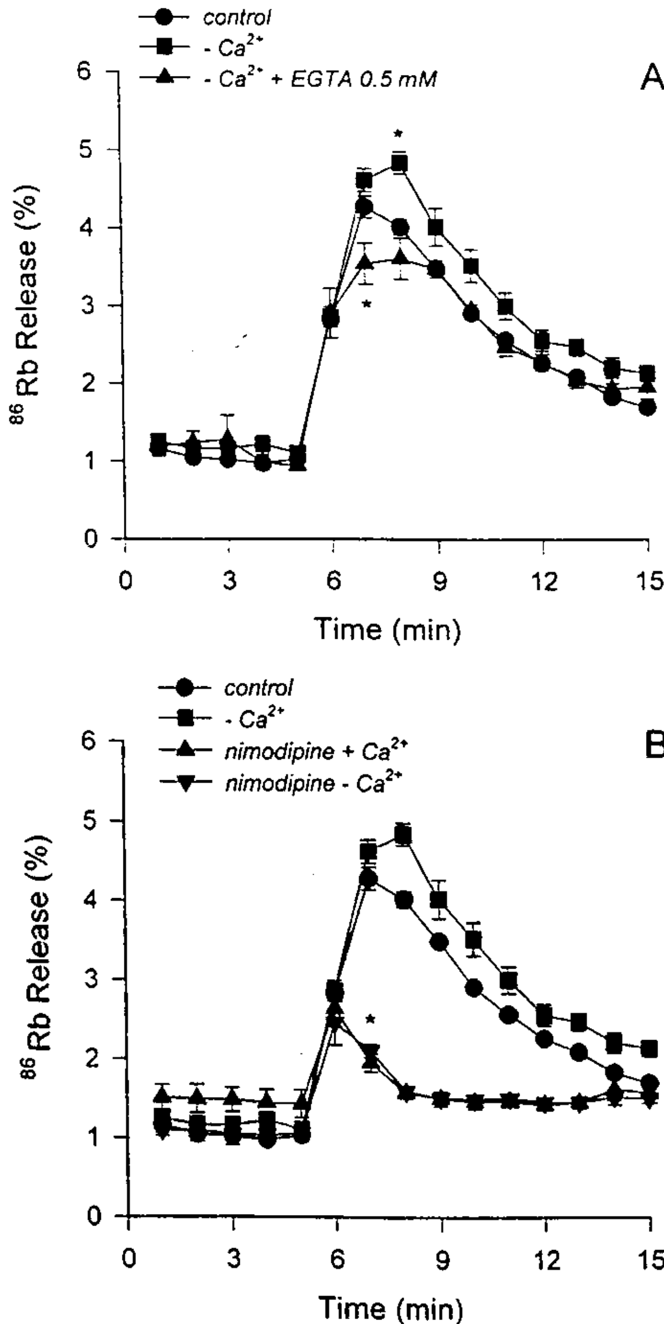

Fig. 6. Effect of Ca^{2+}-free medium and nimodipine on swelling-induced ^{86}Rb release from cultured cerebellar granule neurons. **A:** Cells were exposed sequentially to isosmotic and hyposmotic Ca^{2+}-free media in the absence (■) or presence of 0.5 mM EGTA (▲). **B:** Cells were exposed to isosmotic and hyposmotic media supplemented with 50 μM nimodipine in media with (▲), or without Ca^{2+} (▼). ^{86}Rb release is expresed as in Figure 1. Results are means ± SE of 4–12 experiments. *Significantly different from control, $P < 0.05$.

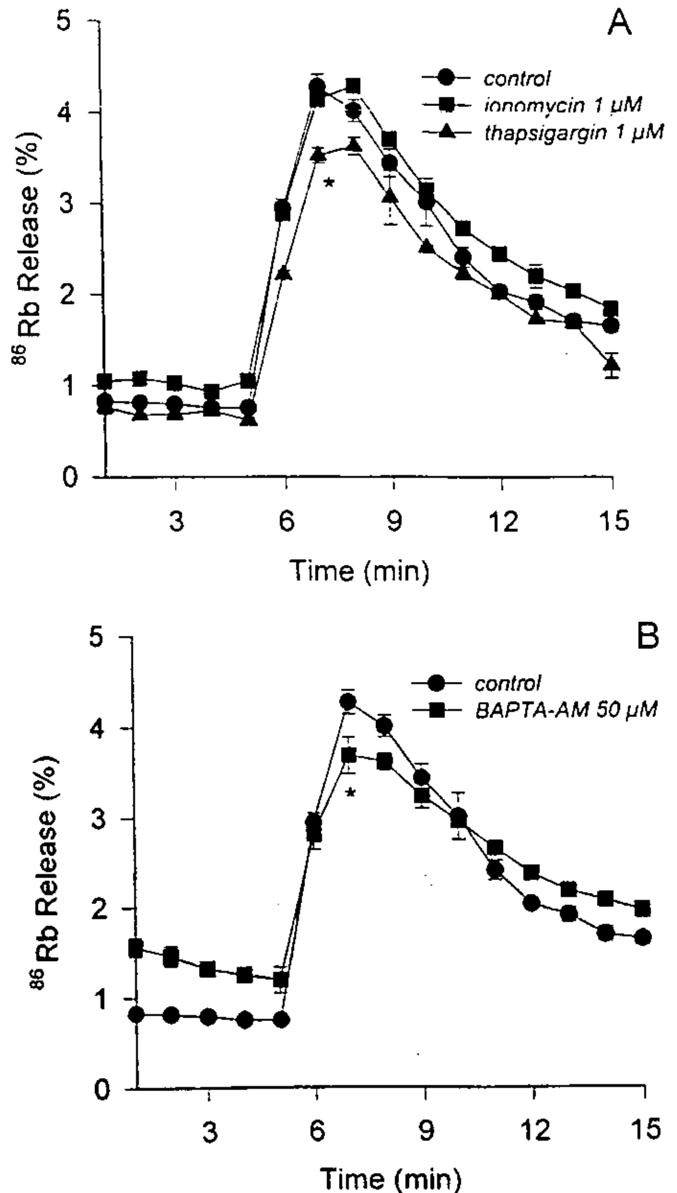

Fig. 7. Effect of ionomycin, thapsigargin, and BAPTA-AM on swelling-induced ^{86}Rb release in cultured cerebellar granule neurons. **A:** Cells were preincubated during 5 min with 1 μM ionomycin or 10 min with 1 μM thapsigargin in isosmotic Ca^{2+}-free medium (plus 0.5 mM EGTA) before exposure to hyposmotic Ca^{2+}-free medium. **B:** Cells were preincubated 30 min with 50 μM BAPTA-AM in isosmotic medium before exposure to the hyposmotic stimulus. Results are means ± SE of 4–12 experiments. *Significantly different from control. $P < 0.05$.

1997; Tilly et al., 1996); but whether these changes are directly modulating K^+ channels related to RVD is at present unclear. Swelling also affects the cell membrane potential according to the nature and the kinetics of the elicited currents. Many cell types depolarize in response to swelling, and this could subsequently activate voltage-gated K^+ channels mediating K^+ efflux during RVD. This has been observed in neuroblastoma cells (Falke and Misler, 1989), where swelling first activates stretch-sensitive unspecific cationic channels depolarizing the cells, and only afterwards Kv channels are activated. In

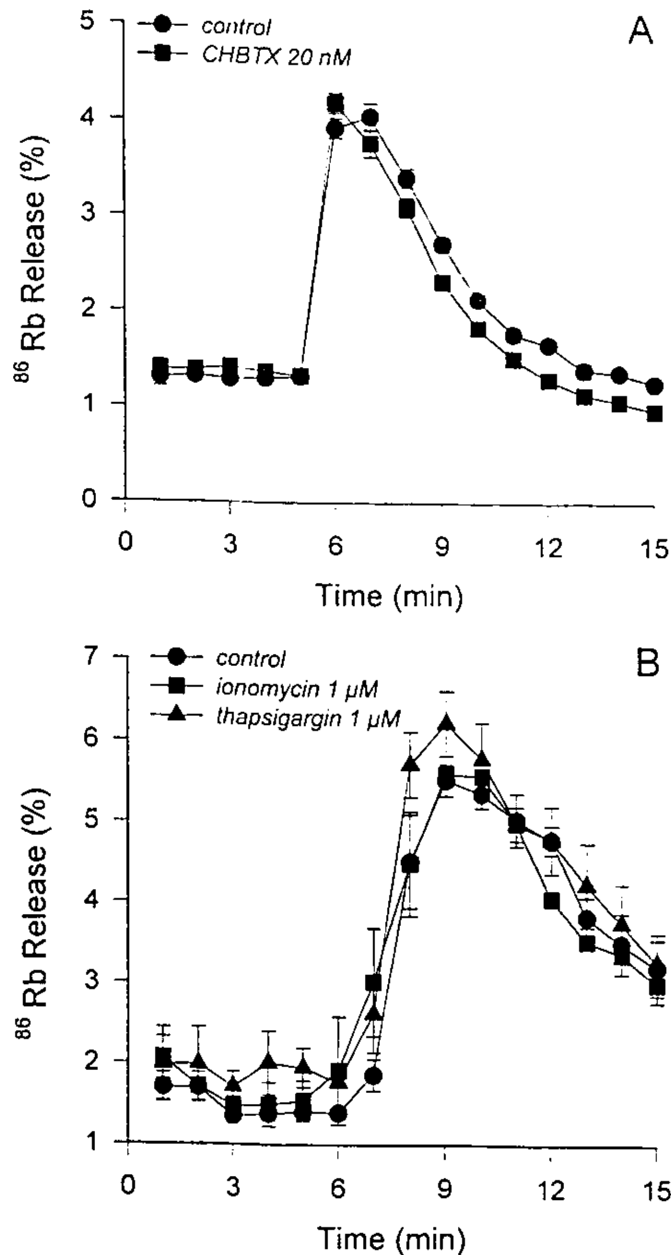

Fig. 8. Effect of charybdotoxin and intracellular Ca^{2+} depletion on ^{86}Rb release induced by swelling from differentiated cerebellar granule neurons (8 DIV). Cells were exposed to hyposmotic media. (**A**) in the presence of 20 nM charybdotoxin (CHBTX); (**B**) after 5 min preincubation in a Ca^{2+}-free medium (0.5 mM EGTA) and 1 μM ionomycin (■), or 10 min with 1 μM thapsigargin (▲). Results are means ± SE of 4–8 experiments.

many cell types, including our cells. Cl^- efflux is immediately elicited by swelling (Sánchez-Olea et al., 1996), leading to depolarization. Kv channels are therefore likely candidates to contribute to K^+ fluxes in these cells. Strong evidence supporting this notion came from a study in T-lymphocytes lacking the ability to regulate volume, which this function restored after transfection and subsequent expression of Kv 1.3 channels (Deutsch and Chen, 1993). This channel subtype seems not involved in K^+ fluxes associated with swelling in our cells; although, since it is markedly sensitive to charybdotoxin, and this toxin did not affect ^{86}Rb efflux (present results), other Kv subtypes may be implicated—as essentially all cloned Kv subtypes are expressed in these cells (Koch et al., 1997). A recent report has shown a direct modulation by hyposmotic solutions of Kv channels of the DR type in hippocampal interneurons, but not in pyramidal cells (Baraban et al., 1997). This raises the question of whether only some Kv channel subtypes are influenced by the change in volume per se, or by cell responses subsequent to swelling. This opens an interesting avenue for future research.

ACKNOWLEDGMENTS

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ARTICULO 4

Journal of Neuroscience Research 57:350–358 (1999)

Influence of Ca^{2+} on K^+ Efflux During Regulatory Volume Decrease in Cultured Astrocytes

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The calcium (Ca^{2+}) dependence of potassium (K^+) efflux activated by hyposmolarity in cultured cerebellar astrocytes was investigated, measuring in parallel experiments ^{86}Rb release and changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Hyposmotic (50%) medium increased $[Ca^{2+}]_i$ from 117 to 386 nM, with contributions of extracellular Ca^{2+} and Ca^{2+} from the endoplasmic reticulum. Hyposmotic medium increased ^{86}Rb efflux rate from 0.015 min^{-1} to a maximal of 0.049 min^{-1} and a net release of 30%. This osmosensitive efflux was inhibited by Ba^{2+} (0.028 min^{-1}), quinidine (0.024 min^{-1}), and charybdotoxin (0.040 min^{-1}), but was unaffected by TEA, 4-AP, or apamin. Removal of external Ca^{2+} from the hyposmotic medium increased ^{86}Rb efflux to a maximal rate constant of 0.056 min^{-1} and a net release of 38% and caused a delay of inactivation. These changes were due to the overlapping of an efflux activated by Ca^{2+} removal in isosmotic medium. This isosmotic ^{86}Rb efflux was unaffected by TEA or 4-AP, reduced by verapamil, and abolished by Ba^{2+}, nitrendipine, and Mg^{2+}. With the swelling-induced $[Ca^{2+}]_i$ rise suppressed by ethyleneglycoltetraacetic acid-acetoxy-methyl ester (EGTA-AM), hyposmotic ^{86}Rb was 30% reduced. The Ca^{2+} entry blockers Cd^{2+}, Ni^{2+}, La^{3+}, and Gd^{3+} did not affect ^{86}Rb efflux. A 40% decrease observed with verapamil and nitrendipine was found unrelated to Ca^{2+}, because these agents did not affect the $[Ca^{2+}]_i$ rise and the inhibition persisted in the absence of external Ca^{2+}. The phospholipase C blocker U-73122 did not affect $[Ca^{2+}]_i$ nor ^{86}Rb efflux. Blockers of Ca^{2+}/calmodulin W7 and KN-93 decreased ^{86}Rb efflux to the same extent as EGTA-AM. Ionomycin markedly potentiated ^{86}Rb release in hyposmotic conditions only when $[Ca^{2+}]_i$ was raised to about 1 μM, suggesting the implication of maxi-K^+ channels at this $[Ca^{2+}]_i$ threshold, which nonetheless, was not attained during hyposmotic swelling. It is concluded that ^{86}Rb efflux in cerebellar astrocytes is largely (70%) Ca^{2+}-independent and the Ca^{2+}-dependent fraction is sustained essentially by Ca^{2+} released from the endoplasmic reticulum and mediated by a mechanism involving Ca^{2+}/calmodulin. J. Neurosci. Res. 57:350–358, 1999.
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Key words: volume regulation; Ca-blockable K channels; volume-sensitive potassium channels

INTRODUCTION

Cultured cerebellar astrocytes respond to swelling evoked by hyposmolar solutions by an active process of cell volume recovery, accomplished by the extrusion of intracellular osmolytes, K^+, Cl^-, and organic molecules, including polyalcohols and amino acids (Kimelberg and Frangakis, 1996; Kimelberg et al., 1990a; Olson et al., 1986; Pasantes-Morales and Schousboe, 1988). The efflux pathways for these osmolytes consist essentially of separate K^+ and Cl^- channels and a leak pathway for the organic molecules, which shares with the anion channel a number of physiologic and pharmacologic features (Jackson and Strange, 1993; Kirk, 1997; Pasantes-Morales, 1996; Roy, 1995). Swelling in astrocytes elicits an immediate and maximal increase in the permeability to Cl^- and amino acids, which represent the initial event in the regulatory volume decrease (RVD). K^+ exit is delayed and is the rate-limiting step in this process (Pasantes-Morales et al., 1994). Increasing the rate of K^+ efflux results in an increase in the rate and efficiency of RVD (Pasantes-Morales et al., 1994; Quesada et al., 1998).

The signal transducing the change in cell volume into the activation of the osmolyte efflux pathways has not been conclusively identified. Ca^{2+} is a likely candidate for this role, because in most cells, including cultured astrocytes, swelling elicits a rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) with contributions of both Ca^{2+} influx and

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Ca^{2+} release from internal sources (Bender et al., 1994; Fischer et al., 1997; Morales-Mulia et al., 1998; O'Connor and Kimelberg, 1993). The osmosensitive K^+ efflux pathway is Ca^{2+} dependent in some cells, particularly in epithelial cells (Sarkadi and Parker, 1991), whereas it seems to be Ca^{2+} independent in other cell types. In cultured astrocytes, this matter is at present unclear, due to some conflicting results. In cortical astrocytes, O'Connor and Kimelberg (1993) reported a blockade of the hyposmotic ^{86}Rb efflux upon removal of external Ca^{2+}. This finding was corrected in a further report showing an increase in ^{86}Rb efflux in Ca^{2+}-free medium (Vitarella et al., 1994). Bender and Norenberg (1994) also showed enhanced hyposmotically stimulated ^{86}Rb efflux in the absence of external Ca^{2+}. Despite these clear results, the conclusion is that ^{86}Rb efflux depends on extracellular Ca^{2+} (Bender and Norenberg, 1994). A partly inhibitory effect of nimodipine of about 20–40% was also considered in support of this conclusion (Bender and Norenberg, 1994; Vitarella et al., 1994). The hyposmotic ^{86}Rb efflux in cortical astrocytes after treatment with 1,2-bis(O-aminophenoxylethane-N,N,N′,N′ tetraacetic acid acetoxy-methyl ester (BAPTA-AM) is reported as partly decreased (Vitarella et al., 1994) or unaffected in Ca^{2+}-free medium (Bender and Norenberg, 1994) or inhibited (40%) if external Ca^{2+} is removed (Bender and Norenberg, 1994). In view of these results, we reexamined the influence of Ca^{2+} from external and internal sources on the swelling-activated ^{86}Rb efflux in cultured cerebellar astrocytes measuring $[Ca^{2+}]_i$ and ^{86}Rb fluxes in parallel experiments. We found results that may provide an explanation for most of the apparent inconsistencies mentioned above.

MATERIALS AND METHODS

Cell Culture

Primary cultures of cerebellar astrocytes were prepared as previously described (Sánchez-Olea et al., 1993). Briefly, the dissociated cell suspensions from cerebellum of 8-day-old rats were plated at a density of 2.1×10^4 cells/cm^2 in plastic dishes. The culture medium consisted of basal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. The culture dishes were incubated at 37°C in atmosphere (5% CO_2/95% air). The enrichment of cultures in astrocytes and the proportion of other cells were assessed by immunocytochemical techniques as previously described (Sánchez-Olea et al., 1993).

^{86}Rb Release Measurements

Cultured cells were incubated in culture medium containing ^{86}Rb (2.5 µCi/ml) for 60 min. After incubation, the medium was replaced with *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-buffered saline containing 135 mM NaCl, 1.0 mM $CaCl_2$, 1.17 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES, and 5 mM glucose, pH 7.4. The medium osmolarity, determined with a freezing-point osmometer (model 5002, Precision Instruments, Natick, MA), was 295 ± 6 mOsm/l. After 15 min of successive washes with this medium, cells were superfused at a rate of 1 ml/min for 5 min, after which stable ^{86}Rb efflux baseline was attained. Then, the superfusion medium was replaced by one of reduced osmolarity (150 mOsm; 50% hyposmotic), and samples were collected for 10 more min. Hyposmotic media were prepared by reducing the NaCl concentration to 60 mM. Control dishes were added with the vehicle used to prepare solutions containing the drugs tested. At the end of the experiments, radioactivity in media and that remaining in the cells was measured in a liquid scintillation counter. Results are expressed as the radioactivity released in each collected sample as percentage of the total radioactivity present in the cells at that time.

Intracellular Free Ca^{2+} Measurement

For Ca^{2+} measurements, cells were detached from dishes by treatment with phosphate-buffered Ca^{2+}-free saline containing 1 ml of ethylenediaminetetraacetic acid (EDTA) and 0.01% trypsin for 2 min and loaded with 5 µM Fura-2/AM at 37°C during 1 hr in basal Eagle's medium. After loading, cells were centrifuged, resuspended in an equal volume of medium, and incubated for an additional 30 min. The cells were then washed, and fluorescence was measured in an Aminco-Bowman luminescence spectrometer (SLM-AMINCO®, Rochester, NY) series 2. Excitation wavelength was alternated between 340 and 380 nm, and fluorescence intensity was monitored at 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the cells with 0.1% Triton X-100 and the minimum fluorescence obtained after buffering the calcium in the solution with 20 mM ethyleneglycoltetraacetic acid (EGTA). The values obtained through this procedure were used to calculate the intracellular calcium concentration according to previously published equations (Grynkiewicz et al., 1995).

RESULTS

$[Ca^{2+}]_i$ Increase by Hyposmolarity

Exposure of cerebellar astrocytes to 50% hyposmotic medium markedly increased $[Ca^{2+}]_i$. This was first examined in individual astrocytes attached to coverslides measured as previously described (Morán et al., 1997). The Ca^{2+} signal largely varied in shape and amplitude

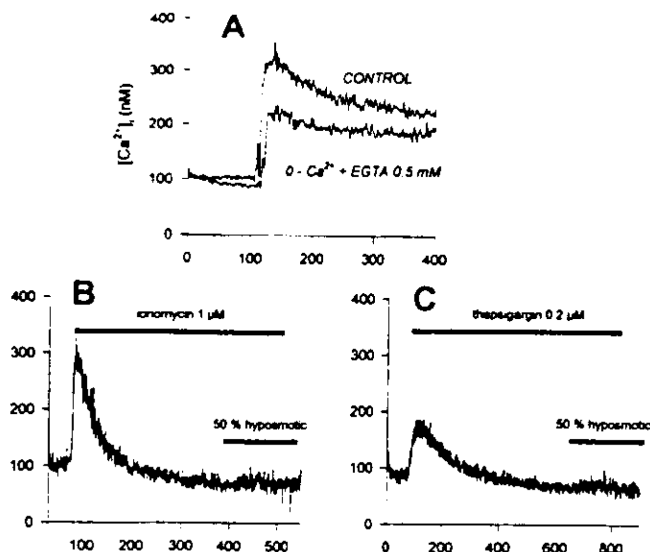

Fig. 1. Intracellular calcium changes in cultured astrocytes induced by hyposmolarity and the effect of ionomycin and thapsigargin exposure before the hyposmotic stimulus. **A:** After 100 sec recording of basal $[Ca^{2+}]_i$ (see Materials and Methods), cells were exposed to 50% hyposmotic medium, either with 1.2 mM extracellular Ca^{2+} (upper trace) or without Ca^{2+} plus 0.5 mM ethyleneglycoltetraacetic acid (EGTA) (lower trace). **B:** Intracellular calcium changes induced by 1 µM ionomycin dissolved in a Ca^{2+}-free/EGTA isosmotic medium (upper bar) and the absence of response to a hyposmotic stimulus (lower bar). **C:** Same as B, but using 0.2 µM thapsigargin as endoplasmic reticulum Ca^{2+}-depleting agent (upper bar). Representative traces of three separate assays are shown.

from essentially no increase, to large, rapid increases up to 800 nM. Due to this variability, we considered a study in a large population of cells in suspension as more representative of the real phenomenon. The average $[Ca^{2+}]_i$ in cells in suspension in isosmotic medium was 117 nM ± 12.4. (n = 16) and raised to 386 ± 36 nM (n = 7) in 50% hyposmotic solutions (Fig. 1A). In Ca^{2+}-free media containing 0.5 mM EGTA, $[Ca^{2+}]_i$ was 87 ± 15 nM in isosmotic medium and 190 ± 26 nM (n = 8) in 50% hyposmotic medium (Fig. 1A), indicating a substantial contribution of Ca^{2+} influx to the $[Ca^{2+}]_i$ elevation. To evaluate the contribution of Ca^{2+} released from endogenous sources, astrocytes were treated with 1 µM ionomycin or 0.2 µM Tg in isosmotic Ca^{2+}-free medium. This treatment elicited a transient increase in $[Ca^{2+}]_i$, and a subsequent hyposmotic stimulus did not evoke any further increase in $[Ca^{2+}]_i$ (Fig. 1B,C). These results in cerebellar astrocytes in suspension were remarkably similar to those reported by Fischer et al. (1997) in their characterization of the hyposmolarity-induced Ca^{2+} response in cultured cortical astrocytes at the single-cell level, showing that the response in astrocytes attached or in suspension is not significantly different.

Influence of Ca^{2+} on ^{86}Rb Efflux

Exposure of cerebellar astrocytes to 50% hyposmotic solutions increased ^{86}Rb release from an efflux rate of 0.022 (min^{-1}) to a maximal rate constant of 0.049 (min^{-1}), attained after 3 min (Fig. 2A). The amount of ^{86}Rb released by the hyposmotic stimulus during the time of the experiment (10 min) was 30 ± 3% (n = 12) of the accumulated radioactivity. Some variations in the response were observed among the different experiments. Therefore, the effects of all drugs or experimental conditions tested were always compared with their corresponding day controls. The osmosensitive release of ^{86}Rb was unaffected by TEA, up to 10 mM, or 1 mM 4AP (results not shown) and was markedly decreased by 1 mM quinidine or barium (Fig. 2A). Charibdotoxin but not apamin inhibited the release, decreasing the maximal rate to 0.040 ± 0.002 (n = 4, $P < 0.05$). Removal of Ca^{2+} from the hyposmotic medium resulted in a significant change in the osmosensitive ^{86}Rb efflux. The time course was modified, showing a delay in the inactivation phase and an increase in the maximal rate constant (Fig. 2B) and in the net amount released (38% in Ca^{2+}-free medium vs. 30% in controls). The possibility was then explored that all these changes were due to the activation of a separate ^{86}Rb efflux elicited by Ca^{2+} removal. This possibility was examined in astrocytes exposed first to isosmotic Ca^{2+}-containing medium and subsequently to isosmotic Ca^{2+}-free (0.5 mM EGTA) medium. Figure 3A shows that Ca^{2+} removal elicited an increase in ^{86}Rb efflux, slowly activating to reach a maximal rate of 0.056 min^{-1} after 10 min, with no detectable inactivation during the time of the experiment. The amount of ^{86}Rb released in 10 min was 36 ± 3% n = 6. This effect of Ca^{2+} removal was essentially the same in solutions in which Ca^{2+} was omitted, containing only the residual Ca^{2+} in water or in those made virtually free of Ca by addition of the chelators BAPTA or EGTA (results not shown). The ^{86}Rb efflux activated by Ca^{2+} removal could be blocked immediately after readdition of Ca^{2+} to the medium. The isosmotic ^{86}Rb release activated by Ca^{2+} removal was unaffected by TEA and 4-AP and markedly inhibited by Ba^{2+} and Mg^{2+} (Fig. 3A). Due to a possible similarity of this pathway with the Ca^{+}-blockable channels described in epithelial cells (Van Driessche et al., 1994; Van Driessche and Erlij, 1994) that are sensitive to dihydropyridines and verapamil, these agents were also tested in the ^{86}Rb release activated by Ca^{2+} removal. Figure 3A shows a marked inhibition of ^{86}Rb efflux by 100 µM verapamil and 25 µM nitrendipine. Also, similar to the Ca^{2+}-blockable channels, isosmotic ^{86}Rb efflux in astrocytes was sensitive to H^{+}, being reduced at external acidic pH (Fig. 3B). This isosmotic ^{86}Rb efflux elicited by Ca^{2+} removal, superimposed to the hyposmotic efflux may

account for the delayed inactivation and the net increase in ^{86}Rb efflux observed in these conditions.

Suppression of all the hyposmotically induced increases in $[Ca^{2+}]_i$ by treatment with 50 μM EGTA-AM during 30 min (Fig. 4A) reduced ^{86}Rb efflux with no change in the activation or inactivation pattern (Fig. 4B). The maximal rate decreased from 0.055 min^{-1} to 0.41 min^{-1} ($P < 0.05$ n = 12), and the amount released from 30% to 24% ($P < 0.05$, n = 12) EGTA-AM was used instead of the more common BAPTA-AM due to some effects of this later, somehow reducing the extent of cell swelling (Morales-Mulia et al., 1998; Jorgensen et al., 1997). Attempts were made to identify the Ca^{2+} source, which sustains this Ca^{2+}-dependent fraction of ^{86}Rb efflux. Because the common approach of external Ca^{2+} removal cannot be used due to the isosmotic activation of ^{86}Rb efflux in this condition, we instead examined the effect of Cd^{2+}, Ni^{2+}, and La^{3+}, which are general blockers of Ca^{2+} channels, and of Gd^{3+}, a known blocker of stretch-activated channels. None of these agents affected ^{86}Rb efflux [maximal rate min^{-1} in controls 0.045 ± 0.004 (n = 8), Cd^{2+} 0.045 ± 0.001, Ni^{2+} 0.044 ± 0.002, La^{3+} 0.040 ± 0.002, and Gd^{3+} 0.042 ± 0.003 (n = 4)]. The L-type Ca^{2+}-channel blockers verapamil and nitrendipine, but not diltiazem, significantly inhibited ^{86}Rb efflux (Fig. 5A). This appears to be an effect unrelated to Ca^{2+}, because these agents did not affect the $[Ca^{2+}]_i$ elevation induced by hyposmolarity (Fig. 5B,C). Besides, when external Ca^{2+} was removed, still nitrendipine inhibited ^{86}Rb efflux (Fig. 5D).

All these results indicate a marginal contribution of external Ca^{2+} to the activation of the osmosensitive ^{86}Rb efflux and rather suggest the involvement of Ca^{2+} release from an endogenous source. This is most likely, the pool stored in the endoplasmic reticulum, as suggested by data showing that upon depletion of this reservoir, there is no Ca^{2+} available to be released by the hyposmotic stimulus. To investigate whether the mechanism of Ca^{2+} release from endoplasmic reticulum involves activation of IP_3 receptors, astrocytes were treated with the phospholipase C (PLC) blocker U-73122. This agent was essentially ineffective (maximal rates: control, 0.048 ± 0.006 min^{-1}; U-73122, 0.046 ± 0.004 min^{-1}; n = 6). The Ca^{2+}/calmodulin blockers W7 and KN-93 decreased ^{86}Rb

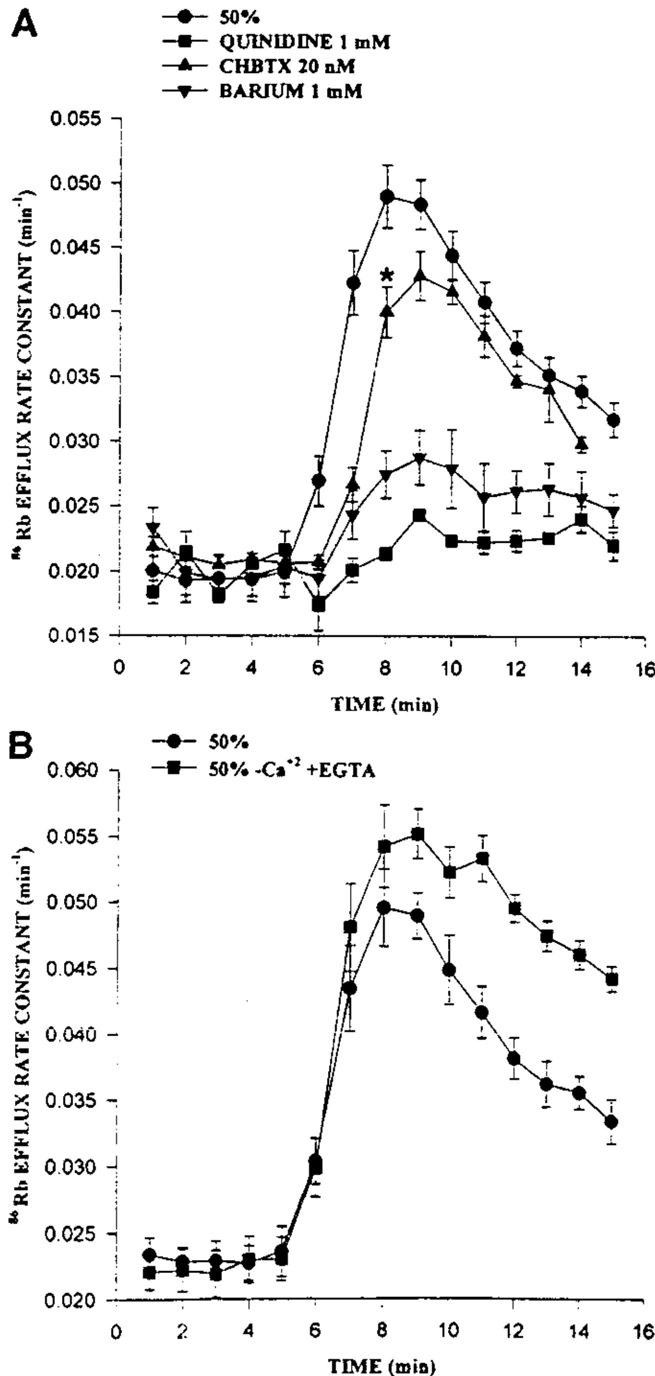

Fig. 2. Time course of the swelling-induced ^{86}Rb release from cultured cerebellar astrocytes. After a 1-hr loading period with 2.5 μCi ^{86}Rb, cells were perfused 15 min with isosmotic medium and fractions were collected each subsequent minute. After a stable efflux was attained, a 50% hyposmotic medium was introduced (sixth min in figure) and fractions were collected for 10 more minutes. A: Swelling-induced ^{86}Rb release without additions (control: circles) and in the presence of 1 mM quinidine (squares), 20 nM charibdotoxin (triangles), or 1 mM barium (inverted triangles) B: Swelling-induced ^{86}Rb release in the presence (circles) or absence (squares) of external Ca plus 0.5 mM ethyleneglycoltetraacetic acid. Data are expressed as efflux rate constant as described in Materials and Methods. Results are means ± SE of 29 (circles) or 6 experiments (all other conditions). *$P < 0.05$; significant differences in A ($P < 0.001$) for quinidine and barium conditions were found from the sixth to fifteenth fraction; symbols were omitted, however, for clarity purposes.

efflux to about the same extent as by the treatment with EGTA-AM (Fig. 6).

Potentiation of ^{86}Rb Efflux by Increasing $[Ca^{2+}]_i$

The osmosensitive efflux of ^{86}Rb from cerebellar astrocytes was dramatically increased by a treatment with 1 μM ionomycin in the presence of Ca^{2+} (Fig. 7A). The magnitude of this increase was clearly dependent on the elevation of $[Ca^{2+}]_i$ due to the ionophore, which in turn increased with increasing the external Ca^{2+} concentration (Fig. 7B). The effect of ionomycin potentiating ^{86}Rb efflux could be observed only when $[Ca^{2+}]_i$ levels increased over 1 μM. In isosmotic conditions ionomycin did not affect ^{86}Rb efflux (results not shown). At variance with results in cortical astrocytes (Bender and Norenberg, 1994), in which ouabain markedly increased the osmosensitive ^{86}Rb efflux, in our preparation this agent did not increase $[Ca^{2+}]_i$ and did not potentiate ^{86}Rb efflux (results not shown).

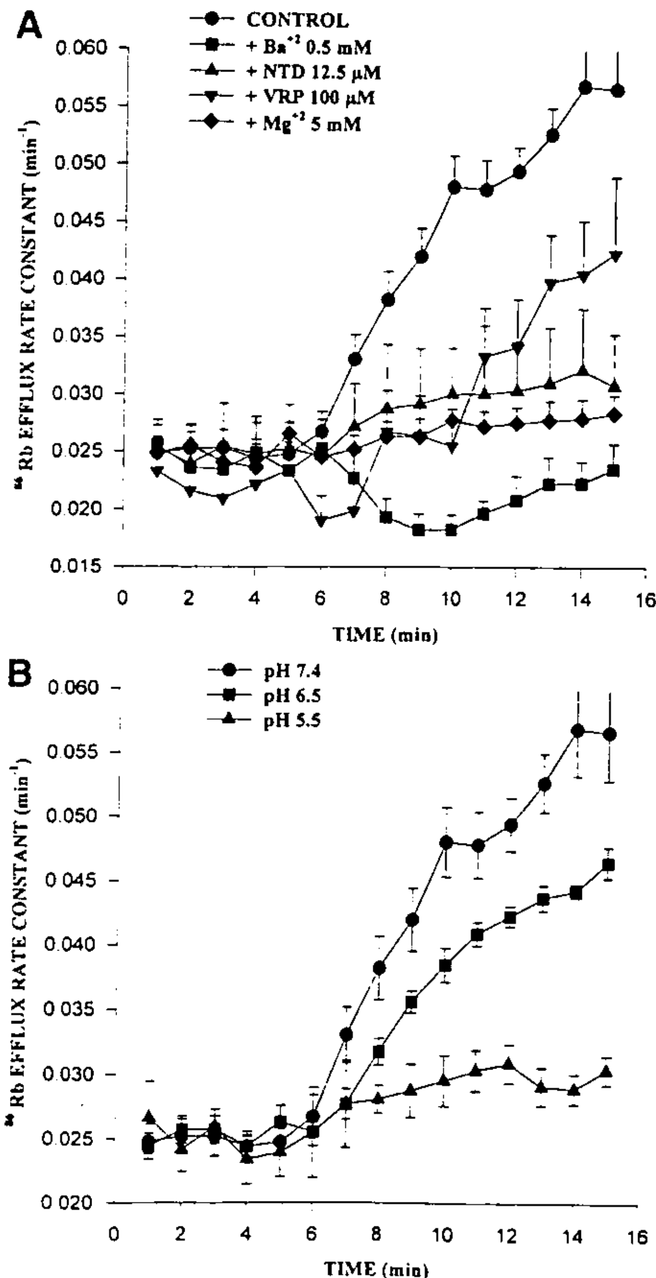

Fig. 3. Time course of the isosmotic ^{86}Rb release activated by Ca^{2+} removal from cultured cerebellar astrocytes. Dishes preloaded with ^{86}Rb were perfused with isosmotic medium until a stable ^{86}Rb efflux was attained. Then, the perfusing medium was switched to an isosmotic Ca^{2+}-free medium added with 0.5 mM ethyleneglycoltetraacetic acid (EGTA). **A:** ^{86}Rb release induced by Ca^{2+}-free media plus EGTA (circles) and supplemented with 0.5 mM barium (squares) 12.5 μM nitrendipine (NTD; triangles) 100 μM verapamil (VRP: inverted triangles) or 5 mM Mg^{2+} (diamonds). Significant differences ($P < 0.05$ for NTD and $P < 0.01$ for VRP and Mg^{2+}) were found. **B:** Sensitivity of the isosmotic ^{86}Rb release to extracellular pH. Control (circles) at pH 7.4 and at pH 6.5 (squares) or 5.5 (triangles). Results are means ± SE of 6–26 experiments. Significant differences ($P < 0.01$ for pH 6.5 curve and $P < 0.001$ for pH 5.5 curve) were found for every point starting from the eighth fraction, compared with the Ca^{2+}-free condition (control); significance symbols were omitted for simplicity.

DISCUSSION

It has been consistently observed that omission of extracellular Ca^{2+} does not decrease but rather increases the osmosensitive ^{86}Rb efflux in astrocytes, a result in conflict with its proposed Ca^{2+} dependence (Bender and Norenberg, 1994; Sánchez-Olea et al., 1993; Vitarella et al., 1994). The finding in the present work of a ^{86}Rb efflux activated by Ca^{2+} removal in isosomotic medium, contributes to clarify this point. It also explains why, in cortical astrocytes, inhibition of the osmosensitive ^{86}Rb efflux by BAPTA-AM is reversed in Ca^{2+}-free medium (Bender and Norenberg, 1994). Besides, this K^+ movement activated in the absence of external Ca^{2+}, by increasing the net amount of K^+ released, explains the apparently paradoxical acceleration of RVD observed in this condition (Morales-Mulia et al., 1998). The mechanism by which Ca^{2+} omission activates ^{86}Rb efflux is unclear. Nonspecific cation channels gated by removal of divalent cations have been described in epithelial cells (Van Driessche et al., 1994; Van Driessche and Erlij, 1994), with features resembling those of the ^{86}Rb efflux activated by Ca^{2+} removal in astrocytes, such as inhibition at low pH and blockade by Ba^{2+}, verapamil, and dihydropyridines. These channels could be those carrying K^+ in the absence of external Ca^{2+}. The occurrence of increased K^+ efflux by Ca^{2+} removal has not been closely exam-

ined in other cell types. This possibility should be considered, though, in all studies in which the Ca^{2+} dependence of osmosensitive K^+ movements is investigated.

In astrocytes, as in many other cell types, hyposmotic swelling leads to a transient increase in $[Ca^{2+}]_i$ with contributions from both extracellular and intracellular sources (Bender et al., 1994; Fischer et al., 1997; Morales-Mulia et al., 1998; O'Connor and Kimelberg, 1993). In cerebellar astrocytes, suppression of the swelling-associated $[Ca^{2+}]_i$ rise by EGTA-AM reduced the osmosensitive ^{86}Rb efflux by 30%. Attempts were made in this work to identify the source of Ca^{2+} involved in this component of ^{86}Rb efflux. Because neither general blockers of Ca^{2+} channels such as Cd^{2+}, Ni^{2+}, and La^{3+}, nor the blocker of stretch-activated channels Gd^{3+}, affected ^{86}Rb efflux, external Ca^{2+} influx appears not influential as a mechanism sustaining the Ca^{2+}-dependent fraction of ^{86}Rb efflux. Verapamil and nitrendipine, but not diltiazem, exhibited an inhibitory action, which, however, cannot be attributable to a blockade of Ca^{2+} entry, because first, these agents did not decrease Ca^{2+} entry during swelling, and second, substantial inhibition persists in the absence of external Ca^{2+}, when there is no Ca^{2+} influx. It is noteworthy that these agents also potently blocked the ^{86}Rb efflux activated by Ca^{2+} removal, a condition which obviously excludes an effect related to Ca^{2+} influx. There is increasing evidence of direct effects of verapamil and dihydropyridines on K^+ channels, including maxi-K^+ channels and voltage-gated channels (Armstrong and Miller, 1990; Fagni et al., 1994; Schlichter et al., 1997). In these later, a Ca^{2+}-binding site at the outer domain of the pore region seems required to stabilize the native conformation of the protein preserving the normal operation of the channel (Armstrong and Miller, 1990). This may be the site where verapamil and dihydropyridines are interacting. In any event, evidence of the direct effect of these agents on at least two types of K^+ channels, suggests caution in interpreting their effects as strictly related to Ca^{2+} dependence in the osmosensitive K^+ pathways.

As for the involvement of Ca^{2+} from internal stores, there is evidence in cortical (Fischer et al., 1997) and

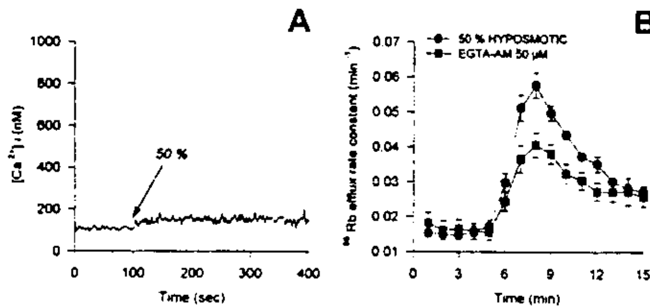

Fig. 4. Effect of ethyleneglycolteuraacetic acid-acetoxy-methyl ester (EGTA-AM) on $[Ca^{2+}]_i$ and ^{86}Rb efflux induced by hyposmolarity. **A:** Suppression of $[Ca^{2+}]_i$ rise in cultured astrocytes preloaded with 50 µM EGTA-AM and exposed to a 50% hyposmotic medium (arrow). Representative trace of three separate determinations. **B:** Time course of the ^{86}Rb efflux triggered by a 50% hyposmotic medium (at the sixth min) from control dishes (circles) and from cells preloaded with EGTA-AM (squares). Results are means ± SE of 6–12 experiments. Significant differences ($P < 0.05$) between traces from the seventh to the twelfth minute were found.

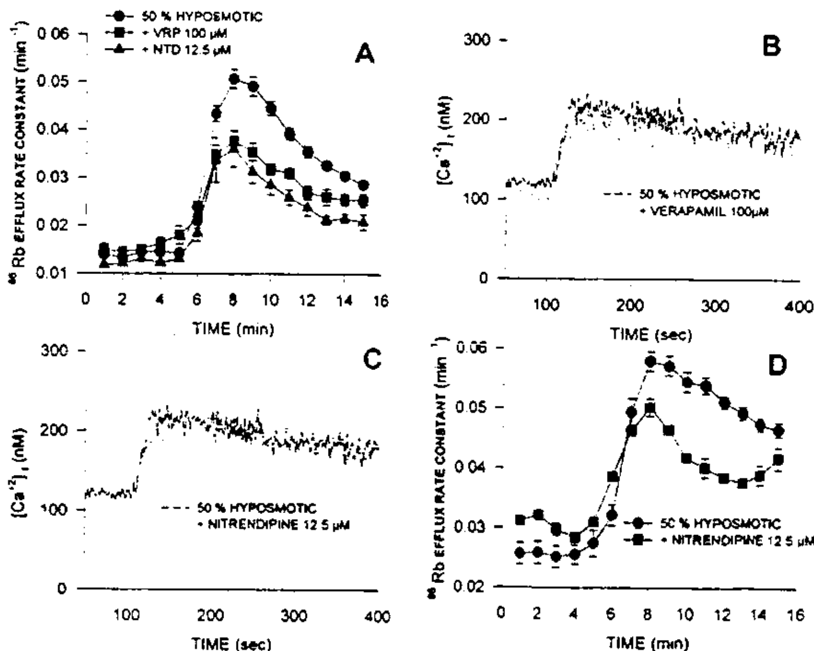

Fig. 5. The effects of verapamil (100 µM) and nitrendipine (12.5 µM) on the $[Ca^{2+}]_i$ increase and the ^{86}Rb efflux induced by swelling. **A:** ^{86}Rb efflux from cells exposed at the sixth minute to a 50% hyposmotic medium without additions (control, circles), plus verapamil (squares), or nitrendipine (triangles). **B:** $[Ca^{2+}]_i$ increase induced by swelling in the presence or absence of verapamil. **C:** Same as in B, but testing the effect of nitrendipine on the swelling-induced $[Ca^{2+}]_i$ increased. **D:** Effect of nitrendipine on the swelling-induced ^{86}Rb efflux in the absence of external Ca^{2+}. Control (circles) and nitrendipine (squares). Results are means ± SE of 6–12 experiments for A and D. B and C are representative pairs of traces of three separate assays.

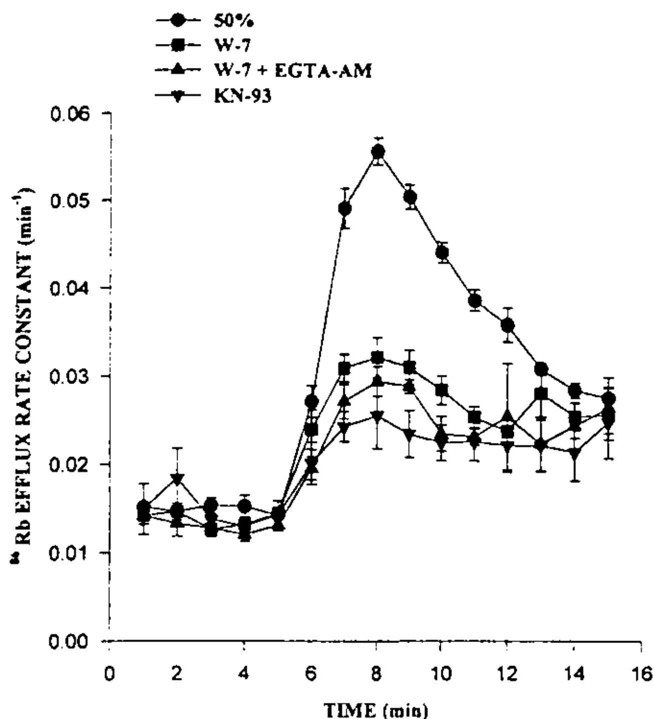

Fig. 6. Effects of the Ca^{2+}-calmodulin complex inhibitors W-7 and KN-93 on the swelling-induced ^{86}Rb release from astrocytes. The hyposmotic stimulus started at the sixth minute. Control condition (circles) with no additions. The effect of KN-93 reagent added with the hyposmotic stimulus is shown (inverted triangles). W-7 (50 μM) was preincubated 10 min and was present during the whole experiment; its effect was tested on untreated cells (squares) and in cells previously loaded with 50 μM ethyleneglycoltetraacetic acid-acetoxy-methyl ester (EGTA-AM; triangles); no significant differences were found between these two conditions. Results are means ± SE of four to six separate experiments.

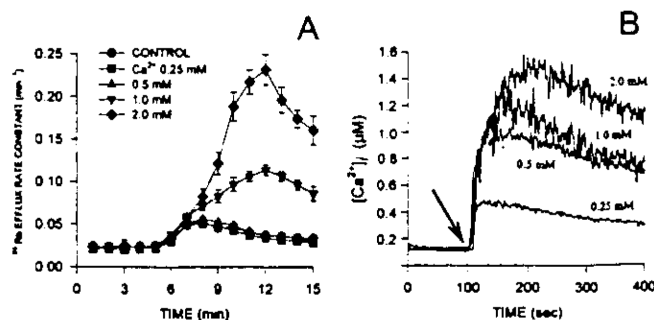

Fig. 7. Potentiation by ionomycin of the $[Ca^{2+}]_i$ changes and ^{86}Rb efflux induced by hyposmolarity. Hyposmotic media containing different $[Ca^{2+}]_o$ were supplemented with 1 μM ionomycin. A: ^{86}Rb efflux induced by 50% hyposmotic media (at the sixth minute in figure), containing the indicated $[Ca^{2+}]_o$. Results are means ± SE of six separate experiments. Notice the ordinate scale change compared with previous figures. B: Time course of the $[Ca^{2+}]_i$ in astrocytes exposed to 50% hyposmotic medium containing 1 μM ionomycin (arrow) and the indicated $[Ca^{2+}]_o$; representative set of traces of two to three separate assays is shown.

cerebellar astrocytes (present results) that points out to the endoplasmic reticulum pool as the source of Ca^{2+} released by the hyposmotic stimulus; therefore, this reservoir should be considered as the main contributor to the Ca^{2+}-dependent fraction of ^{86}Rb efflux. The mechanisms releasing Ca^{2+} from these stores by hyposmotic swelling are still unclear in astrocytes. Although the implication of the endoplasmic reticulum Ca^{2+} pool seems clear (Bender et al., 1993; Fischer et al., 1997; Morales-Mulia et al., 1998), this does not necessarily mean that the release occurs through activation of IP_3 receptors (Bender et al., 1993). In cortical astrocytes, the PLC blocker U-73122 markedly decreased ^{86}Rb efflux, a result supporting the involvement of IP_3 receptors (Bender et al., 1994). In our preparation, this agent had only a marginal inhibitory effect on ^{86}Rb efflux, which besides, was reproduced by its inactive analogue. An additional conflicting evidence is the lack of effect of U-73122 on the $[Ca^{2+}]_i$ rise evoked by swelling in cortical astrocytes (Schliess et al., 1996). Caffeine decreases ^{86}Rb efflux in cortical astrocytes somehow involving also the ryanodine receptor (Bender et al., 1994). Other proposed mechanisms for Ca^{2+} release from the endoplasmic reticulum implicate (1) a direct effect releasing Ca^{2+} by arachidonic acid increased by swelling (Tinel et al., 1997); (2) the osmosensitive mechanical opening of Ca^{2+} channels at the endoplasmic reticulum; and (3) the activation of voltage-gated channels, sensing a voltage change after swelling, between endoplasmic reticulum and cytosol (Jena et al., 1997; McCarthy and O'Neil, 1992; Missiaen et al., 1996).

Reactions mediated by Ca^{2+}/calmodulin have been implicated in the hyposmotically stimulated ^{86}Rb efflux in cortical astrocytes based on the effect of blockers of Ca^{2+}/calmodulin and PKC. It was shown that KN-93 decreased ^{86}Rb release and W7 suppressed it completely (Bender et al., 1994). This later effect is difficult to interpret only in terms of Ca^{2+} dependence, because as shown in the same study (Bender et al., 1994), in astrocytes with all Ca^{2+} chelated by BAPTA-AM, ^{86}Rb efflux decreased only 45%. Thus, its complete blockade by W7 suggests actions additional to those of interruption of the Ca^{2+}/calmodulin-mediated reactions. We also found inhibition of ^{86}Rb efflux by W7 and KN-93 similar to that observed when all $[Ca^{2+}]_i$ is quenched by EGTA-AM, implicating Ca^{2+}/calmodulin in the mechanisms of activation of the Ca^{2+}-dependent fraction of ^{86}Rb efflux in cerebellar astrocytes.

An observation considered as further evidence in support of the osmosensitive Ca^{2+}-dependence of ^{86}Rb

efflux was the large increase induced by ionomycin (Bender et al., 1994). In the present work, the parallel study of changes in $[Ca^{2+}]_i$ allowed us to demonstrate that this increase starts only when $[Ca^{2+}]_i$ reached levels well in excess of those attained during swelling. Thus, it is not strictly Ca^{2+} dependence but a potentiation by a large $[Ca^{2+}]_i$ rise elicited by the ionophore. This may be due to the contribution of additional pathways activated at these Ca^{2+} thresholds. Likely candidates are the Ca^{2+}-activated maxi-K^+ channels, which have the dual requirement for depolarization and a large concentration of $[Ca^{2+}]_i$ (1–2 μM) for activating. Potentiation of ^{86}Rb release by ionomycin in astrocytes was observed when $[Ca^{2+}]_i$ increased to levels close to or above 1 μM and only in association with swelling, which in astrocytes comport a marked depolarization (Kimelberg et al., 1990b; Olson and Li, 1997). In a number of cell lines, particularly in epithelial cells, the K^+ channels activated by hyposmolarity are the Ca^{2+}-activated maxi-K^+ channels (Sarkadi and Parker, 1991).

In conclusion, the swelling-activated ^{86}Rb efflux from astrocytes exhibit a Ca^{2+}-dependent fraction of 30% (cerebellar astrocytes) and 45% (cortical astrocytes; Bender et al., 1994) and a large Ca^{2+}-independent fraction responsible for the remaining 55–70%. The Ca^{2+}-dependent fraction is sustained by Ca^{2+} released from the endoplasmic reticulum, and the mechanism of activation involves Ca^{2+}/calmodulin. It is still undefined whether the Ca^{2+}-dependent and Ca^{2+}-independent components correspond to separate pathways or represent a single pathway with partial Ca^{2+} dependence. It is also unclear at present whether a K^+ channel directly activated by swelling is present in astrocytes or whether K^+ efflux occurs through other types of K^+ channels activated by events concurring with swelling. The increase in $[Ca^{2+}]_i$ and the depolarization known to occur in astrocytes upon swelling (Olson and Li, 1997), may activate maxi-K^+ channels or/and voltage-gated K^+ channels, both contributing to the osmosensitive ^{86}Rb efflux. An electrophysiologic characterization in detail of the K^+ channel(s) activated during swelling is necessary to clarify these points.

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ARTICULO 5

INFLUENCE OF PROTEIN KINASES ON THE OSMOSENSITIVE RELEASE OF TAURINE FROM CEREBELLAR GRANULE NEURONS

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Abstract

The role of phosphorylation events on the activation and modulation of the osmosensitive ^{3}H-taurine release (OTR) was examined in cultured cerebellar granule neurons (CGN) stimulated with 30% hyposmotic solutions. OTR was not decreased when $[Ca^{2+}]_i$ rise evoked by hyposmolarity was prevented by EGTA-AM (50 µM) or depleted by treatment with 1 µM ionomycin in Ca^{2+}-free medium. Accordingly, OTR was not inhibited by Ca^{2+}-dependent signalling events. The calmodulin (CAM) blocker W-7 (50 µM) potentiated OTR while the Ca^{2+}/CAM kinase blocker KN-93 (10 µM) was without effect. Blockade of PKC by H-7, H-8 (50 µM) and Gö6976 (1 µM), as well as activation by phorbol myristate acetate (PMA) (100 nM) did not influence OTR, but chronic treatment to down regulate PKC decreased it by 30%. Forskolin (20 µM) and 8-BrcAMP (10 µM) did not change OTR. Protein tyrosine phosphorylation seems of crucial importance in the activation and modulation of OTR, as it was markedly inhibited (90%) by tyrphostine A23 (50 µM) and potentiated by the tyrosine phosphatase inhibitor ortho-vanadate (100 µM). The PI3 kinase blocker wortmannin 100 nM essentially abolished OTR but LY294002 (10-100 µM) was without effect. This difference may be accounted for PI3K isoforms in neurons with different sensitivity to the blockers. Alternatively, the effect of wortmannin may be exerted not in PI3 kinase but instead on phospholipases, which are also sensitive to this blocker. The hyposmotic stimulus induced activation of Erk1/Erk2, but blockade of this effect by PD 98059 (50 µM) only marginally decreased OTR suggesting that the Erk1/Erk2 is an epiphenomenon not directly involved in OTR activation.

Key words: Tyrosine kinase, Erk1/Erk2, swelling, wortmannin, PI3 kinase

1. Introduction

The active process of volume adjustment allowing cells to recover their normal volume after swelling in hyposmotic solutions seems to be a general response of animal cells. The extrusion of intracellular osmolytes, K^+, Cl^- and organic molecules through difusional pathways, seems to be also a widespread response, common to most cell types (Lang, 1998). An anion channel-like molecule has been proposed as a common pathway for translocation of Cl^- and organic osmolytes, including amino acids, and its properties and molecular characterization have received considerable attention (Strange et al., 1996; Nilius et al., 1997; Pasantes-Morales, 1996; Okada, 1997). Less is known about the molecular signalling mechanisms, which transduce the cell volume change into the activation of these efflux pathways. Calcium, and calcium-activated signalling systems appear as likely candidates, since with few exceptions, hyposmotic swelling leads to an increase in the cytosolic concentration of Ca^{2+} (McCarthy and O'Neil, 1992, Foskett, 1994; Okada, 1997). However, despite this consistently observed response, the osmosensitive Cl^-/taurine fluxes are reported as Ca^{2+}-independent or Ca^{2+}-dependent (Strange et al., 1996), suggesting that the involvement of Ca^{2+} in the mechanisms gating the osmolyte efflux pathway may be cell specific. Increases in the intracellular levels of other messengers such as cAMP and IP_3 in association with a hyposmotic stimulus have also been reported in some cell types (Strange et al., 1996; Nilius et al., 1997; Okada, 1997). These molecules may act as second messengers to turn on protein kinases, potentially involved in the transduction cascade. In addition there is growing evidence showing phosphorylation of a number of tyrosine kinases in response to hyposmotic stress (Schliess et al., 1995, 1996; Sadoshima et al., 1995; Tilly et al., 1996a, 1996b; Sinning et al., 1997; Lepple-Wienhues et al., 1998; van der Wijk et al., 1998; Crépel et al., 1998). Thus, it is not unlikely that the change in cell volume and the osmosensitive Cl^-/taurine channel activation are connected through various tyrosine phosphorylation steps. In fact, general blockers of tyrosine phosphorylation decrease $^{125}I^-$ fluxes elicited by swelling in Intestine 407 cells (Tilly et al., 1996a, 1996b; van der Wijk et al., 1998) and the

osmosensitive Cl^- current cultured cortical astrocytes (Crépel et al., 1998). To contribute to the understanding of the osmotransduction mechanisms, in the present study in CGN, we examined how blockade of Ca^{2+}-dependent and Ca^{2+}-independent protein kinases and of tyrosine kinases, influence taurine fluxes elicited by hyposmotic swelling. Taurine is functions as an osmolyte in a wide variety of cell types and is often used as marker for the Cl^-/amino acid osmosensitive pathway.

2. Experimental procedures

2.1. *Cell cultures*

The dissociated cell suspensions from cerebella of a 7 day old rats were plated at a density of 265 x 10^3 cells/cm^2 in plastic dishes previously coated with poly-L-lysine (5 μg/ml). The culture medium consisted of basal Eagle´s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Cytosine arabinoside (10 μM) was added to the culture medium to prevent replication of non-neuronal cells. The culture dishes were incubated at 37°C in humidified 5% CO_2/95 % air atmosphere. The enrichment of cultures in neurons was determined using polyclonal antibodies against neuron-specific enolase and GFAP, and staining was determined by immunofluorescence. The proportion of neurons in these cultures was 95% and that of glial cells was estimated as 2-4%.

2.2. *^{3}H-taurine efflux*

Cells loaded with ^{3}H-taurine (1 μCi/ml; 1 h) were washed during 15 min with isosmotic medium containing (in mM): 135 NaCl, 5 KCl, 0.6 $MgSO_4$, 1 $CaCl_2$, 10 glucose, 1.7 KH_2PO_4 and 10 HEPES, pH 7.4. The osmolarity calculated was 300 mOsm/kg medium and was verified in a freezing point osmometer (Osmette A. Precision Systems Inc.). Hyposmotic solutions were always prepared by reducing the concentration of NaCl. After washing, ^{3}H-taurine efflux was subsequently measured in a 3 min period in isosmotic and 5 min in hyposmotic medium (30%, 210 mOsmol). At the end of the experiment, cells

were solubilized with 0.4 N NaOH, and radioactivity in the collected samples was measured in a liquid scintillation counter. Results are expressed as efflux rate constants (i.e., the radioactivity present in the medium at each period of time as percent of total radioactivity present in the cells at that time). All drugs were pre-incubate at the indicated times and were present throughout experiments. Control cells were always treated in parallel with the vehicle used to prepare solutions containing the tested drugs, usually DMSO, at a concentration not exceeding 0.1%.

2.3. *Intracellular* Ca^{2+}

For measurements of change in $[Ca^{2+}]_i$, cells were detached by treatment for 1 min with phosphate-buffered Ca^{2+}-free saline containing 1 mM EDTA and 0.01% trypsin and loaded at 37 °C during 1 h in basal Eagle's medium containing 5 μM Fura-2/AM. When cells were exposed to EGTA-AM, it was added 30 min after starting the Fura-2/AM incubation. After loading cells were centrifuged, resuspended in an equal volume of medium and incubated for additional 30 min. The cells were then washed twice and fluorescence was measured in an Aminco-Bowman series 2-luminescence spectrometer. Excitation wavelength alternated between 340/380 nm and fluorescent intensity was monitored at an emission wavelength of 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the cells with 0.1% triton X-100 and the minimum fluorescence obtained after buffering the calcium in the solution with 20 mM EGTA. The values obtained with this procedure were used to calculate the intracellular calcium concentration according to previously published equations (Grynkiewicz, et al., 1985).

2.4. *Erk1/Erk2 kinase activity*

Activity assays were performed according to Alessi et al., (1995) and Hawes et al., (1995) with some modifications. Cells were incubated at 37 °C and treated with isosmotic or hyposmotic media, in the presence or absence 50 μM PD 98059. Cells were lysed in

buffer containing: 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1mM ortho-vanadate, 1 mM sodium β-glycerol phosphate, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% Nonidet-P40, 0.2 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1% β-mercaptoethanol 20 mM Tris-acetate (pH 7.5). A supernatant of this fraction was obtained by centrifugation at 20,000 x g for 15 min, 2.5 mg of this supernatant protein was immunoprecipitated, with a polyclonal antibody to Erk1/Erk2 and protein A sepharose, for 2 h at 4 °C with gentle rocking. Immunoprecipitates were washed with kinase buffer containing: 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, and 1 mM dithiothreitol; then they were pre-incubated with 250 μg/ml myelin basic protein (MBP), 1 μM okadaic acid for 5 min at 30 °C. Reaction was initiated with 20 mM ATP and 10 μCi/ml [γ^{32}P]-ATP. After 30 min, the reaction was stopped by adding Laemmli sample buffer (1970) and the material subjected to 15% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). Gels were stained, dried and exposed to Molecular Dynamics phosphorimager plates.

Duplicated samples of activity were after resolving by 15% SDS-PAGE, transferred to nitrocellulose membrane. Then, membranes were blocked with 5% skim milk in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl (TBS) for 2 h at 22 °C and incubated with anti-Erk1/Er2 antibody at 22 °C for 1 h. After washing, membranes were incubated at room temperature with horseradish peroxidase-conjugated goat anti-rabbit. Protein bands were detected by ECL method.

3. Results

3.1. *The osmosensitive taurine efflux is independent of the $[Ca^{2+}]_i$ rise elicited by hyposmotic swelling.*

Exposure of CGN to 30% hyposmotic medium increased the basal efflux rate of ^{3}H-taurine from 0.005 (min^{-1}) to a maximal rate of 0.04 (min^{-1}), which is attained 3 min after the hyposmotic stimulus. Immediately after onset of the inactivation phase starts and the efflux rate constant progressively decreased (Fig.1A). The decrease in osmolarity raised $[Ca^{2+}]_i$

in CGN, from a basal value of 90 nM to a maximal of 190 nM. This hyposmolarity-dependent $[Ca^{2+}]_i$ increase was abolished by buffering $[Ca^{2+}]_i$ by pre-treatment for 30 min with EGTA-AM (50 μM) (Fig.1B). The same result was obtained when internal Ca^{2+} sources were depleted by pre-incubation with ionomycin (1μM, 5 min) in a Ca^{2+}-free medium (Fig. 1C). None of these treatments modified the basal levels of $[Ca^{2+}]_i$ (Fig. 1). Neither the rate constant nor the time course of OTR from CGN stimulated by hyposmotic medium were affected by the different conditions which prevented the swelling-elicited rise in $[Ca^{2+}]_i$ (Fig. 1A).

3.2. *Ca^{2+}-calmodulin (CaM), CaM kinase, PKC and PKA appear not involved in OTR*

The Ca^{2+}-independence of OTR seems to exclude a role for Ca^{2+}-mediated signalling events in the mechanism of taurine efflux. Accordingly, it was found that KN-93 (10 μM), an agent with potent effect as a blocker of the CAM kinase, failed to modify OTR. Unexpectedly, the CaM inhibitor W-7 markedly potentiated ^{3}H-taurine release (Fig. 2A). OTR was unaffected by the non-selective PK blockers H-7 (not shown) and H-8 (Fig. 2B). The more specific PKC blocker Gö6976 did not influence OTR (Fig. 2B). PKC activation by acute treatment with PMA (100 nM) did not change OTR. However, a chronic treatment to down regulate the enzyme, decreased OTR by 30% (Fig. 2C). Altogether, these results do not point out to a prominent role of PKC in the signalling cascade activating OTR. The role of PKA was further examined by treating neurons with the membrane permeable cAMP analogue 8-BrcAMP (10 μM) or with forskolin (20 μM), a direct stimulator of the adenylyl cyclase. None of these treatments modified OTR, suggesting that the hyposmolarity-activated taurine efflux pathway does not involve a cAMP sensitive signalling step (Fig. 2D).

3.3. *OTR depends on protein tyrosine phosphorylation*

To assess the importance of protein tyrosine phosphorylation on OTR, we examined, first the effect of agents with a general inhibitory effect on tyrosine kinases, such as tyrphostine

A23 and genistein, and second, we investigated the effect of ortho-vanadate, an inhibitor of tyrosine phosphatases, which by this mechanism enhances the phosphorylation reactions. In CGN pretreated with tyrphostine A23 (50 µM), the efflux of ^{3}H-taurine in hyposmotic medium decreased about 90% without modifying the basal efflux in isosmotic conditions. Tyrphostine A1, the inactive analogue of tyrphostine A23, was without effect. Genistein (50 µM), another tyrosine kinase inhibitor, also reduced OTR, although it was less potent than tyrphostine A23 (Fig. 3). In accordance with this observed inhibition by tyrosine kinase blockers, ortho-vanadate (100 µM) elicited a marked potentiation of OTR (Fig. 3). These results clearly implicate tyrosine kinases as elements in the signalling cascade, which activate OTR.

3.4. *Hyposmotic stimulus activates Erk1/Erk2 but this response is unrelated to OTR*

Erk1/Erk2 are serine/threonine kinases, which are commonly activated by tyrosine kinases. The activity of Erk1/Erk2 in CGN was assessed by MBP phosphorylation. The kinase activity was measured in cells in isosmotic and hyposmotic (30%) solutions and in the presence or absence of the MEK inhibitor PD 98059. Erk1/Erk2 amount was essentially the same in all the conditions tested (Fig. 4A lower panel). A marked increase in the MBP phosphorylation was observed in cells stimulated with the hyposmotic solution (Fig 4A upper panel). PD 98059 (50 µM) effectively reduced this enhancement of Erks activation induced by hyposmolarity (Fig.4A). However, this agent, used in the same conditions and at the same concentration, failed to significantly modify OTR (Fig. 4B). These results associate Erks activation with the hyposmotic stimulus but do not link these kinases with the signalling cascade activating OTR in CGN.

3.5. *Effect of PI3 kinase blockers on OTR*

The involvement of PI3 kinase on OTR was assessed by pre-treating neurons with wortmannin, a known inhibitor of this kinase. Incubation (60 min) with 100 nM wortmannin, decreased the stimulated taurine efflux by more than 85% (Fig. 5A). Wortmannin

concentration of 1 μM did not result in more inhibition of OTR. 50 nM wortmannin did not affect OTR (Fig. 5A). Unexpectedly, LY294002, another blocker of PI3K did not affect OTR at concentrations up to 100 μM (Fig. 5C).

4. Discusion

Taurine translocation activated by hyposmotic swelling occurs through a difusional pathway characterized by a notable pharmacological sensitivity to Cl^- channel blockers (Pasantes-Morales et al., 1994). This has raised the suggestion of a common osmosensitive exit route for Cl^-, taurine, amino acids and other organic osmolytes, with properties of an anion channel-like molecule of broad specificity (Strange, 1996). Whereas these features of the osmosensitive taurine transport are consistently found in essentially all cell types, the signalling events that link the change in cell volume and the taurine pathway activation, appear less homogeneous and might be cell specific. This is the case, for instance, for the Ca^{2+}-dependence of the Cl^-/taurine pathway. As shown in the present and previous work (Morán et al., 1997), OTR in CGN is independent of the $[Ca^{2+}]_i$ rise induced by swelling. Taurine and Cl^- fluxes associated with swelling are also Ca^{2+}-independent in other cell types (Rev. in Okada, 1997) including cerebellar astrocytes (Sánchez-Olea et al., 1995), but they are Ca^{2+}-dependent in cortical astrocytes (O'Connor and Kimelberg, 1993), neuroblastoma CHP-100 (Basavappa et al. 1995) and in a tracheal human cell line (Galietta et al., 1996,1997). This is despite the fact that in all cell types, swelling consistently elicits an increase in cytosolic Ca^{2+} (Foskett, 1994). A minimal amount of Ca^{2+} (< 50 nM) referred as permissive Ca^{2+} is required for the activation of osmosensitive Cl^- channels in endothelial cells (Szucs et al., 1996) and in the K^+-activated efflux of taurine (Mongin et al., 1999). In our conditions, this low concentration of $[Ca^{2+}]_i$ was never attained. The presence of minimal amount of intracellular Ca^{2+} may indeed be a requisite for activation of the osmolyte pathways, but in most cells this level is below the normal Ca^{2+} cell content which, in addition is further increased by the hyposmotic stimulus.

In agreement with the observed Ca^{2+} independence of taurine efflux in CGN, CaM kinase appears not to be a signaling element for OTR. However, the CaM complex may somehow modulate the mechanism of release as shown by the effect of W-7, a CaM blocker, which induced a consistent and marked potentiation of taurine efflux in CGN. The potential mechanism of this action of W-7 may be related to a suggested functional coupling of CaM and PLA_2 observed in myocardial A10 cells, where inhibition of CaM by W-7, trifluoperazine and calmidazolium, leads to an increase in arachidonic acid release (Wolf and Gross, 1996). This compound might in turn, directly activates the osmosensitive taurine pathway as has been described in neuroblastoma cells (Basavappa, et al., 1998).

The present results suggest that PKC is not strongly involved in the signaling cascade activating the taurine efflux pathway in CGN. Manipulation of PKC activity by a number of blockers or activators did not affect OTR. However, a mild decrease in taurine efflux was observed after a treatment to downregulate PKC. This effect may be related to changes in the cytoskeleton, which can be seen after this treatment. Some non-specific cell damage as a consequence of the treatment can not be excluded. In any event, the lack of modulation of OTR by other agents tested in the present study seems to exclude PKC as a key element in the mechanism that triggers this process. The PKC blocker chelerythrine inhibits the hyposmotic release from cardiac cells (Song et al., 1998). In studies in rat cerebral cortex in vivo chelerythrine reduces OTR by 28%. Stimulation of PKC by PMA did not affect OTR while PDD mildly stimulated it. The hyposmotic release of other amino acids was not affected by chelerythrine while PMA and PDD exhibited differential effects on the amino acids examined (Estevez et al.1999). With respect to the osmosensitive Cl^- current, the influence of PKC seems clearly cell specific as all the following responses have been described in different cell types: i) no effect of inhibition or activation of PKC on the Cl^- current, ii) down regulation or activation of PKC leads to reduction or stimulation, respectively, of the Cl^- current iii) activation of PKC inhibits the osmosensitive Cl^- current (rev in Okada, 1997; Duan et al., 1999; von Weikerstahl et al., 1999, Leaney et al., 1997).

The present results suggest that PKA is not involved in the signaling mechanisms

activating OTR in CGN. Studies in other cell types also indicate that PKA is not required to activate the osmosensitive Cl^- current, although cAMP may exert a modulatory effect (Okada, 1997).

Results of the present study have clearly shown the involvement of tyrosine kinases in the mechanisms activating taurine efflux in CGN. There is increasing evidence suggesting a role for these enzymes in the signaling cascades activated after hyposmotic stress. Phosphorylation of several proteins upon hyposmotic stimulation is prevented by tyrosine kinase inhibitors in cardiac cells and Intestine 407 cells (Sadoshima et al., 1995; Sorota et al., 1998; Tilly et al., 1996a). This notably includes elements of the MAP kinase pathways. These are prominent cellular signaling cascades by which cells transduce extracellular stimuli into intracellular responses. Activation of the Erk1/Erk2 step of the MAP kinase pathway in a tyrosine-kinase dependent way, appears to be one of the early cell responses to hyposmotic stress in several cell types (Rev. in Nilius, 1997). However, the involvement of Erk1/Erk2 in the osmosensitive transduction leading to activation of the Cl^-/amino acid pathway is at present unclear. It is even possible that its activation is unrelated to osmolyte activation. The link between Erk1/Erk2 activation and the trigger of taurine efflux has not been previously described. In the present work in CGN, a hyposmotic stimulus clearly activated Erk1/Erk2, and PD 98059 blocked this effect, but the inhibitor did not modify the efflux rate or the time course of OTR. As for the Cl^- fluxes, there are conflicting results regarding Erk1/Erk2 connection. In a human intestine 407 cells line, the swelling-activation of Erk1/Erk2 is prevented by PD 98059 but this action has no consequences on the activation and release pattern of $^{125}I^-$ elicited by hyposmolarity (van Der Wijk et al., 1998,1999). In contrast, in primary cultured astrocytes, where the same activation of Erk1/Erk2 occurs upon hyposmotic stress, PD 98059 prevents Erk1/Erk2 phosphorylation as well as the Cl^- conductance activated by swelling (Crépel et al., 1998). The question is then raised if whether, at least in some cell types, activation of Erk1/Erk2 is a response to osmotic stress but is unrelated to mechanisms of cell volume recovery which rely importantly in the compensatory efflux of osmolytes through the Cl^-/amino acid pore.

Other tyrosine kinases potentially interacting with elements of the signaling cascade activating taurine efflux are those forming the routes upstream of Erk1/Erk2, such as those converging on ras, i.e. FAK and Src kinases or via the signaling pathway formed by p21 Rac and p38, some of which are activated by hyposmotic stress (Rev. In Nilius et al., 1997). Alternative interactive routes are those involving the PI3 kinase and the p70s6k pathways. Activation of PI3K in connection with swelling has been observed in C6 glioma cells and the Intestine 407 cell line (Sinning et al., 1997; Tilly et al., 1996a), but the effect of preventing this activation on taurine efflux was not examined in these studies. Inhibition by wortmannin of the osmosensitive Cl^- efflux has been described in Intestine 407 cells (Tilly et al., 1996a). Wortmannin and LY294002 also block cell volume recovery and the osmosensitive Cl^- current in hepatoma cells (Feranchak et al., 1998). We also found a potent inhibitory effect of wortmannin on the taurine fluxes activated by swelling in CGN. However, unexpectedly, no inhibition was observed in the presence of LY294002 up to 100 μM concentration. This might be explained by a different sensitivity to wortmannin and LY294002 of PI3K isoforms present in neurons, as suggested in a study in sensory and sympathetic neurons, where LY294002 was found 100-times less sensitive than wortmannin to inhibit the NGF retrograde axonal transport (Reynolds et al., 1998; Bartlett et al., 1999). Another possibility to explain the difference between the two PI3 kinase blockers on taurine efflux in CGN, is that wortmannin is affecting not PI3 kinase, but instead the activity of one or several phospholipases, in turn involved in the taurine efflux mechanism. A study in fibroblasts has shown inhibition by wortmannin of PLC, PLD and particularly PLA_2 activity. Moreover, inhibition of this latter enzyme occurs with an IC_{50} similar to that reported to inhibit PI3 kinase (Cross et al., 1995). These results are relevant, since there is increasing evidence suggesting a role for PLA_2 in the mechanism of taurine and Cl^- osmosensitive fluxes in a variety of cell types (Margalit et al., 1993; Thoroed et al., 1997; Basavappa et al., 1998). In a neuroblastoma cell line, the release of Cl^-, taurine and arachidonic acid elicited by swelling, were all reduced by PLA_2 blockers, thus providing strong support to the involvement of this enzyme in the mechanisms activating the

osmolyte efflux pathways in these neurons (Basavappa et al., 1998). Considering altogether these observations, it is clear that the site of action of wortmannin has to be precisely identified before assigning a role to PI3K as part of the signaling routes leading to osmolyte activation. A possible cell specific sensitivity of PI3K to LY294002 and wortmannin, which may account for the differences observed between neurons and other cell types, is also worthy to be explored. In summary, results of the present work point to a key role for tyrosine kinases in the mechanisms activating OTR, but the specific kinases involved have not been as yet identified. This is a necessary step in the understanding of the signaling routes involved in the release of taurine associated with swelling.

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Legends of Figures

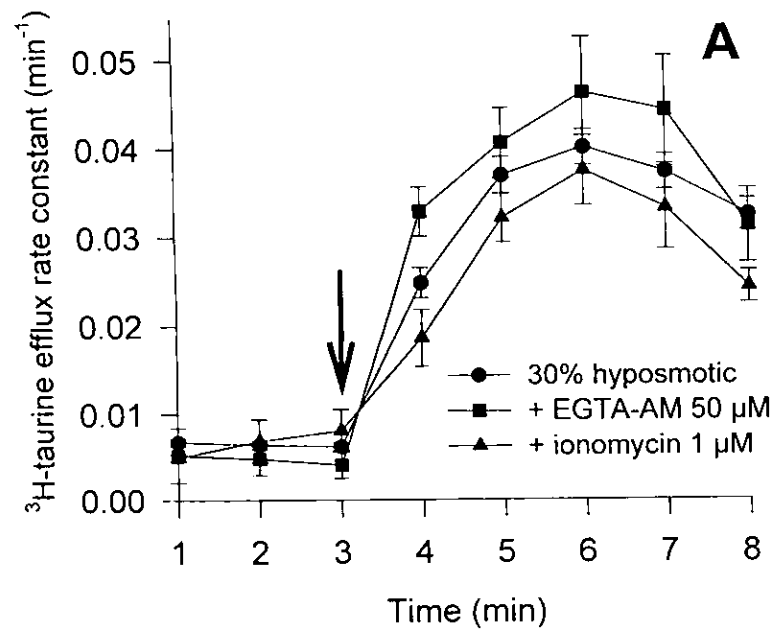
Fig.1. Changes in $[Ca^{2+}]_i$ and ^{3}H-taurine efflux evoked by hypotonic swelling in the presence or absence of Ca^{2+}. **A.** Time course of swelling-induced ^{3}H-taurine efflux from CGN. Control cells superfused with isosmotic medium (3 min) and 30% hyposmotic medium (arrow) during 5 min (●). Cells were incubated during 5 min with 1 µM ionomycin in isosmotic Ca^{2+}-free medium (▲) or with 50 µM EGTA-AM for 30 min in isosmotic medium with Ca^{2+} (■) before exposure to the hyposmotic stimulus. Results are means ± SE of 4-6 experiments. **B.** Cultured cerebellar granule neurons (CGN) in suspension were loaded with fura-2/AM as described in experimental procedures. The basal traces corresponds to $[Ca^{2+}]_i$ in CGN in isosmotic medium (300 mOsmol), in cells pre-treated (30 min) or untreated with 50 µM EGTA-AM. At the time indicated by the arrow, the osmolarity in the medium was reduced to 210 mOsmol (30%) by addition of diluent medium without NaCl. **C.** After 100 sec recording of basal $[Ca^{2+}]_i$, cells were exposed to 1 µM ionomycin in Ca^{2+} -free medium plus 0.5 mM EGTA; 450 sec later (arrow), the osmolarity was reduced to 30%. Traces are representative of 5-10 experiments.

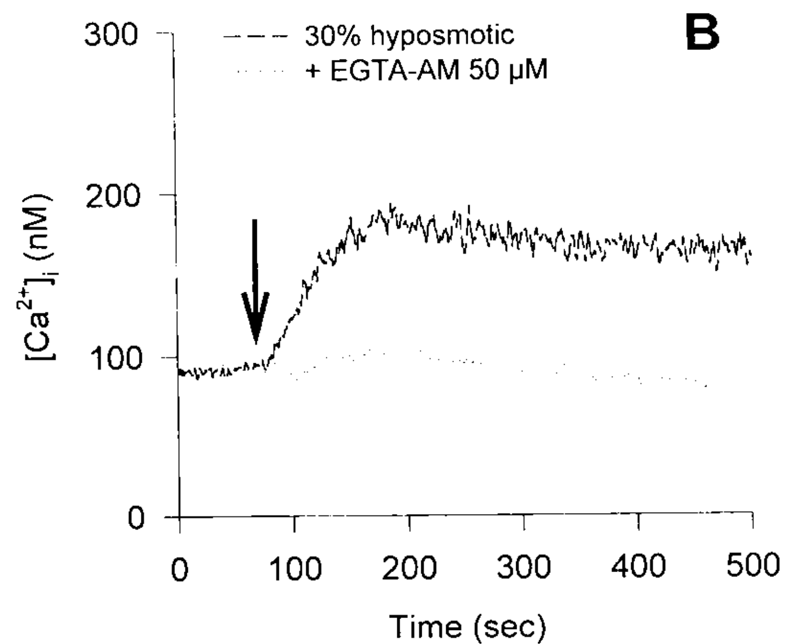
Fig.2. Time course of swelling-induced ^{3}H-taurine efflux from CGN. **A, B.** Cells were exposed to the tested drugs at the concentrations indicated during 10 min. At the arrow, cells were stimulated with 30% hyposmotic medium. **C.** Cells were pre-incubated with 100 nM PMA during 10 min or 24 h before the experiments **D.** Cells were incubated 5 min with 20 µM forskolin or 10 µM cAMP before stimulation with 30% hyposmotic medium (arrow). In all cases the hyposmotic medium contained the drugs tested at the indicated concentrations. Results are means ± SE of 4-8 experiments.
* $P< 0.001$, ** $P< 0.01$, *** $P< 0.05$, significant differences (Student´s test).

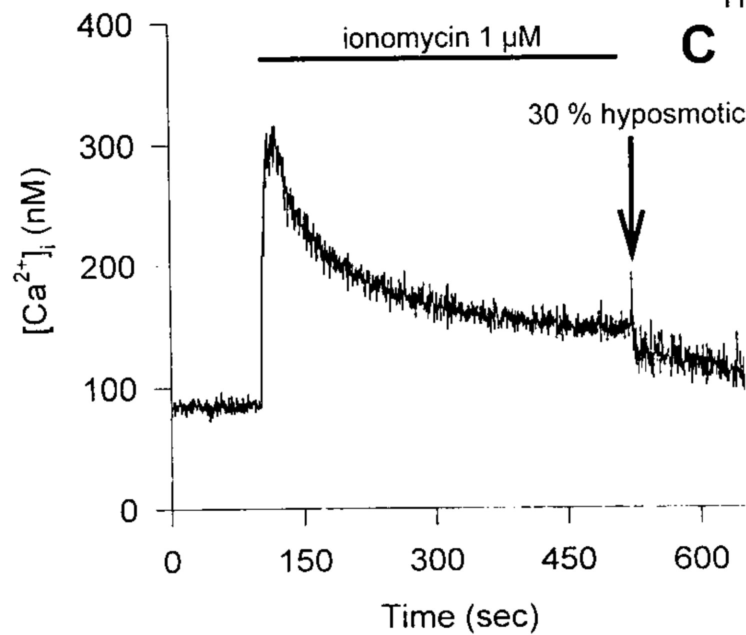
Fig.3. Effect of inhibition of protein tyrosine kinases and tyrosine phosphatases on swelling-induced ^{3}H-taurine efflux from CGN. Cells were pre-incubated with 50 µM tyrphostine A23 (■), A1 (▲), and genistein (▼) during 30 min before the stimulation with 30% hyposmotic medium. In (◆) cells were pre-treated 5 min with 100 µM ortho-vanadate. In all cases the hyposmotic medium contained the drugs tested at the indicated concentrations. Results are means ± SE of 4-6 experiments. * $P< 0.001$, * $P< 0.01$, significant differences (Student´s test).

Figure 4. **A.** Activation of Erk1/Erk2 in response to hyposmotic solution in CGN, determined by MBP phosphorylation. Cells were exposed to isosmotic (Iso) or 30% hyposmotic (Hypo) or 30% hyposmotic + 50 µM PD 98059 (Hypo + PD 98059) media during 5 min. Cells were pre-incubated 60 min with PD 98059. Representative results of three independent experiments are shown. MBP phosphorylation (upper panel) was assayed using the immune-complex against antibody Erk1/Erk2. Western blot of Erk1/Erk2 for the corresponding assays is shown in lower panel. **B.** Cells were pre-incubated (60 min) with 50 µM PD 98059. After this time, cells were superfused with isosmotic or hyposmotic (arrow) media containing the same concentration of the blocker. Results are means ± SE of 6 experiments. *** $P< 0.05$, significant differences (Student´s test).

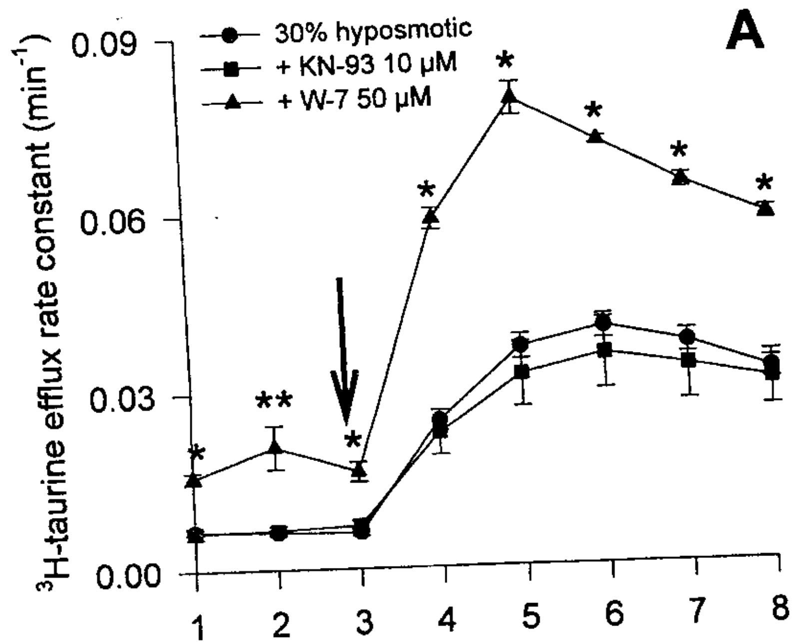
Fig.5. Time course of swelling-induced ^{3}H-taurine efflux from CGN. Cells were pre-incubated during 60 min with 50-100 nM or 1 µM wortmannin (**A**) or with 10, 25, 50 and 100 µM LY294002 (**B**). After this time, cells were superfused with isosmotic medium followed by 30% hyposmotic medium (arrow). All media contained the same concentration of the blocker. Results are means of 4-10 experiments ± SE. * $P< 0.001$, ** $P< 01$, significant differences (Student´s test).

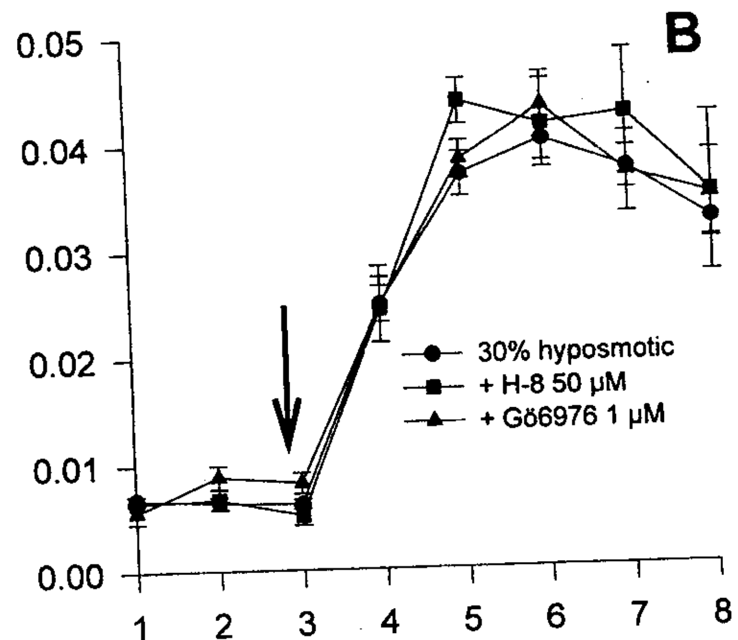


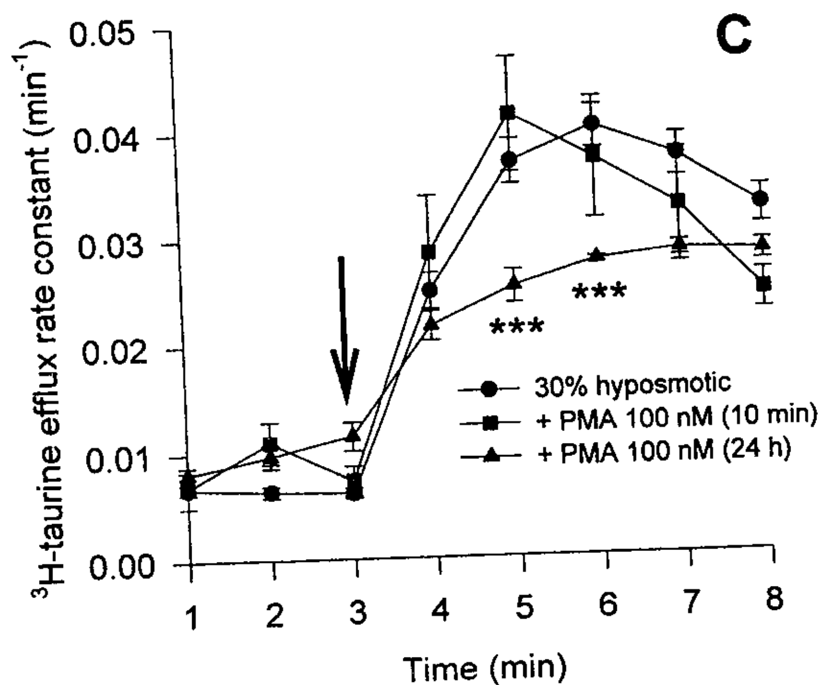


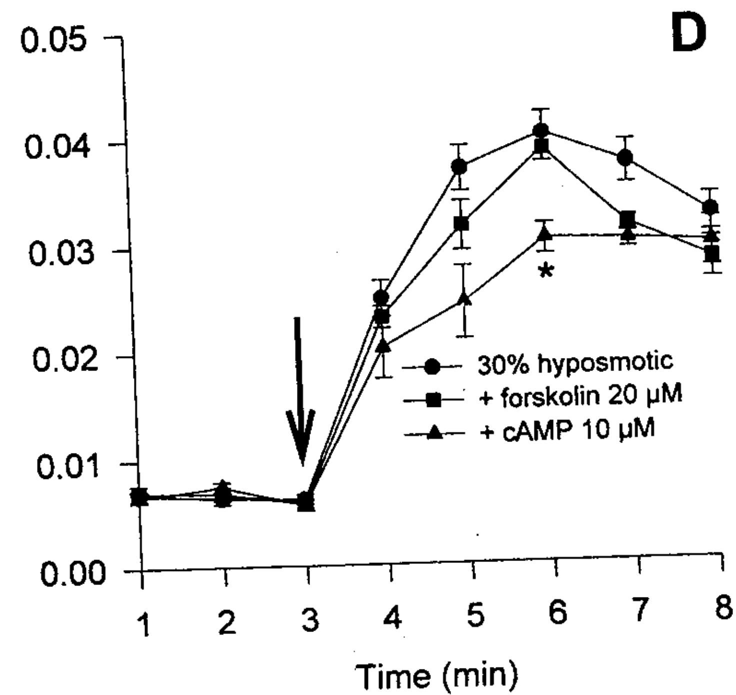


Morales-Mulia et al. Fig. 1.

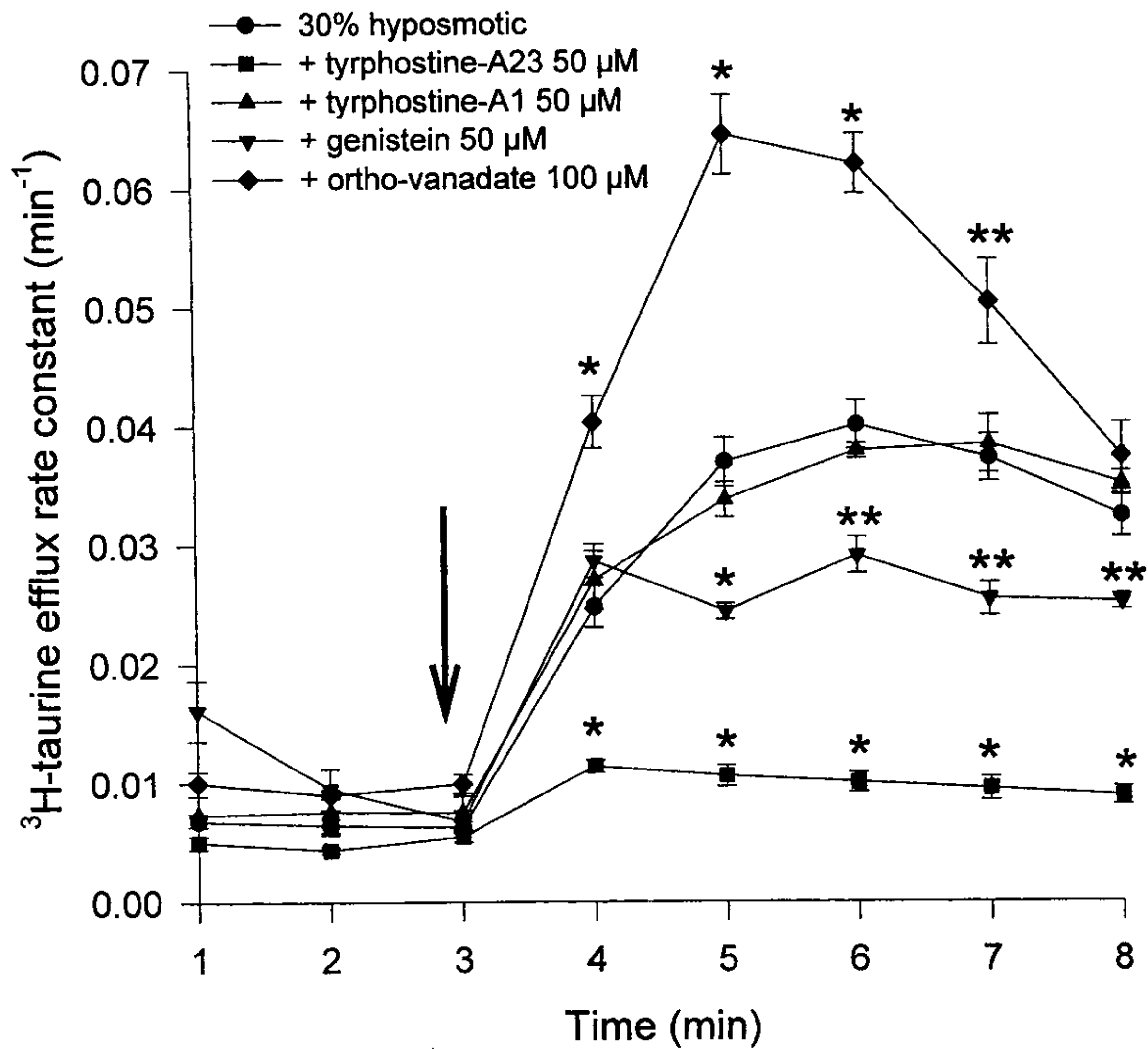








Morales-Mulia et al. Fig. 2



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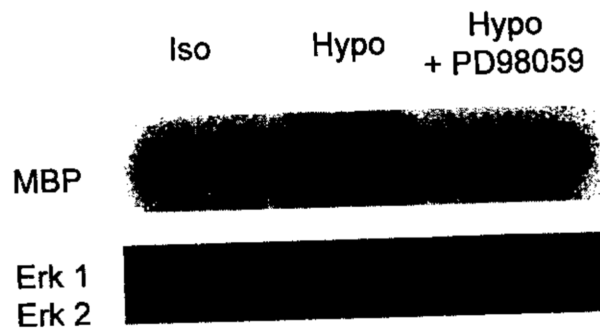
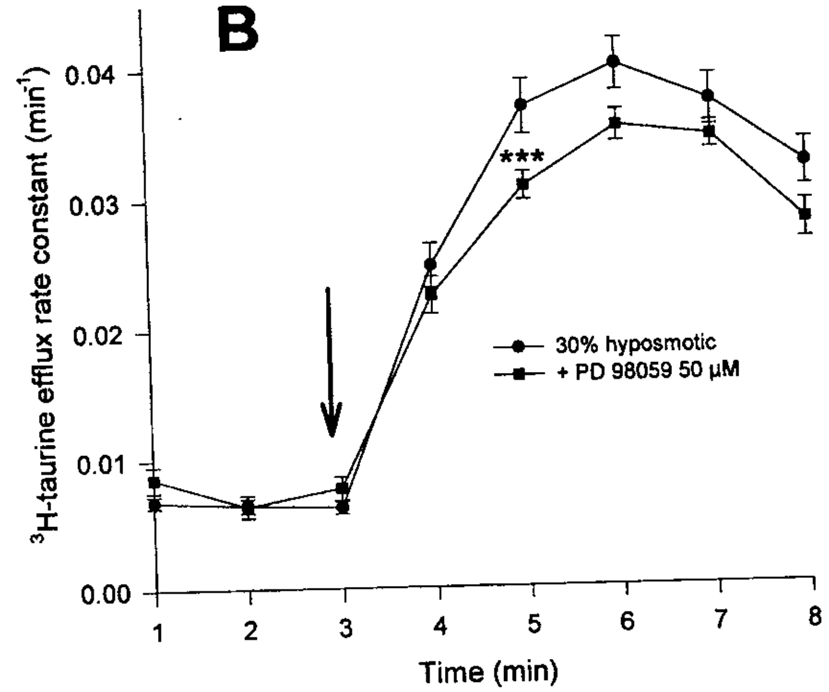
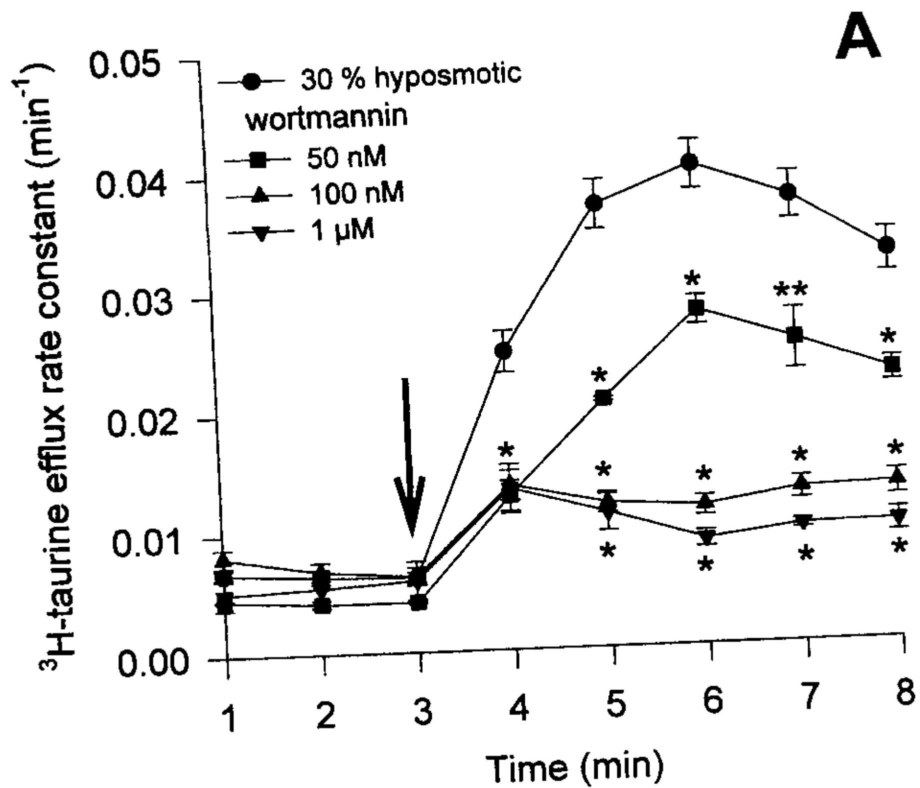
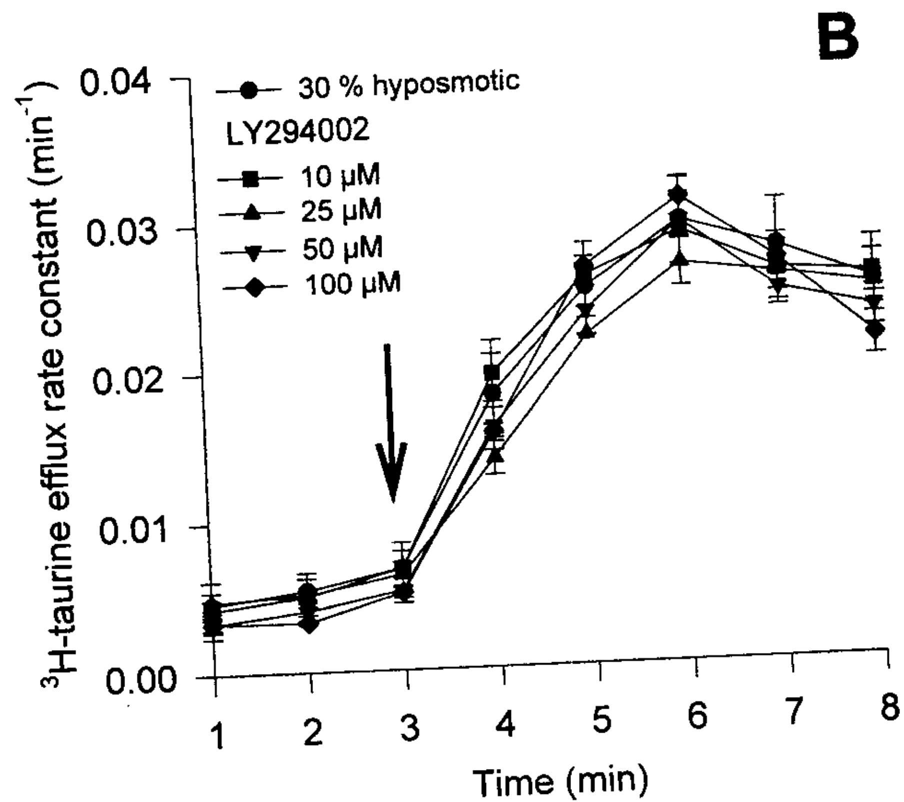

A
Iso
Hypo
Hypo
+ PD98059
MBP
Erk 1
Erk 2


B
0.04
0.03
0.02
0.01
0.00
3H-taurine efflux rate constant (min-1)
***
30% hyposmotic
+ PD 98059 50 µM
1
2
3
4
5
6
7
8
Time (min)





Morales-Mulia et al. Fig. 5

ANEXO 1

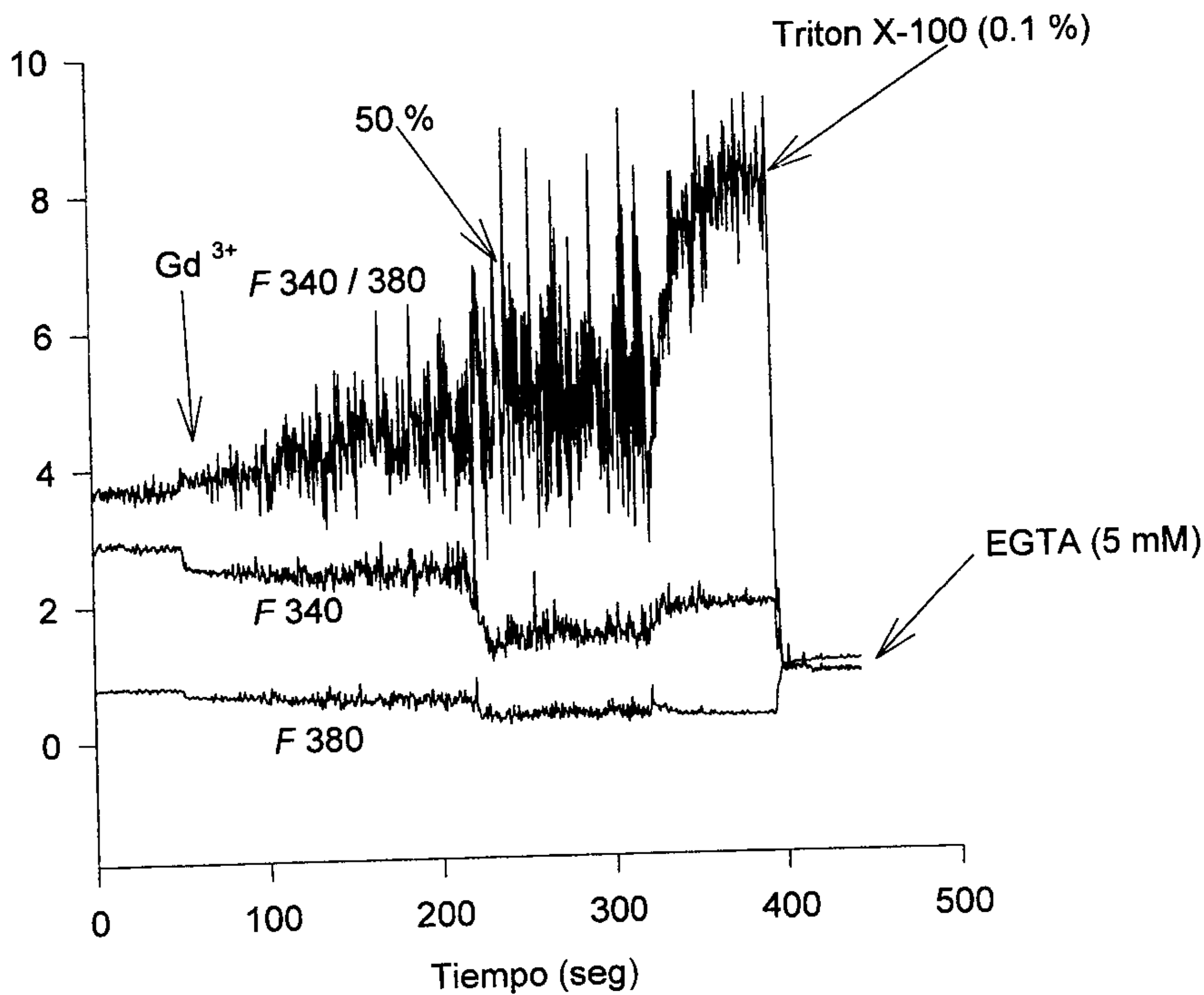


Fig. 1. Efecto de gadolinio sobre la señal de fura2/AM en astrocitos de cerebelo en cultivo.

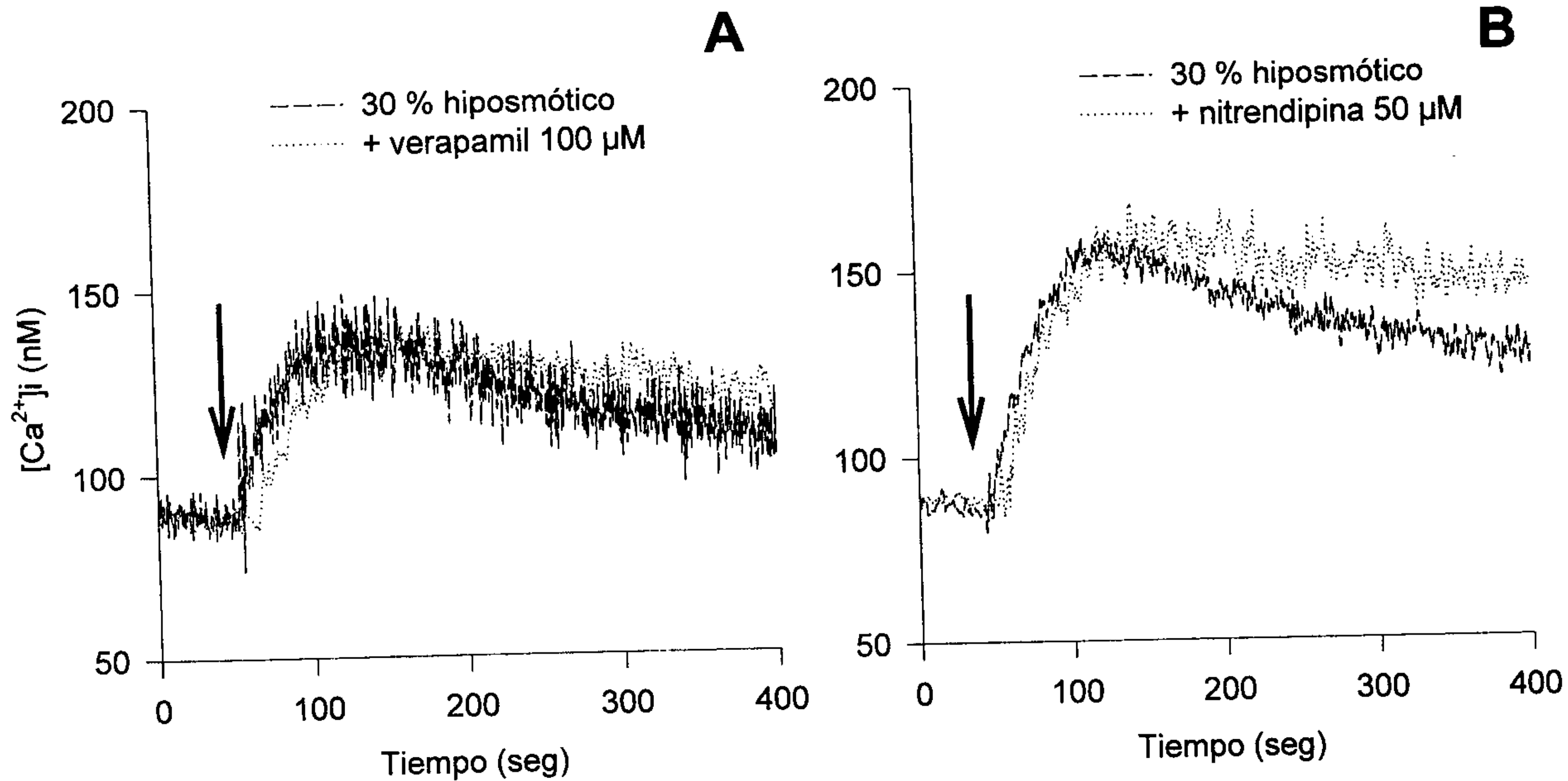


Fig. 2. Efecto de bloqueadores de canales de Ca^{2+} sobre la $[Ca^{2+}]i$ en neuronas granulares de cerebelo de rata en cultivo.

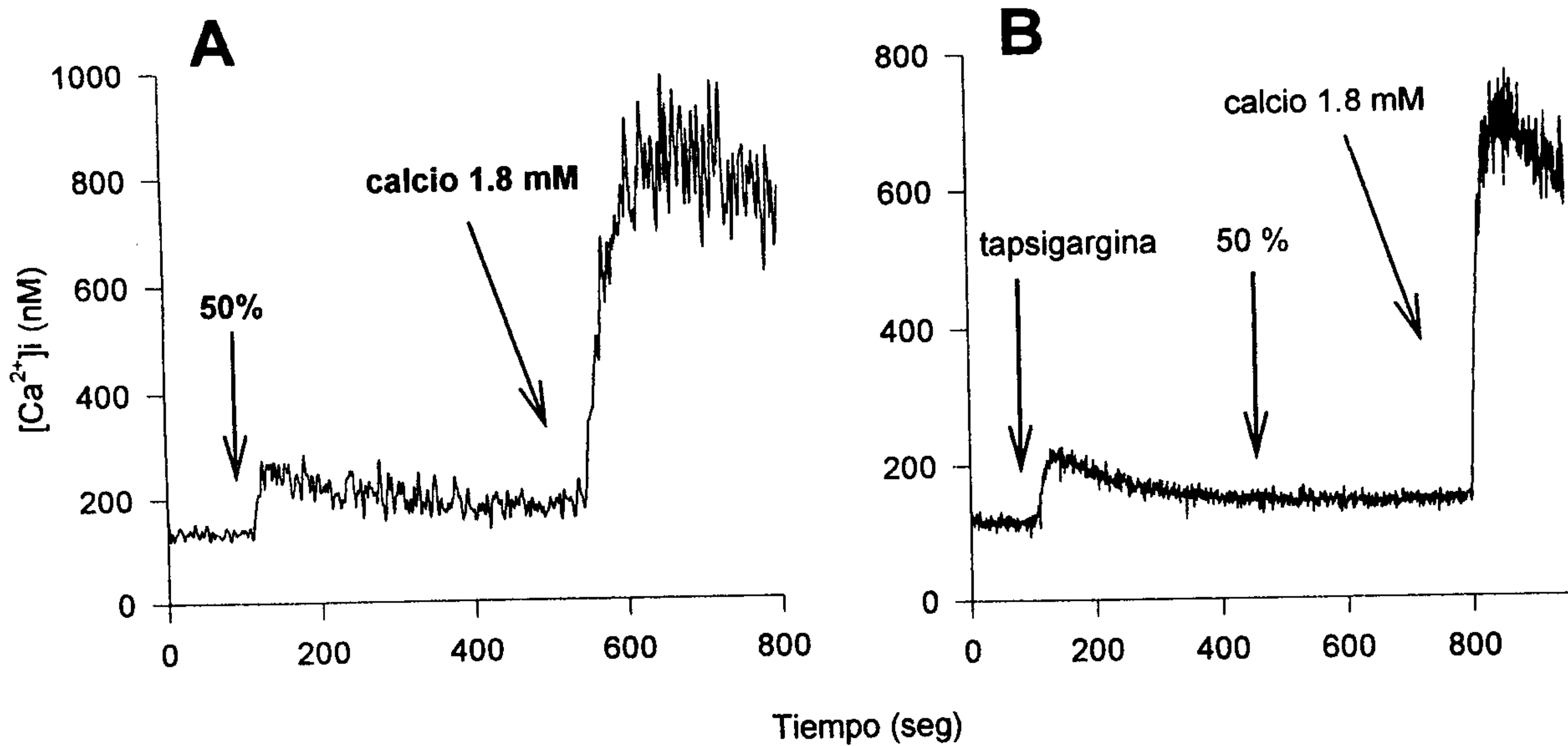
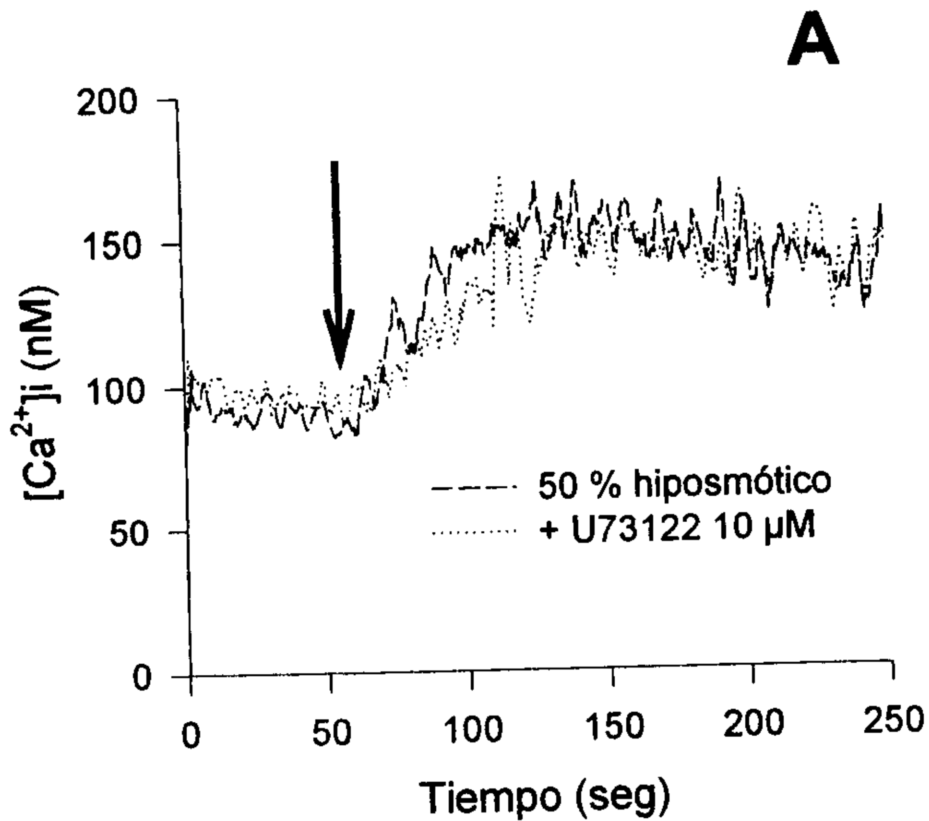


Fig. 3. Permeabilidad al Ca^{2+} extracelular durante el estímulo hiposmótico en astrocitos de cerebelo de rata en cultivo.



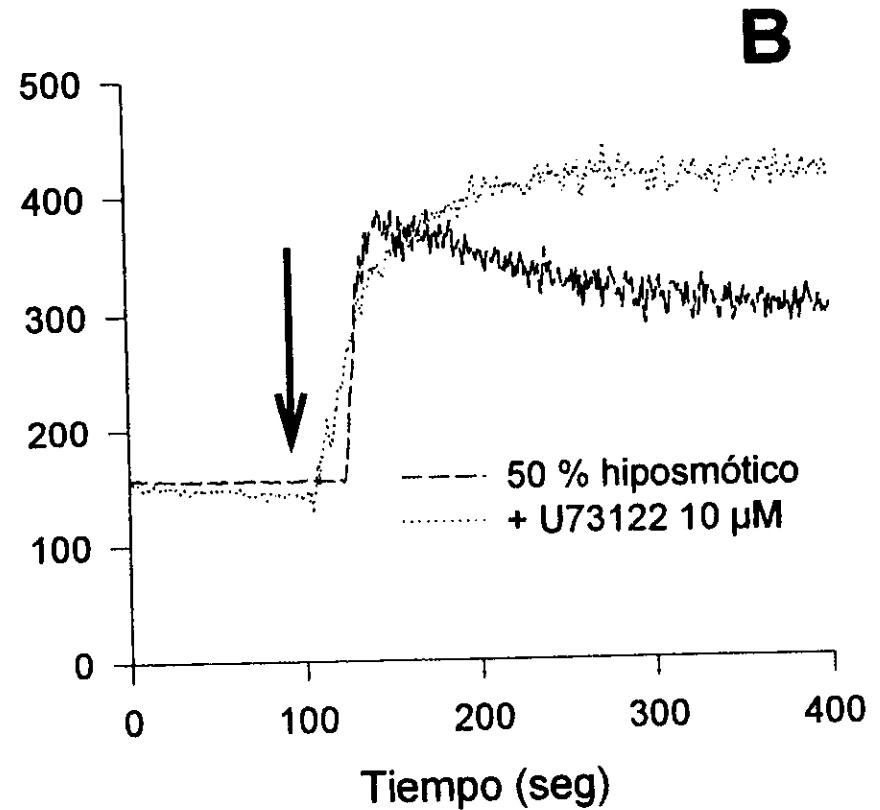


Fig. 4. Efecto de la inhibición de la PLC sobre el incremento en la $[Ca^{2+}]i$ inducida por hiposmolaridad en neuronas (A) y astrocitos (B) de cerebelo de rata en cultivo.

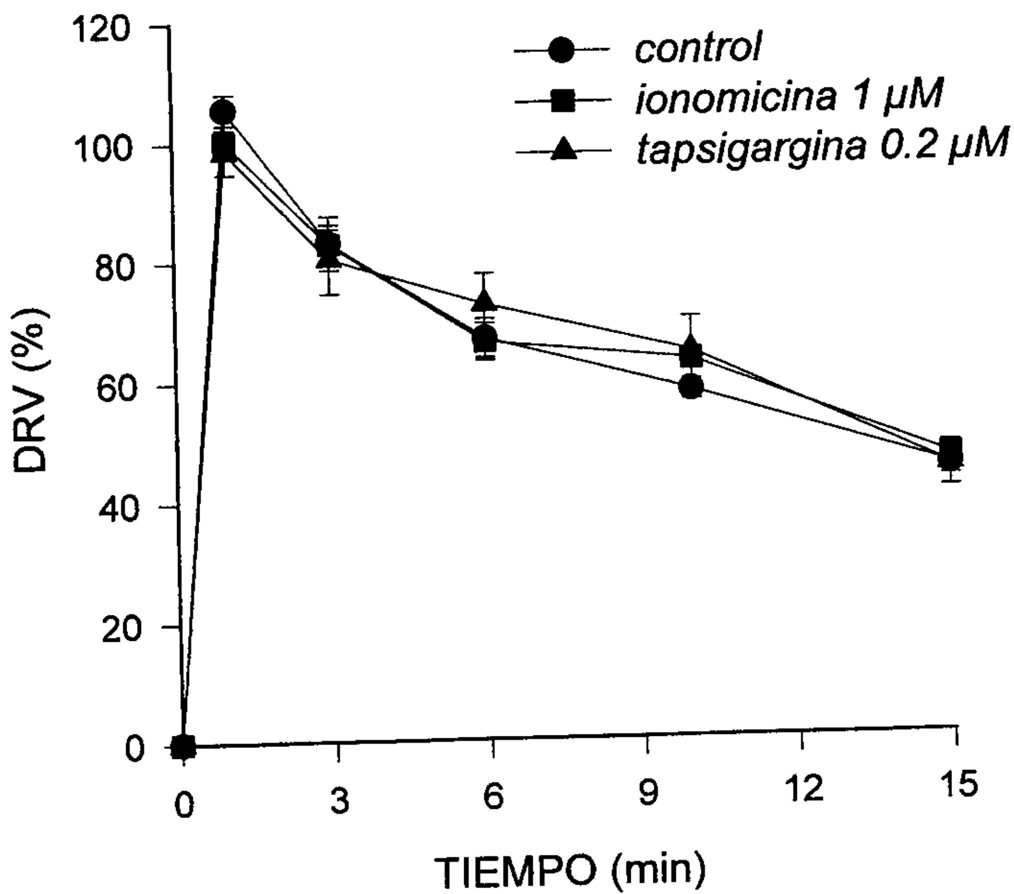


figura 5. Efecto de ionomicina y tapsigargina sobre el DRV y la $[Ca^{2+}]_i$ en neuronas granulares de cerebelo de rata en cultivo.