UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

UNIDAD ACADEMICA DE LOS CICLOS PROFESIONAL

Y DE POSGRADO DEL CCH

"MECANISMOS DE MOVILIZACION DE OSMOLITOS EN CELULAS DEL SISTEMA NERVIOSO EN CULTIVO"

Tesis que para obtener el grado de

DOCTOR EN INVESTIGACION BIOMEDICA BASICA

Presenta el MIBB:

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Octubre de 1993.

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INTRODUCCION

Los individuos pertenecientes a un gran número de especies tanto vegetales como animales, son capaces de vivir en aguas con una concentración variable de sales. Los salmones son tal vez uno de los casos mas conocidos, pero todas las especies de vertebrados e invertebrados que viven en lagunas salobres son otros tantos ejemplos de este interesante fenómeno biológico. Los componentes celulares de los líquidos circulatorios de estos animales (sangre, linfa, hemolinfa) ajustan su volumen a los cambios en salinidad de estos líquidos y en algunas especies también las células que forman los tejidos tienen esta capacidad de adaptación. Durante mucho tiempo se pensó que la propiedad de regulación del volumen estaba restringida a las especies que efectivamente requerían de este control para su supervivencia. Sin embargo, recientemente se ha demostrado que un gran número de células de animales terrestres, como linfocitos humanos (Gristein et al., 1984) y astrocitos de cerebelo de rata en cultivo (Pasantes-Morales y Schousboe, 1988) que están siempre expuestas a fluídos de osmolaridad muy controlada, poseen también esta capacidad. La regulación de la osmolaridad de los líquidos corporales en estos animales se lleva a cabo a través de los distintos tipos de órganos renales, los cuales se encargan de manejar los flujos de agua y solutos en la dirección necesaria para mantener las condiciones isosmóticas en estos líquidos. El número de observaciones que sugieren que los mecanismos de regulación de volumen pueden activarse aún en

condiciones de osmolaridad controlada y de que participan de manera paralela en funciones celulares básicas se incrementa rápidamente (Macknight, 1988). Entre las funciones en las cuales se observa, concomitantemente, una regulación de volumen, se encuentran los procesos de transporte de sustancias nutritivas como azúcares y aminoácidos, las funciones de secreción, los movimientos de organelos intracelulares, de manera particular el citoesqueleto y por supuesto durante las fases de crecimiento y división celular. En todas estas situaciones se crean gradientes osmóticos microscópicos que dan lugar a cambios locales y transitorios en el volumen celular. Es posiblemente por esta razón que los procesos de regulación del volumen se encuentran presentes en prácticamente todos los tipos celulares, independientemente de las características del medio externo en el que se desenvuelve el animal del que forman parte.

PROCESOS DE REGULACION DEL VOLUMEN CELULAR

La membrana plasmática de las células tanto de origen animal como vegetal es muy permeable al agua, por lo que siempre que exista una diferencia en la concentración de los componentes celulares con el exterior, es decir, un gradiente osmótico entre el exterior y el interior de la célula, se observarán movimientos netos de agua. En presencia de un gradiente de presión osmótica el agua se moviliza del compartimento de menor al de mayor presión

osmótica, y como consecuencia, el volumen celular aumenta en soluciones hiposmóticas y disminuye en soluciones hiperosmóticas. Sin embargo, la mayoría de las células animales se comportan en esta forma, es decir, como osmómetros perfectos, únicamente al inicio de la exposición a soluciones anisosmóticas, ya que posteriormente se activan una serie de procesos que permiten la recuperación del volumen inicial a pesar de que persistan dichas condiciones. La regulación de volumen celular que ocurre tanto en condiciones hiposmóticas como hiperosmóticas tiene lugar debido a un cambio, ya sea una disminución o aumento según se requiera, en el contenido de solutos intracelulares osmóticamente activos, de tal manera que la presión osmótica interna tiende a alcanzar el mismo valor que la externa, disminuyendo así el gradiente osmótico.

Cuando las células animales se exponen a un medio hiposmótico inicialmente se observa un aumento rápido en el volumen celular debido a la entrada de agua, seguido de una fase de recuperación más lenta, durante la cual alcanzan un valor muy cercano al volumen original. Este proceso se encuentra mediado por la expulsión de solutos intracelulares osmóticamente activos, con lo cual se reduce la presión osmótica intracelular y por lo tanto el contenido de agua en las células. Entre los solutos importantes en este proceso se encuentran iones inorgánicos, principalmente el cloruro y el potasio (Grinstein et al., 1982; Roy y Sauvé, 1987) y solutos orgánicos como polialcoholes (Wirthensohn et al., 1989), aminoácidos (Smith y Pierce, 1987), metilaminas (Nakanishi et al.,

1988), urea (Dépêche y Schoffeniels, 1975) y azúcares (Bagnasco et al., 1986; Balaban y Burg, 1987).

Igualmente, cuando las células se tratan con soluciones hiperosmóticas se observa una disminución en el volumen, debido a la salida de agua producida en respuesta al gradiente osmótico impuesto. En algunos tipos celulares esta disminución inicial del volumen es seguida de una fase de recuperación en la cual incrementan su contenido de agua como consecuencia de la acumulación de solutos osmóticamente activos. Los solutos implicados en este proceso son los iones potasio, sodio y cloro (Kregenow, 1971; Grinstein et al., 1983) así como compuestos orgánicos como los aminoácidos (Fugelli y Zachariassen, 1976; Fyhn, 1976), sorbitol, mio-inositol, betaína y glicerofosforil colina (Nakanishi et al., 1988).

La regulación del volumen celular es un proceso complejo que implica varios eventos básicos. Primero la célula debe ser capaz de detectar cambios en el volumen. Los sensores del volumen celular no se han identificado pero podrían ser los canales iónicos (generalmente para cationes) activados por el estiramiento de la membrana plasmática (Christensen, 1987; Ubl et al., 1988; Morris y Sigurdson, 1989; Sackin, 1989; Taniguchi y Guggino, 1989) o canales iónicos (selectivos para cationes) inactivados por el estiramiento (Ubl et al., 1988). También se ha sugerido que el volumen celular podría ser detectado indirectamente debido a los

cambios en la concentración de algún componente intracelular, como el calcio (Wong y Chase, 1986) o el pH interno (Lauf, 1985). En segundo lugar la célula debe iniciar un proceso regulador que modifique el contenido intracelular de solutos. Finalmente, debe "recordar" su volumen inicial e inactivar los procesos que se activaron durante el proceso regulador.

REGULACION DE VOLUMEN EN CONDICIONES HIPOSMOTICAS

La recuperación del volumen inicial después de una fase de incremento de volumen producido por soluciones hiposmóticas se ha descrito en una gran variedad de células animales, incluyendo células epiteliales (Beck et al., 1988), eritrocitos (Kregenow, 1981; Cala, 1983), células ascíticas de Ehrlich (Hoffmann et al., 1978), astrocitos en cultivo (Olson y Holtzman, 1982; Kimelberg, 1985), linfocitos humanos (Grinstein et al., 1982) y células MDCK (Roy y Sauvé, 1987). Aunque estos procesos de regulación de volumen se encuentran presentes en la mayoría de las células animales, no son universales ya que ciertos tipos celulares como las células de túbulo renal distal de Amphiuma son incapaces de recuperar su volumen original una vez que éste se ha incrementado con soluciones hiposmóticas (Guggino et al., 1985). Asimismo únicamente una fracción muy pequeña de eritrocitos humanos poseen los mecanismos que permiten una regulación eficaz del volumen (O'Neill, 1989).

Como se mencionó anteriormente esta disminución de volumen celular posterior a un incremento debido a la exposición a un ambiente hiposmótico es posible debido a la expulsión de solutos intracelulares. De manera análoga a la regulación de volumen en condiciones de hiperosmolaridad estos solutos son de dos tipos: iones inorgánicos y compuestos orgánicos, principalmente aminoácidos y polialcoholes. En células de animales terrestres los solutos de mayor importancia son los iones cloruro y potasio, que se expulsan de las células permitiendo la salida de agua (Spring y Ericson, 1982; Grinstein et al., 1982; Eveloff y Warnock, 1987). En células de animales marinos y en las de aquellos con capacidad de vivir en aguas de salinidad variable, los aminoácidos libres y en particular la taurina son los solutos que permiten esta función (Pierce y Greenberg, 1972; Fugelli y Thoroed, 1986; Fincham et al., 1987).

MECANISMOS DE LA SALIDA DE POTASIO Y CLORO ESTIMULADA POR UN INCREMENTO EN EL VOLUMEN CELULAR.

La disminución en el contenido de potasio y cloro intracelular observada en las células en condiciones hiposmóticas implica que estos iones son expulsados del interior celular. El mecanismo de salida del K^+ y el Cl^- depende del tipo celular de que se trate. Un sistema de cotransporte K^+/Cl^- activado por un incremento de volumen participa en expulsión de estos iones en eritrocitos de distintas

especies, incluyendo el pato (McManus y Schmidt, 1978; Kregenow, 1981; Mayer 1985), al borrego (Dunham y Ellory, 1981; Ellory et al., 1985; Lauf, 1985) y algunos peces (Lauf, 1982; Bourne y Cossins, 1984; Borgese et al., 1987). La activación acoplada de los intercambiadores K^+/H^+ y Cl^-/HCO_3^- parece mediar la salida de cloro y potasio en eritrocitos de Amphiuma (Kregenow, 1981; Siebens, 1985; Cala, 1980, 1985a, 1985b). En túbulos proximales de riñón de ratón y de conejo la salida de potasio 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 sistemas de cotransporte electroneutros para la salida de cloro y potasio, el mecanismo más generalizado para expulsar estos iones es la activación de vías electrogénicas constituídas por canales iónicos presentes en la membrana celular. En las células de Ehrlich se ha demostrado que un aumento en el volumen celular produce un incremento en la permeabilidad de la membrana al cloro y al potasio, mientras que se observa una disminución en la permeabilidad para el sodio (Hendil y Hoffmann, 1974; Hoffmann, 1978). Este aumento en la permeabilidad da como resultado la salida de estos iones, disminuyendo el potasio de 580 μmolas/g peso húmedo en condiciones isosmóticas a 450 μmolas/g peso húmedo en condiciones hiposmóticas, y el cloro de un valor de 210 a 140 μmolas/g peso húmedo (Hoffmann et al., 1984), en condiciones isosmóticas e hiposmóticas, respectivamente. Igualmente se ha demostrado que un medio hiposmótico produce la activación de canales iónicos selectivos

para cloro y para potasio en linfocitos humanos (Grinstein et al., 1984), y en la vejiga urinaria de Necturus (Larson y Spring, 1984).

El mecanismo utilizado para disminuir los niveles intracelulares de cloro y potasio determina el curso temporal de la recuperación del volumen. Cuando se encuentra involucrado el sistema de cotransporte K^+/Cl^-, el proceso es muy lento y tarda alrededor de 1 h; si se activan los sistemas de intercambio Cl^-/HCO_3^- y K^+/H^+ tarda cerca de 10 min y si se activan los canales de potasio y cloro, el volumen se recupera en un tiempo menor a los 10 min (Lauf, 1985).

Hipotéticamente, la salida de cloro y potasio podría ocurrir a través de un mismo canal iónico o por canales separados pero interdependientes. En las células de Ehrlich la evidencia experimental en apoyo a esta última alternativa es la siguiente: 1) al inhibirse la salida de potasio con quinidina se inhibe la recuperación del volumen, sin embargo si se provee una vía alterna para la salida de potasio con valinomicina (Hoffmann et al., 1984) o con gramicidina en un medio sin sodio (Hoffmann et al., 1986) el volumen disminuye normalmente, indicando que el canal de cloro se encuentra activo; 2) el curso temporal de la inactivación del canal de cloro es diferente al del canal de potasio: el primero se activa abruptamente con el aumento de volumen y se inactiva en aproximadamente 10 min, mientras que el canal de potasio permanece activado por más tiempo [la permeabilidad al potasio se encontró duplicada en células con 50 min en un medio hiposmótico (Hendil y Hoffmann, 1974; Sarkadi et al., 1984)]; 3) la permeabilidad al

cloro se incrementa más que al potasio, de tal manera que la salida de potasio es el paso limitante en la recuperación del volumen puesto que este proceso se incrementa en presencia de valinomicina (Hoffman et al., 1984) o gramicidina (Hoffman et al., 1986); 4) 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 cloro que para el potasio. Esta despolarización aumenta la fuerza electromotriz para la salida de potasio y puede explicar al menos parte de esta salida (Lang et al., 1987).

COMPUESTOS ORGANICOS EN LA REGULACION DE VOLUMEN EN CONDICIONES DE HIPOSMOLARIDAD.

En animales que se encuentran expuestos en forma natural a variaciones amplias en la osmolaridad del medio externo, como los animales marinos y estuarinos, los elementos que funcionan como osmoefectores determinantes son los compuestos orgánicos. Entre estas moléculas se encuentran la urea, los aminoácidos, compuestos cuaternarios de amonio (Fugelli y Thoroed, 1986), polialcoholes como el sorbitol (Siebens y Spring, 1989) y el mio-inositol (Nakanishi et al., 1988), y metilaminas como la glicerofosforilcolina y la betaína (Nakanishi et al., 1988).

Entre los aminoácidos libres la taurina es el más importante

para una función osmorreguladora debido a que se encuentra presente en concentraciones muy elevadas en la mayoría de las células animales (Jacobsen y Smith, 1968), constituyendo hasta el 70% de la poza total de aminoácidos libres (Pasantes-Morales et al., 1991). La taurina presenta características que la señalan como un osmolito ideal: se encuentra en concentraciones lo suficientemente elevadas (hasta 80 mM en retina, 28 mM en linfocitos, 60 mM en músculo esquelético y cardíaco) como para contribuir de manera importante a la presión osmótica total y es inerte desde un punto de vista fisiológico, ya que no participa en la estructura primaria de proteínas ni en ninguna reacción del metabolismo primario de la célula (Jacobsen y Smith, 1968). Esto permite que los niveles celulares del aminoácido se puedan modificar con el fin de ajustar la presión osmótica interna sin que las variaciones en la concentración intra o extracelular alteren otras funciones celulares. En el cangrejo azul Callinectes sapidus los aminoácidos libres contribuyen con el 70% de la osmolaridad interna; la disminución interna de estos aminoácidos es responsable de un 30% de la reducción intracelular total cuando se exponen a medios hiposmóticos (Gerard, 1975). En eritrocitos y corazón del lenguado Platichthys flesus, los aminoácidos contribuyen con el 55% de la disminución en la osmolaridad intracelular al exponerse a una solución hiposmótica (Fugelli y Zachariassen, 1976). En estas células también se observa el componente iónico, esto es la salida de los iones potasio y cloruro del interior celular, que complementa el proceso de regulación del volumen.

La participación de los iones potasio y cloruro y de compuestos orgánicos se ha demostrado en eritrocitos del poliqueto Glycera (Costa y Pierce, 1983) y del molusco Noetia (Smith y Pierce, 1987) y en músculo de Cancer (Moran y Pierce, 1984). La importancia relativa del componente iónico y orgánico se observa en eritrocitos de Noetia. En estas células la recuperación del volumen en medio hiposmótico se inhibe en un medio sin calcio. La salida de potasio y de cloro sensible a volumen es independiente de calcio (Smith y Pierce, 1987) pero la salida de aminoácidos se reduce drásticamente (Amende y Pierce, 1980; Smith y Pierce, 1987), lo cual sugiere una mayor participación del componente orgánico en la recuperación del volumen. En las células de estos animales marinos el curso temporal de la salida de cloro y potasio es muy distinto al de la salida de los aminoácidos. Los iones salen de la célula inmediatamente después del aumento de volumen, mientras que los aminoácidos muestran un retardo en su liberación (Warren y Pierce, 1982; Moran y Pierce, 1984; Smith y Pierce, 1987).

En las células de animales terrestres el componente iónico se complementa con un componente de osmolitos orgánicos. La presencia del componente orgánico se ha demostrado en células ascíticas de Ehrlich (Hoffmann y Lambert, 1983), en fibras musculares cardíacas (Thurston et al., 1981), cerebro (Thurston et al., 1980) y células de la médula de riñón de mamíferos (Bagnasco et al., 1988; Gullans et al., 1988; Yancey, 1988) así como en células MDCK (Roy y Sauvé, 1987).

SEÑALES QUE ACTIVAN FLUJOS CORRECTORES DE OSMOLITOS

La salida de solutos intracelulares observada en condiciones hiposmóticas puede ser el resultado de una activación directa de la distensión membranal sobre los sistemas de transporte de estos solutos o alternativamente, estos sistemas pueden ser activados por alguna señal intracelular producida como consecuencia de un aumento en el volumen celular. En este sentido, la señal o señales que activan los flujos osmorreguladores podrían ser los propios sensores del cambio o el volumen celular, aunque es posible también que exista una señal adicional que conecte el sensor del volumen y los mecanismos que estimulan la movilización transmembranal de los osmolitos.

Hasta el momento no es muy claro si la salida de solutos en condiciones de hiposmolaridad se encuentra directa o indirectamente asociada con la distensión de la membrana plasmática producida por el aumento de volumen. Se ha demostrado en túbulos renales que si la reducción en la osmolaridad se efectúa de una manera lenta y gradual, el volumen permanece constante aún en condiciones de hiposmolaridad (Lohr y Grantham, 1986). Si el volumen no aumenta entonces no existe una distensión membranal, sin embargo sí ocurre una pérdida de solutos internos puesto que al exponer la preparación a una solución isosmótica se observa una reducción en el volumen celular. En esta investigación no se determinó la naturaleza de los solutos involucrados ni la posible señal mediadora de la disminución intracelular de estos solutos.

Uno de los fenómenos primarios asociados con el incremento en el volumen producido por soluciones hiposmóticas es un aumento en los niveles intracelulares de calcio (Cala et al., 1986; Pierce et al., 1988; Suzuki et al., 1990; Rothstein y Mack, 1990). El aumento en la concentración intracelular de calcio se produce como consecuencia de un aumento en la entrada del catión a la célula. Esta entrada de calcio puede ocurrir a través de canales de calcio activados por la distensión de la membrana producida por el medio hiposmótico (Christensen, 1987) o bien por canales de calcio activados por metabolitos del lípido membranal fosfatidilinositol 1,3-bis fosfato a través de un mecanismo que involucra una proteína G sensible a la toxina pertusis (Suzuki et al., 1990). En algunas preparaciones la regulación de volumen se inhibe en un medio libre de calcio (Amende y Pierce, 1980; Suzuki et al., 1990), lo que sugiere que la salida de solutos observada en soluciones hiposmóticas se encuentra vinculada con el aumento en la concentración interna de calcio. El sitio de acción del calcio puede ser directamente sobre los canales de potasio activados por calcio (Hazama y Okada, 1988), o bien mediante el sistema calcio-calmodulina (Pierce et al., 1989).

La participación de los sistemas de segundos mensajeros en la regulación del volumen celular también tiene un cierto sustento experimental. La exposición a soluciones hiposmóticas induce un aumento en los niveles intracelulares de AMP cíclico a través de una activación directa de la enzima responsable de su síntesis, la

adenilato ciclasa (Watson, 1990). En condiciones isosmóticas el AMP cíclico es capaz de inducir la liberación de potasio (Mills y Skiest, 1985) y taurina (Madelian et al., 1985), sin embargo se desconoce si podría actuar como señal activadora de la salida de estos compuestos en soluciones hiposmóticas.

Existen evidencias que señalan cambios en la organización del citoesqueleto asociados con la regulación de volumen en condiciones de hiposmolaridad (Ziyadeh et al., 1992). Sin embargo, la desorganización de los microtúbulos del citoesqueleto no necesariamente se encuentra asociada con una pérdida en la capacidad de regulación del volumen.

REGULACION DE VOLUMEN EN CONDICIONES HIPEROSMOTICAS.

Como se mencionó previamente, al exponer las células a soluciones hiperosmóticas se observa una disminución de volumen relacionada con el grado de hiperosmolaridad. Algunos tipos celulares responden con un incremento en el volumen que lo aproxima a su volumen original, mediante el proceso denominado Aumento Regulador del Volumen. Este proceso de ajuste se ha demostrado en células de anfibios: en eritrocitos de Amphiuma (Cala, 1980) y células epiteliales de Necturus (Larson y Spring, 1987). Otros tipos celulares se comportan como osmómetros: disminuyen su volumen y no muestran un proceso de recuperación del

mismo mientras permanezcan en condiciones hiperosmóticas. Entre éstas se incluyen las células de Ehrlich (Hoffmann y Simonsen, 1989), los linfocitos humanos (Hempling et al., 1977; Grinstein, 1986), y las células epiteliales de rana (Davis y Finn, 1985; Ussing, 1986). Sin embargo la regulación de volumen se observa en prácticamente todos los tipos celulares si las células son preincubadas en un medio hiposmótico y posteriormente se les regresa a condiciones isosmóticas. En estas condiciones las células han recuperado su volumen después de un incremento inicial a través de una disminución en el contenido intracelular de solutos. De esta manera cuando las células son incubadas en soluciones isosmóticas se observa una disminución en el volumen, que es seguida de una fase reguladora de aumento en el volumen (Hoffmann y Simonsen, 1989; Grinstein y Foskett, 1990). La recuperación del volumen en un medio hiperosmótico se encuentra mediada por un incremento en los niveles intracelulares de solutos osmóticamente activos. Estos solutos son de dos tipos: iones inorgánicos y compuestos orgánicos (Hoffmann y Simonsen, 1989; Grinstein y Foskett, 1990; Bagnasco et al., 1987).

COMPONENTE IONICO DE LA RECUPERACION DE VOLUMEN EN SOLUCIONES HIPEROSMOTICAS.

Los iones involucrados en la regulación de volumen en soluciones hiperosmóticas son el potasio, el sodio y el cloro. Estos iones se transportan al interior celular por diferentes mecanismos dependiendo del tipo celular de que se trate. El sistema de cotransporte $Na^+/K^+/Cl^-$, que es uno de estos mecanismos, se demostró por primera vez en eritrocitos de aves (Orskov, 1954; Parker, 1977). La recuperación del volumen se encuentra mediada por una entrada acoplada de Na^+, K^+ y Cl^- al interior celular (Kregenow, 1971). La captura de Na^+ y de K^+ en estas condiciones no se afecta por la ouabaina (Kregenow, 1971, 1973) y es inhibida por la furosemida (Schmidt y McManus, 1977a, 1977b). El Na^+ que se captura por este sistema no permanece en la célula a menos que la ouabaína se encuentre presente, lo que sugiere que este Na^+ es intercambiado por K^+ a través de la ATPasa $Na+/K^+$ (Hoffmann et al., 1983).

En eritrocitos del anfibio Amphiuma (Cala, 1983a, 1983b, 1985a), y de perro (Parker, 1983, 1986, 1988), así como en linfocitos humanos (Grinstein et al., 1983, 1984), el incremento intracelular de iones tiene lugar a través de dos sistemas de transporte acoplados: el intercambiador Na^+/H^+ y $Cl^-/HCO3^-$. El sistema Na^+/H^+, sensible a amilorida, es el mecanismo primario activado por la disminución en el volumen (Kregenow, 1981; Kregenow et al., 1985; Siebens y Kregenow, 1985; Cala, 1980,

1985a, 1986), aunque en condiciones fisiológicas la actividad de este intercambiador se encuentra regulada por el pH interno. La salida de H^+ es impulsada por el gradiente de Na^+, produciendo un incremento en el pH intracelular y en la concentración de HCO3$^-$. El gradiente de HCO_3^- activa al intercambiador Cl^-/HCO_3^-, dando como resultado una entrada neta de Cl^-. Esta entrada de cloro y sodio es seguida por una entrada de agua que se traduce en un incremento en el volumen celular (Cala, 1980; Siebens y Kregenow, 1985). Es importante hacer notar que los protones que se intercambian por el sodio son osmóticamente inactivos, ya que se encuentran unidos a macromoléculas intracelulares tales como la hemoglobina. Igualmente el bicarbonato que se intercambia por cloro proviene en su mayoría del CO_2. Al incrementarse la concentración de sodio en la célula se activa la ATPasa Na^+/K^+, por lo que parte del sodio entrante se intercambia por potasio (Siebens y Kregenow, 1985) . La entrada de potasio en un medio hiperosmótico se inhibe completamente en presencia de ouabaína (Siebens y Kregenow, 1985).

COMPUESTOS ORGANICOS EN LA REGULACION DE VOLUMEN EN CONDICIONES HIPEROSMOTICAS.

Bricteux-Grégoire y colaboradores (1962) fueron los primeros en señalar la participación de los solutos orgánicos como osmoefectores en tejidos de animales eurihalinos, los cuales habitan naturalmente en medios de osmolaridad cambiante. En el músculo del cangrejo Eriocheir sinensis, el conjunto de aminoácidos libres (glicina, arginina, taurina, alanina, lisina y el ácido glutámico), betaína y oxido de trimetilamina contribuyen con el 40% de la osmolaridad intracelular total. Al mantener los animales en medios hiperosmóticos los niveles de estos compuestos aumentaron en un 100% (Bricteux-Grégoire et al., 1962).

Posteriormente se han realizado estudios con resultados similares tanto en invertebrados eurihalinos como en algunos vertebrados. En general los aminoácidos que muestran las mayores variaciones internas en respuesta a los cambios en la osmolaridad son los aminoácidos no esenciales como la prolina, la glicina, la alanina, el ácido aspártico, el ácido glutámico, la serina, el GABA y la taurina (Schoffeniels y Gilles, 1970; Gilles, 1975).

En animales adaptados a condiciones hiperosmóticas (con niveles de NaCl superiores a los normales) el incremento en los niveles intracelulares de aminoácidos es a partir de la activación de los sistemas de transporte membranales. En crustáceos el aumento de los aminoácidos en los tejidos se encuentra asociado a una

disminución temporal en las concentraciones de estos compuestos en el plasma (Gilles, 1977). En experimentos in vitro realizados con eritrocitos del lenguado, el aumento intracelular de taurina observado en un medio hiperosmótico también es el resultado de una acumulación del aminoácido del medio externo (Fugelli y Reiersen, 1978).

Los aminoácidos no son el único grupo de compuestos orgánicos que son utilizados para fines de control osmótico. En células epiteliales de las papilas renales cultivadas en un medio hiperosmótico, la concentración intracelular de sorbitol se incrementa hasta alcanzar valores de 240 mM (Bagnasco et al., 1988; Balaban y Burg, 1987). El sorbitol es un polialcohol sintetizado a partir de la glucosa por la enzima aldosa reductasa (Bagnasco et al., 1988). El incremento en los niveles de sorbitol se explica principalmente por un aumento en la síntesis de la aldosa reductasa inducida por la condición hiperosmótica (Bagnasco et al., 1988). En varias líneas celulares renales cultivadas en condiciones hiperosmóticas también se observa un aumento en los niveles de sorbitol, mio-inositol, betaína y glicerofosforilcolina (Nakanishi, 1988). La suma de estos compuestos orgánicos es suficiente para compensar el incremento en la osmolaridad externa (Nakanishi, 1988).

VARIACIONES EN EL VOLUMEN CELULAR EN CONDICIONES ISOSMOTICAS.

En condiciones fisiológicas la mayoría de las células de un organismo multicelular no se encuentran expuestas a situaciones anisosmóticas. La osmolaridad de los fluídos corporales de mamíferos es de aproximadamente 285 mOsmolas/l y este valor se mantiene prácticamente constante, mostrando sólo pequeñas fluctuaciones que no exceden a un 3% de este valor (Hoffmann y Simonsen, 1989). Unicamente en condiciones patológicas la osmolaridad del plasma puede oscilar entre 220 y 350 mOsmolas/l, lo que resultaría, en ausencia de mecanismos reguladores de volumen, en un incremento o disminución del volumen de las células sanguíneas de aproximadamente un 30% y un 20%, respectivamente (Macknight, 1983; 1985). En condiciones fisiológicas algunos tipos celulares como las células epiteliales del intestino y las células de la sangre de los capilares intestinales, posiblemente se encuentren en un medio hiposmótico después de una ingesta excesiva de agua (Hoffmann y Simonsen, 1989). Las células renales son otro ejemplo de células que <u>in vivo</u> puedan ser expuestas a soluciones anisosmóticas.

Experimentalmente se puede producir un incremento en el volumen celular por medio de la acumulación intracelular de compuestos no difusibles. En rebanadas de la corteza renal de conejo incubadas en un medio en donde el cloro se sustituye con acetato, el contenido intracelular de agua se aumenta en un 100% (Cooke y Macknight,

1984; Macknight, 1988). Esto se explica en base a que el ácido acético es capaz de atravesar la membrana plasmática e internalizarse en la célula; una vez dentro se disocia y queda atrapado en el interior, debido a que la forma disociada (CH_3-COO^-) no puede cruzar la membrana plasmática. El ión hidrógeno es intercambiado por sodio y se observa una entrada de potasio a las células para compensar la acumulación de las cargas negativas del acetato; la acumulación de estos solutos se acompaña necesariamente de una entrada de agua, produciéndose el incremento en el volumen (Cooke y Macknight, 1984; Macknight, 1988). Igualmente se ha demostrado que la exposición a ácido láctico induce un incremento de volumen por un mecanismo similar, inhibiéndose en un medio libre de sodio y en presencia de amilorida (Macknight, 1988).

Se ha demostrado que concentraciones elevadas de KCl producen un incremento en el volumen celular en numerosas preparaciones, incluyendo rebanadas de cerebro (Bourke y Tower, 1966; Bourke, 1969; Pappius y Elliott, 1956; Moller et al., 1974), retina (Pasantes-Morales et al., 1988), terminales nerviosas aisladas (Kamino et al., 1973) y astrocitos en cultivo (Walz, 1987). Los mecanismos responsables de este proceso incluyen la activación del sistema de cotransporte $2Cl^-/Na^+/K^+$ (Walz y Kimelberg, 1985) o una alteración del equilibrio de Donnan si se incrementa la concentración de K^+ sin reducir la concentración de Cl^- (Boyle y Conway, 1941).

Existen estudios que señalan que ciertos neurotransmisores son capaces de incrementar el volumen celular. En rebanadas de cerebro la norepinefrina y la adenosina incrementan el contenido de agua de una manera dependiente del cloro extracelular. Este efecto se previene con antagonistas sinápticos específicos (Bourke et al., 1983), lo que indica que este proceso está ocurriendo como resultado de la activación de los receptores sinápticos. El aumento en el volumen celular observado en neuronas en cultivo en presencia de aminoácidos excitadores como el ácido glutámico y el ácido aspártico, así como agonistas de los receptores a estos compuestos, también es prevenido en un medio sin sodio o cloro (Choi, 1987).

En general puede afirmarse que cualquier proceso que conduzca a un incremento en la concentración intracelular de solutos osmóticamente activos (iones inorgánicos o compuestos orgánicos) producirá un aumento en el contenido intracelular de agua. Se ha demostrado que la captura de aminoácidos y azúcares, acoplada con la entrada de sodio, produce una activación de los procesos reguladores de volumen en células epiteliales (Lau et al., 1984; Schultz et al., 1985) y en hepatocitos (Kristensen y Folke, 1984; Kristensen, 1986). Ciertos eventos catabólicos celulares como la glucogenólisis en hepatocitos y la lipólisis en adipocitos también pueden conducir a un incremento en los niveles de solutos intracelulares osmóticamente activos (Kristensen, 1986). Igualmente el metabolismo de las fibras musculares durante el ejercicio aumenta los solutos intracelulares libres y activa los procesos

reguladores de volumen (Saltin et al., 1987). Existe evidencia que señala que existen cambios en el volumen asociados con la actividad neuronal en retina (Orkand, et al., 1984) y en rebanadas de corteza cerebral (Lipton, 1973). El aumento de volumen celular es producido por la entrada de iones a la célula inducida por la despolarización y se previene sustituyendo el cloro por un anión impermeante como el gluconato (Lipton, 1973).

TAURINA.

La taurina es un aminoácido sulfonado (ácido 2-aminoetanosulfónico) que se encuentra ampliamente distribuído como constituyente universal de los tejidos animales. Se identificó por primera vez en la bilis del toro (Tiedemann y Gmelin, 1827). Posteriormente se ha encontrado en la mayoría de los phyla del reino animal: poríferos, sipuncúlidos, equinodermos, moluscos, anélidos, artrópodos, y vertebrados, detectándose en anélidos y moluscos las concentraciones más elevadas (Jacobsen y Smith, 1968). La taurina se encuentra presente en todos los tejidos animales aunque en concentraciones muy diferentes que varían desde valores nanomolares en el hígado de conejo hasta milimolares en la retina y corazón del mismo animal (Awapara, 1955). Los niveles más elevados de taurina se observan en los tejidos excitables: músculo liso, esquelético y cardíaco, glándulas endócrinas y exócrinas y en tejido nervioso (Jacobsen y Smith, 1968). En la retina la

taurina se encuentra en concentraciones excepcionalmente elevadas que varían entre 10 y 50 mM (Pasantes-Morales, 1986). Por medio de estudios de fraccionamiento subcelular se ha demostrado que la mayor parte de la taurina se encuentra en la fracción soluble (Rassin et al., 1977).

A pesar de la amplia distribución de la taurina en el reino animal y en los distintos tejidos animales aún no se ha establecido con certeza la función que cumple este aminoácido en la fisiología del organismo.

LA TAURINA COMO OSMOEFECTOR.

La taurina presenta características que lo señalan como el osmolito ideal: se encuentra presente en concentraciones elevadas en prácticamente todos los tejidos animales, no participa en el metabolismo primario de la célula ni se encuentra como constituyente de macromoléculas (Jacobsen y Smith, 1968). Aunque en general todos los aminoácidos libres se movilizan de acuerdo con la osmolaridad del medio extracelular, la taurina es el aminoácido libre que se encuentra en concentraciones mayores y el que principalmente se emplea como osmoefector. Esto se ha demostrado ampliamente tanto en invertebrados (Lange, 1963; Revisado por Schoffeniels, 1976 y por Gilles, 1979) como en vertebrados eurihalinos, específicamente en células cardíacas de teleósteos (Vislie, 1983) y en eritrocitos de peces eurihalinos

(Fugelli y Thoroed, 1986; Fincham et al., 1987).

En células de mamíferos las primeras observaciones que señalan a la taurina como osmoefector se realizaron en las células del tumor ascítico de Ehrlich (Hoffmann y Handel, 1976). Posteriormente se ha sugerido que la taurina cumple la misma función en rebanadas de riñón (Schultze y Neuhoff, 1983), retina (Domínguez et al., 1989) y astrocitos en cultivo (Pasantes-Morales y Schousboe, 1988). En estudios in vivo efectuados en corazón de ratón se ha demostrado que una condición de hipernatremia incrementa los niveles internos de taurina en las fibras musculares (Thurston et al., 1981). En plaquetas de ratas Blattleboro (las cuales derivan de las ratas Long-Evans por una mutación espontánea), que carecen de la capacidad de sintetizar vasopresina y por tanto se encuentran crónicamente deshidratadas, los niveles de taurina endógena así como la acumulación de taurina radiactiva se encuentran incrementados (Nieminen et al., 1988). En investigaciones in vitro la exposición a condiciones de hiperosmolaridad reduce la liberación basal de taurina en tejido muscular esquelético de rata (Lehmann, 1989) e incrementa la acumulación del aminoácido en las células del corazón de fetos de rata (Atlas et al., 1984). La disminución en los niveles intracelulares de taurina observada en condiciones hiposmóticas parece explicarse exclusivamente debido a una liberación del aminoácido y no a una transformación enzimática puesto que la cantidad que disminuye en la célula es recuperado en su totalidad en el medio extracelular (Hoffmann y Lambert, 1983).

OBJETIVO GENERAL:

Determinar utilizando como modelo astrocitos en cultivo, los mecanismos responsables de la movilización de osmolitos orgánicos e inorgánicos que ocurre durante su exposición a condiciones anisosmóticas.

OBJETIVOS ESPECIFICOS:

1) Determinar en astrocitos en cultivo la importancia relativa de osmolitos orgánicos (aminoácidos libres) e inorgánicos (potasio) en los procesos de regulación de volumen observados en condiciones hiposmóticas.

2) Determinar si un incremento en el volumen celular producido por soluciones hiposmóticas se asocia con alteraciones en los niveles intracelulares de calcio, como posible señal mediadora de la liberación de osmolitos.

3) Explorar los mecanismos de transporte involucrados en la salida de potasio y aminoácidos estimulada por soluciones hiposmóticas.

4) Caracterizar la selectividad de la vía de transporte de osmolitos orgánicos activada durante un incremento en el volumen celular.

5) Investigar el mecanismo responsable del incremento en los niveles de taurina observado en astrocitos crecidos en condiciones hiperosmóticas.

TRABAJO 1:

Sánchez-Olea, R., Morán, J., Martínez, A., Pasantes-Morales, H. 1993. Volume-activated Rb^+ transport in astrocytes in culture. Am. J. Physiol. 33:C836-C842.

Volume-activated Rb^+ transport in astrocytes in culture

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Sanchez-Olea, Roberto, Julio Moran, Alejandro Martinez, and Herminia Pasantes-Morales. Volume-activated Rb^+ transport in astrocytes in culture. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C836–C842, 1993.—The involvement of K^+ on the volume regulatory process in astrocytes was investigated by characterizing the hyposmolarity-induced efflux of K^+ using ^{86}Rb as a tracer. About 70 and 30% of the intracellular content of ^{86}Rb was released after reductions in osmolarity from 320 to 160 or 220 mosM, respectively, during the time in which cells exhibit a volume regulatory response subsequent to swelling. No significant increase in ^{86}Rb efflux was observed with lower reductions in osmolarity. The ^{86}Rb efflux was Ca^{2+} independent and insensitive to temperature. It was inhibited by furosemide but not by bumetanide and was unaffected when nitrate, but not gluconate, replaced intracellular Cl^-. The efflux was markedly inhibited by quinidine and by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid. Quinidine also prevented the volume regulatory decrease of cells, and this effect was overcome when a large cation permeability was imposed by gramicidine. In isosmotic conditions ^{86}Rb efflux was not activated by *N*-ethylmaleimide, but this drug strongly inhibited the hyposmolarity-activated release. These findings suggest that ^{86}Rb efflux from astrocytes associated to cell swelling is not mediated by an electroneutral cotransporter and rather favor the implication of a conductive exit pathway that may be a Ca^{2+}-independent K^+ channel.

volume regulation; hyposmolarity; potassium transport; 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; quinidine

IT IS WELL DOCUMENTED that astrocytes counteract swelling induced by hyposmotic conditions by activating mechanisms that restore cell volume, despite the persistence of the anisosmotic situation (13). In other cells this volume regulatory process is accomplished through net extrusion of intracellular solutes accompanied by osmotically obligated water. Intracellular organic molecules as well as inorganic ions participate as osmolytes in this regulatory volume decrease (RVD) (2, 8, 16, 28). The release of free amino acids, notably taurine, associated to cell swelling has been documented in astrocytes (12, 21–23), and the mechanism of its exit pathway has been investigated (27). In contrast, the involvement of inorganic ions in the volume corrective fluxes in these cells is less well known. In the present work we characterized the K^+ efflux activated by exposure of astrocytes in culture to hyposmotic medium using ^{86}Rb (Rb) as a tracer. Swelling-associated release of K^+ may occur either by electroneutral K^+-Cl^- cotransporters or by conductive fluxes that mobilize K^+ and Cl^- separately. A variety of pharmacological agents that block these transporters or channels are available and were used in this work as tools to investigate the mechanism of the K^+ efflux evoked by hyposmolarity. Furosemide and bumetanide were used as inhibitors of the K^+-Cl^- cotransporter. Barium, tetraethylammonium (TEA), 4-aminopyridine (4-AP), and quinidine, known inhibitors of various types of K^+ channels that also block RVD in some cells, were used to investigate whether a K^+ channel is involved in the volume-sensitive exit pathway. The efflux of K^+ might be coupled to an anion conductance even when an electroneutral K^+-Cl^- cotransporter is not involved. Cation and anion channels activated by volume regulatory processes are often separated, but the ion fluxes are interdependent. This possibility was explored in the present work by studying the effect of the inhibitors of Cl^- channels: 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), diphenylamine-2-carboxylate (DPC), and anthracene-9-carboxylate (9-AC) on the volume-activated K^+ fluxes in astrocytes. The Ca^{2+} dependence of the volume regulatory fluxes of K^+ was also examined.

MATERIALS AND METHODS

Primary cultures of astroglial cells from rat cerebellum were obtained as previously described by Morán and Patel (19). Briefly, the dissociated cell suspensions from the cerebella of 8-day-old rats were plated at a density of 225×10^3 cells/cm^2 in 35-mm diameter plastic dishes. The culture medium contained basal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 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. The purity of the cultures was determined by immunostaining counting using a fluorescence microscope. Neurons were stained with a polyclonal antibody against neuron-specific enolase (Dakopatts). Astrocytes were labeled with a polyclonal antibody against glial fibrillary acidic protein (GFAP; Sigma, St. Louis, MO). Fibroblasts were stained with a monoclonal antibody against Thy-1. Cultures were highly enriched in astrocytes containing >95% positive cells for GFAP.

After 2 wk in vitro, cells were incubated in culture medium containing ^{86}Rb (2.0 μCi/ml, original activity) for 60 min. After incubation, the culture medium was replaced with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline containing (in mM) 135 NaCl, 5 KCl, 1.0 $CaCl_2$, 1.17 $MgCl_2$, 10 HEPES, and 5 glucose, pH 7.4. After three successive rapid washes with this medium, cells were superfused as previously described (23) at a rate of 1 ml/min for 5 min, at which time Rb efflux baseline was attained. At the time indicated at each experiment, the superfusion medium was replaced by analogous media of reduced osmolarity. Samples were collected every minute for 20 min. Hyposmotic media were prepared by decreasing NaCl concentration. Some experiments were done in Cl^--free media, prepared replacing KCl and NaCl by their respective gluconate or nitrate salts. Drugs were present sometimes during the incubation period and always throughout the superfusion period. When solutions of drugs were prepared using solvents different from water, controls were exposed to the same concentration of the solvent used. At the end of the experiments radioactivity in media and that remaining in the cells was measured by scintillation spectrometry. Results are expressed as the radioactivity released in each collected sample as percent of total radioactivity present in the cells at that time. Some results are expressed as the difference between the amount of Rb released in the five largest fractions after the hyposmotic stimulus minus the amount released in 5 min before

stimulation. In a typical experiment, radioactivity released per minute in isosmotic conditions was ~600 counts/min (cpm) and ~1,400 after stimulation from a total of 35,000 cpm accumulated by cells. Obviously, the absolute numbers varied according to the half-life of the Rb stocks.

For measurements of cell Cl^- content, cells were incubated with media containing gluconate or nitrate replacing Cl^- for 20 min. At the end of the incubation period, cells were washed, detached from the culture dish, sonicated, and centrifuged. The Cl^- content in the supernatants was measured using a Cl^- electrode (Orion, model 94-17B) (1).

For cell volume measurements, cells were detached from the monolayer by treatment for 10 min with trypsine-EDTA medium (Ca^{2+} and Mg^{2+} free). The detached cells were centrifuged and resuspended in isosmotic medium. At *time 0*, a sample of the cell suspension was diluted ~100-fold with the experimental media, and the cell volume was followed in a Coulter Counter with a channelizer (model ZM 256). The changes in volume for the cell population were measured from the shift in median size based on calibration with latex microspheres of 9.63 μm diameter. To explore the effect of replacing Cl^- by gluconate or nitrate, cells were incubated in media containing the different anions, and cell volume measurements were carried out in media with the corresponding anion replacements.

Lactic acid dehydrogenase (LDH) activity in the medium or in cells was measured spectrophotometrically as reduction of NAD followed by changes in absorbancy at 340 nm. The statistical significance of the results was determined by Student's *t* test.

RESULTS

Cell volume changes. Reducing the medium osmolarity from 320 mosM (isosmotic condition) to 160 or 220 mosM elicited an immediate increase in astrocyte volume that was proportional to the hyposmolarity reduction. Increases in astrocyte volume of 62 and 35% were observed in solutions of 160 or 220 mosM, respectively. In all cases, swelling was followed by RVD, returning cell volume to 18–20% above control (Fig. 1). The decline in cell volume consists of a rapid phase occurring within the first 5 min and a slower phase during the subsequent 10 min. These results were similar to those reported previously (11, 23).

Effect of hyposmolarity on ^{86}Rb efflux. The time course of K^+ exit evoked by hyposmolarity was measured following the unidirectional efflux of Rb as a tracer for K^+. Rb release at the baseline in isosmotic conditions was ~1.4%/min. Reducing osmolarity of media from 320 mosM (isosmotic) to 96 or 160 mosM (i.e., reductions of 70 and 50%, respectively) led to a rapid release of Rb, markedly increasing the efflux observed in isosmotic conditions. A decrease in osmolarity from 320 to 220 mosM (30% reduction) evoked a noticeable but not significant increase on Rb efflux, whereas a decrease from 320 to 272 mosM (15% reduction) did not affect Rb release (Fig. 3). The efflux of Rb evoked by hyposmolarity reached a peak after 2–3 min after stimulation and then declined slowly, despite the persistence of the hyposmotic conditions (Fig. 2). About 30–35% of Rb in cells was released by 50% reduction in hyposmolarity during the 15 min subsequent to the stimulus, the time in which RVD occurs. Efflux was unchanged in the presence of 0.5–1 mM ouabain (results not shown).

Effect of conditions affecting K^+-Cl^- cotransporters. Electroneutral K^+-Cl^- cotransporters activated by swelling in a variety of cells are inhibited by furosemide and bumetanide. These carriers are strictly dependent on the presence of Cl^- (or Br^-) and therefore are inactivated when nitrate or gluconate replace Cl^- in the medium (8, 15). The K^+-Cl^- cotransporter in red blood cells stimulated by swelling is also activated under isosmotic conditions by the sulfhydryl-reactive reagent *N*-ethylmaleimide (NEM) (15). To assess the possibility that the Rb efflux evoked by hyposmolarity in astrocytes is mediated by this mechanism, the influence of all the above mentioned conditions and inhibitors on Rb efflux was examined.

Furosemide exerted a concentration-dependent inhibitory effect on Rb release that was maximal at 2 mM.

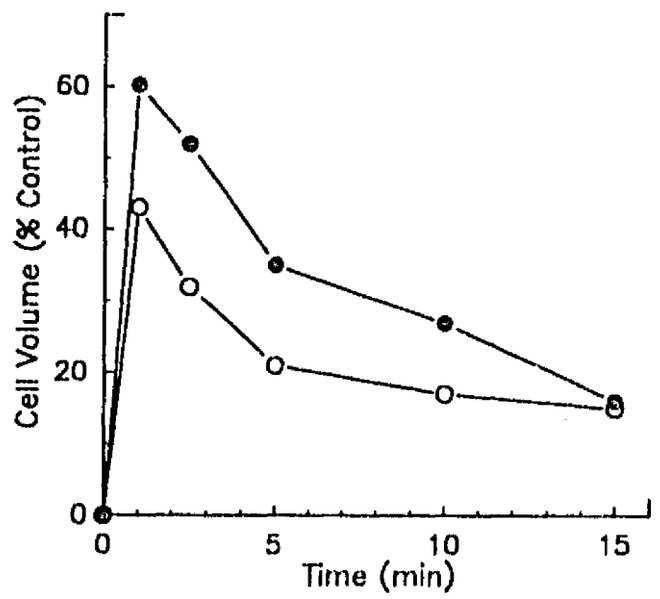

Fig. 1. Regulatory volume decrease of cultured astrocytes in hyposmotic media: 160 mosM (●) and 220 mosM (○). Reduction in osmolarity was obtained by decreasing concentration of NaCl from 135 to 60 or 90 mM. Astrocytes were detached from culture dish as described in MATERIALS AND METHODS, and cell volume was measured in Coulter Channelizer. Changes in cell volume are expressed as percentage increase over volume in isosmotic conditions (320 mosM). Mean volume of astrocytes was 1.126 ± 0.02 pl/cell. Results are means of 6–18 experiments, with bars representing SE when exceeding symbol size.

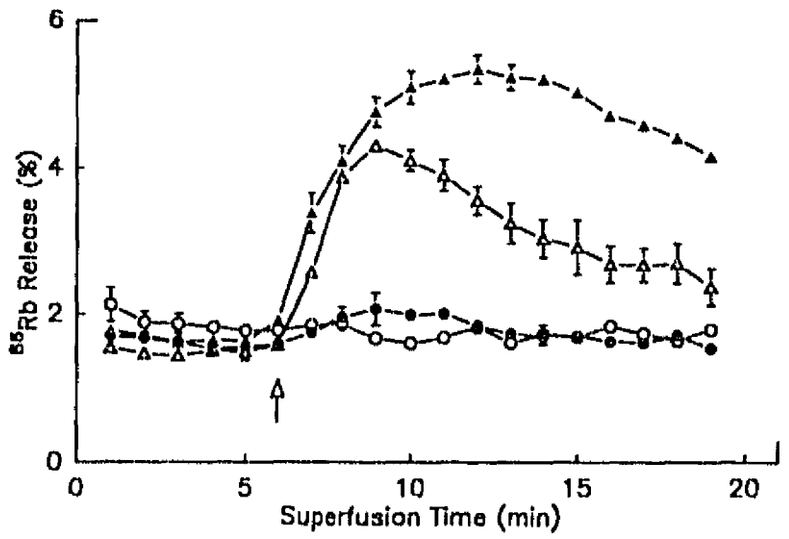

Fig. 2. Time course of ^{86}Rb efflux stimulated by hyposmolarity. Cells were preloaded with ^{86}Rb, washed, and superfused with isosmotic medium (320 mosM). At time indicated by arrow superfusion was continued with hyposmotic media of 96 (▲), 160 (△), 220 (●), and 272 mosM (○). At end of superfusion, radioactivity remaining in cells and that in collected samples was measured. Fractional efflux of ^{86}Rb is expressed as radioactivity released at each fraction as percent of total radioactivity present in cells at that time. Results are means ± SE of 4–12 experiments.

Bumetanide was ineffective at concentrations of 50–250 μM (Fig. 3). Replacing NaCl and KCl in the medium by the corresponding gluconate salts reduced Rb efflux by ~30%. No inhibition was observed when Cl^- was replaced by nitrate (Fig. 4). The intracellular concentration of Cl^- during an incubation period of 20 min in the presence of gluconate decreased by 80% and by >95% in the presence of nitrate (results not shown). Astrocytes incubated in isosmotic medium in which Cl^- was replaced by gluconate showed a moderate reduction in cell volume of 20%. On exposure to hyposmotic medium, volume in these cells was higher than in controls, and RVD was clearly impaired. When nitrate replaced Cl^-, no cell shrinking was observed, and RVD was essentially the same as in controls (Fig. 5). The efflux of Rb under isosmotic conditions was not stimulated by NEM, but, in contrast, the hyposmolarity-evoked release was markedly inhibited (Fig. 6).

Effect of blockers of K^+ channels. The effect of inhibitors of K^+ channels (TEA, 4-AP, barium, and quinidine) was examined on the Rb efflux evoked by hyposmolarity. Of all these drugs, only quinidine exhibited a clear concentration-dependent inhibitory effect, reducing Rb efflux by ~60% at 0.6 mM concentration (Fig. 7). Higher concentrations cannot be used without causing cell damage. Quinidine, at the concentrations that decreased Rb release, showed an inhibitory effect on RVD that was abolished when high cation permeability was imposed by gramicidin (Fig. 8). RVD in controls was also accelerated by gramicidin (Fig. 8).

TEA and 4-AP did not affect Rb efflux stimulated by hyposmolarity. Rb release from cells exposed to TEA and 4-AP was 13.3 ± 1.25 (n = 4) and 16.6 ± 2.01 (n = 4), respectively, which was not significantly different from release in controls (14.9 ± 1.01, n = 12). TEA and 4-AP did not affect RVD (results not shown). Barium (5 mM), markedly increased the spontaneous release of Rb and did not affect the stimulated release of Rb but prevented the decline of efflux typically observed in normal conditions. Volume in cells exposed to barium slowly increased in isosmotic conditions up to 21% over cell volume in controls with a time course parallel to that of Rb^+ release. Cell viability was not affected by barium as assessed by LDH release. LDH activity in the medium was 0.14 $\mu mol \cdot h^{-1} \cdot ml^{-1}$ in controls and 0.15 $\mu mol \cdot h^{-1} \cdot ml^{-1}$ in cells exposed to barium. This amount represents ~2% of total LDH.

Effect of blockers of Cl^- channels. The stilbene disulfonate DIDS had a marked inhibitory effect on Rb release that was concentration dependent. At the highest concentration examined (400 μM) DIDS markedly decreased (85%) the Rb release evoked by hyposmolarity. Reductions of 75 and 66% were observed at 200 and 100 μM DIDS, respectively. At lower concentrations of 50 and 25 μM, DIDS still had an inhibitory effect of 51 and 42%, respectively (Fig. 9). The Cl^- channel blockers 9AC and

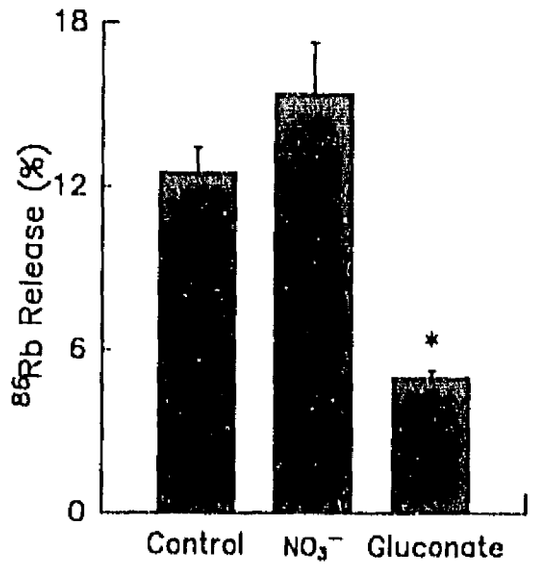

Fig. 4. Effect of chloride replacement on hyposmolarity stimulated efflux of ^{86}Rb. Astrocytes preloaded with ^{86}Rb were superfused with normal isosmotic or hyposmotic (160 mosM) media or with media (isosmotic or hyposmotic) in which NaCl and KCl were replaced by corresponding gluconate or nitrate salts. Results are expressed as in Fig. 3 and are means ± SE of 4–8 experiments. Significant difference from control, * $P < 0.001$ (Student's t test).

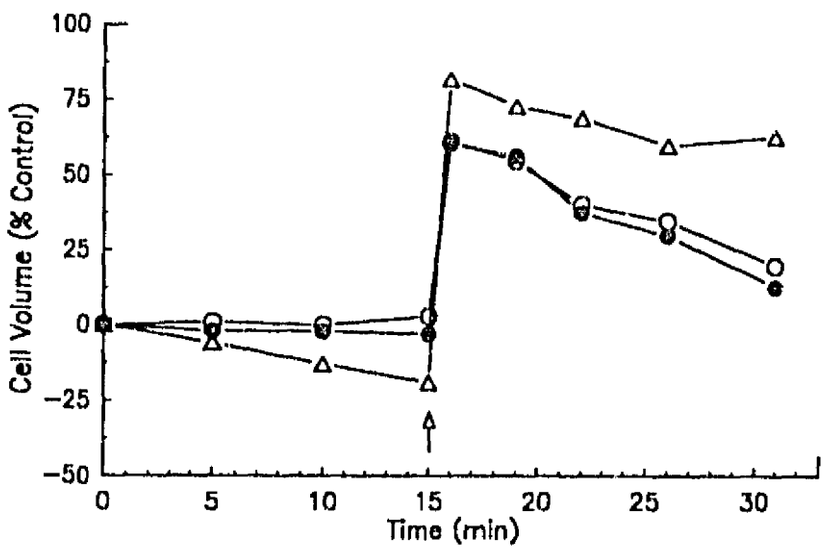

Fig. 5. Effects of anion replacement on astrocyte volume in isosmotic and hyposmotic conditions. Astrocytes were incubated in normal isosmotic medium (●) or in isosmotic media in which nitrate (○) and gluconate (△) replaced chloride and volume was measured as in Fig. 1. At time indicated by arrow, cells were transferred to hyposmotic medium (50%) and RVD was followed at indicated times. Results are expressed as percentage changes of initial volume of preparation. Results are means of 4 experiments.

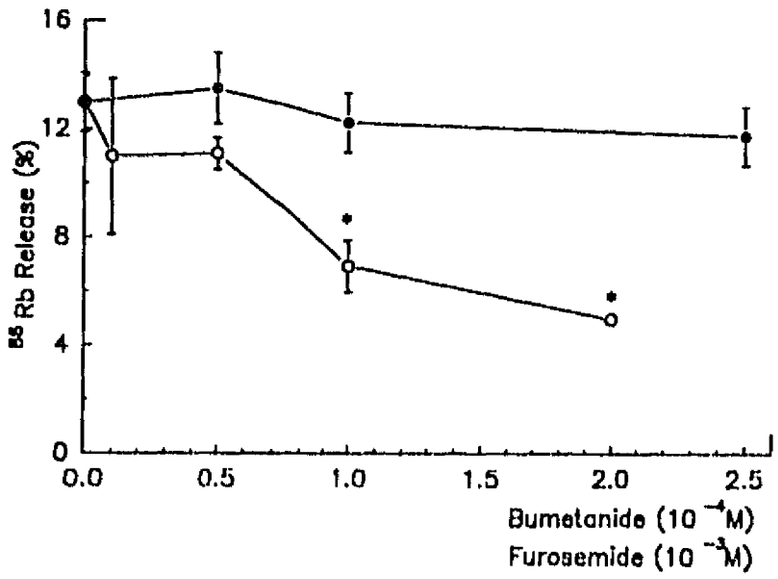

Fig. 3. Effect of furosemide and bumetanide on hyposmolarity-evoked efflux of ^{86}Rb. Cells were preloaded and superfused as described in Fig. 2 and stimulated with hyposmotic medium (160 mosM). Bumetanide (●) and furosemide (○) at concentrations indicated were present throughout superfusion period. Release of ^{86}Rb is expressed in percentage, and points represent amount released in 5 largest stimulated fractions minus amount released in 5 basal fractions prior to hyposmotic stimulus. Results are means ± SE of 4–10 experiments. Significant difference from control, * $P < 0.001$ (Student's t test).

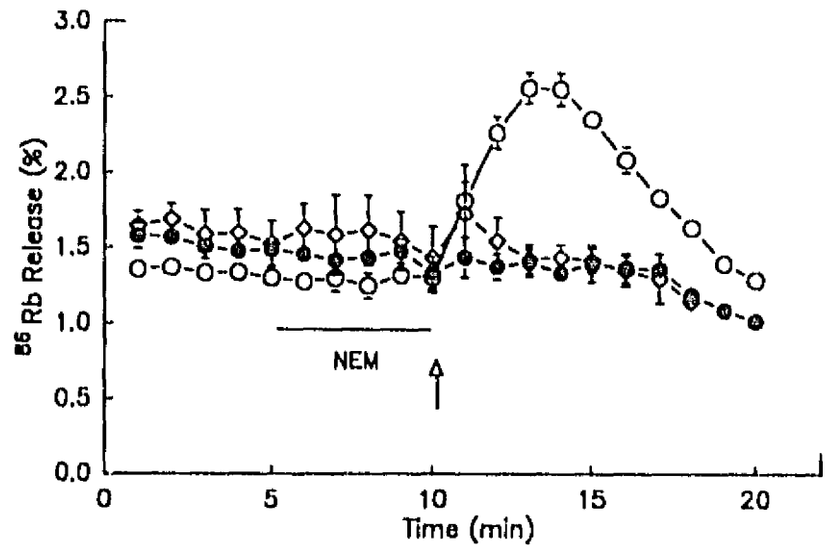

Fig. 6. Effect of *N*-ethylmaleimide (NEM) on Rb release in isosmotic and hyposmotic conditions. Cells were preloaded, washed, and superfused with isosmotic medium. From min 5–10, as indicated by bar, isosmotic medium contained 100 μM (●) or 50 μM (◇) of NEM. At time indicated by arrow superfusion continued with hyposmotic medium (160 mosM) without NEM. Controls not exposed to NEM (○). Points are means ± SE of 4 experiments.

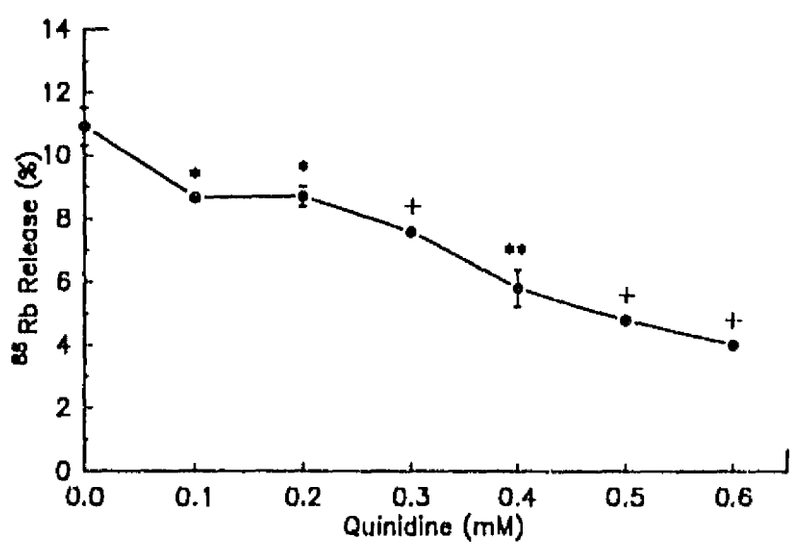

Fig. 7. Effect of quinidine on hyposmolarity stimulated efflux of ^{86}Rb. Cells were preloaded, washed, and superfused in isosmotic or hyposmotic media containing quinidine at concentrations indicated. Results are expressed as in Fig. 3 and are means ± SE of 2–10 experiments. $^{+}n = 2$. Significant difference from control, * $P < 0.01$; ** $P < 0.001$ (Student's t test).

DPC decreased by 15–20% the release of Rb (Fig. 9). None of these compounds showed inhibitory effects on the efflux of Rb in isosmotic conditions. The effects of simultaneous exposure to DIDS (100 μM) and quinidine (200 μM) were additive (results not shown).

Effect of extracellular Ca^{2+}. When cells were incubated in a Ca^{2+}-free medium containing EGTA (0.5 mM), a delayed increase in Rb efflux in isosmotic conditions was observed, but the rapid efflux evoked by hyposmolarity was unchanged (Fig. 10). No changes in cell volume or cell leakage were observed in these conditions.

Effect of temperature. Reducing the temperature of the superfusion medium from 37 to 23 or 15°C did not decrease the hyposmolarity-sensitive efflux of Rb. At 4°C the efflux was essentially abolished (Fig. 11).

DISCUSSION

A large variety of cells mobilize K^+ and Cl^- to adjust to changes in cell volume occurring in anisosmotic conditions (8, 28). A question addressed in this work was whether K^+ also participates preferentially in the mechanism of volume regulation in astrocytes. This might not be the case, in view of the key role played by K^+ to maintain transmembrane potentials in excitable cells, which demands a strict control of its concentration in both intracellular and extracellular compartments. Moreover, the regulatory function of astrocytes in clearing K^+ from the extracellular space (7) makes it unlikely that this ion is preferentially involved as an osmolyte in the nervous tissue. Results of the present study seem to support this notion because significant Rb^+ release was observed only when external osmolarity is markedly (70 or 50%) decreased (present results and Ref. 12). No release of Rb^+ could be observed in response to reductions in osmolarity of 30 or 15%. This is in contrast to that observed in a large number of cells, including renal cells and other epithelial cells, Ehrlich ascites cells and lymphocytes (5, 8, 24, 28), in which small changes in cell volume

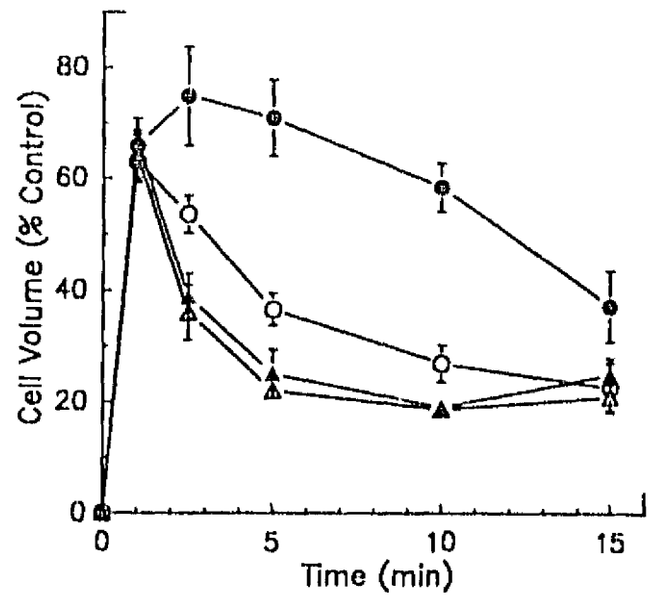

Fig. 8. Effects of quinidine and gramicidin on regulatory volume decrease. Astrocyte volume was measured and results expressed as in Fig. 1. ●, Cells were exposed to 0.2 mM quinidine throughout experiment. ▲, Cells were similarly exposed to quinidine and after first measurement of cell volume after stimulation with hyposmotic medium (min 1), 1 μM gramicidin was added to solution. ○, Control; △, control plus gramicidin. Results are means ± SE of 3–6 experiments.

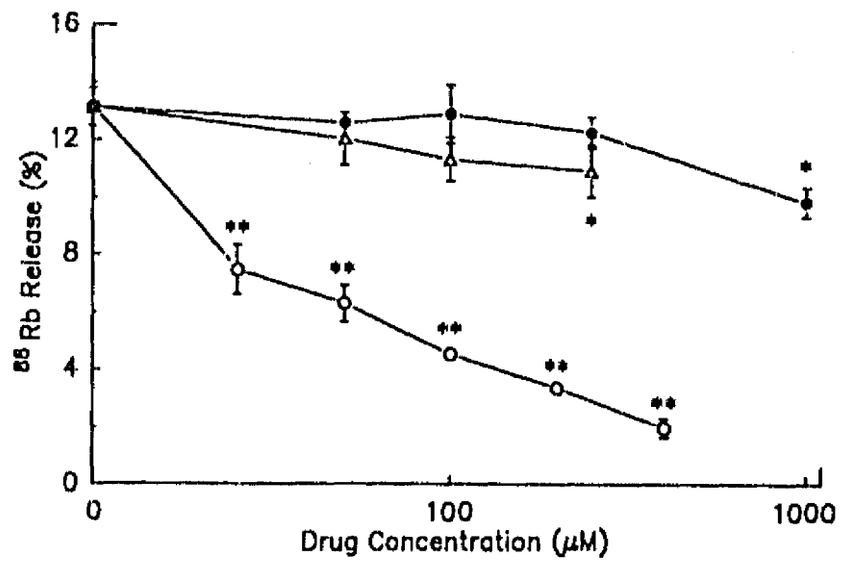

Fig. 9. Effect of inhibitors of Cl^- channels on hyposmolarity stimulated ^{86}Rb efflux. Cells were preloaded, washed, and superfused with isosmotic and hyposmotic (160 mosM) media containing DIDS (○), DPC (△), or 9-AC (●) at indicated concentrations. Astrocytes were exposed to DIDS during last 10 min of incubation period and to all drugs throughout superfusion period. Results are expressed as in Fig. 3 and are means ±SE of 8–16 experiments. Significant difference from control, * $P < 0.05$; ** $P < 0.001$ (Student's t test).

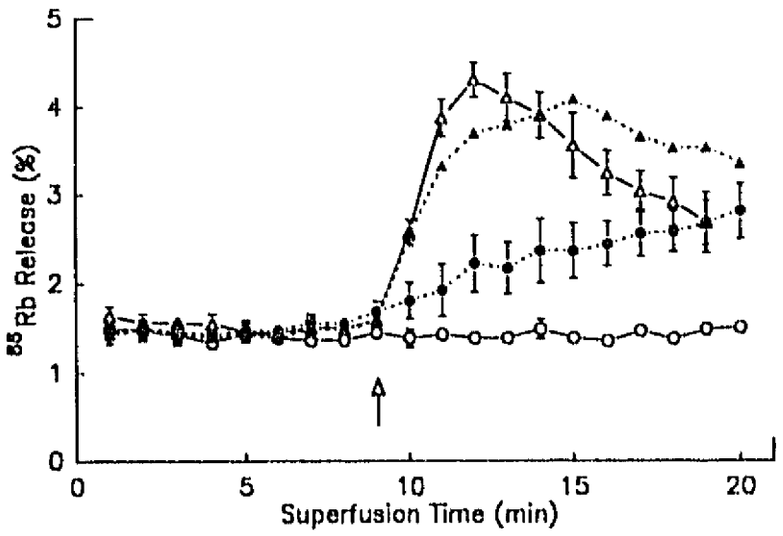

Fig. 10. Effect of omission of Ca^{2+} and addition of EGTA on hyposmolarity-stimulated efflux of ^{86}Rb. Cells were superfused with isosmotic (○) and hyposmotic (△) media containing CaCl, or with isosmotic (●) or hyposmotic (▲) (160 mosM) media in which $CaCl_2$ was omitted and 0.5 mM EGTA was added. Curves represent fractional ^{86}Rb efflux as defined in MATERIALS AND METHODS and are means ± SE of 6-8 experiments.

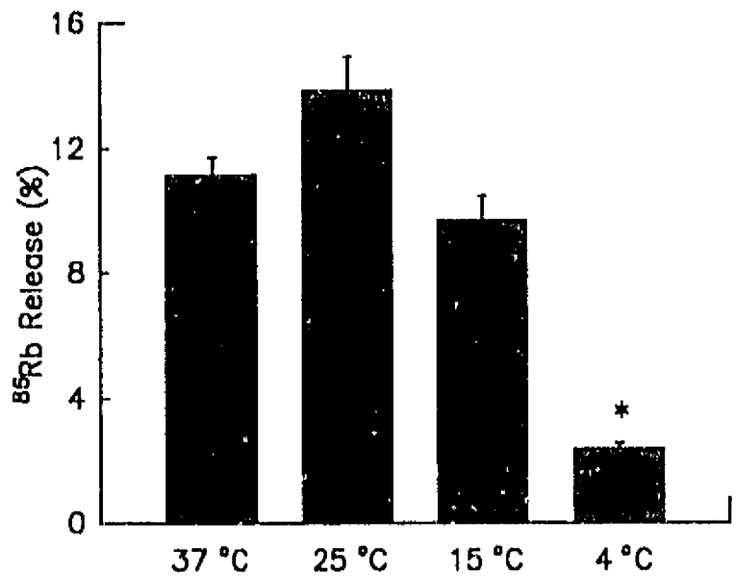

Fig. 11. Effect of temperature on hyposmolarity-activated efflux of ^{86}Rb. Superfusion of cells was as described in Fig. 1, except that temperature of media, both isosmotic and hyposmotic (160 mosM), was decreased to values indicated in figure. Results are expressed as in Fig. 3 and are means ± SE of 6-12 experiments. Significant difference from 37°C (control), * $P < 0.001$ (Student's t test).

are sufficient to activate the efflux of K^+. These findings suggest that the release of K^+ from astrocytes may be a mechanism activated only when large changes in cell volume occur, at variance with that observed in nonexcitable cells.

From our results the hyposmolarity-evoked Rb efflux from astrocytes may be characterized. The process was *1*) sensitive to quinidine (59% inhibition); *2*) inhibited by Cl^- channel blockers, 59% by 9-AC, 85% by DIDS, and 51% by furosemide; and *3*) Cl^- dependent (51% inhibited in gluconate medium), but Cl^- may be replaced by nitrate.

The analysis of these properties provides some indications about the possible mechanism of Rb efflux activated by swelling in astrocytes. Essentially two mechanisms have been shown to operate for swelling-activated K^+ fluxes in vertebrate cells: *1*) electroneutral K^+-Cl^- cotransporters, as observed in red blood cells, gallbladder epithelium, and kidney tubules (8, 28) and *2*) volume-sensitive channels for K^+ and Cl^- separated but interconnected, as found in lymphocytes, Ehrlich ascites cells, various epithelial cells, platelets, granulocytes, hepatocytes, and cell lines of osteosarcoma (5, 6, 8, 14, 18, 28). From these results the possibilities for these two mechanisms of being responsible for Rb efflux from astrocytes can be discussed.

Evidence against the electroneutral cotransport as the mechanism responsible for swelling activated Rb efflux include *1*) Cl^- independence: a rigorous specificity for Cl^- is a property of the cotransporter. In all cells so far examined, the K^+-Cl^- cotransport is blocked when other anions with the exception of Br^-, replace Cl^- (8, 15, 28). *2*) Insensitivity to bumetanide: even at a concentration of 250 μM, which is much higher than that required to inhibit the K^+-Cl^- cotransporter, Rb efflux from astrocytes was unaffected. *3*) Temperature independence. *4*) Insensitivity to NEM: In red blood cells, the K^+-Cl^- cotransporter activated by swelling is also stimulated by NEM in isosmotic conditions (15). This was not observed in astrocytes, but in contrast NEM caused the suppression of the swelling-evoked release of Rb. *5*) Sensitivity to DIDS: the K^+-Cl^- cotransporter in red blood cells and in astrocytes is not affected by stilbene sulfonates (10, 15). The inhibition by furosemide is the only observation suggesting the involvement of the cotransporter. However, it is known that the effects of furosemide are not specific as this drug, apart from being a blocker of the cotransporter, also inhibits anion exchangers as well as Cl^- channels (29).

An early study by Kimelberg and Frangakis (10) in cultured astrocytes described a K^+-Cl^- cotransporter that could be, in principle, the mechanism activated by hyposmolarity. However, this cotransporter was maximally inhibited by both furosemide and bumetanide, at concentrations of bumetanide much lower (1 μM) than those that were ineffective in our study. In addition, the cotransporter was not inhibited by 1 mM 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), in clear contrast with the present results.

Evidence supporting the involvement of a conductive pathway on Rb release in astrocytes include the following findings: *1*) the insensitivity of the process to temperature; *2*) the inhibitory effect of quinidine, a well known blocker of K^+ channels, in both Rb efflux and in RVD, which is overcome when the K^+-blocked conductance is bypassed by addition of gramicidin; *3*) the possibility of replacing Cl^- by nitrate as the anion accompanying the efflux of Rb; and *4*) the transient membrane depolarization of astrocytes observed after lowering osmolarity (13).

Activation of conductive K^+ transport pathways by hyposmolarity has been shown in a variety of cells (8, 28). However, marked differences have been observed in the ionic selectivity and the properties of these channels. Three types of K^+ channels have been identified in association with mechanisms of cell volume regulation: Ca^{2+}-independent, unselective cation channels (25, 26); Ca^{2+}-dependent, K^+-selective channels (3); and stretch-activated, Ca^{2+}-insensitive and K^+-selective channels (22). Because the pharmacology of these different channels is still unknown, it is not possible to identify from

the results of the present work the type of channel presumably involved in RVD in astrocytes, although the Ca^{2+} independence of Rb efflux exclude at least one of the channels. The dominant K^+ channel type in cultured astrocytes exhibit one voltage-independent and two voltage-dependent modes and at least three different conductances for outward currents (9, 20). It is unclear whether they correspond to different types of K^+ channels or to one single type with complex behavior. These channels appear to be less sensitive to various pharmacological agents than the corresponding channels described in other systems because only small inhibitory effects of TEA, 4-AP, and barium are observed (20).

An increase in Rb^+ release in isosmotic conditions was observed in cells exposed to 5 mM barium. This release can easily be related to the increase in cell volume that occurred with the same time course as the barium-stimulated Rb efflux but there is no obvious explanation for this later effect of barium.

The hyposmolarity-activated Rb release from astrocytes was inhibited by Cl^- channel blockers, suggesting an interdependence of the two ionic fluxes. In most cells, fluxes of K^+ and Cl^- associated to changes in cell volume seems to be interconnected even if the transport occurs through separate conductive pathways (8, 17, 18, 28). Available evidence on Cl^- channels in astrocytes is not sufficient to identify a Cl^- channel, presumably involved in volume regulatory fluxes, that could be associated to K^+ conductances. Large-conductance Cl^- channels, similar to those described in muscle cells, are present in astrocytes together with small conductance channels opened by hyperpolarization (20), corresponding to neurotransmitter-gated channels. Most Cl^- channels in astrocytes appear to be nonfunctional at rest, and it is possible that one of these conductances is activated by cell swelling. Alternatively, the effect of anion channel blockers on Rb efflux may be explained, considering the possibility that K^+ is transported through a type of Cl^- channel with dual anion-cation permeability. These channels have been described in neurons, muscles, epithelial cells, and gland cells (4), all of which show processes of cell volume regulation. They are inhibited by 9-AC, DPC, and SITS, drugs that also partially inhibited Rb efflux. This is an interesting possibility to be explored in astrocytes.

The data of the present study support the view that astrocytes have the possibility to correct changes in cell volume by activation of K^+ fluxes normally quiescent in isosmotic conditions. The high threshold of activation of these K^+ fluxes suggests that astrocytes utilize this mechanism preferentially in extreme conditions of cell swelling. It is likely that other osmolytes, such as organic molecules, that do not disturb nervous excitability are preferred for purposes of cell volume control.

The expert technical assistance of Claudia Rodriguez and Irma López Martinez is acknowledged.

The work was supported by a grant from DGAPA-UNAM (IN-204589).

Received 31 August 1992; accepted in final form 26 October 1992.

REFERENCES

1. **Alvarez-Leefmans, F. S., F. Giraldez, and J. H. Russell.** Methods for measuring chloride transport across nerve, muscle and glial cells. In: *Chloride Channels and Carriers in Nerve, Muscle, and Glial Cells*, edited by F. J. Alvarez-Leefmans and J. M. Russell. New York: Plenum, 1990, p. 3–66.
2. **Chamberlin, M. E., and K. Strange.** Anisosmotic cell volume regulation: a comparative view. *Am. J. Physiol.* 257 (*Cell Physiol.* 26): C159–C173, 1989.
3. **Christensen, O.** Mediation of cell volume regulation by Ca^{2+} influx through stretch-activated channels. *Nature Lond.* 330: 66–68, 1987.
4. **Franciolini, F., and A. Petris.** Chloride channels of biological membranes. *Biochim. Biophys. Acta* 1031: 247–259, 1990.
5. **Grinstein, S., A. Rothstein, B. Sarkadi, and E. W. Gelfand.** Responses of lymphocytes to anisotonic media: volume-regulating behavior. *Am. J. Physiol.* 246 (*Cell Physiol.* 15): C204–C215, 1984.
6. **Hazama, A., and Y. Okada.** Ca^{2+} sensitivity of volume-regulatory K^+ and Cl^- channels in cultured human epithelial cells. *J. Physiol. Lond.* 402: 687–702, 1988.
7. **Hertz, L.** An intense potassium uptake into astrocytes, its further enhancement by high concentrations of potassium and its possible involvement in potassium homeostasis at the cellular level. *Brain Res.* 145: 202–208.
8. **Hoffmann, E. K., and L. O. Simonsen.** Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* 69: 315–382, 1989.
9. **Jalonen, T., and I. Holopainen.** Properties of single potassium channels in cultured primary astrocytes. *Brain Res.* 484: 177–183, 1989.
10. **Kimelberg, H. K., and M. V. Frangakis.** Furosemide- and bumetanide-sensitive ion transport and volume control in primary astrocyte cultures from rat brain. *Brain Res.* 361: 125–134, 1985.
11. **Kimelberg, H. K., and M. Frangakis.** Volume regulation in primary astrocyte cultures. *Adv. Biosci.* 61: 177–186, 1986.
12. **Kimelberg, H. K., S. K. Goderie, S. Higman, S. Pang, and R. A. Waniewski.** Swelling induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J. Neurosci.* 10: 1583–1591, 1990.
13. **Kimelberg, H. K., and H. Kettenmann.** Swelling-induced changes in electrophysiological properties of cultured astrocytes and oligodendrocytes. I. Effects on membrane potentials, input impedance and cell-cell coupling. *Brain Res.* 529: 255–261, 1990.
14. **Khademazad, M., B. X. Zhang, P. Loessberg, and S. Muallem.** Regulation of cell volume by the osteosarcoma cell line UMR-106-01. *Am. J. Physiol.* 261 (*Cell Physiol.* 30): C441–C447, 1991.
15. **Lauf, P. K.** K^+:Cl^- Cotransport: sulfhydryls, divalent cations, and the mechanism of volume activation in a red cell. *J. Membr. Biol.* 88: 1–13, 1985.
16. **Law, R. O., and M. B. Burg.** The role of organic osmolytes in the regulation of mammalian cell volume. In: *Advances in Comparative and Environmental Physiology*, edited by R. Gilles. Berlin: Springer-Verlag, 1991, vol. 9, p. 189–225.
17. **Lindstrom, P., L. Norlund, and J. Sehlin.** Potassium and chloride fluxes are involved in volume regulation in mouse pancreatic islet cells. *Acta Physiol. Scand.* 128: 541–546, 1986.
18. **MacLeod, R. J., and J. R. Hamilton.** Separate K^+ and Cl^- transport pathways are activated for regulatory volume decrease in jejunal villus cells. *Am. J. Physiol.* 260 (*Gastrointest. Liver Physiol.* 23): G405–G415, 1991.
19. **Moran, J., and A. J. Patel.** Stimulation of the *N*-methyl-D-aspartate receptor promotes the biochemical differentiation of cerebellar granule neurons and not astrocytes. *Brain Res.* 486: 15–25, 1989.
20. **Nowak, L., P. Ascher, and Y. Berwald-Netter.** Ionic channels in mouse astrocytes in culture. *J. Neurosci.* 7: 101–109, 1987.
21. **Pasantes-Morales, H., and M. Martin del Rio.** Taurine and mechanisms of cell volume regulation. In: *Taurine: Functional Neurochemistry, Physiology, and Cardiology*, edited by H. Pasantes-Morales, D. L. Martin, W. Shain, and R. Martin del Rio. New York: Wiley, 1990, p. 317–328.

22. **Pasantes-Morales, H., J. Moran, and A. Schousboe.** Volume-sensitive release of taurine from cultured astrocytes: properties and mechanisms. *Glia* 3: 427–432, 1990.
23. **Pasantes-Morales, H., and A. Schousboe.** Volume regulation in astrocytes: a role for taurine as an osmoeffector. *J. Neurosci. Res.* 20: 505–509, 1988.
24. **Roy, G., and R. Sauve.** Effect of anisotonic media on volume, ion and amino-acid content and membrane potential of kidney cells (MDCK) in culture. *J. Membr. Biol.* 100: 83–96, 1987.
25. **Sachs, F.** Mechanical transduction by membrane ion channels: a mini review. *Mol. Cell. Biochem.* 104: 57–60, 1991.
26. **Sackin, H.** A stretch-activated K^+ channel sensitive to cell volume. *Proc. Natl. Acad. Sci. USA* 86: 1731–1735, 1989.
27. **Sanchez-Olea, R., J. Morán, A. Schousboe, and H. Pasantes-Morales.** Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. *Neurosci. Lett.* 130: 233–236, 1991.
28. **Sarkadi, B., and J. C. Parker.** Activation of ion transport pathways by changes in cell volume. *Biochim. Biophys. Acta* 1071: 407–427, 1991.
29. **Stoddard, J. S., G. A. Altenberg, M. L. Ferguson, and L. Reuss.** Furosemide blocks basolateral membrane Cl^- permeability in gallbladder epithelium. *Am. J. Physiol.* 258 (*Cell Physiol.* 27): C1150–C1158, 1990.

TRABAJO 2:

Sánchez-Olea, R., Pasantes-Morales, H., Schousboe, A. 1993. Neurons respond to hyposmotic conditions by an increase in intracellular free calcium. Neurochem. Res. 18:147-152.

Neurochemical Research, Vol. 18, No. 2, 1993, pp. 147-152

Neurons Respond to Hyposmotic Conditions by an Increase in Intracellular Free Calcium

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(Accepted May 20, 1992)

The effect of hyposmotic conditions on the concentration of intracellular free calcium ($[Ca^{2+}]_i$) was studied in cultured cerebellar granule cells and cerebral cortical neurons after loading of the cells with the fluorescent Ca^{2+} chelator Fluo-3. It was found that in both types of neurons exposure to media with a decrease in osmolarity of 20 to 50% of the osmolarity in the isosmotic medium (320 mOsm) led to a dose dependent increase in $[Ca^{2+}]_i$ with a time course showing the highest value at the earliest measured time point, i.e. 40 s after exposure to the hyposmotic media and a subsequent decline towards the basal level during the following 320 s. The response in the cortical neurons was larger than in the granule cells but both types of neurons exhibited a similar increase in $[Ca^{2+}]_i$ after exposure to 50 mM K^+ which was of the same magnitude as the increase in $[Ca^{2+}]_i$ observed in the cortical neurons exposed for 40 s to a medium with a 50% reduction in osmolarity. In both types of neurons the blocker of voltage gated Ca^{2+} channels verapamil had no effect on the hyposmolarity induced increase in $[Ca^{2+}]_i$. On the contrary, this increase in $[Ca^{2+}]_i$ was dependent upon external calcium and could be inhibited partly or completely by the inorganic blockers of Ca^{2+} channels Mg^{2+} and La^{3+}. Dantrolene which prevents release of Ca^{2+} from internal stores had no effect. The results show that exposure of neurons to hyposmotic conditions leading to swelling results in a large increase in free intracellular Ca^{2+} which represents an influx of Ca^{2+} rather than a release of Ca^{2+} from internal, dantrolene sensitive stores.

KEY WORDS: Swelling; neurons; intracellular Ca^{2+} Fluo-3.

INTRODUCTION

Exposure of neurons to depolarizing conditions such as high concentrations of potassium or excitatory amino acids is known to lead to large increases in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) either by an increase of calcium influx or release of calcium from internal stores or both (1-7). Such an increase in intracellular calcium may trigger a large number of events such as transmitter release (8), activation of different sond messenger systems (9), and if the increased level of calcium persists it may even have lethal consequences (10). Exposure of neurons to elevated potassium concentrations is also known to lead to an increase in cell volume (11). It has been suggested that in astrocytes changes in cell volume which activate the efflux of osmotically active compounds (12-15) may be associated with changes in the intracellular calcium concentration (16). It was therefore of interest to investigate whether exposure of neurons to hyposmotic conditions also leading to cell swelling and release of osmotically active

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0364-3190/93/0200-0147$07.00/0

substances (11,17) might influence $[Ca^{2+}]_i$. The effect of hyposmotic conditions on $[Ca^{2+}]_i$ was accordingly studied in cerebral cortical neurons as well as in cerebellar granule cells utilizing the fluorescent Ca^{2+} chelator Fluo-3 (3,18). In order to obtain information about possible mechanisms by which changes in cell volume may affect $[Ca^{2+}]_i$, the effects of blockers of calcium channels such as verapamil, La^{3+} and Mg^{2+} (19-21) as well as dantrolene which prevents release of calcium from internal stores (5,6,22,23) were investigated.

EXPERIMENTAL PROCEDURE

Materials. Pregnant mice (15 gestational day) were obtained from the animal quarters at the Panum Institute, University of Copenhagen (Denmark). Plastic tissue culture dishes were purchased from NUNC A/S (Denmark) and foetal calf serum from Sera-Lab Ltd. (Sussex, UK). Cytosine arabinoside, trypsin, soybean trypsin inhibitor, DNAse, amino acids, dantrolene and vitamins were obtained from Sigma Chemical Company (St. Louis, Mo.). the acetoxymethyl ester of Fluo-3 (Fluo-3/AM) and Pluronic F-127 were purchased from Molecular Probes Inc. (Eugene, Or.). All other chemicals were of the highest purity available from regular commercial sources.

Cell Culture. Cerebral cortical neurons and cerebellar granule cells were cultured essentially as described previously (24-26). The cells were seeded at a density of 3×10^6 cells/ml in NUNCLON 96-well microtiter plates (100 µl per well). Astrocytic proliferation was curtailed by the addition of 20 µM of the mitotic inhibitor cytosine arabinoside after 48 h of culture and onwards (25,26). The neurons were routinely cultured for 7-9 days before experiments were performed. For further details regarding the characteristics of these cultures, see Schousboe et al. (27).

Measurements of $[Ca^{2+}]_i$. Fluo-3/AM loading and fluorescence measurements were performed according to the procedure of Wahl et al. (3) and Frandsen and Schousboe (5,6). In order to allow calculations of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), cells were incubated with the ionophore A-23187 (10 µM) in HEPES-buffered saline (135 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 6 mM glucose, pH 7.4) allowing sufficient influx of Ca^{2+} to attain the saturation level of binding with the intracellularly trapped Fluo-3 ligand (F_{max}). Subsequently, the fluorescence was quenched with 2 mM $CuCl_2$ dissolved in 0.9% (w/v) NaCl plus 10 µM A-23187 to obtain the minimum fluorescence signal (F_{min}). Fluorescence was blanked against unloaded cells. The observed relative fluorescence values for the cells were used in the following equation to calculate the free cytosolic Ca^{2+} concentration: $[Ca^{2+}]_i = K_D (F - F_{min})/(F_{max} - F)$, where the K_D is 450 nM and F the observed fluorescence, which increases upon binding of Ca^{2+} without shifts in excitation or emission wavelengths (18). The neurons were exposed to hyposmotic conditions by reducing the NaCl concentration in the HEPES buffered saline, i.e. a reduction to 60 mM corresponds to 50% osmolarity. In some cases $CaCl_2$ was exchanged with $MgCl_2$ or $LaCl_3$ as indicated.

RESULTS

Hyposmolarity Induced Increase in $[Ca^{2+}]_i$. Exposure to a hyposmotic medium (50% reduction in osmolarity) led to a rapid increase in Fluo-3 fluorescence corresponding to an increase in $[Ca^{2+}]_i$ in both cerebellar granule cells and cerebral cortical neurons (Figure 1 A,B). The actual level of fluorescence was higher in cortical neurons than in cerebellar granule cells but the time course of the increase in $[Ca^{2+}]_i$ was similar in the two types of neurons (Figure 1 A,B). After the initial increase in $[Ca^{2+}]_i$ the highest value of which was seen at the earliest measured time point, i.e. after 40 s of exposure to the hyposmotic medium, the fluorescence decreased during the following 320 s approaching a level only somewhat higher than that corresponding to the level in neurons kept under isosmotic conditions which was similar to that at the beginning of the experiment.

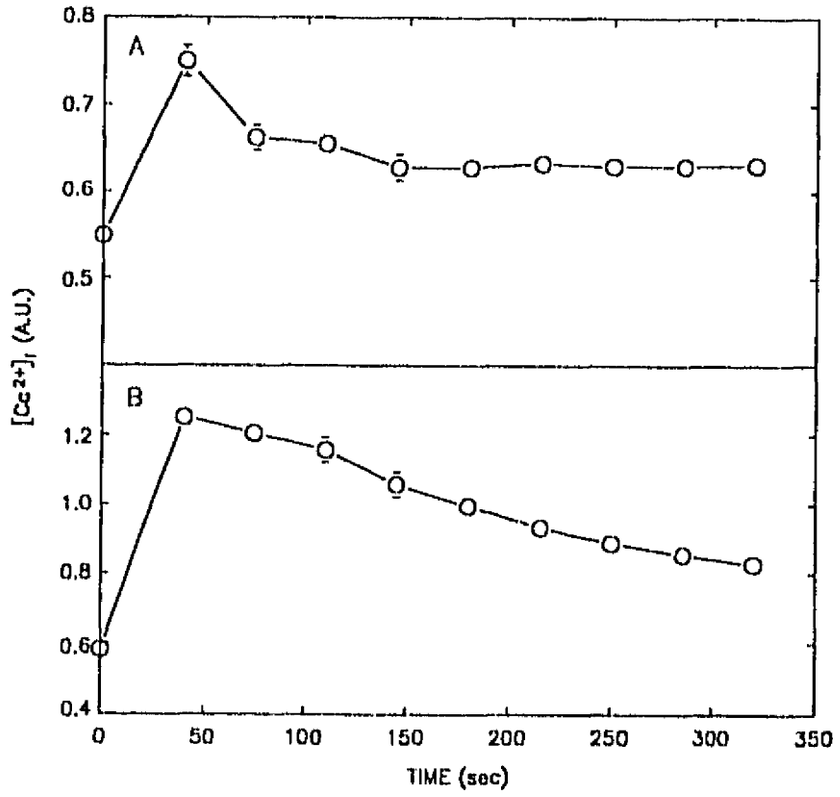

Figure 1. Time course of the increase in $[Ca^{2+}]_i$ expressed in arbitrary fluorescence units (A.U.) following exposure of cerebellar granulle cells (A) or cerebral cortical neurons (B) to a medium with a 50% reduction in osmolarity achieved by reducing the concentration of NaCl from 135 mM to 60 mM. Neuronal cultures were prepared as detailed in Experimental Procedures and grown for 7-9 days before experiments were performed. Prior to the experiment cells were loaded with the fluorescent Ca^{2+} chelator Fluo-3 by incubation of the cultures with the AM-ester of Fluo-3 as detailed in Experimental Procedures. After loading, cultures were washed to remove excess Fluo-3-AM ester and fluorescence was read in a Fluoscan fluorometer to obtain the basal fluorescence level. Subsequently the media were changed to hyposmotic media and fluorescence read as indicated. At the end of the experiment values for F_{min} and F_{max} fluorescence were determined as described in Experimental Procedures. Based on these values the basal fluorescence in granule cells was calculated to correspond to a $[Ca^{2+}]_i$ of 146 nM and that in cortical neurons to 107 nM. Results are averages of 8 experiments (except for the basal values where n = 368 (granule cells) and 279 (cortical neurons)) and SEM values are shown by vertical bars if they extend beyond the symbols. All fluorescence values measured after exposure of the neurons to hyposmotic media were statistically different from the basal level of fluorescence ($P < 0.001$; Student's *t* test).

ginning of the experiment. Since the highest increase in fluorescence was observed after 40 s exposure to hyposmotic conditions, all subsequent experiments were conducted using this exposure period. Figure 2 A,B. shows that in both types of neurons the increase in $[Ca^{2+}]_i$ observed after exposing the cells for 40 s to hyposmotic media increased with decreasing osmolarity. It is seen that a 20% reduction in osmolarity led to a significant increase in $[Ca^{2+}]_i$ in the granule cells but the corresponding increase observed in the cortical neurons did not reach the statistically significant level (Figure 2B). However, at 70% osmolarity the increase in $[Ca^{2+}]_i$ was highly significant in both types of neurons. A reduction in the osmolarity of only 10% did not change the fluorescence signal significantly in any of the neurons (results not shown). For the sake of comparison increases in fluorescence for the two types of neurons after exposure to 50 mM K^+ are also shown in Figure 2 A,B. It is seen that in case of the cerebellar granule cells (Figure 2A) the response to 50 K^+ was much larger than that seen during exposure to a medium with a 50% reduction in osmolarity whereas for the cerebral cortical neurons the fluorescence signal under these two conditions was almost the same (Figure 2B). It can also be seen that exposure to 50 mM K^+ increased the intracellular Ca^{2+} concentration to the same extent in the two types of neurons. Since reductions in osmolarity as well as elevation of the external concentration of K^+ involved reductions in the external concentration of NaCl it was studied if removal of NaCl per se affected the intracellular Ca^{2+} concentration . This was found not to be the case since the fluorescence observed after exposure to an isosmotic medium with 60 mM NaCl (0.638 ± 0.031, n = 8) was similar to that observed in cells exposed to isosmotic media with the normal NaCl concentration (cf. Table I).

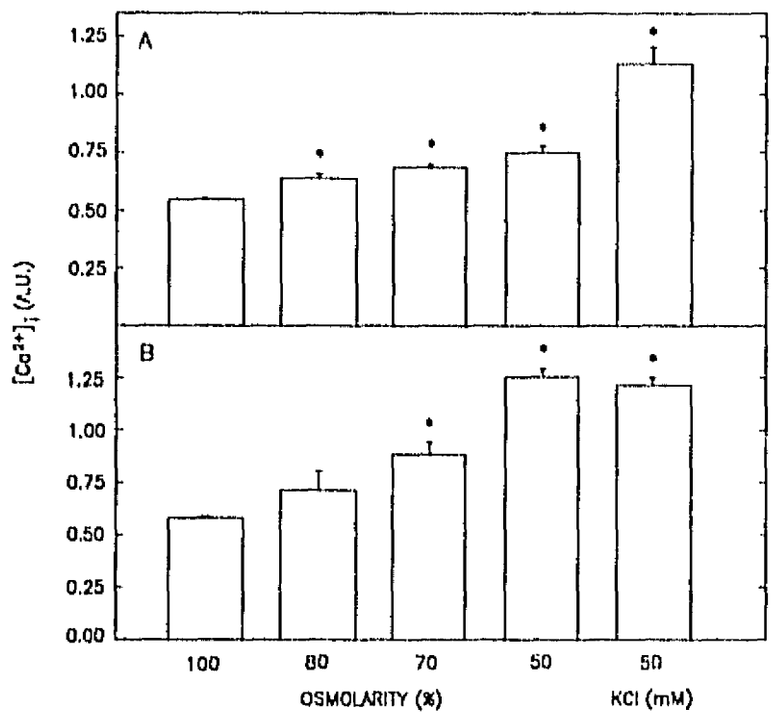

Figure 2. Levels of $[Ca^{2+}]_i$ expressed in A.U. (cf. Fig. 1) in cerebellar granule cells (A) or cerebral cortical neurons (B) after 40 s exposure to media with different reduction in osmolarity achieved by removal of NaCl (cf. Experimental Procedures) or to media in which the KCl concentration was increased to 50 mM with a corresponding reduction in the NaCl concentration to maintain isosmolarity. Preparation of cell cultures, loading of cells with Fluo-3 and fluorescence measurements were performed as described in Fig. 1 and Experimental Procedures. Based on measurements of F_{min} and F_{max}, the values of fluorescence observed after exposure of the neurons to 50 mM K^+ could be converted into values for $[Ca^{2+}]_i$ and they were 544 nM and 556 nM for respectively cerebellar granule cells and cortical neurons. Results are averages of 8-12 experiments and SEM values are shown by vertical bars. In case of the basal levels of fluorescence the numbers of experiments were 368 and 279 for granule cells and cortical neurons, respectively. Statistically significant differences from basal levels (isosmotic media with 5 mM KCl) are indicated by asterisks (* $P < 0.001$, Student's *t*-test).

Effects of Dantrolene and Ca^{2+}-Channel Blockers. The effect on the hyposmolarity induced increase in $[Ca^{2+}]_i$ in the two types of neurons of different compounds or conditions known to affect Ca^{2+} homeostasis was studied. Table I shows that a decrease in the extracellular calcium concentration from 1.0 to 0.1 mM only marginally reduced the increase in fluorescence observed during exposure of the granule cells to hyposmotic conditions (50% osmolarity) whereas in the cortical neurons the corresponding effect was significant. Exposure of the neurons to media nominally free of Ca^{2+} reduced the hyposmolarity induced increase in Fluo-3 fluorescence by 40-70% and if EGTA (200 μM) was added to these media no increase in fluorescence was observed after exposure of the neurons to media with a 50% reduction in osmolarity. An increase in extracellular Ca^{2+} from 1.0 to 2.5 mM had no effect on the ability of hyposmolar media to increase $[Ca^{2+}]_i$. In agreement with this Ca^{2+} dependency of the hyposmolarity induced increase in $[Ca^{2+}]_i$ it was found that Mg^{2+} (2.5 mM) in the absence of Ca^{2+} could block the hyposmolarity induced increase in $[Ca^{2+}]_i$ in both types of neurons (Table II). In agreement with this, La^{3+} inhibited the response in cortical neurons whereas in the granule cells La^{3+} had no effect (Table II). Verapamil had no significant effect on the increase in fluorescence observed after exposure of the cells to media with a 50% reduction in the osmolarity and also dantrolene was found not to have any effect (Table II).

DISCUSSION

In agreement with previous studies of the ability of elevated K^+ concentration to elevate intracellular Ca^{2+} in cortical neurons and cerebellar granule cells (3,5) it

Table I. Effect of External Ca^{2+} on the Hyposmolarity Induced Increase in $[Ca^{2+}]_i$ in Cerebellar Granule Cells and Cerebral Cortical Neurons

Exp. Condition Ext. Ca^{2+} (mM)		$[Ca^{2+}]_i$ (A. U.)			
Isosm.	Hyposm.	Granule cells		Cortical neurons	
1.0	-	0.549 ± 0.004	(368)	0.583 ± 0.007	(279)
-	2.5	0.763 ± 0.038	(8)	1.355 ± 0.038	(8)
-	1.0	0.748 ± 0.029	(8)	1.253 ± 0.040	(8)
-	0.1	0.686 ± 0.029	(8)	1.037 ± 0.042*	(8)
-	0	0.610 ± 0.010**	(8)	0.971 ± 0.017**	(8)
-	0+EGTA (200μM)	0.515 ± 0.015**	(8)	0.612 ± 0.021**	(24)

Cerebellar granule cells and cerebral cortical neurons were cultured and loaded with Fluo-3 as specified in Experimental Procedures. Subsequently, the fluorescence was determined and the measurements repeated after 40 s exposure to hyposmotic media (50% osmolarity) containing different concentrations of Ca^{2+}. Control experiments in isosmotic media showed that the changes in external Ca^{2+} did not per se affect the fluorescence. Values for $[Ca^{2+}]_i$ are expressed in arbitrary fluorescence units (A.U.). For conversion of these values into nM concentrations for the two cell types incubated in isosmotic media with 1.0 mM Ca^{2+}, see the legend to Fig. 1. Values are averages ± SEM of the number of experiments shown in parentheses. Asterisks indicate statistically significant differences from the fluorescence values in the two types of neurons observed after exposure to a hyposmotic medium containing 1.0 mM Ca^{2+} (*$P < 0.01$; **$P < 0.001$; Student's *t*-test).

Table II. Effects of Organic and Inorganic Ca^{2+} Channel Blockers and Dantrolene on the Hyposmolarity Induced Increase in $[Ca^{2+}]_i$ in Cerebellar Granule Cells and Cerebral Cortical Neurons

Exp. Condition	$[Ca^{2+}]_i$ (A. U.)			
	Granule cells		Cortical neurons	
Isosmotic media	0.549 ± 0.004	(368)	0.583 ± 0.007	(279)
Hyposmotic media	0.748 ± 0.029	(8)	1.253 ± 0.040	(8)
Hyp. + Dantrolene (30μM)	0.735 ± 0.009	(8)	1.099 ± 0.089	(8)
Hyp. + Verapamil (30μM)	0.712 ± 0.015	(8)	1.138 ± 0.037	(8)
Hyp. + La^{3+} (100μM)	0.713 ± 0.027	(8)	1.060 ± 0.029*	(8)
Hyp. + Mg^{2+} (2.5 mM)	0.538 ± 0.015**	(8)	0.711 ± 0.001**	(88)

Cerebellar granule cells and cerebral cortical neurons were cultured and loaded with Fluo-3 as specified in Experimental Procedures. Subsequently, the fluorescence was determined and the measurements repeated after 40 s exposure to hyposmotic media (50% osmolarity) without additions or containing dantrolene, verapamil, La^{3+} or Mg^{2+} as indicated. The Mg^{2+} containing medium was Ca^{2+} free. Values for $[Ca^{2+}]_i$ are expressed in arbitrary fluorescence units (A.U.). For conversion of these values into nM concentrations for the two cell types incubated in isosmotic media, see the legend to Fig. 1. Values are averages ± SEM of the number of experiments shown in parentheses. Asterisks indicate statistically significant differences from the fluorescence observed in the two types of neurons after exposure to the hyposmotic medium without additions (*$P < 0.01$; **$P < 0.001$; Student's *t*-test).

was found in the present study that 50 mM K^+ elicited a pronounced increase in Fluo-3 fluorescence corresponding to a large increase in $[Ca^{2+}]_i$ in both types of neurons. In the cerebral cortical neurons the fluorescence signal seen after exposure to a hyposmotic medium was of the same magnitude as that seen after exposure to 50 mM K^+ whereas in the granule cells the hyposmolarity induced increase in $[Ca^{2+}]_i$ was much smaller than that mediated by 50 mM K^+. The finding that both types of neurons responded to the same extent to K^+ shows that the difference regarding the response to hyposmolar conditions cannot be explained on the basis that the neurons differ with regard to the ability to buffer similar Ca^{2+} overloads. The difference in the magnitude of the response to hyposmolarity is difficult to explain at present since the basic properties of the swelling associated in-

crease in $[Ca^{2+}]_i$ appeared to be the same in the two types of neurons. The time courses of the increase in $[Ca^{2+}]_i$ were identical in the two types of neurons. The very rapid increase in $[Ca^{2+}]_i$ followed by a gradual decrease following exposure to hyposmotic conditions may suggest either that the cells have the ability to buffer long lasting changes in internal $[Ca^{2+}]_i$ or that exposure to the hyposmotic condition leads only to a transient increase in $[Ca^{2+}]_i$. The explanation based on the ability to buffer internal Ca^{2+} can probably be discarded on the basis that these neurons are known to be essentially unable to buffer a Ca^{2+} overload induced by glutamate (2,3). The transient increase in $[Ca^{2+}]_i$ induced by continuous exposure of these neurons to 50 mM K^+ is presumably due to the involvement of voltage gated Ca^{2+}-channels which rapidly inactivate (2,21). This might indicate that Ca^{2+} channels could also be involved in the swelling induced increase in $[Ca^{2+}]_i$. However, the finding that verapamil which is a blocker of such channels (20) and inhibits the K^+ stimulated increase in $[Ca^{2+}]_i$ in these neurons (2) had no effect on the hyposmolarity induced increase in $[Ca^{2+}]_i$ strongly argues against the involvement of similar channels in this process. The findings that the hyposmolarity induced increase in $[Ca^{2+}]_i$ was dependent on external Ca^{2+} and could be completely inhibited by the Ca^{2+} channel blocker Mg^{2+} (cf. (19)) may, however, indicate that some type of Ca^{2+} channels may be involved. The exact nature of such channels remains, however, to be elucidated. This uncertainty regarding the nature of the Ca^{2+} channel is underlined by the surprising observation that the other inorganic Ca^{2+} channel blocker La^{3+} (cf. (19)) had no effect in the granule cells and only marginally inhibited the hyposmolarity induced increase in $[Ca^{2+}]_i$ in the cortical neurons. Another mechanism responsible for the increase in $[Ca^{2+}]_i$ could conceivably involve release of Ca^{2+} from internal stores. It has recently been shown that increases in $[Ca^{2+}]_i$ in these neurons elicited by excitatory amino acids, which are known to affect intracellular Ca^{2+} stores (cf. (7)), can be prevented by dantrolene (2,5,6). Dantrolene is known to decrease mobilization of intracellular calcium stores in several cell types (22,23) but the exact nature of the Ca^{2+} store affected by this drug in neurons is not known. The finding in the present study that dantrolene had no effect on the hyposmolarity-induced increase in $[Ca^{2+}]_i$, however, strongly indicates that the increase in $[Ca^{2+}]_i$ is not related to release of Ca^{2+} from internal stores.

It has been previously shown that swelling induced by hyposmotic conditions or elevated concentrations of potassium in astrocytes and neurons leads to a large increase in efflux of taurine and certain other amino acids such as glycine and glutamate (11-15,17,28). This taurine release is in most preparations independent of external Ca^{2+} (cf. (15,17)). Since the increase in $[Ca^{2+}]_i$ was found to be dependent upon external Ca^{2+} it is presently difficult to reach any conclusion as to whether the swelling induced increases in $[Ca^{2+}]_i$ might be functionally related to the swelling induced release of taurine observed in these neurons (11,17). It should, however, be pointed out that the swelling induced efflux of glutamate and GABA could possibly represent neurotransmitter release from vesicular pools in respectively the cerebellar granule cells and the cortical neurons (29) as a result of the increase in $[Ca^{2+}]_i$. Further experimentation is needed in order to obtain a better understanding of the possible functional significance of the swelling associated increase in intracellular calcium in neurons.

ACKNOWLEDGMENTS

The technical assistance of Ms. Charlotte F. Andersen and Inge Damgaard is gratefully acknowledged. The work has been supported financially by grants from the Danish State Biotechnology Program (1991-95), the Lundbeck and Dir. Ib Henriksen Foundations, Mrs. Jenny Vissing, DGAPA (024589) and PADEP (DCH 9163) UNAM.

REFERENCES

1. Kudo, Y., and Ogura, A. 1986. Glutamate-induced increase in intracellular Ca^{2+} concentration in isolated hippocampal neurones. Br. J. Pharmacol. 89:191–198.
2. Bouchelouche, P., Belhage, B., Frandsen, A., Drejer, J., and Schousboe, A. 1989. Glutamate receptor activation in cultured cerebellar granule cells increases cytosolic free Ca^{2+} by mobilization of cellular Ca^{2+} and activation of Ca^{2+} influx. Exp. Brain Res. 76:281–291.
3. Wahl, P., Schousboe, A., Honoré, T., and Drejer, J. 1989. Glutamate-induced increase in intracellular Ca^{2+} in cerebral cortex neurons in transient in immature cells but permanent in mature cells. J. Neurochem. 53:1316–1319.
4. Perney, T. M., Dinerstein, R. J., and Miller, R. J. 1984. Depolarization-induced increases in intracellular free calcium detected in single cultured neuronal cells. Neurosci. Lett. 51:165–170.
5. Frandsen, Aa., and Schousboe, A. 1991. Dantrolene prevents glutamate cytotoxicity and Ca^{2+} release from intracellular stores in cultured cerebral cortical neurons. J. Neurochem. 56:1075–1078.
6. Frandsen, Aa., and Schousboe, A. 1992. Mobilization of dantrolene-sensitive intracellular calcium pools is involved in the cytotoxicity induced by quisqualate and *N*-methyl-D-aspartate but not by 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate and kainate in cultured cerebral cortical neurons. Proc. Natl. Acad. Sci. USA 89:2590–2594.
7. Schousboe, A., Frandsen, Aa., Wahl, P., and Krogsgaard-Larsen, P. 1991. Excitatory amino acid induced cytotoxicity in cultured neurons: role of intracellular Ca^{2+} homeostasis. Pages 137–152. *in* Ascher, P., Choi, D., and Christen, Y. (eds.), Glutamate, Cell Death and Memory, Springer-Verlag, Berlin.

8. Cohen, I., and Van der Kloot, W. 1982. Calcium and transmitter release. Int. Rev. Neurobiol. 27:299–336.
9. Berridge, M. J. 1987. Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann. Rev. Biochem. 56:159–193.
10. Siesjö, B. K., Bengtsson, F., Grampp, W., and Theander, S. 1989. Calcium, excitotoxins, and neuronal death in the brain. Ann. N.Y. Acad. Sci. 568:234–251.
11. Schousboe, A., Moran, J., and Pasantes-Morales, H. 1990. Potassium-stimulated release of taurine from cultured cerebellar granule neurons is associated with cell swelling. J. Neurosci. Res. 27:71–77.
12. Pasantes-Morales, H., and Schousboe, A. 1988. Volume regulation in astrocytes: A role for taurine as an osmoeffector. J. Neurosci. Res. 20:505–509.
13. Kimelberg, H. K., Goderie, S. K., Higman, S., Pang, S., and Waniewski, R. A. 1990. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. J. Neurosci. 10:1583–1591.
14. Martin, D. L., Madelian, V., Seligmann, B., and Shain, W. 1990. The role of osmotic pressure and membrane potential in K^+-stimulated taurine release from cultured astrocytes and LRM55 cells. J. Neurosci. 10:571–577.
15. Schousboe, A., and Pasantes-Morales, H. 1992. Role of taurine in neural cell volume regulation. Can. J. Physiol. Pharmacol., in press.
16. Bender, A. S., Neary, J. T., Blicharska, J., Norenberg, L.-O. B., and Norenberg, M. 1991. Role of calmodulin and protein kinase C in astrocytic cell volume regulation. J. Neurochem. 58, 1874–1882.
17. Schousboe, A., Apreza, C. L., and Pasantes-Morales, H. 1992. GABA and taurine serve as respectively a neurotransmitter and an osmolyte in cultured cerebral cortical neurons. Pages 391–392. *in* Lombardini, J. B., Schaffer, S. W., and Azuma, J. (Eds.), Taurine: Nutritional Value and Mechanisms of Action, Plenum Press, New York.
18. Minta, A., Kao, J. P. Y., and Tsien, R. Y. 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein. J. Biol. Chem. 264:8171–8178.
19. Nichols, R. A., and Nakajama, Y. 1975. Effects of manganese and cobalt on the inhibitory synapse of the crustacean strech receptor neurons. Brain Res. 89:493–498.
20. Miller, R. J. 1987. Multiple calcium channels and neuronal function. Science 235:46–52.
21. Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R., and Fox, A. P. 1988. Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci. 11:431–438.
22. Kojima, I., Kojima, K., Kreutter, D., and Rasmussen, H. 1984. The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. J. Biol. Chem. 259:14448–14457.
23. Ward, A., Chaffman, M. O., and Sorkin, E. M. 1986. Dantrolene: a review of its pharmacological and therapeutic use in malignant hyperthermia, the neuroleptic malignant syndrome and an update of its use in muscle spasticity. Drugs 32:130–168.
24. Frandsen, Aa., and Schousboe, A. 1990. Development of excitatory amino acid induced cytotoxicity in cultured neurones. Int. J. Devl. Neurosci. 8:209–216.
25. Hertz, E., Yu, A. C. H., Hertz, L., Juurlink, B. H. J., and Schousboe, A. 1989. Preparation of primary cultures of mouse cortical neurons. Pages 183–196. *in* Shahar, A., De Vellis, J., Vernadakis, A., and Haber, B. (eds.), A Dissection and Tissue Culture Manual of the Nervous System, Alan R. Liss, Inc., New York.
26. Schousboe, A., Meier, E., Drejer, J., and Hertz, L. Preparation of primary cultures of mouse (rat) cerebellar granule cells. Pages 203–206. *in* Shahar, A., De Vellis, J., Vernadakis, A., and Haber, B. (eds.), A Dissection and Tissue Culture Manual of the Nervous System, Alan R. Liss, Inc., New York.
27. Schousboe, A., Drejer, J., Hansen, G. H., and Meier, E. 1985. Cultured neurons as model systems for biochemical and pharmacological studies on receptors for neurotransmitter amino acids. Devl. Neurosci. 7:252–262.
28. Pasantes-Morales, H., and Schousboe, A. 1989. Release of taurine form astrocytes during potassium-evoked swelling. Glia 2:45–50.
29. Ehrhart-Bornstein, M., Treiman, M., Hansen, G. H., Schousboe, A., Thorn, N. A., and Frandsen, Aa. 1991. Parallel expression of synaptophysin and evoked neurotransmitter release during development of cultured neurons. Int. J. Devl. Neurosci. 9:439–447.

TRABAJO 3:

Schousboe, A., Sánchez-Olea, R., Morán, J., Pasantes-Morales, H. 1991. Hyposmolarity-induced taurine release in cerebellar granule cells is associated with diffusion and not with high-affinity transport. J. Neurosci. Res. 30:661-665.

Journal of Neuroscience Research 30:661–665 (1991)

Hyposmolarity-Induced Taurine Release in Cerebellar Granule Cells Is Associated With Diffusion and Not With High-Affinity Transport

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The effects of hyposmotic conditions on taurine uptake and release were studied in mice cultured cerebellar granule cells. The effect of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) and of the divalent cations Mg^{++} and Mn^{++} on the hyposmolarity-induced changes in these parameters was investigated. Mg^{++} (20 mM) and Mn^{++} (5 mM) inhibited by 25% and 41%, respectively, the release of taurine observed in 30% hyposmolar media. DIDS (100 μM) inhibited this release by 46%. Taurine efflux evoked by 50% hyposmolar solutions was reduced about 40% by Mg^{++} and 55% by Mn^{++}. Taurine uptake into the granule cells could be resolved into a high-affinity carrier-mediated component plus a nonsaturable diffusion component. The kinetic constants (K_m and V_{max}) for the high-affinity uptake were unaffected by a 50% decrease in the osmolarity. The diffusion constant for the nonsaturable taurine uptake was increased from 1.5×10^{-4} in isosmotic media to 4.6×10^{-4} ml × min^{-1} × mg^{-1} in hyposmotic (50%) media. This increase in the diffusional component of taurine uptake elicited by the hyposmotic condition was inhibited approximately 25% by either 100 μM DIDS or 5 mM Mn^{++}. These results strongly suggest that the increase in taurine release induced by swelling under hyposmotic conditions is mediated by a diffusional process and not by a reversal of the high-affinity taurine carrier.

Key words: taurine, swelling, uptake, volume, neurons, DIDS

INTRODUCTION

Several lines of experimental evidence have suggested that taurine may play a role as an osmotically active substance in the brain (Thurston et al., 1980; Van Gelder, 1983, 1989; Walz and Allen, 1987; Pasantes-Morales and Schousboe, 1988, 1989; Solis et al., 1988; Nieminen et al., 1988; Wade et al., 1988; Trachtman et al., 1988; Law, 1989; Pasantes-Morales et al., 1990a,b; Schousboe et al., 1990; Martin et al., 1990; Kimelberg et al., 1990). Conditions leading to swelling of neurons and astrocytes elicit a prominent release of taurine that, under extreme conditions, may account for a loss of up to 80% of the cellular taurine pool (Pasantes-Morales and Schousboe 1988, 1989; Schousboe et al., 1990). Release of other amino acids also occurs but to a much lesser extent than that of taurine (Pasantes-Morales and Schousboe, 1988; Schousboe et al., 1990, Kimelberg et al., 1990) suggesting that the taurine pool is more accessible to this release process.

Previous observations (Pasantes-Morales et al., (1990b) on the sodium and temperature independence of the swelling-evoked release of taurine suggest that the high-affinity taurine carrier may not be directly involved in the release process and that it can be alternatively accounted for by diffusional fluxes that are also present in these cells (Schousboe et al., 1976; Larsson et al., 1986; Holopainen et al., 1987). In order to obtain more direct information concerning the respective roles of the taurine carrier and the diffusion processes in the swelling-associated taurine efflux, the uptake of taurine was examined now in cerebellar granule cells under isosmotic and hyposmotic conditions. Since it has been shown that DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) is an inhibitor of swelling induced taurine release (Pasantes-Morales and Schousboe, 1989; Pasantes-Morales et al., 1990b; Schousboe et al., 1990; Kimelberg et al., 1990) the effect of this compound on taurine uptake and release under iso- and hyposmotic conditions was also studied. In addition, the effects of Mn^{++} and Mg^{++} were studied, since these divalent cations appear to have some inhibitory effects on taurine release in neurons and

Received January 29, 1991; revised April 22, 1991; accepted April 23, 1991.

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astrocytes presumably associated with swelling (Philibert et al., 1988; Schousboe and Pasantes-Morales, 1989).

MATERIALS AND METHODS

Materials

Seven-day-old mice were obtained from the animal quarters at the Panum Institute or the National University of Mexico. Plastic tissue culture multiwell plates were purchased from NUNC A/S (Denmark) or Costar (Mexico) and fetal calf serum was from Seralab Ltd. (Sussex, UK) or from Microlab (Mexico). Polylysine (mol. wt. > 300,000), trypsin, trypsin inhibitor, DNase, and amino acids were obtained from Sigma Chemical Comp. (St. Louis, MO.), insulin from NOVO-Nordisk (Denmark), and penicillin from Leo (Denmark). [^{3}H]Taurine (specific radioactivity 21 Ci/mmol) was purchased from du-Pont–New England Nuclear (Boston). All other chemicals were of the purest grade available from regular commercial sources.

Cell Cultures

Granule neurons were cultured from cerebellae of 7-day-old mice in 24-well multitest dishes essentially as described by Schousboe et al. (1989) and Drejer and Schousboe (1989). Cultures grown under these conditions are devoid of other types of neurons than the granule cells characterized by an intense stimulus-coupled glutamate release (Drejer and Schousboe, 1989; Schousboe and Pasantes-Morales, 1989) and by ultrastructural features of cerebellar granule neurons (Hansen et al., 1984; Schousboe et al., 1989). Cells were routinely cultured for 7–10 days, at which time they have properties similar to those of mature granule neurons (Schousboe and Hertz, 1987).

Uptake of Taurine

Kinetics of taurine uptake were studied essentially as described by Schousboe et al. (1976) and Larsson et al. (1986). Cells in the 24-well multitest dishes were incubated in isotonic or hypotonic (50% osmolarity) media containing 100 mM sucrose, 55 mM NaCl, 5 mM KCl, 0.5 $MgCl_2$, 1.0 mM $CaCl_2$, 6.0 mM glucose, 10 mM Tris–HCl, pH. 7.0 (isotonic), or the same composition without sucrose (hypotonic). Where indicated the media contained either DIDS (100 μM) or $MnSO_4$ (5 mM). In addition, the media contained taurine at different concentrations in the range 5–1,000 μM (8–10 different concentrations). After 5-min preincubation (37°C) to permit swelling (hypotonic conditions) the media were exchanged with corresponding media containing in addition [^{3}H]taurine (2–4 μCi/ml), and the incubation was continued for 1–2 min. Uptake was terminated by rapid wash of the cultures in phosphate-buffered saline (PBS) (Larsson et al., 1986) and radioactivity and protein (Lowry et al., 1951) in the cultures were subsequently determined after solubilization in 1.0 M KOH (Schousboe et al., 1976). In all experiments, radioactivity present in cultures briefly exposed (1–2 sec) to [^{3}H]taurine at 0°C was subtracted from the experimental values obtained at 37°C. The carrier mediated and diffusion related parts of the taurine uptake were determined as described previously (Schousboe et al., 1976; Larsson et al., 1986).

Effects of DIDS (100 μM) or Mn^{++} (5 mM) on the diffusion-related taurine uptake were determined in separate experiments performed in a similar manner at 500 and 1,000 μM taurine from which the diffusion component of the uptake could be calculated. It was verified in another set of experiments that these compounds had no effect on the high-affinity carrier-mediated taurine uptake.

Taurine Release

For release experiments, cells were incubated with [^{3}H]taurine (5 μM, 0.5 μCi/ml) for 30 min. After incubation, the culture medium was replaced with Krebs–Hepes medium containing (in mM) NaCl 130, KCl 5, $CaCl_2$ 1.0, $MgCl_2$ 0.5, Hepes 10, glucose 5, pH 7.4. After three successive rapid washes, cells were further washed for 10 min with the isosmotic medium or with media of different osmolarities brought about by reductions in the concentration of NaCl. For experiments on the effect of DIDS, the drug was added at the end of the loading period for 15 min and was present during all incubation periods, including the washing period. At the end of the experiments radioactivity in media and that remaining in the cells was measured by scintillation spectrometry. Results are expressed as fractional release, i.e. the radioactivity present in the medium at each incubation period as percent of total radioactivity initially present in the cells at the start of the incubation periods, i.e., after the washing period. The statistical significance of the effect of inhibitors was determined by one-way analysis of variance (ANOVA) followed by a Fisher's test where statistical significance was set at $P < 0.005$.

RESULTS

The effects of DIDS, Mg^{++}, and Mn^{++} on the hyposmolarity-evoked release of [^{3}H]-taurine are shown in Figure 1. It is seen that the release evoked by a 30% decrease in osmolarity was inhibited by 36% in the presence of 100 μM DIDS. A similar reduction was seen in the presence of Mn^{++}, whereas Mg^{++} inhibited to a lesser extent. The two divalent cations inhibited taurine release induced by 50% hyposmolar solutions by about 40%. Whereas DIDS did not affect the release under

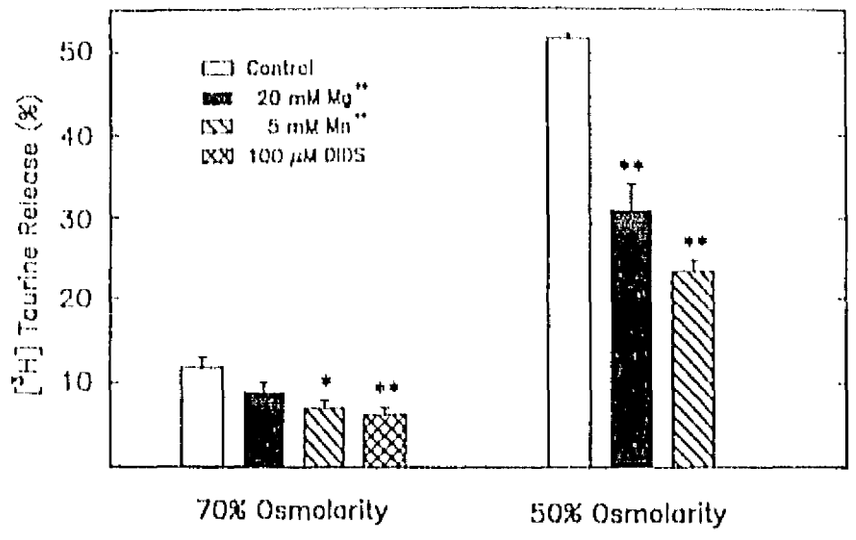

Fig. 1. Effects of DIDS (100 μM) and the divalent cations Mg^{++} (20 mM) and Mn^{++} (5 mM) on taurine release from cerebellar granule cells evoked by a 30 or 50% reduction in osmolarity. Cells were preloaded with [^{3}H]taurine (5 μM, 0.5 μCi/ml) for 30 min; release of [^{3}H]taurine was subsequently determined during 10-min periods in isotonic or hypotonic media containing DIDS or the divalent cations. Taurine release is expressed as fractional release during 10 min, i.e., as the percentage of the accumulated radioactivity released during this period. For further details, see Materials and Methods. Results are averages ±SEM of 3–8 experiments. Data were analyzed by 1-way analysis of variance (ANOVA) followed by Fisher's test. Asterisks indicate significant differences from the corresponding controls (* $P < 0.005$; ** $P < 0.001$).

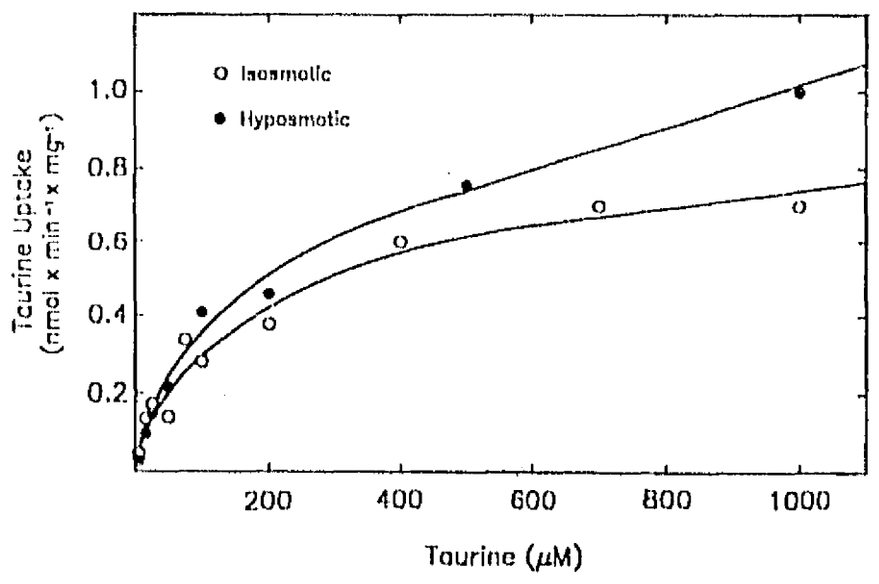

Fig. 2. Taurine uptake into cerebellar granule cells incubated in isosmotic or hyposmotic (50%) media. Cells were incubated for 5 min in the experimental solutions containing different concentrations of taurine and subsequently for 1–2 min in analogous solutions containing [^{3}H]taurine (2–4 μCi/ml). For further details, see Materials and Methods. The curves were fitted to the experimental points as described by Schousboe et al. (1976) and Larsson et al. (1986). Results are averages of 6–16 experiments with SEM less than 10%.

isosmotic conditions, this release was somewhat enhanced by the divalent cations (results not shown).

The kinetics of [^{3}H]taurine uptake into granule neurons in isotonic and hypotonic (50% osmolarity) media are shown in Figure 2. It is seen that exposure to hypotonic conditions leading to cell swelling resulted in an increase in the uptake of taurine accounted for by diffusion, whereas the saturable component of the uptake was unaffected by the osmolarity of the incubation media. This is further illustrated (Table I) by the K_m, V_{max}, and the diffusion constants (K_{diff}) derived from the graphs in Figure 2. The effects of DIDS and Mn^{++} on the diffusional component of [^{3}H]taurine uptake are shown in Table II. It is seen that both DIDS and Mn^{+} inhibited the diffusion-related component of taurine uptake. On the other hand, these compounds were found to have no effect on the saturable component of the uptake (results not shown).

DISCUSSION

Under isosmotic conditions, taurine uptake into granule neurons could be accounted for by a saturable component plus a diffusion-related nonsaturable component as previously described for taurine uptake into different preparations of cultured astrocytes and neurons (Schousboe et al., 1976; Larsson et al., 1986; Holopainen et al., 1987; Abraham and Schousboe, 1989). The values for K_m, V_{max}, and K_{diff} were also comparable to previously published values (cf. above). To evaluate the effect of hyposmolarity on taurine uptake, an experimental design was used in this work, by which the transmembrane fluxes of taurine in hyposmotic conditions were equilibrated by preincubation with unlabeled taurine during the initial period of cell swelling and the concomitant massive release of intracellular taurine. By the end of this period, while the intracellular volume is still high and the cell membrane remains distended, cells were exposed for a short time to [^{3}H]taurine for tracing changes in taurine uptake caused by swelling. It is nevertheless possible that the effects of hyposmolarity on taurine uptake are indeed stronger than those observed under these conditions.

The finding that the kinetic characteristics (K_m and V_{max}) of the saturable component of the uptake were unaffected by exposure of the cells to hyposmotic conditions leading to pronounced swelling (Pasantes-Morales and Schousboe, 1988; Schousboe et al., 1990) strongly suggests that the swelling induced release of taurine is not mediated by a reversal of the high-affinity carrier. This conclusion is consistent with the observation in astrocytes that swelling induced release of taurine is independent of the presence of Na^+ in the incubation media, whereas the taurine carrier is Na^+ dependent (Schousboe, 1982). Moreover, the previous findings that

TABLE I. Kinetic Constants for Taurine Uptake into Cultured Cerebellar Granule Cells Under Isosmotic and Hyposmotic Conditions*

Experimental conditions	Kinetic constants		
	K_m (μM)	V_{max} (nmol × min^{-1} × mg^{-1})	$K_{diff.}$ (ml × min^{-1} × mg^{-1})
Isosmotic media	111	0.64	1.5×10^{-4}
Hyposmotic media (50%)	87	0.59	4.6×10^{-4}

*Values were calculated from the graphs presented in Figure 2 using the procedures described by Schousboe et al. (1976) and Larsson et al. (1986).

TABLE II. Effects of DIDS (100 μM) and Mn^{++} (5 mM) on the Increase in Diffusional Taurine Uptake in Cultured Cerebellar Granule Cells Evoked by Hyposmotic Conditions†

Experimental conditions	Diffusional taurine uptake (% of control)
Isosmotic medium (control)	100 ± 6
Hyposmotic medium (50%)	307 ± 25
Hyposmotic medium + DIDS (100 μM)	234 ± 19*
Hyposmotic medium + Mn^{++} (5 mM)	224 ± 18*

†Cultured cerebellar granule cells were incubated under the different experimental conditions at 500 μM and 1,000 μM taurine as detailed under Materials and Methods. The diffusion constants were calculated as described in the legend to Table I and results are expressed as percentage of these values. Results are averages ±SEM of 8 experiments.
*Statistically significant differences from the value obtained under hyposmotic conditions ($P < 0.05$).

the release of taurine is insensitive to ouabain and reduced temperature (Pasantes-Morales et al., 1990b), both of which inhibit the taurine carrier (Schousboe et al., 1976; Sieghart and Karobath, 1976) is also compatible with the notion that the release is independent of the carrier.

In contrast to the lack of effect of swelling on the saturable taurine uptake it was observed that the nonsaturable component of taurine uptake was greatly increased by the hyposmolarity-induced swelling. This may suggest that a diffusion-related process is involved in the swelling induced taurine release from the cells. The finding that the swelling induced increase in the nonsaturable component of taurine uptake could be inhibited by DIDS or Mn^{++}, both of which were found to inhibit the swelling induced taurine release also supports the conclusion that taurine release observed after swelling in cerebellar granule cells may be mediated by a diffusion-related process. The swelling associated release of taurine observed in several nervous tissue preparations might also occur through a similar diffusion-related mechanism, since in all cases the release of taurine has been found to be inhibited by DIDS (Pasantes-Morales et al., 1988, 1990b; Dominguez et al., 1989; Pasantes-Morales and Schousboe, 1989; Schousboe et al., 1990; Kimelberg et al., 1990). The mechanism of the inhibitory effect of DIDS is not clearly understood. It might be independent of an action on an anionic site, since other blockers of anion conductances have no effect on taurine release (Pasantes-Morales et al., 1990b). No information is at available regarding the mechanism of the inhibitory effect of Mn^{++} on taurine fluxes under hyposmotic conditions. The fact that release of small molecules during swelling is not strictly limited to taurine but also includes other amino acids (Pasantes-Morales and Schousboe, 1988; Schousboe et al., 1990; Kimelberg et al., 1990) may also be in line with a mechanism involving a diffusion-related process. It should be stressed, however, that taurine release is increased by swelling to a much larger extent than the release of other amino acids. This would suggest that the release process exhibits a certain degree of specificity that could be associated with facilitated diffusion. Alternatively, the taurine pool may be more accessible to diffusion.

A preferential release of taurine in response to changes in cell volume relative to other amino acids may be advantageous for the cell in the light of the fact that taurine in contrast to other amino acids is metabolically inert. The involvement of taurine in cell volume regulation may be of general occurrence in nervous tissue as taurine release associated to cell swelling has been observed in a large variety of nervous tissue preparations in vitro as well as in vivo (cf. Introduction). Moreover, cell volume regulation so far observed only in astrocytes, may also occur in neurons as suggested by a recent report by Babila et al. (1990) showing volume regulation in isolated nerve endings, with properties similar to those found in astrocytes. There is a possibility that the widespread occurrence of taurine in many different populations of nervous tissue cells is related to its function in cell volume regulation.

ACKNOWLEDGMENTS

The technical assistance of Mrs. Inge Damgaard, Susanne Johannessen, Charlotte F. Andersen, and Claudia Rodriguez is highly appreciated. This work was supported by grants to A.S. from the Danish Biotechnology Program (1987–90 and 1991–95), the Danish Medical

Research Council (42-0021), and the Lundbeck Foundation, and to H.P.M. from the Stiftung Volkswagenwerk and DGAPA-UNAM (IN-204589).

REFERENCES

Abraham JH, Schousboe A (1989): Effects of taurine on cell morphology and expression of low-affinity GABA receptors in cultured cerebellar granule cells. Neurochem Res 14:1031–1038.

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

Domínguez L, Montenegro J, and Pasantes-Morales H (1989): A volume-dependent chloride-sensitive component of taurine release stimulated by potassium from retina. J Neurosci Res 22:356–361.

Drejer J, Schousboe A (1989): Selection of a pure cerebellar granule cell culture by kainate treatment. Neurochem Res 14:751–754.

Hansen GH, Meier E, Schousboe A (1984): GABA influences the ultrastructure composition of cerebellar granule cells during development in culture. Int J Dev Neurosci 2:247–257.

Holopainen I, Malminen O, Kontro P (1987): Sodium-dependent high-affinity uptake of taurine in cultured cerebellar granule cells and astrocytes. J Neurosci Res 18:479–483.

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

Larsson OM, Griffiths R, Allan IC, Schousboe A (1986): Mutual inhibition kinetic analysis of γ-aminobutyric acid, taurine and β-alanine high affinity transport into neurons and astrocytes: Evidence for similarity between the taurine and β-alanine carriers in both cell types. J Neurochem 47:426–433.

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

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Martin DL, Madelian V, Seligmann B, Shain W (1990): The role of osmotic pressure and membrane potential in K^+-stimulated taurine release from cultured astrocytes and LRM55 cells. J Neurosci 10:571–577.

Pasantes-Morales H, Schousboe A (1988): Volume regulation in astrocytes: A role for taurine as an osmoeffector. J Neurosci Res 20:505–509.

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

Pasantes-Morales H, Domínguez L, Montenegro J. Morán J. (1988): A chloride-dependent component of the release of labelled GABA and taurine from the chick retina. Brain Res 459:120–130.

Pasantes-Morales H, Morán J, Schousboe A (1990a): Taurine release associated to cell swelling in the nervous system. In Pasantes-Morales H, Shain W, Martin D, Martin del Rio R (eds): "Functional Neurochemistry of Taurine." New York: Alan R. Liss, Inc., pp 355–363.

Pasantes-Morales H, Moran J, Schousboe A (1990b): Volume-sensitive release of taurine from cultured astrocytes: Properties and mechanism. Glia 3:427–432.

Philibert RA, Rogers KL, Allen AJ, Dutton GR (1988): Dose-dependent, K^+-stimulated efflux of endogenous taurine from primary astrocyte cultures is Ca^{2+}-dependent. J Neurochem 51: 122–126.

Schousboe A, Fosmark H, Svenneby G (1976): Taurine uptake in astrocytes cultured from dissociated mouse brain hemispheres. Brain Res 116:158–164.

Schousboe A (1982): Glial marker enzymes. Scand J Immunol 15(Suppl 9):339–356.

Schousboe A, Hertz L (1987): Primary cultures of GABAergic and glutamatergic neurons as model systems to study neurotransmitter functions. II. Developmental aspects. In Vernadakis A, Privat A, Lauder JM, Timiras P, and Giacobini E (eds): "Model Systems of Development and Aging of the Nervous System." Amsterdam: Martinus Nijhoff Publishing, pp 33–42.

Schousboe A, and Pasantes-Morales H (1989): Potassium-stimulated release of [^{3}H]taurine from cultured GABAergic and glutamatergic neurons. J Neurochem 53:1309–1315.

Schousboe A, Meier E, Drejer J, Hertz L (1989): Preparation of cultures of mouse (rat) cerebellar granule cells. In Shahar A, De Vellis J, Vernadakis A, Haber B (eds): "A Dissection and Tissue Culture Manual for the Nervous System." New York: Alan R. Liss, Inc. pp 203–206.

Schousboe A, Morán J, Pasantes-Morales H (1990): Potassium-stimulated release of taurine from cultured cerebellar granule neurons is associated with cell swelling. J Neurosci Res 27:71–77.

Sieghardt W, Karobath M (1976): Uptake of taurine into subcellular fractions of C-6 glioma cells. J Neurochem 26:981–986.

Solis JM, Herranz AS, Herreras O, Lerma J, Martin R (1988): Does taurine act as an osmoregulatory substance in the rat brain? Neurosci Lett 91:53–58.

Thurston JH, Hauhart RE, Direo JA (1980): Taurine: A role in osmotic regulation of mammalian brain and possible clinical significance. Life Sci 26:1561–1568.

Trachtman H, Barbour R, Sturman JA, Finberg L (1988): Taurine and osmoregulation: Taurine is a cerebral osmoprotective molecule in chronic hypernatremic dehydration. Pediatr Res 23:35–39.

Van gelder NM (1983): A central mechanism of action for taurine: Osmoregulation, bivalent cations and excitation threshold. Neurochem Res 8:687–699.

Van Gelder NM (1989): Brain taurine content as a function of cerebral metabolic rate: Osmotic regulation of glucose derived water production. Neurochem Res 14:495–498.

Wade JV, Olson JP, Samson FE, Nelson SR, Pazdernik TL (1988): A possible role for taurine in osmoregulation within the brain. J Neurochem 51:740–745.

Walz W, Allen AF (1987): Evaluation of the osmoregulatory function of taurine in brain cells. Exp Brain Res 68:290–298.

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

Neuroscience Letters, 130 (1991) 233–236

ADONIS 030439409100519T

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Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion

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(Received 20 March 1991; Revised version received 11 June 1991; Accepted 12 June 1991)

Key words: Volume regulation; Astrocyte; Taurine; Diffusion; Osmoregulation

In order to obtain information about the mechanism responsible for swelling associated taurine release in astrocytes, the kinetics of taurine uptake in cultured astrocytes from mouse cerebral cortex was studied under isosmotic and hyposmotic (50% osmolarity) conditions. It was found that the V_{max} for the high affinity component of taurine uptake was unaffected by exposure of the astrocytes to hyposmotic conditions and that the K_m value was somewhat increased. Contrary to V_{max}, the non-saturable component of the uptake was greatly increased (2.5-fold) after exposure of the cells to hyposmotic media leading to cell swelling. In addition to the kinetic characterization of taurine uptake the actual intracellular taurine content after incubation (15 min) in isosmotic or hyposmotic media with different taurine concentrations (0–100 mM) under Na^+-free conditions was determined. At taurine concentrations < 30 mM corresponding to the intracellular content in cells not exposed to taurine, exposure to hyposmotic media led to a decrease in the intracellular taurine content. At higher external taurine concentrations (> 30 mM) the intracellular taurine contents were dramatically increased after exposure to hyposmotic conditions. The increase in intracellular taurine seen under hyposmotic conditions at 100 mM external taurine could be significantly reduced by 100 μM DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate). Altogether these results suggest that a diffusional process rather than the high affinity taurine carrier is involved in the swelling induced increase in astrocytic taurine influx and efflux.

It is known that astrocytes possess a volume regulatory mechanism which allows the cells to re-establish the original volume during exposure to conditions leading to cell swelling [4]. It has recently been shown that taurine release is greatly increased by such conditions with a time course superimposable on that of the volume regulatory process [5, 6, 8, 9, 11]. The mechanism of this release of taurine has not been clarified but it is likely that it might involve either a reversal of the high affinity taurine carrier or a diffusional process. The observation that taurine release is sodium independent and temperature and ouabain insensitive [11] does not support the former mechanism but rather suggests that it occurs through diffusion. In order to provide direct support for this possibility, the effect of hyposmolarity on the kinetics of taurine uptake was examined. Such studies allow the distinction between the carrier mediated and the diffusion related influx [15]. In addition, the effect of swelling on the intracellular content of taurine as a function of the extracellular taurine concentration was investigated under sodium-free conditions in order to demonstrate that the volume activated fluxes are directed by the transmembrane concentration gradient. So far, the only agent consistently exerting a significant inhibition of the swelling-evoked release of taurine is DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) [1, 5, 9–11, 16]. Therefore the effect of this compound on the hyposmolarity induced changes in the intra/extracellular distribution ratio of taurine was also studied.

Astrocytes dissociated from cerebral cortex of newborn mice were cultured in 24-well multitest dishes (Costar, Mexico) as detailed by Hertz et al. [3]. Kinetics of taurine uptake was studied essentially as described by Schousboe et al. [15]. Cells were incubated in isotonic or hypotonic (50% osmolarity) media containing (in mM): sucrose 100, NaCl 55, KCl 5, $MgCl_2$ 0.5, $CaCl_2$ 1, glucose 6.0, Tris-HCl 10, pH 7.3 (isotonic) or the same composition without sucrose (hypotonic). In addition, the media contained taurine at different concentrations (5–1000 μM, 8 different concentrations). After 5 min preincubation (37°C) to allow swelling (hypotonic conditions) the media were exchanged with corresponding media containing in addition [^{3}H]taurine (2–4 μCi/ml) and the

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incubation was continued for 1–2 min. Uptake was terminated and radioactivity and protein determined as previously described [15]. For experiments concerning the effect of hyposmolarity on intracellular taurine contents as a function of the extracellular taurine concentration, cultured cells were incubated in a medium with a composition similar to that used for uptake experiments except that NaCl and sucrose were replaced by choline chloride. In addition, the media contained increasing concentrations of taurine (1–100 mM) with the osmolarity maintained by equiosmolar reductions in choline chloride. Cells were incubated for 15 min at 37°C under the different conditions and subsequently washed and extracted with 70% (v/v) ethanol for determination of free amino acids by HPLC [2]. In order to avoid inaccuracies in the measurement of taurine content due to the very high concentrations of taurine in the media used in these experiments, cells were briefly exposed (1–2 s) to media containing taurine, then washed and values of taurine found under these conditions were subtracted from those measured in incubated cells. Significant amounts of taurine were found only in cells briefly exposed to media containing taurine concentrations higher than 15 mM. In experiments with DIDS, cells were preincubated for 20 min in culture media containing 100 μM DIDS and subsequently in the above mentioned media containing the same concentration of DIDS.

The kinetics of taurine uptake into astrocytes incubated in isotonic or hypotonic (50%) media (Fig. 1) clearly show that the uptake could be separated into a high affinity, carrier mediated component and a non-saturable, diffusional component. The characteristics of the uptake were similar to those previously reported in different preparations of astrocytes [13]. While the exposure to hyposmotic conditions did not affect the V_{max} (0.17 nmol × min^{-1} × mg^{-1}) of the saturable component and only marginally increased the K_m (from 38 to 71 μM), the diffusion constant for taurine uptake was increased about 3-fold after incubation in hyposmotic media (Fig. 1). The virtual lack of effect of hyposmolarity on the carrier mediated component of the uptake suggests that the taurine carrier does not play a major role in the swelling associated release of taurine. This conclusion is compatible with the previous observations that the release process is sodium independent and ouabain and temperature insensitive [11] since the carrier is sodium dependent and inhibited by ouabain and decreased temperatures [14, 15, 17]. Moreover, it is also in line with the previous finding that no homoexchange of taurine occurs in these cells [12]. The large effect of hyposmolarity on the diffusion component strongly suggests the involvement of a diffusion process in the swelling related taurine efflux.

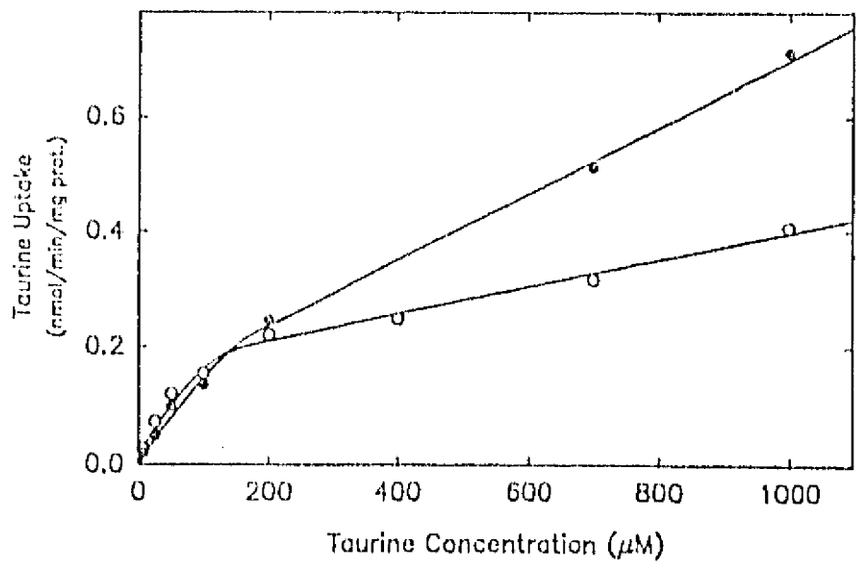

Fig. 1. Taurine uptake (nmol × min^{-1} × mg^{-1}) into cultured astrocytes incubated under isosmotic (○) or hyposmotic (●) conditions in media containing different concentrations of [³H]taurine. Cells were preincubated for 5 min in isosmotic or hyposmotic (50% osmolarity) media containing unlabeled taurine and subsequently for 1–2 min in analogous media containing in addition [³H]taurine (2–4 μCi/ml). Rates of uptake were calculated from the amount of radioactivity in the cells after the incubation and the specific radioactivity of taurine in the incubation media. Curves were fitted to the experimental points using the Enzfitter program from Elsevier/Biosoft assuming a saturable plus a diffusional component of the uptake [15]. Results are averages of 6–12 experiments with S.E.M. values < 10%. Under isosmotic conditions the K_m and V_{max} were respectively 38 μM and 0.189 nmol × min^{-1} × mg^{-1} and the diffusion constant (k) 2.16 × 10^{-4} ml × min^{-1} × mg^{-1} and after exposure to hyposmotic conditions the corresponding values were: K_m, 71 μM; V_{max}, 0.162 nmol × min^{-1} × mg^{-1}; k, 5.5 × 10^{-4} ml × min^{-1} × mg^{-1}.

This notion is further supported by the results of the experiments in which the extra-/intracellular taurine transmembrane gradient was changed while the carrier was inhibited by removal of sodium (Fig. 2). It was found that under isosmotic conditions, the intracellular concentration of taurine was increased subsequent to exposure to increasing external taurine concentrations (Fig. 2). Exposure to hyposmotic conditions at low external taurine concentrations (< 30 mM) resulted in a marked decrease in the intracellular taurine pool, as consistently observed previously [7]. As the extracellular concentration of taurine was increased, the intra-/extracellular ratio was dramatically changed so that at an external concentration of 30 mM no decrease in intracellular taurine was observed under hyposmotic conditions and moreover, at higher extracellular taurine concentrations a net increase in intracellular taurine was seen after exposure to hypotonic media. An intracellular taurine concentration of 30 mM was calculated from a taurine content of 140.7 nmol/mg protein (Fig. 2) and a cell volume under isosmotic conditions of 4.7 μl/mg protein. The volume of cells in hyposmotic media containing 30 mM or 50 mM extracellular taurine was about 7.8 μl/mg protein and the taurine content was 348.8 and 417.9

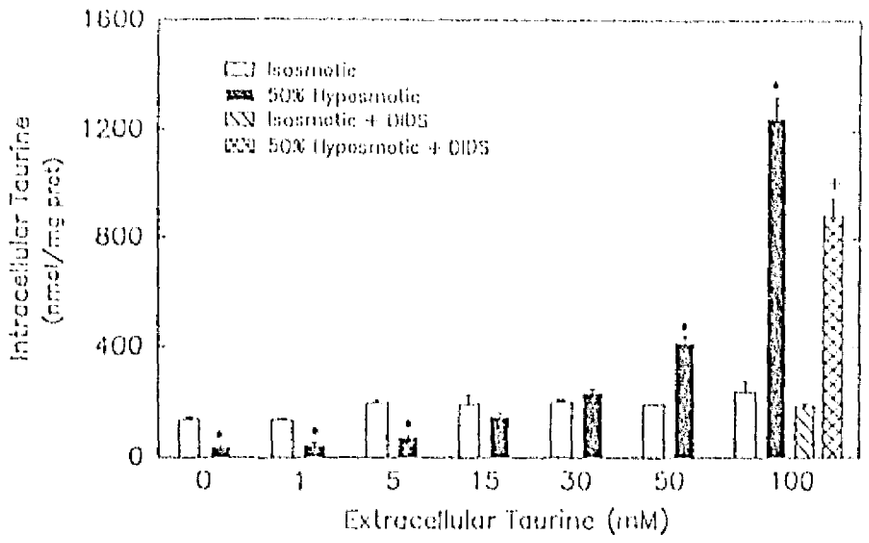

Fig. 2 Intracellular content (nmol/mg) of taurine in cultured astrocytes after incubation in Na^+-free, isosmotic (open columns) or hyposmotic (black columns) media containing increasing taurine concentrations. At 100 mM external taurine the effect of exposure to 100 μM DIDS (hatched column, isosmotic and cross-hatched column, hyposmotic) was also investigated. Cells were incubated for 15 min in the different, Na^+-free media and subsequently the intracellular taurine contents were determined by HPLC analysis as detailed in the text. Results are averages of 12 experiments with SEM values shown as vertical bars. Statistically significant differences between cells incubated in isosmotic and hyposmotic (50% osmolarity) media ($P < 0.001$, Student's t-test) are shown by asterisks and the difference between DIDS treated and untreated cultures by a cross ($P < 0.001$, Student's t-test).

nmol/mg protein, respectively (Fig. 2). Under these conditions, the calculated intracellular concentrations of taurine of 32 mM and 53 mM, respectively, were in equilibrium with the extracellular concentrations of taurine. At 100 mM external taurine, however, a significant deviation was observed since the intracellular taurine content calculated as above was 160 mM, clearly exceeding the extracellular taurine concentration. The reason for this anomalous behavior is unclear at present. Similar results have been reported by Walz and Allen [18] who observed that in cultured astrocytes exposed for 5 h to high extracellular concentrations of taurine under isosmotic conditions, the intracellular concentration may be as high as 120 mM, without noticeable changes in cell volume or in fluxes of other osmolytes. It is suggested that taurine accumulated under these conditons is removed from the osmotically active space, presumably being sequestered into intracellular organelles. This may also be the case at the very high extracellular concentrations of taurine used in our experiment. Although this may be an interesting point to clarify, it is not relevant for the purpose of this study which was to demonstrate that the hyposmolarity activated fluxes of taurine are determined primarily by the transmembrane gradient. An inhibitory effect of DIDS (100 μM) similar to that observed on the hyposmolarity induced release on taurine [5, 11] was found on the hyposmolarity activated fluxes of taurine (Fig. 2). Since in the absence of sodium this accumulation is likely to result from diffusion, the effect of DIDS is presumably exerted on the diffusional component of taurine uptake. Altogether these observations suggest that taurine release associated with swelling occurs mainly by means of diffusional processes.

The expert technical assistance of Ms. Claudia Rodriguez is acknowledged. The work has been supported by grants to H.P.-M. from the Organization of American States (PRDCyt) and to A.S. from the Danish Biotechnology Program (1987–90 and 1991–95) and the Lundbeck Foundation.

1 Dominguez, L., Montenegro, J. and Pasantes-Morales, H., A volume-dependent chloride-sensitive component of taurine release stimulated by potassium from retina, J. Neurosci. Res., 22 (1989) 356–361.
2 Geddes, J.W. and Wood, J.D., Changes in the amino acid content of nerve endings (synaptosomes) induced by drugs that alter the metabolism of glutamate and γ-aminobutyric acid, J. Neurochem., 42 (1984) 16–24.
3 Hertz, L., Juurlink, B.H.J., Hertz, E., Fosmark, H. and Schousboe, A., Preparation of primary cultures of mouse (rat) astrocytes. In A. Shahar, J. De Vellis, A. Vernadakis and B. Haber (Eds.), A Dissection and Tissue Culture Manual of the Nervous System, Liss, New York, 1989, pp. 105–108.
4 Kimelberg, H.K. and Ransom, B.R., Physiological and pathological aspects of astrocytic swelling. In S. Fedoroff and A. Vernadakis (Eds.), Astrocytes, Vol. 3, Academic Press, New York, 1986, pp. 129–166.
5 Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S. and Waniewski, R.A., Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures, J. Neurosci., 10 (1990) 1583–1591.
6 Martin, D.L., Madelian, V., Seligmann, B. and Shain, W., The role of osmotic pressure and membrane potential in K^+-stimulated taurine release from cultured astrocytes and LRM55 cells, J. Neurosci., 10 (1990) 571–577.
7 Pasantes-Morales, H. and Martin del Rio, M., Taurine and mechanisms of cell volume regulation. In H. Pasantes-Morales, D.L. Martin, W. Shain and R. Martin del Rio (Eds.), Taurine: Functional Neurochemistry, Physiology, and Cardiology, Wiley-Liss, New York, 1990, pp. 317–328.
8 Pasantes-Morales, H. and Schousboe, A., Volume regulation in astrocytes: a role for taurine as an osmoeffector, J. Neurosci. Res., 20 (1988) 505–509.
9 Pasantes-Morales, H. and Schousboe, A., Release of taurine from astrocytes during potassium-evoked swelling, Glia, 2 (1989) 45–50.
10 Pasantes-Morales, H., Dominguez, L. and Montenegro, J., A chloride-dependent component of the release of labelled GABA and taurine from chick retina, Brain Res., 459 (1988) 120–130.
11 Pasantes-Morales, H., Moran, J. and Schousboe, A., Volume-sensitive release of taurine from cultured astrocytes: properties and mechanism, Glia, 3 (1990) 427–432.
12 Schousboe, A., Glutamate, GABA and taurine in cultured, normal glia cells. In E. Schoffeniels, G. Franek, L. Hertz and D.B. Tower (Eds.), Dynamic Properties of Glia Cells, Pergamon, Oxford, 1978, pp. 173–182.
13 Schousboe, A., Metabolism and function of neurotransmitters. In S.E. Pfeiffer (Ed.), Neuroscience Approached Through Cell Culture, Vol. 1, CRC Press, Boca Raton, FL, 1982, pp. 107–141.

14 Schousboe, A., Glial marker enzymes, Scand. J. Immunol., 15, Suppl. 9 (1982) 339–356.
15 Schousboe, A., Fosmark, H. and Svenneby, G., Taurine uptake in astrocytes cultured from dissociated mouse brain hemispheres, Brain Res., 116 (1976) 158–164.
16 Schousboe, A., Moran, J. and Pasantes-Morales, H., Potassium-stimulated release of taurine from cultured cerebellar granule neurons is associated with cell swelling, J. Neurosci. Res., 27 (1990) 71–77.
17 Sieghardt, W. and Karobath, M., Uptake of taurine into subcellular fractions of C-6 glioma cells, J. Neurochem., 26 (1976) 981–986.
18 Walz, W. and Allen, A., Evaluation of the osmoregulatory function of taurine in brain cells, Exp. Brain Res., 68 (1987) 290–298.

TRABAJO 5:

Pasantes-Morales, H., Murray, R.A., Sánchez-Olea, R., Morán, J. 1993. Regulatory volume decrease in cultured astrocytes: II. Changes in membrane permeability to amino acids and polyols. Am. J. Physiol. Manuscrito en segunda revision.

Regulatory volume decrease in cultured astrocytes: II. Changes in membrane permeability to amino acids and polyols.

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ABSTRACT

The permeability of the pathway activated by hyposmolarity to amino acids and polyols in cultured astrocytes was examined following the change in rate and direction of RVD when increasing the extracellular concentration of the osmolytes up to reverse their intracellular: extracellular concentration gradient. Accordingly, upon swelling activation of the pathway, those permeable osmolytes will enter the cell and inhibit RVD. Following this device, the pathway was found permeable to neutral and acidic amino acids but not to basic amino acids. Aspartate was more permeable than glutamate and K^+ and not Na^+ must be the accompanying cation. Beta-amino acids (ß-alanine = taurine > GABA) permeate better than α-amino acids. No significant differences in permeability were observed between glycine, alanine, threonine, phenylalanine and asparagine but proline permeate more easily and glutamine less easily than the rest of the α-amino acids. It seems that the amino acid pore activated by hyposmolarity excludes basic amino acids and has a dimension at the limit of glutamate-glutamine size. Influx rather than efflux of amino acids was observed when extracellular > intracellular concentration, with differences in the amount accumulated by cells, which correlate with their efficacy as RVD blockers. Influx of taurine (as representative of permeable amino acids), was inhibited by Cl^- channel blockers/exchangers DIDS (40%) and dypiridamole (85%), suggesting that amino acids could permeate through an anion channel. The polyalcohols sorbitol and mannitol exhibited a small inhibitory effect on the later phase of RVD whereas inositol slightly accelerated RVD. The efflux mechanism for these compounds seems different from the dissipative pathway of amino acids.

Key words: osmoregulation, organic osmolytes, hyposmolarity. taurine, DIDS, dypiridamole.

INTRODUCTION

It was considered for some time that the involvement of organic osmolytes in volume regulatory processes was essentially restricted to invertebrate cells whereas K^+ and Cl^- were in charge of this function in vertebrate cells. However, evidence has been

accumulating over the last decade which stresses the importance of organic osmolytes for both regulatory volume decrease (RVD) and regulatory volume increase (RVI) in mammalian tissues, including brain (2,6,9). Free amino acids, polyhydric alcohols (polyols) and methylamines are among the most common organic osmolytes. Free amino acids are highly concentrated in brain and are good candidates to serve a role of osmolytes. The involvement of free amino acids, particularly taurine, in brain volume regulation is well documented both *in vivo* and *in vitro*. Taurine levels in brain *in vivo* decrease subsequent to plasma hyponatremia (5, 22) or as consequence of local alterations in the extracellular osmolarity by microdialysis perfusion with hyposmotic solutions (19,25).

Corresponding to the decrease in tissue taurine, an increased efflux of the amino acid evoked by cell swelling has been observed in a variety of nervous tissue preparations including astrocytes and neurons in culture (8,9,16), excised retina (6), brain slices (17) and isolated nerve endings (14). Aspartate and glutamate efflux is also activated by hyposmolarity (4) and the brain content of these and other amino acids is also modified in response to changes in osmolarity (3). Regarding polyols, inositol is the only one found at sufficiently high levels in nervous tissue (9-15 mM in rat brain) to significantly contribute to osmoregulatory processes (23). The involvement of this molecule in RVI in cultured astrocytes is well documented (20,21) and there is also a report about a decrease of inositol brain levels during hyponatremia (24).

The mechanism responsible for the hyposmolarity activated efflux of amino acids is not elucidated although it has clearly shown in cultured astrocytes that it is different from the Na-dependent carrier which accumulates taurine into the cell in isosmotic conditions. Rather, the amino acid pathway activated by hyposmolarity seems to be a diffusional pore through which amino acid fluxes occur following the direction of the concentration gradient (13). Based on this study, the permeability properties of a diffusional pathway for amino acids and polyols were examined in the present work with an experimental design consisting in measuring RVD when the driving force for osmolyte membrane translocation favors influx rather than efflux, i.e. by a reversal of the intracellular : extracellular gradient. The ability of the presumed osmolytes to block RVD will then be an indication of their ability to permeate through a similar diffusional pathway and counteract the efflux of the main intracellular osmolyte

MATERIALS AND METHODS

All experimental details for cell culture preparation, volume measurement and solutions are described under Materials and Methods in the preceding paper in this issue (12). Briefly, rat cerebellar astrocytes were cultured in 60 mm Petri dishes with a basal Eagle's medium supplemented with 10% fetal calf serum. For volume measurements, cells after 2-3 weeks in culture were detached by treatment with a Ca^{2+}-free saline containing 1 mM EDTA and 0.01% trypsin. The cell suspension was centrifuged and resuspended in a

small volume of Krebs-HEPES medium. At zero time a sample of the cell suspension was diluted about 100-fold with isosmotic or 50% hyposmotic media containing the indicated concentrations of the amino acids. Exactly one min after, cell volume was measured at the indicated times by electronic sizing. To examine the effect of amino acids, polyols and saccharides, hyposmotic media were prepared by replacing NaCl by equiosmolar concentrations of these compounds.

For amino acid influx experiments, cells were washed twice and then incubated during 15 min with isosmotic or 50% hyposmotic Krebs-HEPES medium containing the indicated concentrations of amino acids. At the end of the incubation, cells were washed twice with isosmotic fresh medium and the endogenous amino acids were extracted with ethanol (70% (v/v)). The analysis of ethanol extracts was carried out after amino acid derivatization with O-phtaldialdehyde by reversed phase HPLC in a Beckman chromatograph equipped with an Ultrasphere column.

To test the effect of Cl^- channel blockers on taurine influx stimulated by hyposmolarity, cells were first briefly exposed to 50% hyposmotic medium to deplete cells of taurine and then preincubated 10 min in isosmotic medium containing DIDS (400 μM) or dipyridamole (150 μM). At the end of the preincubation period cells were exposed during further 10 min to isosmotic or 50% hyposmotic media added with 60 mM taurine and the same concentration of DIDS or dipyridamole. Finally cells were washed and endogenous taurine was measured by HPLC analysis.

RESULTS

Effect of high extracellular concentrations of amino acids on RVD.

The extracellular concentration of NaCl in hyposmotic solutions is 60 mM. In the companion paper (12) it was shown that external NaCl can be replaced by various cations, excepted K^+ and Rb^+ without affecting RVD. In the following experiments, NaCl was replaced by equiosmolar amounts of amino acids. Three groups of amino acids were examined: basic, neutral and acidic amino acids. Among the neutral group, amino acids investigated were both α-amino acids and ß-amino acids. In each one of this groups, amino acids with polar or non polar side groups were tested. Results in Fig. 1 show the effect on RVD of increasing the extracellular concentration of taurine, glycine and alanine. Table 1 shows results of the same type of experiments for ß-alanine, GABA, proline, phenylalanine, cysteine, threonine glutamine and asparagine. When all NaCl was replaced by amino acids, i.e. amino acid extracellular concentration of 120 mM, RVD was essentially blocked in all cases. The same total blockade of RVD was observed in the presence of taurine and ß-alanine at 90 mM concentration and still an effect in reducing RVD was observed at 60 mM. The inhibition was not complete for any other amino acid at 90 mM and essentially no effect was observed at 60 mM concentration. No inhibition was observed for any of the amino acids at 30 mM (Table 1). Glutamine permeability was the lowest among the α-amino acid group. The inefficiency of glutamine to block RVD was not due to

the presence of the amido group since asparagine at the same concentration, blocked RVD (Table 1). The basic amino acid lysine did not affect RVD, even at the highest concentration of 120 mM. In the presence of arginine (120 mM), the swelling phase was reduced but RVD still occurred (Fig. 2). The acidic amino acids, aspartate and glutamate, as K^+ salts inhibited RVD but were without effect when the accompanying cation was Na^+ (Fig. 3). Aspartate blocked RVD more efficiently than glutamate. None of the amino acids examined, with the exception of arginine, affected astrocyte volume in isosmotic conditions.

Effect of polyalcohols on RVD.

The effect of the polyhydric alcohols, sorbitol, mannitol and inositol on RVD is shown in Fig. 4. Sorbitol and mannitol had a small inhibitory effect on the last phase of RVD. The effect of mannitol showed some variability. In most experiments, RVD was affected, as shown in Fig. 4, but in a short number of cells (about 15%) mannitol slightly increased cell volume in isosmotic conditions and had a stronger inhibitory effect on RVD. Inositol 120 mM caused an acceleration of RVD (Fig. 4).

Influx of amino acids in hyposmotic conditions following the concentration gradient.

The inhibitory effect of amino acids on RVD above described may be explained if upon cell swelling, those amino acids which permeate through the hyposmolarity-activated pathway enter the cell by the driving force of the concentration gradient. The increased intracellular concentration of these amino acids will then cancel the decrease of intracellular content of othe osmolytes, caused by the hyposmolarity activated efflux of these osmolytes and decrease the efficacy of the volume regulatory process. To verify this interpretation, we examined the accumulation of amino acids in astrocytes exposed to hyposmotic solutions containing increasing concentrations of various amino acids. The tested amino acids were four neutral amino acids (alanine, taurine, ß-alanine and glutamine), one acidic amino acid, glutamic acid (as potassium or sodium salt), and two basic amino acids, lysine and arginine. The intracellular concentration of taurine and ß-alanine increased almost linearly with increasing the external concentration of the amino acids. At the highest concentration tested of 120 mM, the intracellular levels of these two amino acids were about 1200 nmol/mg protein. Some increase in the cell content of taurine and ß-alanine was also observed in isosmotic conditions. This increase was also linearly dependent on the external concentration, but was much lower than that observed in hyposmotic conditions (Fig. 5A). Alanine also accumulated into the cells in hyposmotic conditions, by a process linearly dependent of the external concentration, but the amount accumulated by cells. The amount of alanine accumulated at the highest external concentration of alanine was 980 nmol/mg protein (Fig. 5B). This amount was somewhat lower than that of taurine or ß-alanine.

Glutamine content in astrocytes is high (160 nmol/mg prot)

and this concentration increased to 440 nmol/mg protein when increasing the extracellular levels of glutamine to 120 mM in isosmotic conditions. Only a moderate increase over the value in isosmotic conditions was observed at the highest extracellular concentration of glutamine of 120 mM in hyposmotic conditions. Changes in glutamine content in both isosmotic and hyposmotic conditions were observed only at concentrations up to 30 mM (Fig. 5B). The basic amino acids, lysine and arginine were also somewhat permeable in isosmotic conditions and very low increases in amino acid accumulation were observed in hyposmotic conditions (Fig. 5C). Glutamate as sodium or potassium salts were similarly accumulated in isosmotic conditions but a substantial difference was observed between the two salts in hyposmotic conditions. At the highest possible concentration of 60 mM, glutamate present as sodium salt, increased the intracellular concentration of glutamate to 339 nmol/mg protein whereas as potassium salt the intracellular concentration raised to 560 nmol/mg protein (Fig. 5D).

To investigate whether amino acids could interfere with each other at the permeability pathway opened by hyposmolarity we examined the effect of a number of amino acids over taurine accumulation by astrocytes. These experiments were carried out measuring taurine content in astrocytes after 3 min of exposure to hyposmotic solutions. At this time, the influx process has almost reached a maximum. Two neutral amino acids, one ß (ß-alanine) and one Ó (glycine), an acidic (glutamate) and a basic amino acid (lysine) were used for these competence experiments. In any case the influx of taurine was affected by the presence of other amino acids (Table 2). The same type of experiment using inositol and sorbitol, gave similar negative results (Table 2).

Effect of Cl- channel blockers on taurine influx activated by hyposmolarity.

The anion channel blockers DIDS and dypiridamole inhibited RVD as shown in the companion paper. In a previous study it was shown that these drugs markedly reduced the hyposmolarity stimulated efflux of taurine, D-aspartate and Rb (15). The effect of these two inhibitors was examined on the reversal taurine pathway, i.e. on taurine accumulation observed in hyposmotic conditions at high external concentration of the amino acid (60 mM). In order to further favor the influx of taurine by the concentration gradient in these experiments cells were first depleted of taurine by a hyposmotic shock, then recover in isosmotic medium subsequently exposed to hyposmotic medium containing 60 mM taurine. Fig.6 shows that DIDS (400 µM) reduced taurine influx by almost 50% and dypiridamole inhibited the process by 85%. None of the inhibitors affected the influx in isosmotic conditions.

DISCUSSION

Previous observations on the properties of the hyposmolarity-induced release of taurine from astrocytes, such as low sensitivity to temperature and Na⁺-independence, have suggested that this efflux

occurs through a diffusion pathway, different from the energy-dependent carrier (10). Studies on the kinetics of amino acid transport always consistently a diffusional component, in addition to the saturable, Na-dependent component. This has been observed for taurine in cultured astrocytes and neurons and also that the diffusional but not the saturable component increases in hyposmotic conditions (13,16).

The diffusional nature of taurine fluxes activated by hyposmolarity was confirmed in a study showing that these fluxes are directed only by the concentration gradient and therefore, efflux or influx of taurine occur subsequent to hyposmolarity depending on the amino acid concentration in the intracellular or the extracellular compartments (13).

As could be predicted from these studies, in the present work it was found that the reversal of the concentration gradient for taurine results in blockade of RVD in astrocytes. The similarities between taurine and other amino acids in this respect suggest that the conclusions raised for taurine may be valid for other amino acids as well. This was also confirmed in the present work, where it was shown that influx rather than efflux of a number of amino acids is observed in hyposmotic conditions when the extracellular concentration exceeds the intracellular content. Marked differences were observed however, among the various amino acids. Some highly permeable amino acids, rapidly attain high intracellular levels and counteract perhaps not only their own hyposmolarity-stimulated efflux but that of other osmolytes as well. As a result, RVD is blocked. The facility of amino acids to permeate through the pathway opened by hyposmolarity correlates well with their ability to block RVD. Amino acids which accumulate rapidly and at high amounts in cells have also the highest efficiency as RVD blockers. Taurine and ß-alanine were the most permeable amino acids. The influx of these amino acids seems to be a non saturable process up to 120 mM in further support to the diffusional nature of the pathway. The lack of interference of other amino acids with taurine influx suggests that separate, independent pathways exist for the different amino acids or alternatively, that the pore is sufficiently large to allow the passage of various amino acids without hindrance.

The experimental device used in this study provides a useful tool to characterize the diffusion pathway for amino acids activated by hyposmolarity. According to their efficacy to block RVD together with the intracellular accumulation when the concentration gradient is reversed, the permeability properties of the hyposmolarity-sensitive pathway to amino acids can be depicted. The pathway is permeable to acidic amino acids accompanied by K^+, but not by Na^+. It is permeable to neutral amino acids, both α and ß amino acids but not to the basic amino acids arginine and lysine. However, the differences observed between aspartate and glutamate in one hand, between α and ß amino acids in the other hand and between various amino acids from the α and ß groups, suggest that features other than the charge are important for permeation through this pathway. Aspartate which is smaller than

glutamate, permeated more easily and the ß-amino acids get through more easily than α-amino acids. From the ß-amino acid series, the efficiency order of permeation through the pathway was ß-alanine = taurine > GABA. At the α-amino acid group, the efficiency order was proline > glycine = alanine = cysteine= threonine = phenylalanine > glutamine. Asparagine was more permeable than glutamine, suggesting that the amido group is not a restriction to the passage through this pathway but rather the amino acid size is influential. These results altogether suggest that the amino acid pore activated by hyposmolarity excludes basic amino acids, and has a size at the limit of the glutamine-glutamate size.

The differences observed in the ability of amino acids to block RVD which reflect their facility of getting into the cell, have their counterpart on the easiness of free amino acids to leave the cell after the hyposmotic stimulus. In a previous study on the efflux of the main constituents of the free amino acid pool in astrocytes it was shown that 50% reduction in osmolarity releases about 60% of the intracellular pool of taurine, aspartate and ß-alanine, 40% of that of glutamate and 10-15% of that of glutamine, alanine and glycine (11).

In the companion paper, using a similar experimental paradigm it was shown that fluxes of K^+ accompanied by a number of anions occur through a pathway opened by hyposmolarity only following the concentration gradient. Two separate but interconnected pathways for K^+ and Cl^- seems to operate for the dissipative fluxes of the ionic osmolytes. In that study it was also shown that RVD was strongly inhibited by the Cl^- channel blockers dypiridamole and DIDS which probably exert their action blocking primarily Cl^- fluxes. These compounds also block the hyposmolarity stimulated efflux of taurine, D-aspartate and Rb (15) and the influx of taurine when the concentration gradient for this amino acid was reversed. These observations suggest that one bidirectional pathway carries the efflux and the influx of taurine. The inhibitory effects of blockers of Cl^- channels on taurine influx and efflux also raise the question as whether the amino acid pathway is the same as the anion channel. Experimental evidence in support to this option comes from a recent report in MDCK cells which describes a non selective anion channel which is permeable to neutral and acidic amino acids (1). It cannot be ruled out that several types of Cl channels, sensitive to DIDS and dypiridamole may be implicated in RVD. One type of channel may be associated to K conductance and another type (s), perhaps of larger capacity could represent the permeation pathway for amino acids. The occurrence of Cl- channels blocked by DIDS has been shown in cultured astrocytes. It remains to be demonstrated that these channels are activated by volume changes and sensitive to the above mentioned blockers of Cl^- channels and taurine efflux. Alternative explanations for the amino acid-Cl- connection observed in this work are first, that the Cl^- channel blockers are acting solely on the anion pathway but that Cl and amino acid fluxes are so closely interconnected that inhibition of Cl^- fluxes leads to blockade of amino acid fluxes, second, that these blockers act on a common

target activating all osmolyte fluxes.

Polyols exhibited a response different from amino acids on RVD in astrocytes. Increasing extracellular concentrations of sorbitol and mannitol had only marginal effects on RVD whereas an acceleration of the process was observed in the presence of myo-inositol. If we consider that osmolyte efflux occurs through a diffusional pathway directed only by the concentration gradient, these results should be taken as evidence that polyols do not permeate through such pathway. If this is also an indication that these compounds are not implicated in RVD remains to be demonstrated. This is unlikely, however, if we consider results of a study *in vivo* where was shown that the concentration of inositol is decreased in about the same proportion and with the same time-course as that of free amino acids in conditions of hyponatremia (24). The inability of inositol to block RVD only indicates that this compound does not permeate through a pathway opened by hyposmolarity allowing the fluxes of other osmolytes, K^+, Cl^- and amino acids, which importantly contribute to the regulatory process occur but it cannot be excluded that inositol may also leave the cell in response to swelling via a different pathway, perhaps involving an active process rather than simple diffusion.

Acknowledgements. The excellent technical assistance of Ms. Claudia Rodríguez and Claudia Peña is gratefully acknowledged. This work was supported in part by a grant from DGAPA-UNAM (IN-204589)

REFERENCES

1. BANDERALI, U. AND G. ROY. Anion channels for amino acids in MDCK cells. Am. J. Physiol. 32:C1200-C1207, 1992.
2. CHAMBERLIN, M.E., AND K. STRANGE. Anisosmotic cell volume regulation: a comparative view. Amer. Physiol. Soc. C159-C172, 1989.
3. CHAN, P.H., AND R.A. FISHMAN. Elevation of rat brain amino acids, ammonia and idiogenic osmoles induced by hyperosmolality. Brain Res. 161:293-301, 1979.
4. KIMELBERG, H.K., GODERIE, S.K., HIGMAN, S., PANG, S.AND WANIEWSKI, R.A. Swelling induced release of glutamte, aspartate and taurine from astrocyte cultures. J. Neurosci. 10:1583-1591, 1990.
5. LAW, R.O. The effects of pregnancy on the contents of water, taurine, and total amino nitrogen in rat cerebral cortex J. Neurochem. 53:300-302, 1989.
6. LAW, R.O. AND M.B. BURG. The role of organic osmolytes in the regulation of mammalian cell volume. In: Advances in comparative and environmental physiology, Vol. 9, edited by R. Gilles et al. Springer Verlag. p. 189-225, 1991.
7. MORAN, J., S. HURTADO, AND H. PASANTES-MORALES. Similar properties of taurine release induced by potassium and hyposmolarity in rat retina. Exp. Eye Res. 53, 347-352 1991.
8. PASANTES-MORALES, H. AND A. SCHOUSBOE. Volume regulation in

astrocytes: a role for taurine as an osmoeffector, J. Neurosci. Res. 20:505-509, 1988.
9. PASANTES-MORALES, H. AND M. MARTIN DEL RIO. Taurine and mechanisms of cell volume regulation. In. H. Pasantes-Morales, D.L. Martín, W. Shain and R. Martín del Río (Eds.) Taurine: Functional Neurochemistry, Physiology, and Cardiology, Wiley- Liss, New York, pp. 317-328, 1990.
10. PASANTES-MORALES, H., J. MORAN, AND A. SCHOUSBOE. Volume-sensitive release of taurine from cultured astrocytes: Properties and mechanisms. Glia. 3:427-432, 1990.
11. PASANTES-MORALES, H., ALAVEZ, S., SANCHEZ-OLEA, R. AND MORAN, J. Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture. Neurochem. Res. 18:445-452, 1993.
12. PASANTES-MORALES, H., R.A. MURRAY, L. LILJA, AND J. MORAN. Regulatory volume decrease in astrocytes: I. Potassium and chloride activated permeability. This issue.
13. SANCHEZ-OLEA, R., J. MORAN, A. SCHOUSBOE, AND H. PASANTES-MORALES. Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. Neurosc. Lett. 130:233-236, 1991.
14. SANCHEZ-OLEA, R., AND H. PASANTES-MORALES. Taurine and volume regulation in isolated nerve endings. In: Taurine: Nutritional value and mechanisms of action. (J.B. Lombardini and SW Schaffer, eds.) Advances in Exp. Med. Plenum Press, New York, Vol. 315, pp 381-389, 1992.
15. SANCHEZ-OLEA, R., PEÑA, C., MORAN, J. AND PASANTES-MORALES, H. Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl- transport in cultured astrocytes. Neurosci. Lett., In press, 1993.
16. SCHOUSBOE, A., R. SANCHEZ-OLEA, J. MORAN, AND H. PASANTES-MORALES. Hyposmolarity-induced taurine release in cerebellar granule cells is associated with diffusion and not with high-affinity transport. J. Neurosci. Res. 30:661-665, 1991.
17. SCHOUSBOE, A., C. LOPEZ-APREZA AND H. PASANTES-MORALES. GABA and taurine serve as respectively a neurotransmitter and an osmolyte in cultured cerebral cortical neurons. In: Taurine: Nutritional value and mechanisms of action. (J.B. Lombardini and SW Schaffer, eds.) Advances in Exp. Med. Plenum Press, New York, Vol. 315, pp 391-397, 1992.
18. SCHOUSBOE, A. MORAN, J. AND PASANTES-MORALES, H. Potassium-stimulated release of taurine from cultured cerebellar granule neurons is associated with cell swelling. J. Neurosci. Res. 27:71-77, 1990.
19. SOLIS, J., A. HERRANZ, O. HERRERAS, J. LERMA, AND J. Martín del Río, R. Does taurine act as an osmoregulatory substance in rat brain? Neurosci. Lett. 91:5358, 1988.
20. STRANGE, K. AND R. MORRISON. Volume regulation during recovery from chronic hypertonicity in brain glial cells. Am. J. Physiol. 263, C412-C419, 1992.
21. STRANGE, K., R. MORRISON, C.W. HEILIG, S. DiPIETRO AND S.R. GULLANS. Upregulation of inositol transport mediates inositol

accumulation in hyperosmolar brain cells. Am. J. Physiol. C784-C790, 1991.
22. THURSTON, J.H., R.E. HAUHART, J.A. DIRGO. Taurine: a role in osmotic regulation of mammalian brain and possible clinical significance. Life Sci. 26:1561-1568, 1980.
23. TRACHTMAN, H., S. FUTTERWEIT, E. HAMMER, T.W. SIEGEL., AND P. OATES. The role of polyols in cerebral cell volume regulation in hypernatremic and hyponatremic states. Life Sci. 49, 677-688, 1991.
24. VERBALIS, J.G. AND S.R. GULLANS. Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. Brain Res. 567:274-282, 1991.
25. WADE, J., J. OLSON, F. SAMSON, S. NELSON, T. PAZDERNIK. A possible role of taurine in osmoregulation within the brain. J. Neurochem. 45:335-344, 1988.

Table 1. Effect of extracellular amino acids on RVD in cerebellar astrocytes

	% of RVD		
Amino acid	60 mM	90 mM	120 mM
ß-Alanine	62.3	32.3	-3.6
GABA	--	61.5	15.8
Cysteine	69.0	68.0	-15.3
Threonine	75.0	71.0	9.5
Phenylalanine	72.0	72.0	4.1
Proline	74.0	68.0	-13.9
Glutamine	75.0	72.6	46.1
Asparagine	--	--	-19.6

Cell volume was measured in cultured cells as described in **Materials and Methods.** Cells were resuspended in isosmotic media and RVD was measured in 50% hyposmotic media containing the indicated concentrations of amino acids. Results are expressed as percentage of RVD, i.e. the % of recovery of cell volume between min 1 (maximal volume) and min 15. In control cells the percentage of RVD was 75.04 ± 1.2 %.

Table 2. Swelling-induced accumulation of taurine in the presence of amino acids and polyols.

Condition	Taurine Content (nmol/mg prot)
Taurine (60 mM)	321 ± 27.3
+ ß-alanine	353 ± 31.5
+ glycine	329 ± 29.8
+ lysine	316 ± 29.3
+ glutamate (K^+)	297 ± 26.4
+ inositol	339 ± 32.8
+ sorbitol	307 ± 35.6

After removal of the culture medium, cells were exposed during 3 min to 50% hyposmotic media containing 60 mM taurine plus 60 mM sorbitol, inositol or the indicated amino acids, replacing NaCl. At the end of the incubation, cells were washed and endogenous taurine was extracted and analyzed by HPLC as described in **Materials and Methods**. In the control (taurine alone), the osmolarity was adjusted with mannitol. Results are means ± SEM of 4 experiments.

Legends of the Figures

Fig. 1. Effect of taurine (A), alanine (B) and glycine (C) on RVD in cerebellar astrocytes. Cell volume was measured as described in Methods. Isosmotic and hyposmotic media were prepared by replacing NaCl by equiosmolar concentrations of the amino acids. Results are expressed as relative volume considering cell volume in isosmotic conditions as 1. Values are means ± SEM of 4-8 experiments.

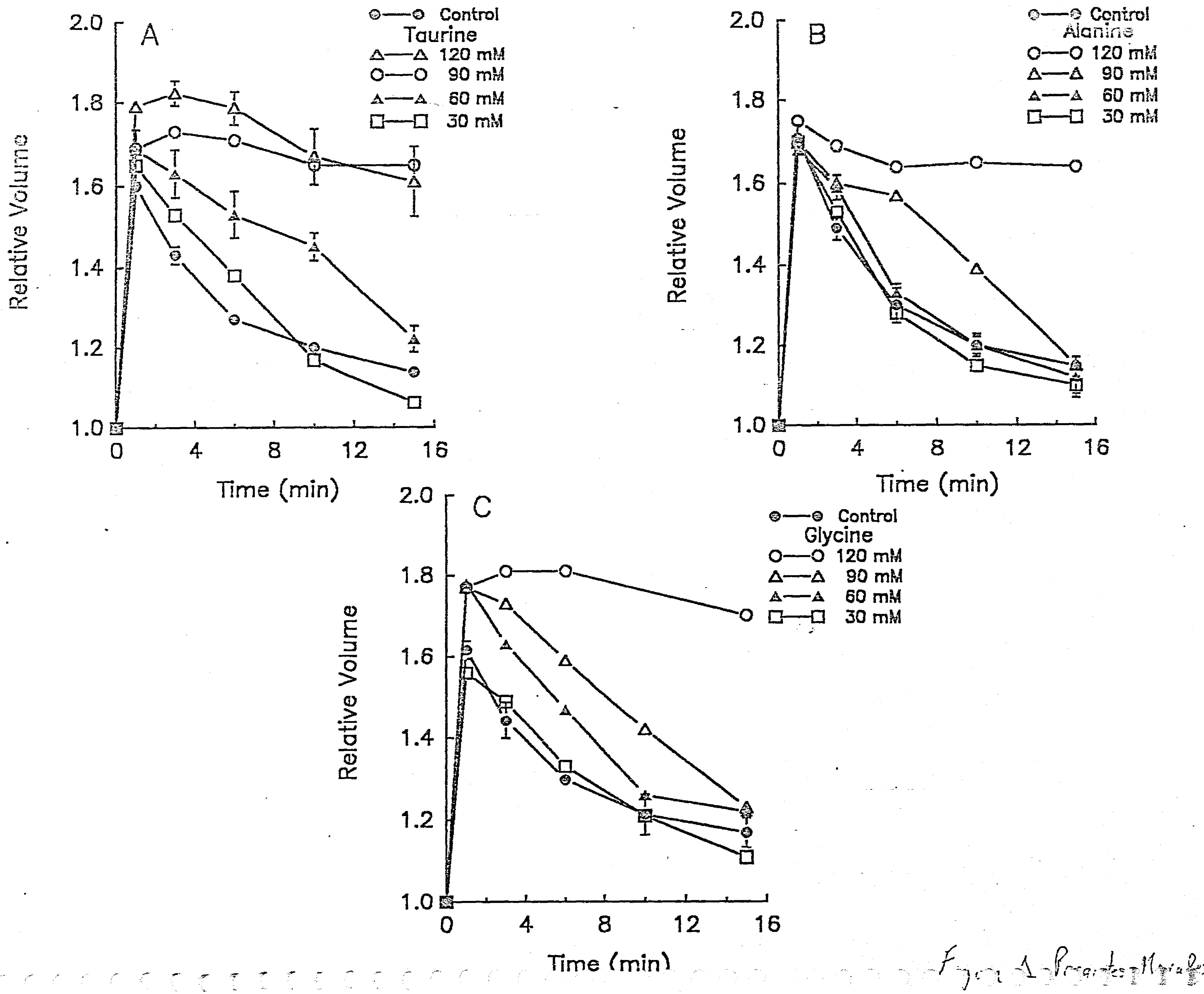
Fig 2. Effect of lysine and arginine on RVD in cerebellar astrocytes. RVD was measured in 50% hyposmotic media containing 120 mM lysine or arginine. Results are expressed as relative volume considering cell volume in isosmotic conditions as 1. Values are means ± SEM of 4-6 experiments.

Fig. 3. Effect of glutamate and aspartate on RVD in cerebellar astrocytes. RVD was measured in 50% hyposmotic media in which NaCl was replaced by 60 mM of the Na^+ salt of glutamate or aspartate (A) or 60 mM of the K^+ salt of glutamate or aspartate (B). None of the amino acids examined affected astrocyte volume under isosmotic conditions. Results are expressed as relative volume considering cell volume in isosmotic conditions as 1. Values are means ± SEM of 4-6 experiments. $^*P< 0.001$, $^{**}P< 0.01$ (ANOVA test).

Fig 4. Effect of polyols on RVD in cerebellar astrocytes. RVD was measured in 50% hyposmotic media containing 120 mM mannitol, sorbitol or inositol. Results are expressed as relative volume considering cell volume in isosmotic conditions as 1. Values are means ± SEM of 4-8 experiments.

Fig. 5. Intracellular content of amino acids in astrocytes exposed to isosmotic or hyposmotic solutions with increasing concentrations of the amino acid. After removal of the culture medium, cells were exposed during 15 min to isosmotic (--) or 50% hyposmotic ( ) media, containing the investigated amino acids at the indicated concentrations. The osmolarity was adjusted with mannitol, when necessary. At the end of the experiment, amino acids were extracted and analyzed by HPLC as described in Methods. Results are means of 6 experiments.

Fig. 6. Effects of DIDS and dypiridamole on the influx of taurine stimulated by hyposmolarity. Astrocytes treated as described in Methods were preincubated during 10 min with DIDS (400 µM) or dypiridamole (150 µM) in isosmotic medium and then in 50% hyposmotic medium containing 60 mM taurine. After the experiment, taurine content in cells was measured by HPLC as described in Methods. Results are means ± SEM of 6-8 experiments. $^*P< 0.001$ (Student's t-test).


A
Control
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90 mM
60 mM
30 mM
B
Control
Alanine
120 mM
90 mM
60 mM
30 mM
C
Control
Glycine
120 mM
90 mM
60 mM
30 mM
Relative Volume
Time (min)
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1.4
1.6
1.8
2.0
0
4
8
12
16

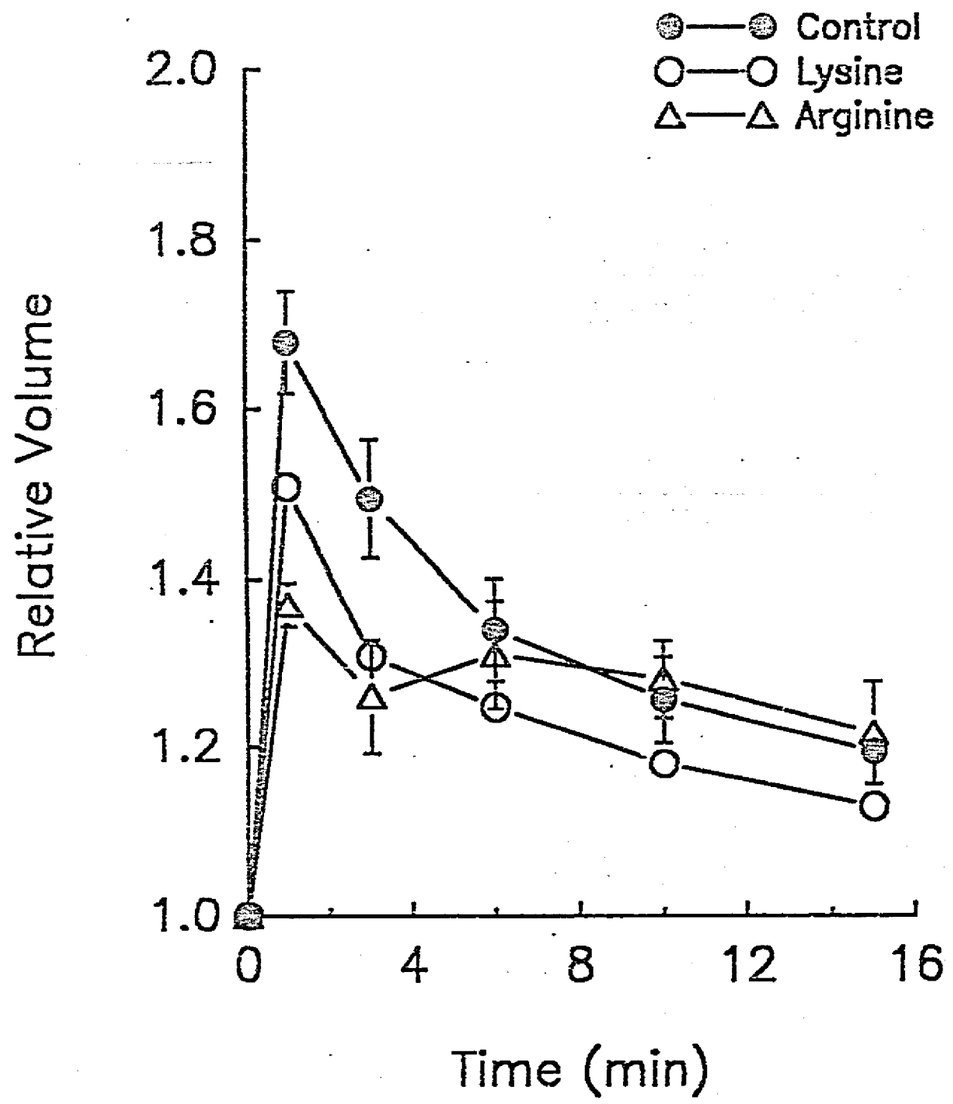


Fig. 2 Portales-Morales et al

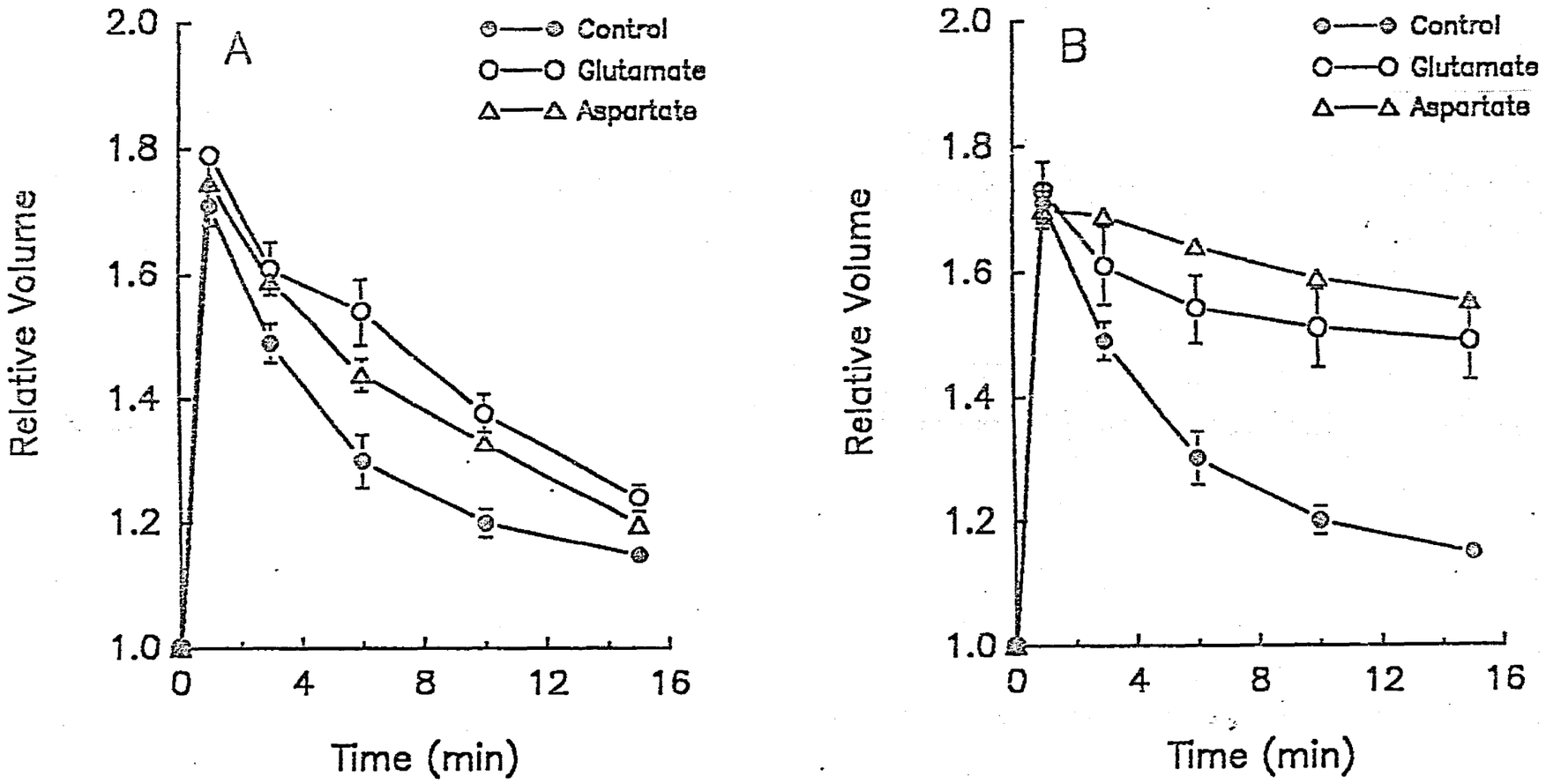
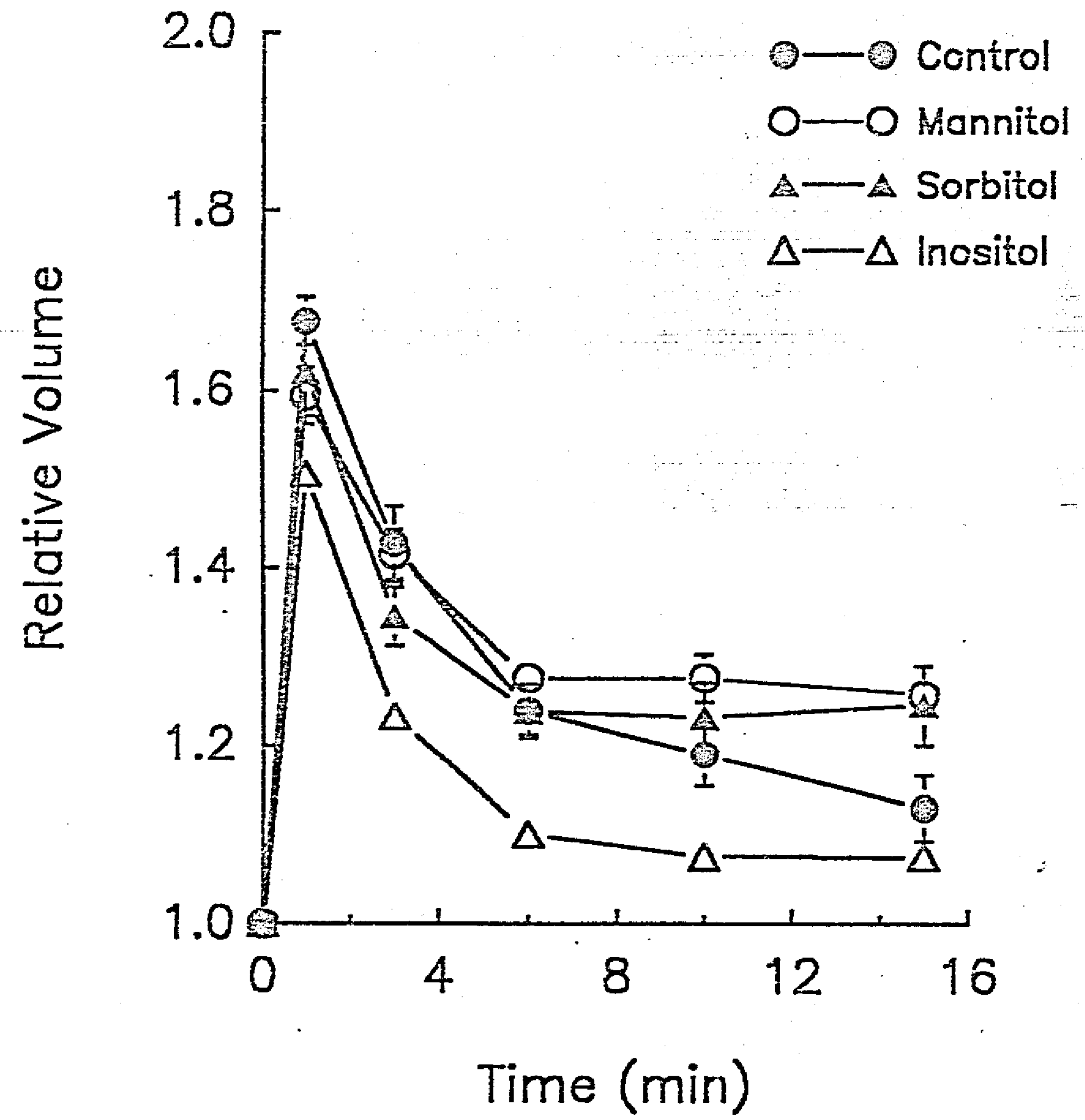


Fig 3 Davis-Meeks



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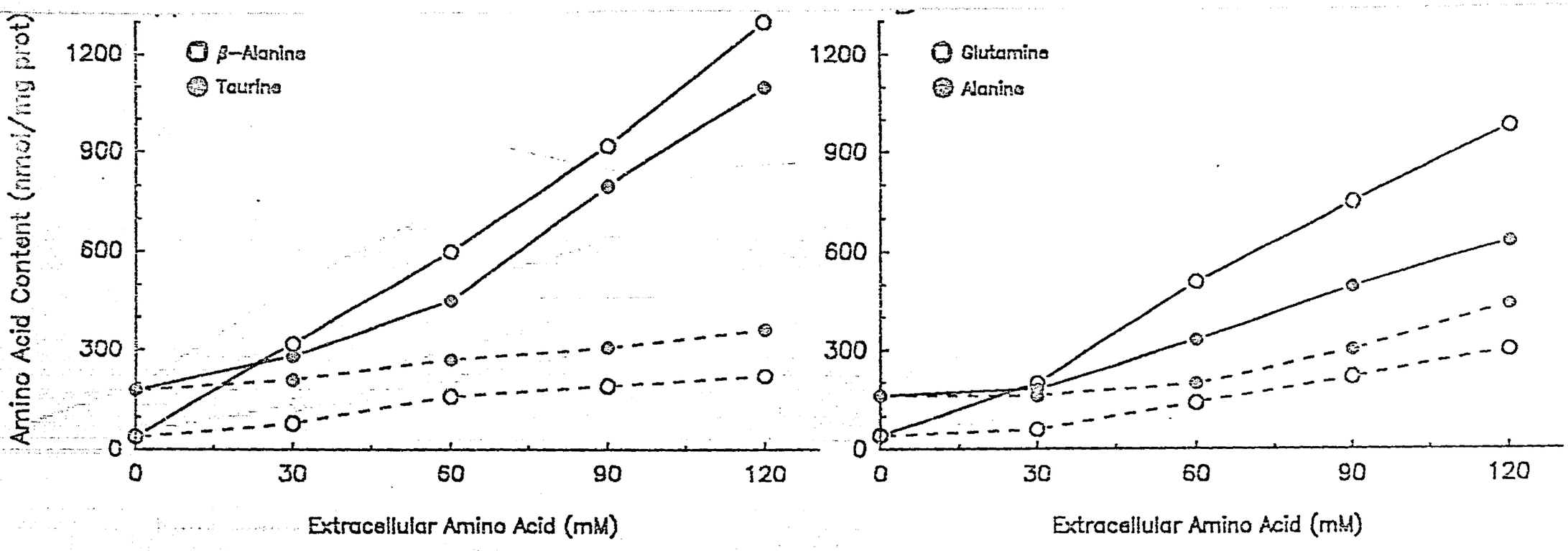

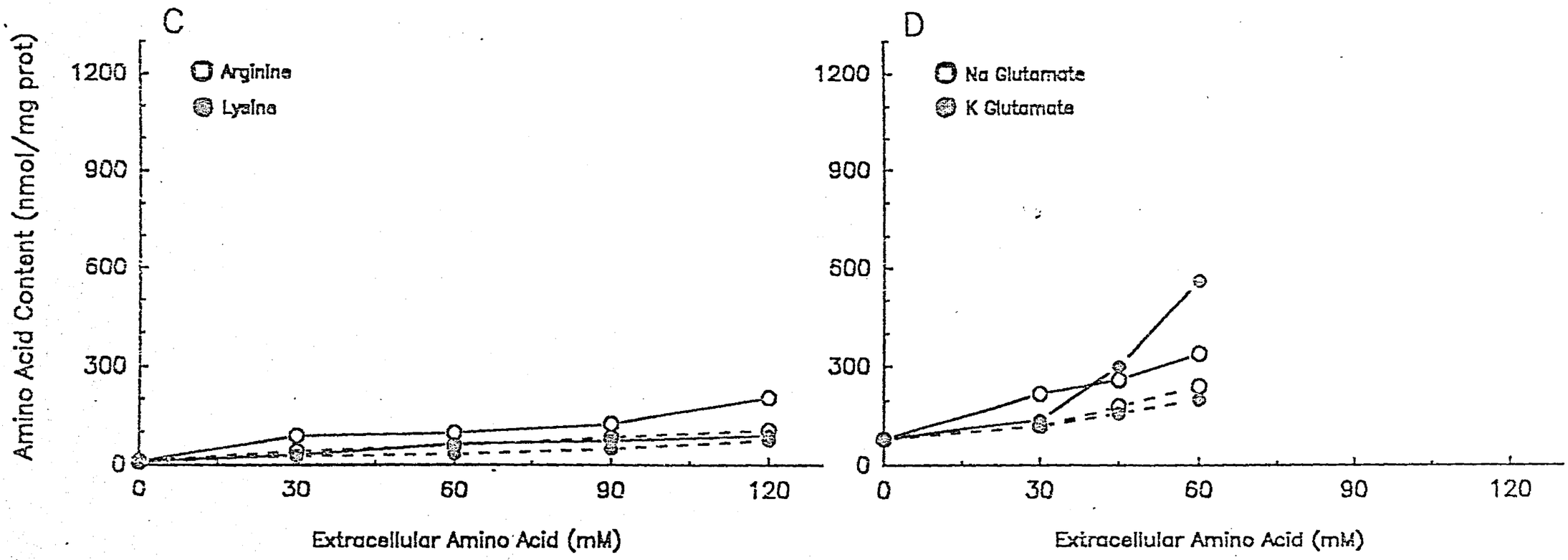

Figura 5. Posadas-Morales et al.

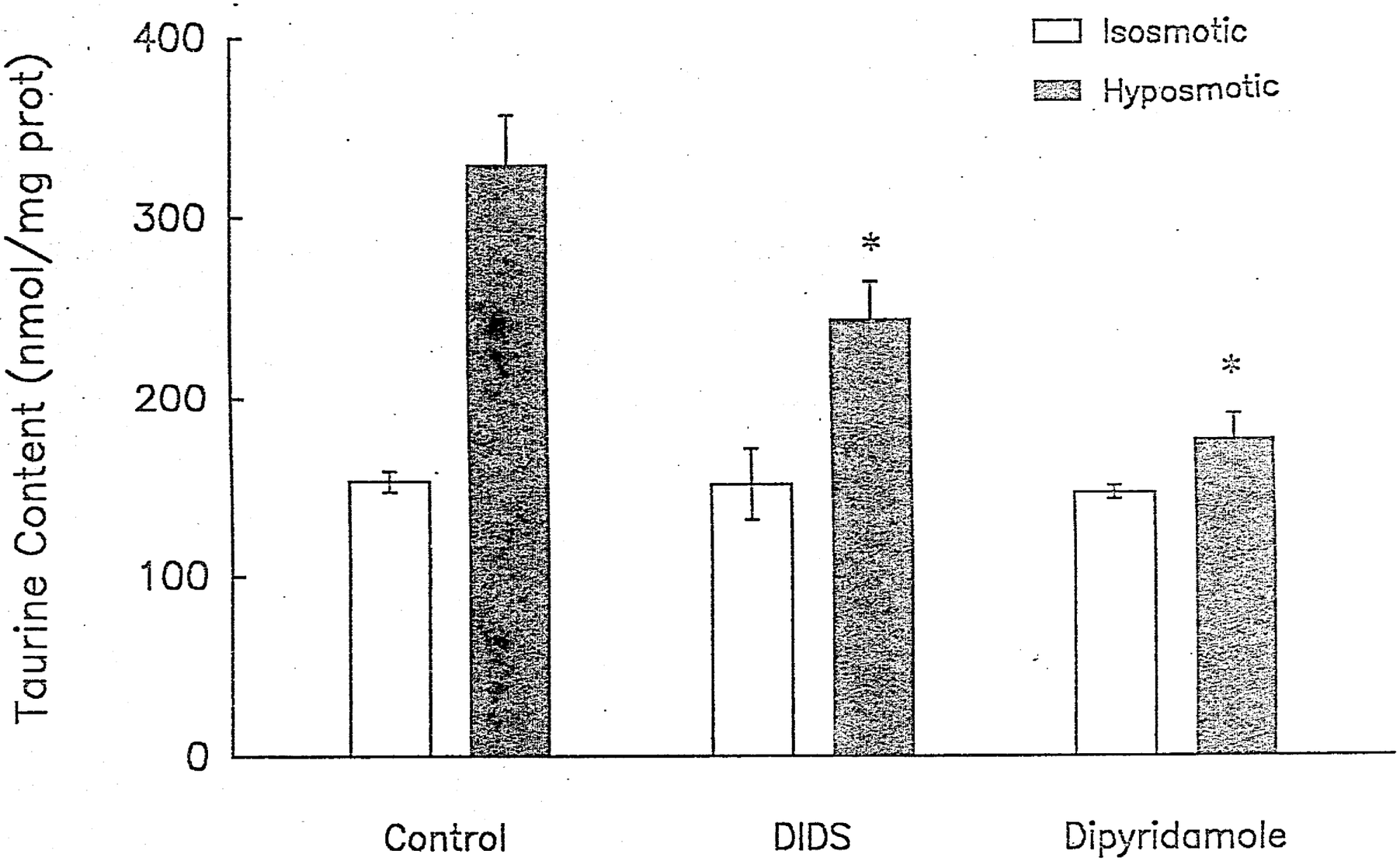

Figura 6.
Pasantes-Morales et al.

TRABAJO 6:

Sánchez-Olea, R., Peña, C., Morán, J., Pasantes-Morales, H. 1993. Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl^- transport in cultured astrocytes. Neurosci. Lett. En prensa.

Neuroscience Letters, 000 (1993) 1–000

NSL 09616 Author: please give journal name Ref. 7.

Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl^- transport in cultured astrocytes

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(Received 25 February 1993; Revised version received 1 April 1993; Accepted 2 April 1993)

Key words: Volume regulation; Osmolyte; Astrocyte; Niflumic acid; Dipyridamole; DIDS; Anion channel; Anion exchanger

Regulatory volume decrease (RVD) in astrocytes was inhibited by the Cl^- exchanger blockers 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), dipyridamole and niflumic acid but not by the Cl^- channel inhibitors diphenylamine-2-carboxylate (DPC) and anthracene-9-carboxylate (9-AC). The volume activated efflux of [^{3}H]taurine and [^{3}H]D-aspartate (as marker for glutamate) was similarly affected by these compounds. However, neither RVD nor osmolyte fluxes were significantly reduced by removal of external Cl^-, suggesting that an anion exchanger activity is not required for the volume regulatory process. Alternatively, these results suggest that the anion exchanger molecule may function as an unidirectional Cl^- channel possibly permeable also to amino acids.

Astrocytes in culture swell after exposure to solutions of reduced osmolarity and then recover cell volume despite the persistence of the hyposmotic condition [12, 19]. This volume regulatory process, known as regulatory volume decrease (RVD), takes place by activation of transmembrane fluxes of intracellular osmolytes followed by osmotically obliged water. In astrocytes as in many other cells, K^+ and Cl^- as well as organic molecules are implicated in this process. Free amino acids and notably taurine are among the organic osmolytes importantly contributing to RVD [13, 19, 20, 22]. The nature of the dissipative pathways for the various osmolytes is not fully understood in astrocytes. Previous studies suggested that these fluxes occur through diffusional pathways (possibly channels) rather than via carriers or cotransporters [23, 24]. These studies also showed that Rb^+ and taurine fluxes were significantly inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) but only marginally affected by diphenylamine-2-carboxylate (DPC) and anthracene-9-carboxylate (9-AC) [20, 24]. DIDS is a well known inhibitor of the anion exchanger [2–4] but also inhibits Cl^- channels [5], including volume-activated Cl^- channels [16]. In fish red blood cells [7, 8], the volume-sensitive efflux of taurine and K^+ is inhibited by niflumic acid, another blocker of the anion exchanger. An anion exchanger molecule (AE_3) is present in brain that performs the same functions as band 3 in red cells [11], and might be involved in volume regulatory processes in this tissue. Apart from the mentioned studies on the effect of DIDS on osmolyte fluxes, there is no information available about the effect of Cl^- channel or exchanger blockers on RVD in astrocytes. These points were addressed in the present work investigating the effect of a number of blockers of Cl^- channels as well as of conditions and compounds which inhibit the anion exchanger, on both RVD and amino acid efflux in astrocytes.

Astrocytes dissociated from cerebella of 8-day old rats were cultured for two weeks as previously described in detail [19]. For release experiments cells grown in 24-well multitest dishes were loaded with [^{3}H]taurine or [^{3}H]D-aspartate as described in the legend to Fig. 2. After 4 successive washes with Krebs-HEPES medium, cells were further incubated during 5 min with isosmotic (basal release) or 50% hyposmotic (stimulated release) media in the presence or absence of the tested drugs. At the end of the experiments, radioactivity in media and that remaining in the cells was measured by scintillation spectrometry. For volume measurements, cells were trypsinized as described in Fig. 1 and resuspended in isosmotic or 50% hyposmotic media with or without the drugs. Cell volume was measured by electronic sizing in a Coulter counter coupled to a Channelizer, as previ-

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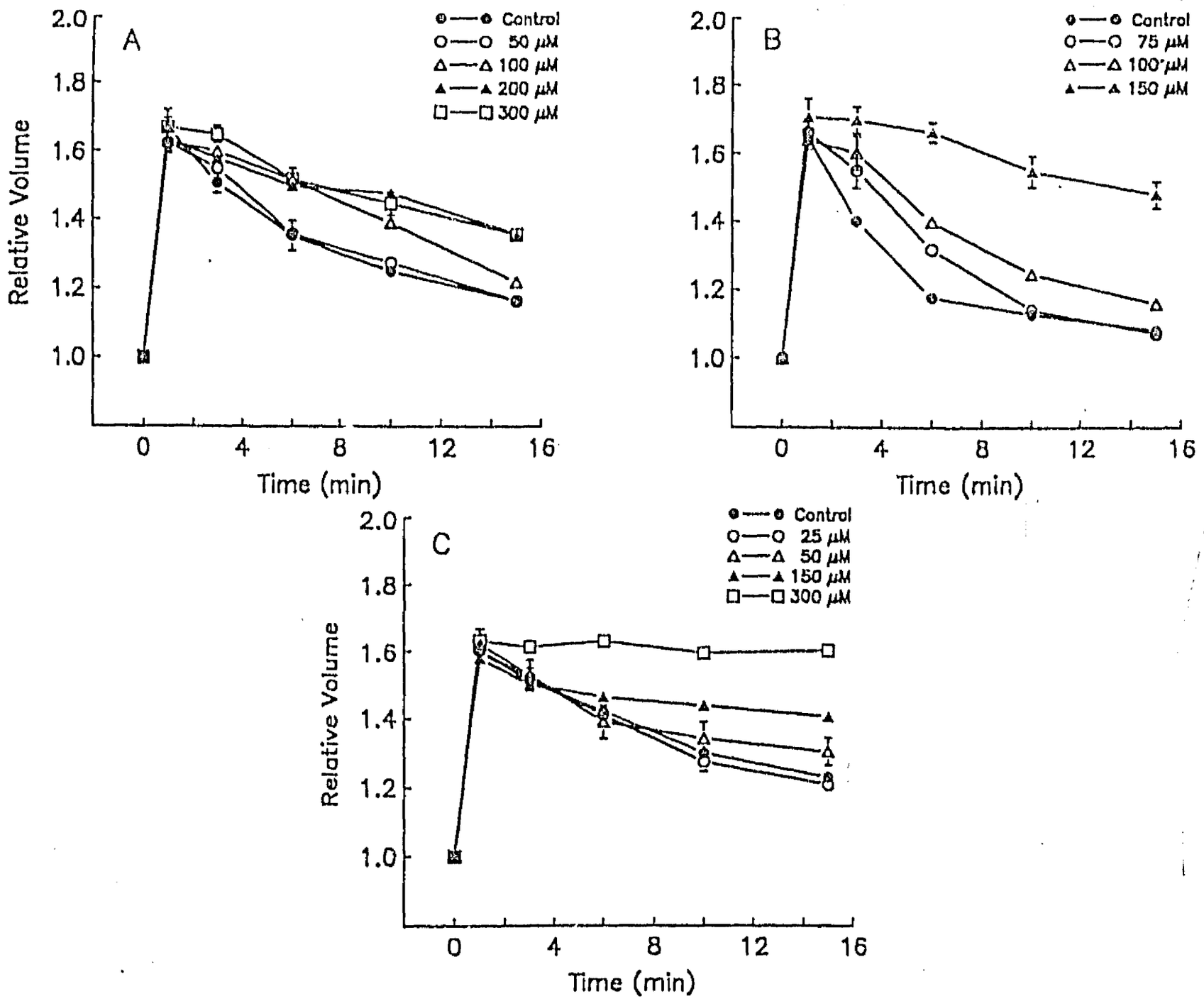

Fig. 1. Effect of DIDS (A), dipyridamole (B) and niflumic acid (C) on the regulatory volume decrease in astrocytes under hyposmotic conditions. Cerebellar astrocytes of rat brain were cultured in 60 mm Petri dishes with a basal Eagle's medium containing 10% fetal calf serum. After 2 weeks, cells were detached by treatment with a Ca^{2+}-free phosphate-buffered saline supplemented with 1 mM EDTA and 0.01% trypsin and cell suspension was centrifuged and resuspended in a small volume of isosmotic Krebs-HEPES medium (containing, in mM: NaCl 135, KCl 5, $MgSO_4$ 0.6, $CaCl_2$ 1, KH_2PO_4 1.7, glucose 10, HEPES 10, 320 mOsm). At zero time a sample of the suspension was diluted about 100-fold with isosmotic or 50% hyposmotic media (160 mOsm) in the presence or absence of the tested drugs at the indicated concentrations and cell volume was measured by electronic sizing with a Coulter counter coupled to a particle analyzer (Channelizer model 256). Osmolarity of hyposmotic media was decreased by reducing the concentration of NaCl. Results are expressed as percentage change with time over the volume recorded in isosmotic conditions. Results are means ± S.E.M. of 4–8 experiments.

ously described [21]. Hyposmotic medium was prepared reducing the NaCl concentration from 135 to 60 mM.

The following compounds were examined for their ability to block RVD in astrocytes after exposure to hyposmotic (50%) medium: 9-AC, DPC, DIDS, dipyridamole and niflumic acid. From this group of drugs, 9-AC and DPC are Cl^- channel blockers but have not been implicated in the functioning of the anion exchanger. DIDS and dipyridamole have reported actions on both Cl^- channels and exchangers whereas niflumic acid is a well known inhibitor of the anion exchanger but has not been recognized as a Cl^- channel blocker. Fig. 1 shows the effect of these drugs on RVD. DPC and 9-AC did not significantly affect RVD (not shown). Dipyridamole exhibited a concentration dependent inhibitory effect on RVD causing a marked reduction at 150 μM, which is the highest concentration that could be used without affecting cell viability. Niflumic acid was the most potent inhibitor totally blocking RVD at 300 μM (Fig. 1B,C). DIDS up to 400 μM produced only a partial inhibition of RVD (Fig. 1A). These drugs also decreased the hyposmolarity-activated taurine efflux with about the same potency order as for RVD. Compounds with the strongest effect were niflumic acid and dipyridamole, followed by DIDS (Fig. 2A), whereas DPC and 9-AC caused only a weak inhibition (not shown). The swelling activated fluxes of another amino acid, D-aspartate, (used as a non metabolized analogue of glutamate) [18], were also

C

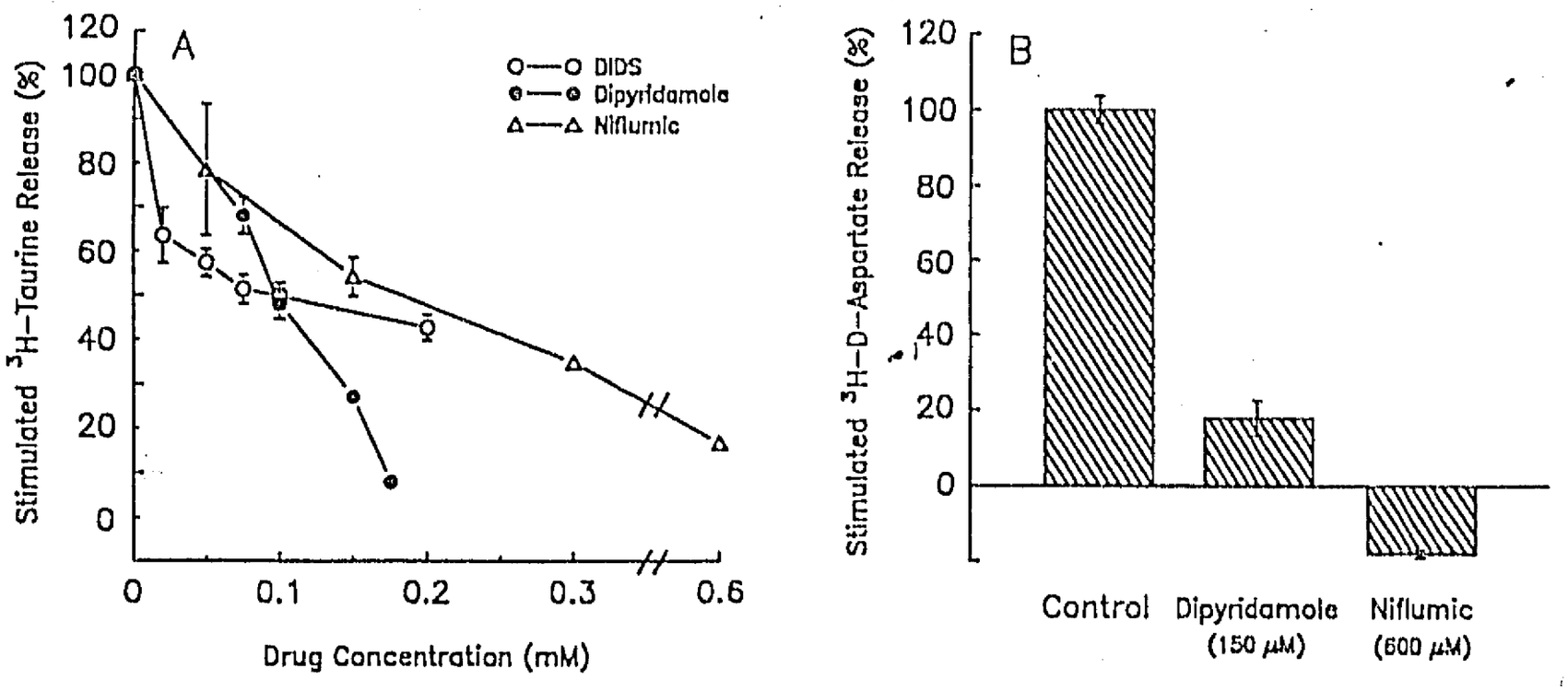

Fig. 2. Effect of dipyridamole and niflumic acid on the hyposmolarity-induced release of labeled taurine (A) and D-aspartate (B) from astrocytes. Cerebellar astrocytes were cultured in 24-well multitest dishes as described in Fig. 1. Prior to release experiments, cells were incubated 1 h with [^{3}H]taurine (1 μCi/ml) or 30 min with [^{3}H]D-aspartate (0.5 μCi/ml) in culture medium. After 4 washes of 10 min, cells were incubated during 5 min with isosmotic (basal release) or 50% hyposmotic (stimulated release) Krebs-HEPES medium containing the indicated concentrations of the tested drugs. Drugs were present during the last 20 min prior to stimulation. At the end of the experiments, radioactivity in media and that remaining in the cells was measured by scintillation spectrometry. Results are expressed as net stimulated release, i.e the difference between basal and stimulated release considerind the stimulated release in control as 100%. Basal and stimulated release of [^{3}H]taurine represented 13.4 ± 0.7% and 64.2 ± 2.2% of total radioactivity, respectively. For basal and stimulated [^{3}H]D-aspartate release values were 17.8 ± 0.6% and 59.8 ± 2.1%, respectively. Values are means ± S.E.M. of 4–24 experiments.

strongly inhibited by niflumic acid and dipyridamole (Fig. 2B).

From these results it seems clear that among the Cl^- channel blockers, those with the highest potency to inhibit RVD and osmolyte fluxes in astrocytes are those also related to the anion exchanger, supporting a role for this molecule in RVD. However, experiments of anion replacement next described were inconsistent with this notion. When Cl^- was replaced by the impermeant anion gluconate or by *N*-methyl-D-glucamine$^+$, a very large and impermeant cation, RVD was not inhibited but rather slightly accelerated (results not shown). This ion replacement is without effect on the volume activated taurine efflux [20]. Since in the absence of an external anion, the exchanger activity must be markedly reduced, the results on ion replacement and the pharmacological sensitivity of RVD and osmolyte fluxes are in apparent contradiction. An interpretation that could reconcile this discrepancy is that the anion exchanger is in fact involved in RVD but that it functions essentially as an unidirectional Cl^- channel rather than as an exchanger. Reports exist indicating that this may indeed occur [6, 14, 15]. Alternatively, other Cl^- channels independent of any exchanger activity but sensitive to the drugs tested here may be involved in RVD.

The pharmacological sensitivity of RVD and Cl^- fluxes to Cl^- channel inhibitors widely varies among the different cells. DIDS is only a partial inhibitor of RVD and Cl^- fluxes in most cells and may be even totally ineffective as in Ehrlich ascites cells [10]. Our present knowledge about the meaning of these differences in terms of the nature of the Cl^- channels involved in the various cell types is still limited.

The reported effects of niflumic acid and dipyridamole, so far the most potent blockers of RVD in astrocytes, are of much interest and emphasize the role of Cl^- pathways in the volume regulatory process in these cells. Chloride channels of large capacity (about 400 pS) sensitive to DIDS have been described in astrocytes [9, 17]. These channels could participate in RVD although an effect of changes in cell volume on their activity has not been reported so far. In view of the present results it would be of interest to examine the sensitivity to niflumic acid of volume associated Cl^- channels in astrocytes and in other cells. Another interesting observation is that the efflux of taurine was markedly inhibited by the Cl^- channel/exchanger blockers, suggesting either that taurine efflux occurs through the Cl^- pathway itself or that the two fluxes are closely interconnected. The first possibility is not unlikely since a recent report in renal cells shows that amino acids including even the neutral amino acids may permeate through Cl^- channels [1]. If this is the case in astrocytes, these Cl^- channels permeable to amino acids, would represent a most important pathway for volume regulation in astrocytes. The effect of Cl^- channel blockers was not examined directly on Rb^+ (K^+) efflux in this

work but the marked inhibition observed on RVD suggest that K^+ fluxes passively following Cl^- are not occurring in the presence of the drugs and therefore, all osmolyte fluxes are directly or indirectly blocked. Another interpretation of the present results is that niflumic acid and dipyridamole were blocking a common signal for activation of all osmolyte fluxes. This possibility cannot be experimentally confirmed because little is presently known about the mechanisms regulating RVD in astrocytes.

The excellent technical assistance of Ms. Claudia Rodriguez is gratefully acknowledged. This work was supported in part by a grant from DGAPA-UNAM (IN-204589).

1 Banderalli, U. and Roy, G., Anion channels for amino acids in MDCK cells, Am. Physiol. Soc., 263 (1992) 1200–1207.
2 Falke, J.J. and Chan, S.I., Molecular mechanisms of band 3 inhibitors. 1. Transport site inhibitors, Biochemistry, 25 (1986) 7888–7894.
3 Falke, J.J. and Chan, S.I., Molecular mechanisms of band 3 inhibitors. 2. Channel blockers, Biochemistry, 25 (1986)7895–7898
4 Falke, J.J. and Chan, S.I., Molecular mechanisms of band 3 inhibitors. 3. Translocation inhibitors, Biochemistry, 25 (1986) 7899–7906.
5 Franciolini, F. and Petris, A. Chloride channels of biological membranes, Biochim. Biophys. Acta, 1031 (1990) 247–259.
6 Frohlich, O. and King, P.A., Mechanisms of anion transport in the human erythrocyte. In R.B. Gunnard and J.C. Parker (Eds.), Cell Physiology of Blood, Rockefeller Univerity Press, New York, 1988, p Cell Physiology of Blood, pp. 182–192.
7 Garcia-Romeu, F., Cossins, A.R. and Motais, R., Cell volume regulation by trout erythrocytes: characteristics of the transport systems activated by hypotonic swelling, 440 (1991) 547–567.
8 Goldstein, L. and Brill, S.R., Volume-activated taurine efflux from skate erythrocytes: possible band 3 involvement, Am. J. Physiol., 260 (1991) R1014–R1020.
9 Gray, P.T.A. and Ritchie, J.M., A voltage-gated chloride conductance in rat cultured astrocytes, Proc. Soc. Lond. B, 228 (1986) 267–288.
10 Hoffmann, E.K. and Simonsesn, L.O., Membrane mechanisms in volume and pH regulation in vertebrate cells, Physiol. Rev., 69 (1990) 315–382.
11 Kay, M.M.B., Hughes, J., Zagon, I. and Lin, F., Brain membrane protein band 3 performs the same functions as erythrocyte band 3, Proc. Natl. Acad. Sci. USA, 88 (1991) 2778–2782.
12 Kimelberg, H.K., Frangakis, M., Volume regulation in primary astrocyte cultures, Adv. Biosci., 61 (1986) 177–186.
13 Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S. and Waniewski, R.A., Swelling induced release of glutamate, aspartate, and taurine from astrocyte cultures, J. Neurosci., 10 (1990) 1583–1591.
14 Knauf, P.A., Law, F.Y. and Marchant, P.J., Relationship of net chloride flow across the human erythrocyte membrane to the anion exchange mechanism, J. Gen. Physiol., 81 (1983) 95–126.
15 Lepke, S., Becker, A. and Passow, H., Mediation of inorganic anion transport by the hydrophobic domain of mouse erythroid band 3 protein expressed in oocytes of *Xenopus laevis*, Biochim. Biophys. Acta, 1106 (1992) 13–16.
16 McEwan, G.T.A., Brown C.D.A., Hirst, B.H. and Simmons, N.L., Hypo-osmolar stimulation of transepithelial Cl^- secretion in cultured human T_{84} intestinal epithelial layers, Biochim. Biophys. Acta, 1135 (1992) 180–183.
17 Nowak, L., Ascher, P. and Berwald-Netter, Ionic channels in mouse astrocytes in culture, J. Neurosci., 7 (1987) 101–109.
18 Palaiologos, G. Hertz, L. and Schousboe, A., Role of aspartate aminotransferase and mitochondrial dicarboxylate transport for release of endogenously and exogenously supplied neurotransmitter in glutamatergic neurons, Neurochem. Res., 14 (1989) 459–466.
19 Pasantes-Morales, H. and Schousboe, A., Volume regulation in astrocytes: a role for taurine as an osmoeffector, J. Neurosci. Res., 20 (1988) 505–509.
20 Pasantes-Morales, H., Morán, J. and Schousboe, A., Volume-sensitive release of taurine from cultured astrocytes: properties and mechanisms, Glia, 3 (1990) 427–432.
21 Pasantes-Morales, H., Maar, T.E. and Morán, J., Cell volume regulation in cultured cerebellar granule neurons, J. Neurosci. Res., 34 (1993) 219–224.
22 Pasantes-Morales, H., Alavez, S., Sánchez-Olea, R. and Morán, J., Contribution of organic and inorganic osmolytes to volume regulation in rat brain cells in culture, Neurochem. Res., 18 (1993) 445–452.
23 Sánchez-Olea, R., Morán, J., Schousboe, A. and Pasantes-Morales, H., Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion, Neurosci. Lett., 130 (1991) 233–236.
24 Sánchez-Olea, R., Morán, J., Martínez, A. and Pasantes-Morales, H., Volume-activated Rb^+ transport in astrocytes in culture, Am. J. Physiol., 264 (1993) in press.

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TRABAJO 7:

Sánchez-Olea, R., Morán J., Pasantes-Morales, H. 1992. Changes in taurine transport evoked by hyperosmolarity in cultured astrocytes. J. Neurosci. Res. 32:86-92.

Journal of Neuroscience Research 32:86–92 (1992)

Changes in Taurine Transport Evoked by Hyperosmolarity in Cultured Astrocytes

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Cultured astrocytes grown chronically (1–3 days) in medium made hyperosmotic (450 mOsm) with NaCl or sucrose showed an increase in taurine concentration from 294 to 501 nmol/mg protein in NaCl and to 382 nmol/mg protein in sucrose. The effect of hyperosmolarity on taurine uptake and release was examined to investigate whether or not changes in these processes may account for the increase observed in cell taurine content. Hyperosmolarity significantly affected the two components of taurine uptake (i.e., the Na^+-dependent and the diffusional component). The Vmax of the Na^+-dependent, active transport increased 50%, whereas no change was observed in the Km. The diffusion coefficient was markedly decreased by hyperosmolarity, being 2.2×10^{-4} and 6.6×10^{-6} ml/min/mg protein in isosmotic and hyperosmotic conditions, respectively, indicating a blockade of the leak pathway. These changes in the active and passive components of taurine transport were opposite to those induced by hyposmolarity. The effect of hyperosmolarity increasing cell taurine content was insensitive to cycloheximide and colchicine. The basal efflux of taurine from astrocytes also decreased in cells exposed to hyperosmotic medium, indicating that alterations in both influx and efflux of taurine are involved in the mechanism responsible for the increase in taurine levels induced by hyperosmolarity in astrocytes. © 1992 Wiley-Liss, Inc.

Key words: taurine, hyperosmolarity, cultured astrocytes, free amino acids

INTRODUCTION

Increasing evidence suggests an association of taurine with the mechanisms of cell volume regulation in the nervous system (reviewed in Pasantes-Morales and Martin del Rio, 1990). A response of nervous tissue cells to hyposmolarity by massive release of taurine is documented in a variety of preparations, ranging from cultured cells to the intact brain (Pasantes-Morales and Schousboe, 1988; Wade et al., 1988; Solis et al., 1988; Morán et al., 1991). There is also evidence indicating that the adaptive response of brain to hyperosmolarity involves an increase in the intracellular concentration of free amino acids, particularly taurine. An early study by Thurston et al. (1980) in chronic hypernatremic dehydrated mice showed that a decrease in water in the brain is followed by a significant increase in the concentration of some free amino acids, with the largest change corresponding to taurine. Similarly, in Brattelboro rats chronically dehydrated by vasopressin deficiency, taurine concentration in brain is markedly enhanced (Niemminen et al., 1988). A recent study by Olson and Goldfinger (1990) in cultured astrocytes demonstrated an increase in taurine concentration in cells exposed to solutions made hyperosmotic with NaCl. The mechanism implicated in this adaptive response is unknown. The increase in cell taurine may result from stimulation of the taurine uptake system which in cultured astrocytes occurs via active and diffusional components. Alternatively, or in addition, a decreased efflux may contribute to the observed changes. These possibilities may be better explored in an in vitro system as that of cell culture, in which the influence of certain components of the integrated preparation, such as the blood brain barrier, can be avoided. In the present work we examined the features of taurine uptake and release in astrocytes chronically exposed to hyperosmotic conditions.

MATERIALS AND METHODS

Primary cultures of astroglial cells from mouse forebrain were obtained as previously described by Patel and Hunt (1985) and Morán and Patel (1989). Briefly, the dissociated cell suspensions from the forebrain of newborn mice were plated at a density of 225×10^3 cells/cm^2 in 35 mm diameter plastic dishes or 24 multiwell dishes. The culture medium contained Basal Eagle's

Received September 5, 1991; revised November 20, 1991; accepted November 27, 1991.

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Medium Supplemented with 10% heat-inactivated fetal calf serum (Gibco), 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. The purity of cultures was determined by immunostaining counting using a fluorescence microscope. Neurons were stained with a polyclonal antibody against neuron-specific enolase (Dakopatta, CA). Astrocytes were labeled with a polyclonal antibody against glial fibrillary acidic protein (GFAP) (Sigma Co., St. Louis, MO). Fibroblasts were stained with a monoclonal antibody against Thy-1. Cultures were highly enriched in astrocytes containing more than 95% positive cells for GFAP.

After two weeks in vitro, cells were treated with NaCl at the indicated concentrations for different periods of time. At the end of the treatment cells were washed twice with 37°C Krebs-HEPES medium containing the same NaCl concentration as in the treatment (NaCl, 5 mM KCl, 1.17 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES), pH 7.6, and the endogenous amino acids were extracted with ethanol (70% (v/v)). Some cultures were treated with 2 mM guanidinoethane sulfonate (GES) for 72 hr after NaCl treatment. The analysis of ethanol extracts was carried out after amino acid derivatization with O-phtaldialdehyde by reversed phase HPLC according to Geddes and Wood (1984) in a Beckman chromatograph equipped with an Ultrasphere column.

In ^{3}H-taurine uptake experiments, astrocytes grown in either isosmolar or hyperosmolar culture media for 72 hr were incubated in Krebs-HEPES medium with 210 mM NaCl or 150 mM sucrose. Uptake of ^{3}H-taurine (2–4 μCi/ml) was examined in media containing taurine at different concentrations in the range 5–1,000 μM. After incubation for 5 min, uptake was terminated by three successive rapid washes (2 sec) of the cultures. Radioactivity and protein (Lowry et al., 1951) in the cultures were subsequently determined after cell solubilization in 0.4 N NaOH. In all experiments, radioactivity present in cultures briefly exposed (1–2 sec) to ^{3}H-taurine at 0°C was subtracted from the experimental values obtained at 37°C. For release experiments, cells maintained for 72 hr in isosmolar or hyperosmolar culture medium were incubated with ^{3}H-taurine (5 μM, 0.5 μCi/ml) for 30 min. After incubation, the culture medium was replaced with Krebs-HEPES medium containing (in mM) KCl, 5; $CaCl_2$, 1.17; $MgCl_2$, 0.5; HEPES, 10; glucose, 5; and the same NaCl concentration as in culture media. Cells were then superfused for 10 min (wash period) with media of the corresponding osmolarity and superfusion continued for further 50 min. At the end of the experiments, radioactivity in the collected samples, excluding the wash period, and that remaining in the cells was measured by scintillation spectrometry. 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).

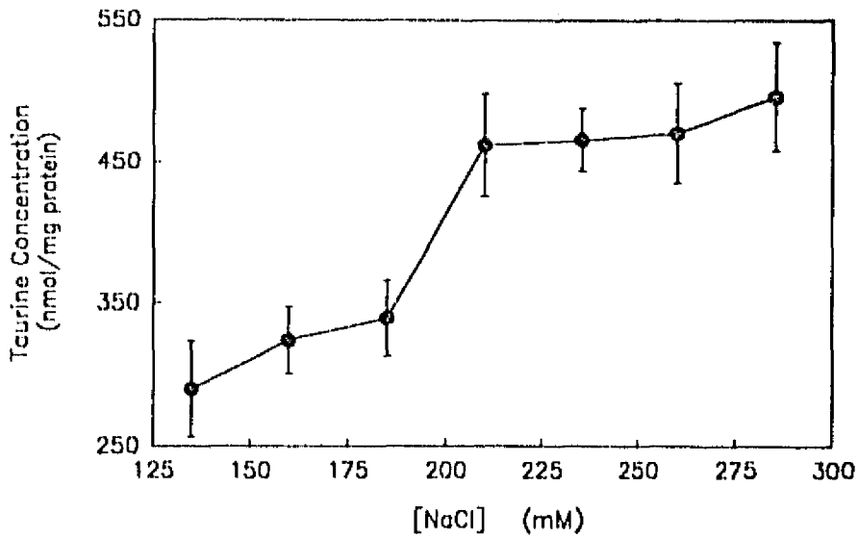

Fig. 1. Effect of hyperosmolarity of solutions with increasing concentrations of NaCl on the taurine content of mouse cerebral cortex astrocytes. Cells were grown in isosmotic conditions (culture medium) for 2 weeks and then NaCl, at the indicated concentrations, was added to the medium to increase osmolarity from 300 mOsmol (135 mM NaCl) to 600 mOsmol (285 mM NaCl). After 24 hr the taurine content of astrocytes was measured in extracts prepared in 70% ethanol and analyzed by HPLC. Results are means ± SEM of 6 cultures.

RESULTS

Effect of Hyperosmolarity on the Concentration of Taurine and Free Amino Acids

The effect of increasing medium osmolarity with NaCl (135 to 285 mM), on taurine levels in astrocytes is shown in Figure 1. Increasing NaCl from 135 mM to 185 mM caused only a small increase in cell taurine content. A marked rise in taurine levels was observed at NaCl concentration of 210 mM (65%) and this is practically a maximum effect, since further increases, up to 285 mM only elicited an increase of 71% (Fig. 1). From these results, hyperosmotic media made by adding 75 mM NaCl (210 mM final concentration, 450 mOsmol) were selected for further experiments to examine the effect of hyperosmolarity. Table I shows the effect of hyperosmolarity on the free amino acid content of astrocytes. The concentration of taurine increased by more than 70% in these conditions and the glutamine content was also markedly enhanced, by about 100%. The concentration of glutamate, alanine + tyrosine, phenylalanine, and glycine + threonine also increased, whereas that of serine, histidine, leucine, isoleucine, valine, and lysine were not affected by hyperosmolarity (Table I). The con-

TABLE I. Free Amino Acid Content of Cultured Cerebral Cortex Astrocytes Grown in Hyperosmolar Conditions*

Amino acid	Amino acid concentration (nmol/mg protein)		
	Isosmolar NaCl 135 mM 300 mOsmol	Hyperosmolar NaCl 210 mM 450 mOsmol	Hyperosmolar Sucrose 150 mM 450 mOsmol
Glutamate	100.5 ± 10.3	135.4 ± 8.4	—
Serine + histidine	23.1 ± 1.5	24.6 ± 2.3	22.2 ± 1.8
Glutamine	228.5 ± 11.9	457.8 ± 45.3	396.3 ± 27.9
Glycine + threonine	32.5 ± 2.2	54.5 ± 4.2	46.5 ± 3.9
Taurine	290.0 ± 19.2	501.7 ± 36.2	382.0 ± 19.5
Alanine + tyrosine	41.8 ± 8.7	58.3 ± 8.9	59.4 ± 10.0
Phenylalanine	5.9 ± 1.5	10.0 ± 1.4	11.5 ± 1.6
Valine	9.7 ± 2.4	8.6 ± 1.2	8.9 ± 1.6
Isoleucine	5.3 ± 0.8	5.6 ± 3.9	3.9 ± 0.7
Lysine	4.9 ± 0.3	4.3 ± 0.6	4.1 ± 0.4

*Cells were grown in isosmolar medium for 2 weeks. After that period, 75 mM NaCl or 150 mM sucrose was added to the culture medium. After 24 hr, the free amino acid content of cells was measured in extracts prepared in 70% ethanol and assayed by HPLC. Results are means ± SEM of 6–12 cultures.

centration of taurine and free amino acids in medium made hyperosmotic with sucrose is also shown in Table I. The same amino acids were affected by hyperosmolarity in this condition, but the observed increases were not as pronounced as those in medium with high NaCl concentration.

The time course of taurine increase induced by hyperosmolarity is shown in Figure 2. A significant rise in cell taurine content was observed only after 6 hr of exposure to the hyperosmotic medium. The maximal elevation occurred at about 12 hr and after this time a plateau was reached (Fig. 2). Associated with the increase in cell taurine content in response to hyperosmolarity, a decrease in taurine levels in the culture medium from 18 μM to 11 μM was observed.

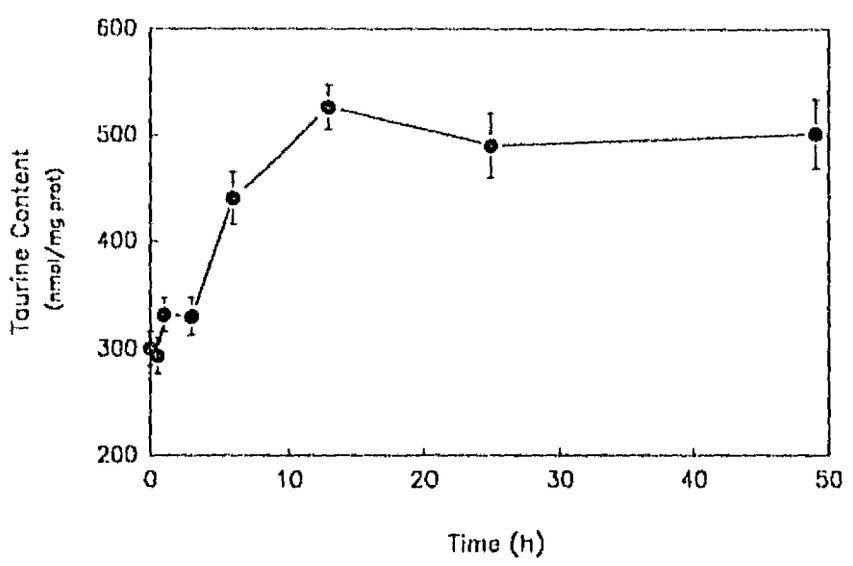

Fig. 2. Time course of hyposmolarity induced increase in taurine content of astrocytes. Cells were grown for 2 weeks in culture medium (isosmotic) and then 75 mM NaCl was added for the period of time indicated. At the end of the treatment the taurine concentration in cells was measured by HPLC. Results are means ± SEM of 12 cultures.

Taurine Uptake

Since hyperosmotic solutions were prepared by increasing the concentration of NaCl, accumulation of ^{3}H-taurine (25 μM) by cells growing in isosmotic conditions was examined in media containing increasing concentrations of NaCl from 0 to 285 mM. Figure 3 shows that taurine uptake was markedly activated by NaCl, with the highest increase observed between 0 and 100 mM. At higher NaCl concentrations there is still a small but continuous increase of taurine uptake, so that at the NaCl concentration used to prepare hyperosmotic solutions (210 mM), taurine uptake was about 30% higher than that observed at the Na concentration of isosmotic medium (135 mM).

The effect of hyperosmotic conditions on the kinetics of ^{3}H-taurine uptake is shown in Figure 4. For these experiments, cells were grown for 2 weeks and then exposed for 3 days to culture medium containing additional 75 mM NaCl (210 mM NaCl, final concentration 450 mOsmol). After the hyperosmotic treatment, the culture medium was replaced by a Krebs-HEPES medium, also containing 210 mM NaCl. Controls were grown in isosmolar medium but for the uptake experiments the medium also contained 210 mM NaCl in order to avoid differences due to the concentration of Na^+, which may influence the uptake process. The kinetic curves of astrocytes exposed to hyperosmolar solutions were markedly different from those in controls (Fig. 4). An increase of the saturable component was observed in the curve, a change reflected by the higher value of the Vmax shown in Table II. The Km value, in contrast, was not affected by hyperosmolarity (Table II). The most

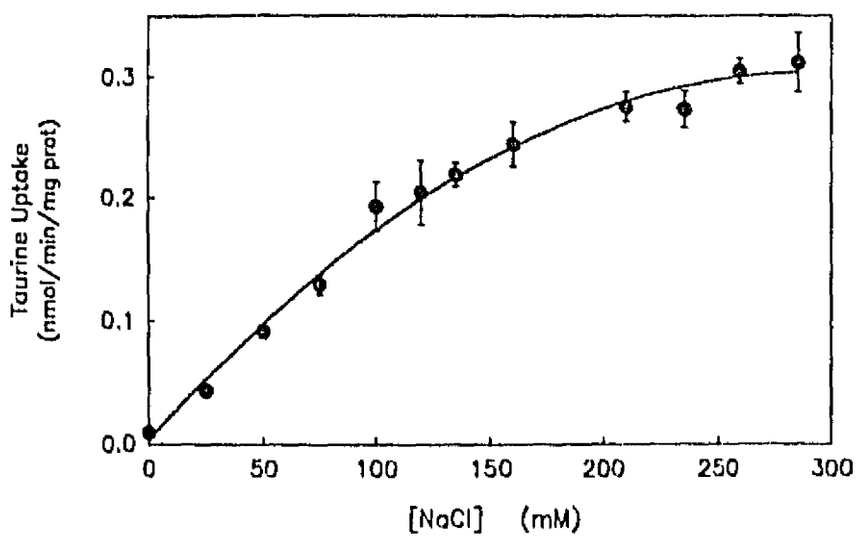

Fig. 3. Sodium-dependence of ^{3}H-taurine uptake by cultured astrocytes. Cells were grown in 24-well multitest as described in Materials and Methods. For uptake experiments, the culture medium was replaced by Krebs-HEPES medium containing the indicated concentrations of NaCl. When the concentrations of NaCl were lower than 150 mM, the osmolarity was maintained with choline chloride. Solutions containing NaCl concentrations higher than 150 mM were correspondingly hyperosmotic. Cells were incubated with ^{3}H-taurine (3 μCi/mol, 25 μM) for 5 min at 37°C. After this time, cells were washed and accumulated radioactivity was determined by scintillation spectrometry. The points in the curve are means ± SEM of 6 cultures.

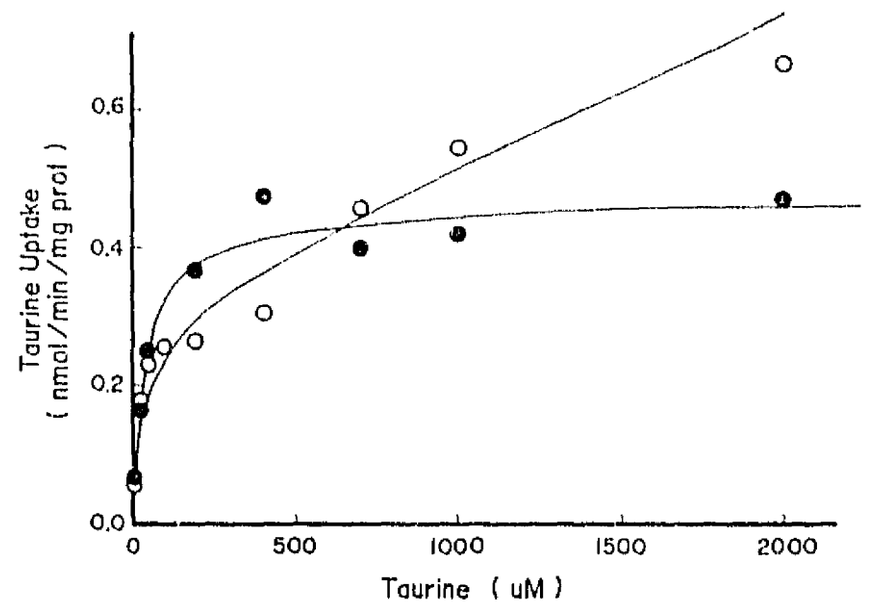

Fig. 4. Effect of hyperosmolarity on ^{3}H-taurine uptake by cultured astrocytes. Cells were grown for 2 weeks and then exposed for 3 days to culture medium made hyperosmotic with NaCl (450 mOsm). After this time, the culture medium was replaced by media containing ^{3}H-taurine (3–6 μCi/ml and unlabelled taurine in concentrations ranging 5–2,000 μM and the same concentration of NaCl as in the culture medium. Controls were cells grown in isosmotic medium, but taurine uptake was measured in media made hyperosmotic with NaCl. (○) control; (●) hyperosmotic. Results are means of 12 cultures.

striking difference, however, was detected in the diffusional component, which was markedly reduced in cells exposed to hyperosmotic conditions (Fig. 4, Table II). In other experiments cells were exposed to hyperosmotic medium for 3 days and uptake experiments were carried out in media made hyperosmotic with sucrose. Results were essentially similar to those obtained in hyperosmotic medium with NaCl.

TABLE II. Effect of Hyperosmolarity on the Kinetic Parameters of Taurine Uptake by Cultured Astrocytes*

Kinetic parameters	Isosmolar 300 mOsmol	Hyperosmolar 450 mOsmol
Km (μM)	38.6	41.0
Vmax (nmol/min/mg protein)	0.30	0.45
K (ml/min/mg protein)	2.26×10^{-4}	6.58×10^{-6}

*Cells were grown in isosmolar medium for 2 weeks. After that period 75 mM NaCl was added to the culture medium, and after 3 days of this treatment, the culture medium was replaced by Krebs-HEPES medium containing an additional 75 mM NaCl for cells grown in both isosmolar and hyperosmolar conditions. ^{3}H-taurine uptake was measured as described in Figure 4. Results are means ± SEM of 12 cultures.

The observed changes in the kinetic constants suggest an increase in active transporters either exposed or newly synthesized. To investigate these possibilities, the effect of hyperosmolarity on astrocyte taurine content was examined in cells treated for 24 hr with cycloheximide (10 μg/ml) to block protein synthesis or with colchicine (100 μM) to disrupt carrier transport processes mediated by the cytoskeleton. Although these treatments decreased the intracellular taurine levels in isosmotic conditions, none of the drugs affected the increase evoked by hyperosmolarity (Results not shown).

Taurine Efflux

The decrease observed in the diffusion component of taurine uptake suggests that the passive transmembrane fluxes of the amino acid were affected by hyperosmolarity. Since this leak pathway may mobilize taurine in both directions, hyperosmolarity could affect taurine efflux as well. To further examine this possibility, the basal release of ^{3}H-taurine from cells cultured in normal or hyperosmotic media was compared and results are shown in Figure 5. The spontaneous efflux of taurine was lower in cells grown in hyperosmotic conditions as shown by the significant differences in the efflux rate constants observed particularly during the first 10 min of superfusion (Fig. 5). This difference persisted through all the superfusion period, although it decreased with time. As result of this lower efflux, the total amount of taurine released in the period examined (50 min) was significantly less in cells exposed to hyperosmotic solutions (7.2% ± 0.57) than in controls in isosmotic conditions (13.6% ± 0.87). In these release experiments, the initial concentration of taurine was higher in cells exposed to hyperosmotic conditions than in isosmotic conditions and this difference might affect the efflux

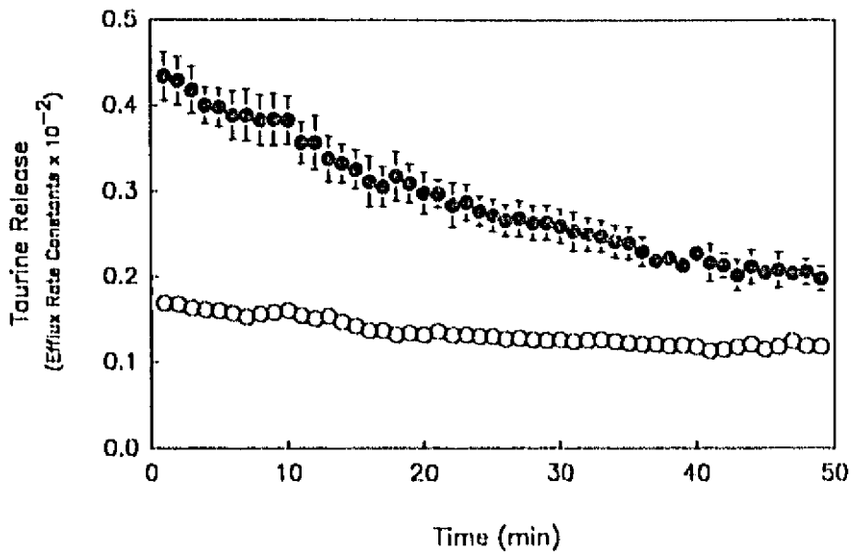

Fig. 5. Effect of hyperosmolarity on the efflux of ^{3}H-taurine from astrocytes. Cells treated as described in Figure 3 were loaded with ^{3}H-taurine and then superfused (0.8 ml/min) with isosmotic (●) or hyperosmotic (○) medium for 50 min. Results represent efflux rate constants and are means ± SEM of 12 cultures.

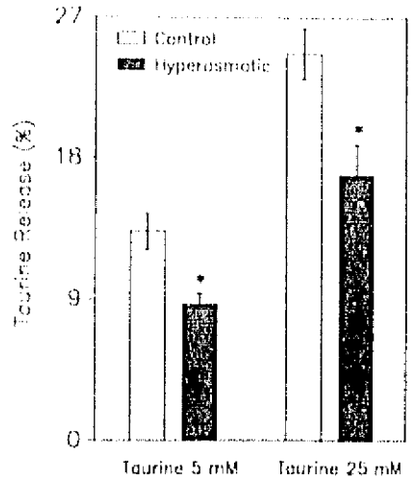

Fig. 6. Effect of increasing intracellular taurine content on the efflux of taurine induced by hyperosmolarity. Cells were exposed for 3 days to hyperosmotic medium and then 5 mM or 25 mM taurine was added to culture medium for a period of 2 hr. After this time, cells were incubated in isosmotic (open bars) or hyperosmotic (filled bars) Krebs-HEPES medium for 5 min. Taurine content was measured at the end of the incubation by HPLC. Results are means ± SEM of 6 cultures. *$P < 0.001$.

rate. To examine this possibility, cells were incubated with external taurine in order to increase the cell taurine content in controls to levels present in cells in hyperosmolar conditions. After an incubation period of 2 hr in the presence of 5 mM or 25 mM taurine, the intracellular concentration of taurine increased from 290 to 494 and 800 nmoles/mg protein in controls and from 500 to 637 and 1161 nmoles/mg protein in cells exposed to hyperosmolar conditions. Then, cells were incubated for 5 min in isosmotic or hyperosmotic medium. The amount of taurine released to the medium and that remaining in the cells was measured and the release expressed as percentage of the initial taurine content. Figure 6 shows that in all cases taurine efflux in hyperosmotic medium was lower than in controls, despite the large differences in initial intracellular taurine levels.

To further investigate the effect of hyperosmolarity on taurine release, the efflux of taurine was followed indirectly by measuring the decrease in cell taurine in conditions of a total blockade of the influx component. This was achieved using a very potent blocker of taurine uptake, guanidino ethanesulphonate (GES), which at 2 mM concentration practically abolished taurine uptake by astrocytes (Morán and Pasantes-Morales, 1991). As a consequence of the blockade of taurine accumulation, the intracellular concentration of the amino acid markedly decreased and the extent of this decrease indicates the rate of loss of cell taurine. Figure 7 compares the decrease in intracellular taurine in cells grown in hyperosmotic or isosmotic conditions and then exposed to GES for 3–6 hr. In cells grown in isosmolar conditions, GES treatment resulted in a decrease of taurine from an initial level of 294 nmol/mg protein to 186 and 107 nmol/mg of protein after 3 and 6 hr, respectively. This change represents an efflux corresponding to 36.7% and 63.6% of the original concentration of taurine. In cells exposed for 3 days to a hyperosmotic medium, the loss of taurine in GES treated cells was significantly lower, from 507 to 415 and 291 nmol/mg protein after 3 and 6 hr of exposure, respectively, to GES (i.e., an efflux corresponding to 18% and 41% more of the intracellular taurine content than that in cells grown in isosmolar media) (Fig. 7).

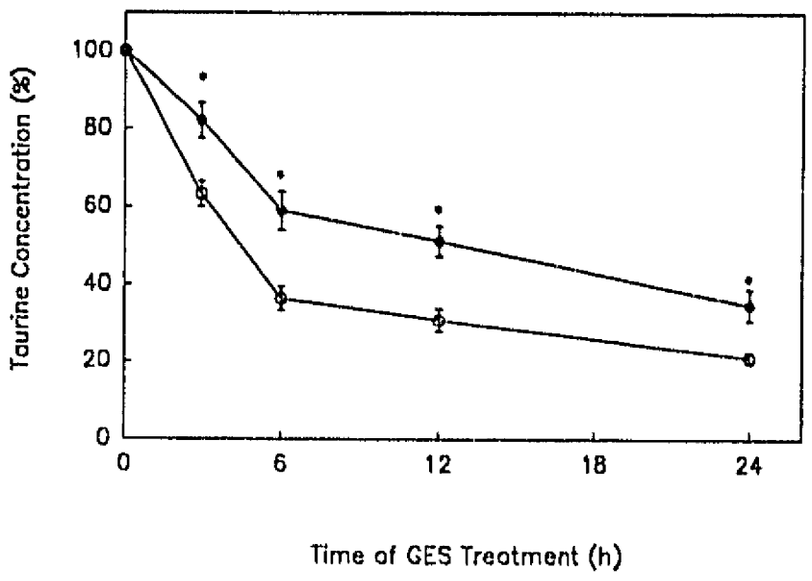

Fig. 7. Effect of hyperosmolarity on the concentration of taurine in GES-treated astrocytes. Cells were exposed to isosmotic (○) or hyperosmotic (●) media and then treated with 2 mM GES. At the time indicated, cell taurine content was measured by HPLC. Results are means ± SEM of 6 cultures. *$P < 0.001$.

DISCUSSION

Our present results confirm the observation of Olson and Goldfinger (1990) on the increase of taurine and

other free amino acids in astrocytes exposed to hyperosmotic conditions. A difference in the cell content of these amino acids found between the two reports is most likely due to differences in the paradigm to increase osmolarity in the culture medium and to the extraction procedure.

From the results of the present study it seems clear that changes in the mechanisms of taurine transport are associated with and presumably account for the increase in taurine content in astrocytes exposed to hyperosmolar conditions. Taurine uptake in most cells, including astrocytes, occurs via a Na^+- and energy-dependent component, with high affinity for the amino acid, and a nonsaturable process in which the flux of taurine is a linear function of the external taurine concentration (Schousboe et al., 1976; Holopainen et al., 1987). The present work has shown that hyperosmolarity stimulates the sodium-dependent active carrier concomitant with a marked decrease in efflux through the diffusible pathway. The observed change in the Na^+-dependent component of taurine uptake seems not be mediated by modifications in the affinity of taurine for the carrier, since the Km was unaffected by hyperosmolarity. In contrast, the increase in the Vmax suggests that the turnover rate of the transporter has increased in these cells. It is noteworthy that remarkably similar changes in the transport of other organic osmolytes have been observed in renal cells in culture. When these cells are grown in hyperosmotic medium, an increase in uptake of sorbitol, inositol, and betaine is observed. This change is due to increases in the Vmax of the transporters without any change in Km (Nakanishi et al., 1989).

For taurine, the observed increase in the Vmax may be a consequence in part, of changes in the transmembrane potential caused by the high concentrations of NaCl present in the hyperosmotic media, since taurine uptake was stimulated to certain extent by increasing extracellular NaCl in isosmotic solutions. This stimulatory effect may account for the early increase observed in taurine concentration (i.e., from 1–3 hr). The observed adaptive changes to hyperosmolarity of taurine uptake observed over the long term cannot be interpreted as result of synthesis de novo of the protein carrier, since cycloheximide did not prevent the effect of hyperosmolarity on the cell's taurine content. Another possibility is that the number of transporters increase by insertion in the membrane of preformed carriers from a cytoplasmic pool, as has been shown to occur in renal cells in studies by Chesney et al. (1989). An upregulation of taurine transporters not involving synthesis de novo has been demonstrated in these cells and the process depends on the cytoskeleton and, consequently, is sensitive to colchicine. This drug was ineffective in counteracting the effect of hyperosmolarity on taurine levels in astrocytes, suggesting either that there is no upregulation of the carriers in astrocytes or that the process of transporter insertion does not require the integrity of the cytoskeleton. At present, the mechanism activating the Na^+-dependent taurine uptake by hyperosmolarity remains to be clarified.

The most striking effect of hyperosmolarity on the kinetics of taurine uptake observed in this study was the marked reduction of the nonsaturable component of taurine transport. This observation suggests that the transmembrane fluxes of taurine occurring via this transport mechanism are inhibited by hyperosmolarity. This assumption is supported by the decrease in the efflux of ^{3}H-taurine observed in cells exposed to hyperosmolarity. Along the same line is the finding that in cells treated with hyperosmotic solutions in the presence of GES, which eliminates taurine uptake, there is a delay in the efflux of intracellular taurine.

It seems clear that both the increase in taurine uptake through the sodium-dependent carrier and the decrease in the efflux presumably occurring through a leaky pathway are responsible for the increase in taurine levels in response to hyperosmolarity. It is interesting to note that the changes observed in the active and the diffusional components of taurine transport in hyperosmolar conditions are essentially opposite of those caused by hyposmolarity. In astrocytes exposed to hyposmolar solutions, a rapid release of taurine occurs which markedly reduces the intracellular taurine content (Pasantes-Morales and Schousboe, 1988; Pasantes-Morales et al., 1990). It was shown that in these conditions, the energy dependent component of taurine transport is slightly inhibited, whereas the diffusional component is markedly increased (Sánchez Olea et al., 1991). Moreover, in experiments carried out in the absence of sodium (i.e., in a condition in which the active carrier is not functional), the taurine fluxes activated by hyposmolarity are directed only by the concentration gradient of taurine, indicating that the hyposmolarity sensitive transmembrane fluxes of the amino acid take place essentially via the diffusional pathway (Sánchez Olea et al., 1991).

The properties of the diffusional component of taurine transport which appears to be importantly involved in the adaptive response of astrocytes to changes in external osmolarity remain to be determined. It is unclear whether or not it represents a passive, protein-mediated bidirectional transport or a simple transbilayer bidirectional diffussive translocation.

The increase in cell taurine concentration subsequent to hyperosmolarity is suggestive of its involvement in the adaptive response of astrocytes to this situation. The mechanism to enhance cell taurine content described in this work may be operating also in the intact brain, although in this preparation, other factors, such as the development of the blood-brain barrier, have to be con-

sidered. The nature of the potential beneficial actions of taurine has to be determined. It may be related to a role as an osmotically active solute and/or to a function as an osmoprotectant (i.e., counteracting the perturbing actions of high concentrations of inorganic solutes). This is likely to occur in conditions when hyperosmolarity is obtained, increasing the concentration of NaCl, which may have adverse effects on protein functions. This effect of organic osmolytes as compatible solutes is well documented in other preparations (Yancey et al., 1982). Studies are now in progress to further examine these possibilities for taurine in astrocytes.

ACKNOWLEDGMENTS

The expert technical assistance of Ms. Claudia Rodriguez and Ms. Irma López Martínez is highly appreciated. We thank Dr. Hiram Olivera for the program used to calculate the kinetic parameters. This study was supported by grants from DGAPA-UNAM (IN-024589) and from the Stiftung Volkswagenverk. We gratefully acknowledge the support of Prof. Bernd Hamprecht, who acted as the German partner in the "Program of Partnerships" of the Stiftung Volkswagenverk.

REFERENCES

Chesney RW, Jolly K, Zelikovic I, Iwahashi C, Lohstroh P (1989): Increased Na^+-taurine symporter in rat renal brush border membranes: Preformed or newly synthesized? FASEB J 3: 2081–2085.

Geddes JW, Wood JD (1991): Changes in the amino acid content of nerve endings (synaptosomes) induced by drugs that alter the metabolism of glutamate and gamma-aminobutyric acid. J Neurochem 42:16–23.

Holopainen I, Malminen O, Kontro P (1987): Sodium-dependent high-affinity uptake of taurine in cultured cerebellar granule cells and astrocytes. J Neurosci Res 18:479–483.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275.

Morán J, Pasantes-Morales H (1991): Taurine-deficient cultured cerebellar astrocytes and granule neurons obtained by treatment with guanidinoethane sulfonate. J Neurosci Res 29:533–537.

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

Morán J, Hurtado S, Pasantes-Morales H (1991): Similar properties of taurine release induced by potassium and hyposmolarity in the rat retina. Exp Eye Res 53:347–352.

Nakanishi TR, Turner RJ, Burg MB (1989): Osmoregulatory changes in myo-inositol transport by renal cells. Proc Natl Acad Sci USA 86:6002–6006.

Nieminen ML, Tuomisto L, Solatunturi E, Eriksson L, Paasonen MK (1988): Taurine in the osmoregulation of the Brattleboro rat. Life Sci 42:2137–2143.

Olson JE, Goldfinger MD (1990): Amino acid content of rat cerebral astrocytes adapted to hyperosmotic medium in vitro. J Neurosci Res 27:241–246.

Pasantes-Morales H, Martín del Río M (1990): Taurine and mechanisms of cell volume regulation. In: Pasantes-Morales H, Martín DL, Shain W, Martin del Río R, (eds): "Taurine: Functional Neurochemistry, Physiology, and Cardiology." New York: Wiley-Liss Inc., pp 317–328.

Pasantes-Morales H, Schousboe A (1988): Volume regulation in astrocytes: A role for taurine as an osmoeffector. J Neurosci Res 20:505–509.

Pasantes-Morales H, Morán J, Schousboe A (1990): Volume-sensitive release of taurine from cultured astrocytes: properties and mechanism. Glia 3:427–432.

Patel AJ, Hunt A (1985): Observations on cell growth and regulation of glutamine synthetase by dexamethasone in primary cultures of forebrain and cerebellar astrocytes. Dev Brain Res 18:175–184.

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

Schousboe A, Fosmark H, Svenneby G (1976): Taurine uptake in astrocytes cultured from dissociated mouse brain hemispheres. Brain Res 116:158–164.

Solís JM, Herranz AS, Herrera O, Lerma J, Martín del Río M (1988): Low chloride-dependent release of taurine by a furosemide sensitive process in the in vivo rat hippocampus. Neuroscience 24:885–891.

Thurston JH, Hauhart RE, Direo JA (1980): Taurine: A role in osmotic regulation of mammalian brain and possible clinical significance. Life Sci 26:1561–1568.

Wade JV, Olson JP, Samson, Nelson SR, Pazdernik TL (1988): A possible role for taurine in osmoregulation within the brain. J Neurochem 51:740–745.

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982): Living with water stress: Evolution of osmolyte systems. Science 217:1214–1222.

DISCUSION GENERAL.

1) IMPORTANCIA RELATIVA DE OSMOLITOS ORGANICOS E INORGANICOS EN EL PROCESO DE REGULACION DE VOLUMEN EN CELULAS NERVIOSAS.

La capacidad de regulación del volumen celular en condiciones anisosmóticas se ha documentado en la mayoría de las células animales en las que se ha estudiado este proceso, incluyendo células tan diversas como eritrocitos (Kregenow, 1971; Cala et al., 1986), linfocitos (Grinstein y Foskett, 1990), células de Ehrlich (Hoffmann y Simonsen, 1989), células renales (Larson y Spring, 1987; Roy y Sauvé, 1987). Sin embargo la presencia de este proceso no es universal, ya que sólo una población limitada de eritrocitos humanos es capaz de recuperar su volumen en condiciones hiposmóticas (O'Neill, 1989) y esta propiedad se encuentra ausente en células del túbulo renal distal (Guggino et al., 1985). Las células nerviosas en cultivo, tanto neuronas como astrocitos, poseen los mecanismos necesarios para recuperar su volumen inicial después que éste se ha incrementado por exposición a soluciones hiposmóticas (Pasantes-Morales y Schousboe, 1988; Pasantes-Morales et al., 1992; trabajo 1, figura 1).

La recuperación del volumen en condiciones de hiposmolaridad es posible a través de una disminución en la concentración de solutos osmóticamente activos (osmolitos) en el interior de la célula, lo que determina una reducción inmediata en el contenido intracelular de agua. Estos osmolitos pueden ser clasificados en dos grandes

grupos: 1) iones inorgánicos, principalmente potasio y cloruro, y 2) compuestos orgánicos pequeños, como aminoácidos libres (Gilles, 1979; Pasantes-Morales et al., 1988), poliaminas (Yamauchi, 1992) y polialcoholes (Siebens y Spring, 1989). En los organismos acuáticos este último grupo de osmolitos desempeña un papel fundamental en los procesos de recuperación de volumen, mientras que los iones inorgánicos cumplen una función complementaria (Smith y Pierce, 1987). En células de organismos terrestres la situación parece ser la inversa: los iones potasio y cloruro son los que mayoritariamente contribuyen al proceso regulador de volumen y los osmolitos orgánicos cumplen una función secundaria. En células renales en cultivo (MDCK) (Roy y Sauvé, 1987; Sánchez-Olea et al., 1991) y en linfocitos humanos (Pasantes-Morales et al., 1991) se ha calculado que los aminoácidos libres constituyen entre un 25 y 30% de la disminución total de solutos en condiciones hiposmóticas.

En los resultados obtenidos en el presente trabajo es evidente que en los astrocitos la liberación de potasio (utilizando rubidio como marcador) es poco sensible a incrementos en el volumen celular producidos por la exposición a soluciones hiposmóticas. La salida de potasio se incrementa sobre el valor basal únicamente con reducciones extremas en la osmolaridad externa (50% o mayores), mientras que disminuciones de 15 o 30% no estimulan significativamente la liberación espontánea (Trabajo 1, figura 2). Estos resultados contrastan con la gran sensibilidad que muestran otros tipos celulares para liberar potasio en respuesta a

incrementos pequeños en el volumen celular: en células MDCK una reducción del 15% es suficiente para estimular la liberación de potasio (Roy y Sauvé, 1987); en hepatocitos un incremento en el volumen celular (aprox. 15%) producido por la acumulación de alanina (internalizada simultáneamente con sodio y cloro) estimula la liberación de potasio (Kristensen, 1980; Kristensen y Folke, 1984); igualmente, en células intestinales el aumento de volumen (de aproximadamente 5%) producido por la acumulación de glucosa estimula la salida de potasio (MacLeod y Hamilton, 1991).

El alto valor umbral que presentan los astrocitos para la liberación de potasio posiblemente se explique con base en el papel fisiológico que desempeñan estas células dentro del sistema nervioso. Se ha propuesto que una de las funciones de los astrocitos es precisamente el de mantener concentraciones reducidas de potasio (aproximadamente 5 mM) en el espacio intercelular (Hertz, 1978; Walz y Hinks, 1985). De acuerdo con esta propuesta se han descrito un gran número de canales y transportadores por los cuales el potasio es acumulado activamente en el interior de los astrocitos (Gardner-Medwin et al., 1981, Walz, 1987). Es muy probable que el empleo del potasio como un osmolito en el sistema nervioso se encuentre limitado debido a que la excitabilidad general de las células nerviosas depende en gran medida de la concentración extracelular de este catión (el potencial de membrana muestra siempre un valor muy próximo al potencial de equilibrio electroquímico para el potasio, calculado de acuerdo a la ecuación de Nernst).

En los astrocitos y neuronas en cultivo la liberación de aminoácidos libres, a diferencia del potasio, muestra una alta sensibilidad a disminuciones en la osmolaridad del medio externo (Pasantes-Morales et al., 1993). Reducciones en la osmolaridad tan pequeñas como del 5-10% son suficientes para estimular la liberación de taurina sobre el valor basal observado en condiciones isosmóticas (Pasantes-Morales y Schousboe, 1988). El papel fisiológico de la taurina como osmolito se encuentra ampliamente documentado en el sistema nervioso, tanto en estudios *in vitro* (Pasantes-Morales y Schousboe, 1988) como *in vivo* (Thurston et al., 1980). Es interesante mencionar que la exposición de los astrocitos en cultivo a condiciones hiperosmóticas produce un incremento notable en los niveles intracelulares de aminoácidos libres (Olson y Goldfinger, 1990; Ver última sección), lo cual apoya la propuesta del papel de los aminoácidos como osmolitos en el sistema nervioso.

2) MECANISMO DE LIBERACION DE POTASIO EN RESPUESTA A INCREMENTOS EXTREMOS EN EL VOLUMEN CELULAR.

A pesar de que en astrocitos en cultivo el potasio no parece ser utilizado como osmolito en respuesta a disminuciones pequeñas en la osmolaridad, es evidente (Trabajo 1, figura 2) que con reducciones de 50% o mayores en la osmolaridad externa efectivamente se activa un mecanismo de transporte que produce una liberación de este

catión. El mecanismo involucrado en la liberación de potasio en respuesta a un incremento en el volumen celular depende del tipo celular de que se trate. Como se mencionó en la introducción, existen al menos tres mecanismos posibles: 1) El cotransportador $K^+/Na^+/2Cl^-$ o K^+/Cl^- (Cala, 1990); 2) la activación simultánea de dos antiportadores: Cl^-/HCO_3^- y K^+/H^+ (Cala, 1983); o 3) la salida a través de un canal iónico (Hoffmann, 1987; Grinstein et al., 1987). En los astrocitos el cotransportador $K^+/Na^+/2Cl^-$ o K^+/Cl^- parece no ser el mecanismo de salida de potasio debido a que la liberación no se afectó en presencia de bumetanida (Trabajo 1, figura 3), un fármaco que selectivamente inhibe este sistema de transporte (Kimelberg y Frangakis, 1985); otro resultado que descarta la participación de este sistema de transporte es el que la liberación de potasio ocurre normalmente en astrocitos donde el cloro intracelular se ha reemplazado por nitrato (Trabajo 1, figura 4), mientras que la actividad del cotransportador $K^+/Na^+/2Cl^-$ o K^+/Cl^- se inhibe por completo cuando el cloro es substituído por este anión (Hoffmann y Simonsen, 1989; Sarkady y Parker, 1991). Finalmente, la poca sensibilidad del proceso de liberación del potasio a la temperatura (Trabajo 1, figura 11) también es un resultado que descarta la participación de un transportador.

La participación de un canal iónico en la salida de potasio sensible a volumen en los astrocitos es sugerida por el efecto inhibidor de la quinidina (Trabajo 1, figura 7), un fármaco que interactúa con canales de potasio. En las células en donde la

liberación de potasio en respuesta a un aumento de volumen ocurre a través de un canal iónico, la salida de potasio es interdependiente de la salida de cloro (Hoffmann y Simonsen, 1989). En los astrocitos es muy claro que existe una relación muy estrecha entre la movilización de potasio y la de cloro. Por una parte, la liberación de potasio se inhibió, de manera dependiente de la concentración, en presencia de inhibidores de canales de cloro como DIDS y furosemida (Trabajo 1, figuras 9 y 3, respectivamente). Además, la salida de potasio se redujo significativamente al disminuir los niveles intracelulares de cloro a través de la incubación en un medio en donde el cloro se substituyó por el anión impermeante gluconato (Trabajo 1, figura 4). Sin embargo, el reemplazamiento de cloro intracelular por el anión permeante nitrato no disminuyó la salida de rubidio (Trabajo 1, figura 4), lo que sugiere que la salida del catión debe acoplarse con la movilización de algún anión, independientemente de que éste sea cloruro o nitrato.

Es muy posible que la liberación de potasio sensible a volumen ocurra a través de los canales que se han descrito se activan por la distensión de la membrana celular (Guharay y Sachs, 1984; Ubl et al., 1988; Falke y Misler, 1989; Sakin, 1989; Sachs, 1991). Recientemente, se describió en astrocitos en cultivo un canal selectivo para potasio que se activa en respuesta a un incremento de volumen producido por soluciones hiposmóticas (Islas et al., 1993). En este trabajo se demostró que el cloro no es capaz de

utilizar el mismo canal que el potasio, lo que sugiere que, a pesar de que la salida de potasio y cloro sensible a volumen es interdependiente, los iones son movilizados a través de canales distintos.

3) CAMBIOS EN LOS NIVELES INTRACELULARES DE CALCIO COMO POSIBLE SEÑAL INTRACELULAR EN EL PROCESO DE LIBERACION DE OSMOLITOS.

Aún no se ha determinado si la liberación de osmolitos observada en condiciones hiposmóticas es iniciada únicamente por la distensión en la membrana celular producida por el incremento de volumen, o si estos mecanismos de liberación son activados por alguna señal intracelular producida como consecuencia del incremento de volumen. Algunos investigadores han propuesto que la activación de canales iónicos sensibles a la distensión membranal es suficiente para iniciar el proceso de regulación de volumen (Guharay y Sachs, 1984; Falke y Misler, 1989; Sakin, 1989; Sachs, 1991; Islas et al., 1993). Igualmente, se ha sugerido que la participación de segundos mensajeros como el AMP cíclico (Mills y Skiest, 1985; Watson, 1990) o el calcio (Pierce y Politis, 1990) podría desempeñar una función crítica en la liberación de osmolitos.

La exposición a soluciones hiposmóticas produce un aumento en la permeabilidad de la membrana celular al calcio, medido como entrada de ^{45}Ca (Wong y Chase, 1986; Pierce et al., 1988). A través de

medidas directas de los niveles de calcio con compuestos fluorescentes, se ha demostrado que esta entrada de calcio resulta en un incremento en la concentración interna del catión (Cala et al., 1986; McEwan et al., 1992). Los resultados obtenidos en el presente trabajo muestran que la exposición de neuronas de corteza cerebral y neuronas granulares de cerebelo (Trabajo 2, figuras 1 y 2) a soluciones hiposmóticas efectivamente resulta en un incremento en los niveles de calcio libre intracelular. Ambos tipos celulares responden con un incremento rápido en los niveles de calcio, seguido de una fase de disminución lenta en la que se estabiliza a niveles ligeramente superiores a los observados en condiciones isosmóticas (Trabajo 2, figura 1). Este proceso se aumenta con disminuciones progresivas en la osmolaridad del medio externo (Trabajo 2, figura 2).

El curso temporal y la curva vs. osmolaridad de los cambios en los niveles de calcio podrían ser compatibles con la idea de que el calcio podría funcionar como una señal para la liberación de osmolitos en células nerviosas. Desafortunadamente, el proceso de liberación de osmolitos se encuentra mejor caracterizado en astrocitos, donde no fue posible examinar los cambios en calcio intracelular, debido a que el compuesto fluorescente utilizado (fluo-3) no se retuvo en estas células aún en condiciones isosmóticas. En neuronas el incremento en el calcio intracelular es dependiente de la presencia de calcio extracelular (Trabajo 2, tablas I y II), pero en astrocitos la liberación de osmolitos, tanto de potasio (Trabajo 1, figura 10) como de aminoácidos

(Pasantes-Morales y Schousboe, 1988) es un proceso que ocurre en ausencia de calcio extracelular. En células de organismos eurihalinos la liberación de aminoácidos sensible a volumen ocurre por un proceso completamente dependiente de calcio (Amende y Pierce, 1980; Smith y Pierce, 1987), lo que podría sugerir la existencia de diferencias en los mecanismos de movilización de osmolitos orgánicos entre células de animales acuáticos y terrestres. Actualmente se tiene en desarrollo un proyecto en el laboratorio para determinar en células de sistema nervioso la importancia del calcio y de los sistemas de segundos mensajeros en los procesos de salida de osmolitos y de regulación del volumen celular.

4) MECANISMO DE LIBERACION DE OSMOLITOS ORGANICOS.

La utilización de compuestos orgánicos como osmolitos se encuentra ampliamente documentada en diversas preparaciones experimentales, incluyendo células de organismos acuáticos (Pierce y Politis, 1987) y terrestres (Pasantes-Morales y Schousboe, 1988; Hoffmann y Simonsen, 1989). Entre estos osmolitos se encuentran aminoácidos libres (Gilles, 1979), poliaminas (Yamauchi et al., 1992) y polialcoholes como el sorbitol (Siebens y Spring, 1989) y el inositol (Jackson y Strange, 1993). Como se esperaría para un compuesto que funciona como osmolito, los niveles intracelulares de aminoácidos libres, sorbitol e inositol se disminuyen en soluciones

hiposmóticas y se incrementan en condiciones de hiperosmolaridad.

La disminución en los niveles celulares de aminoácidos libres producida por un incremento en el volumen celular se acompaña de la aparición de estos compuestos en el medio de incubación (Pasantes-Morales et al., 1991), lo que demuestra que los aminoácidos no son degradados ni incorporados a proteínas, sino que son movilizados al exterior por algún sistema de transporte presente en la membrana celular que se activa como consecuencia del incremento de volumen. A pesar de que este mecanismo se encuentra presente en todos los tipos celulares donde se ha estudiado, no se ha determinado con certeza la naturaleza de la proteína involucrada.

Es interesante mencionar que aparentemente los diferentes aminoácidos que se movilizan en respuesta a un aumento en el volumen celular parecen utilizar una vía común. En células MDCK la liberación de todos los aminoácidos endógenos sensible a volumen se inhibe en presencia de DIDS y quinidina (Sánchez-Olea et al., 1991). Estos compuestos también inhiben la salida de sorbitol (Siebens y Spring, 1992) e inositol (investigación en proceso), lo que indica la posible existencia de un mecanismo de transporte único para las diferentes clases de osmolitos orgánicos. En el presente trabajo se utilizó principalmente a la taurina como representante para estudiar la via de movilización de los osmolitos orgánicos y los resultados se discuten en la siguiente sección.

A) Naturaleza del mecanismo de transporte involucrado en la liberación de aminoácidos sensible a volumen.

La principal evidencia que sugiere la participación de una proteína en el proceso de liberación de aminoácidos sensible a volumen, es que tanto en hepatocitos (Ballatory y Boyer, 1992), como en astrocitos (investigación en proceso) la salida de taurina se inhibe en presencia de N-etil-maleimida, un compuesto que reacciona específicamente con los grupos sulfihídrilos de las proteínas. Igualmente, en astrocitos en cultivo la modificación de argininas con 1,2-ciclohexanediona o fenilglioxal, resulta en una disminución de la liberación de taurina sensible a volumen que es dependiente de la concentración del fármaco utilizada (Pasantes-Morales, investigación en proceso). Sin embargo aún no se conoce la identidad de esta proteína.

En prácticamente todos los tipos celulares la taurina es acumulada en el interior celular a través de un transportador saturable y dependiente de sodio, el cual se clonó recientemente de células MDCK y de cerebro de rata (Smith et al., 1992). La participación de este acarreador en la liberación de taurina sensible a volumen es poco probable, debido a que en astrocitos este proceso es independiente de sodio y poco sensible a la temperatura (Pasantes-Morales et al., 1990). Sin embargo, esta posibilidad se exploró directamente a través de la determinación del efecto de un incremento en el volumen sobre los parámetros cinéticos del sistema de captura de taurina tanto en células

granulares (Trabajo 3, figura 2) como en astrocitos en cultivo (Trabajo 4, figura 1). Los resultados obtenidos en las dos preparaciones muestran que un aumento de volumen no resulta en una activación del transportador de taurina sino que se observa una reducción ligera que podría ser el resultado de la despolarización que se ha reportado ocurre en astrocitos en condiciones hiposmóticas (Kimelberg y Kettenmann, 1990).

Además del transportador saturable de taurina existe otro mecanismo que participa en la acumulación intracelular de este aminoácido. Este mecanismo es un componente difusional (y por lo tanto no saturable) e independiente de sodio y se ha descrito en diversas preparaciones, incluyendo neuronas y astrocitos en cultivo (Schousboe et al., 1976; Larsson et al., 1986; Holopainen et al., 1987). Tanto en neuronas (Trabajo 3, figura 2) como en astrocitos (Trabajo 4, figura 1), un incremento de volumen celular estimuló marcadamente la magnitud de este componente difusional. Diferentes condiciones que inhiben la liberación de taurina sensible a volumen, como concentraciones elevadas de cationes divalentes (manganeso, magnesio) y el inhibidor de canales de cloro DIDS, también inhibieron la estimulación de este componente difusional por condiciones hiposmóticas (Trabajo 3, figura 1), lo que sugiere un mecanismo común para el componente difusional de la captura de taurina y la liberación de este aminoácido sensible a volumen.

Con el fin de examinar si efectivamente los movimientos de taurina sensibles a volumen ocurren puramente por un proceso

difusional, se determinaron los niveles celulares de taurina de astrocitos incubados en presencia de concentraciones extracelulares crecientes de taurina, tanto en condiciones isosmóticas como hiposmóticas. Este experimento se realizó en un medio libre de sodio para inactivar al transportador saturable (el cual funciona únicamente en presencia de este catión), con el fin de registrar los flujos de taurina mediados exclusivamente por el componente difusional. Los resultados obtenidos muestran que efectivamente la dirección y la magnitud de los flujos de taurina producidos por un aumento de volumen se encuentran determinados por el gradiente de concentración para este aminoácido. Con una concentración de taurina externa de aproximadamente 30 mM, un aumento de volumen no induce movimientos netos del aminoácido en ninguna dirección, mientras que a concentraciones menores o mayores se observa una disminución o un incremento en los niveles celulares de taurina (Trabajo 4, figura 2). En células MDCK, la concentración a la cual no se observan flujos netos de taurina al incrementar el volumen celular fue de aproximadamente 5 mM, consistente con los niveles menores de taurina en estas células (resultados no publicados).

Considerando que la liberación de taurina sensible a volumen es independiente de sodio y poco dependiente de la temperatura, así como los resultados de la cinética de captura, resulta poco probable que el transportador de taurina dependiente de sodio constituya el mecanismo responsable de la movilización de taurina observada en condiciones hiposmóticas. Los resultados obtenidos en

astrocitos demuestran claramente que, una vez que se ha activado la vía de transporte, los flujos de taurina se encuentran determinados por el gradiente de concentración del aminoácido, lo cual sería consistente con la participación de un "canal" o "poro" en este proceso.

B) Selectividad de la vía activada por un incremento en el volumen celular.

En investigaciones previas del laboratorio se demostró que tanto en linfocitos humanos (Pasantes-Morales et al., 1991) como en células MDCK (Sánchez-Olea et al., 1991) la exposición a soluciones hiposmóticas estimula la salida de distintos aminoácidos endógenos. Por otra parte se ha reportado que en células renales un aumento de volumen induce la activación de un mecanismo de transporte que moviliza sorbitol (Siebens y Spring, 1989) e inositol (Jackson y Strange, 1993). Con estos antecedentes se decidió examinar en una preparación única (astrocitos) la selectividad de la vía sensible a volumen.

Considerando que en los astrocitos los flujos de taurina a través de la vía activada por un incremento de volumen se encuentran determinados únicamente por el gradiente de concentración del aminoácido, se decidió examinar el efecto de concentraciones externas elevadas de distintos aminoácidos y polialcoholes sobre el proceso de recuperación de volumen, con el

fin de determinar la selectividad del mecanismo de transporte activado por esta condición. El presupuesto fue que si el soluto de interés era capaz de utilizar la vía activada por el aumento de volumen entonces la pérdida de osmolitos internos se vería compensada por la entrada de este soluto y el proceso de regulación de volumen resultaría inhibido; si el soluto no es movilizado por este vía entonces el proceso de regulación de volumen ocurriría normalmente.

Como se puede observar en el trabajo 5 (Figura 1 y tabla 1) en astrocitos el proceso de recuperación de volumen observado en soluciones hiposmóticas se inhibe por completo en presencia de una concentración externa de 120 mM de distintos aminoácidos neutros, incluyendo taurina, alanina, y glicina (Trabajo 5, figura 1), ß-alanina, GABA, prolina, fenilalanina, cisteina, treonina y asparagina y sólo un 39% en presencia de glutamina (Trabajo 5, tabla 1). Utilizando una concentración de 90 mM únicamente la taurina y la ß-alanina todavía fueron capaces de inhibir la regulación de volumen, mientras que utilizando una concentración de 60 mM de diversos aminoácidos este poceso ocurrió prácticamente de manera normal. Al examinar el efecto de aminoácidos con carga eléctrica neta se determinó que los aminoácidos ácidos (glutámico o aspártico) pero no los básicos (arginina o lisina) son capaces de inhibir el proceso de regulación de volumen normalmente observado en condiciones hiposmóticas, indicando que los aminoácidos básicos no son capaces de utilizar la vía activada en soluciones hiposmóticas. Interesantemente, el efecto inhibidor de los

aminoácidos ácidos (glutámico y aspártico) únicamente se observa cuando el catión principal en las soluciones es el K^+ (para el que la membrana es muy permeable, especialmente en condiciones hiposmóticas), pero no en presencia de Na^+ (para el que la membrana es impermeable, aún en condiciones hiposmóticas), lo cual indica que la entrada del aminoácido debe acompañarse de la entrada simultánea de un catión, resultando en un proceso eléctricamente neutro (Trabajo 5, figura 3).

Para corroborar la propuesta de que la habilidad de los distintos aminoácidos para inhibir el proceso de recuperación de volumen se fundamenta en la capacidad de la vía sensible a volumen para transportar estos solutos, se examinaron directamente los niveles celulares de los aminoácidos y se encontró que existe una relación directa entre la capacidad de los aminoácidos de inhibir el proceso de regulación de volumen y la de incrementar sus niveles como consecuencia de la exposición a soluciones hiposmóticas (Trabajo 5, comparar las figuras 1 y 5). Igualmente, se demostró que la entrada de ácido glutámico sensible a volumen es mayor en presencia de potasio que de sodio como principal catión en el medio externo (Trabajo 5, figura 5).

Un aumento en el volumen celular además de estimular la liberación de aminoácidos induce también la salida de polialcoholes como el sorbitol (Siebens y Spring, 1989) y el inositol (Jackson y Strange, 1993), lo que demuestra que existe una vía de transporte

para estos compuestos que se estimula en condiciones hiposmóticas. Sin embargo, una concentración externa elevada (120 mM) de sorbitol sólo muestra un pequeño efecto inhibidor sobre la regulación de volumen y en presencia de inositol (120 mM) se observa una clara estimulación de este proceso (Trabajo 5, figura 4). Permanece por determinar si la vía responsable de la liberación de polialcoholes es unidireccional y sólo permite la movilización de estos compuestos del interior celular al exterior y no en dirección opuesta o, alternativamente, si la velocidad de entrada de estos compuestos es más lenta que la salida de los osmolitos intracelulares, lo que explicaría la falta de un efecto inhibidor en presencia de concentraciones elevadas de estos compuestos.

C) Existe un mecanismo de transporte común para la liberación ed cloro y aminoácidos en respuesta a un incremento de volumen?

Un aumento de volumen celular producido por exposición a soluciones hiposmóticas produce la activación de canales de cloro en diversos tipos celulares, incluyendo linfocitos humanos (Grinstein et al., 1984), células de Ehrlich (Hoffmann y Simonsen, 1989), células MDCK (Roy y Sauvé, 1984), células T84 (Worrell et al., 1989), keratinocitos (Rugolo et al., 1992), células epiteliales humanas (Kubo y Okada, 1992; Rasola et al., 1992), etc. Las propiedades de estos canales de cloro se han estudiado con

diferentes enfoques, incluyendo la liberación de cloro radiactivo sensible a volumen (Rugolo et al., 1992), estudiando el efecto de inhibidores de canales de cloro sobre el proceso de regulación de volumen (Grinstein et al., 1984), y recientemente, con técnicas electrofisiológicas (Kubo y Okada, 1992; Rasola et al., 1992; Roy y Malo, 1992). Los canales de cloro activados por un incremento de volumen muestran características comunes en los distintos tipos celulares donde se han descrito: estos canales, una vez activados por el aumento de volumen conducen mayor corriente a voltajes positivos que negativos (Chan et al., 1992; Kubo y Okada, 1992; Rugolo et al., 1992; Yantorno et al., 1992), presentan un proceso de inactivación a potenciales de membrana mayores a +60 mV (Kubo y Okada, 1992; Rasola et al., 1992; Worrel et al., 1989) y son inhibidos por diversos inhibidores de canales de cloro, incluyendo DIDS, SITS, 1,9-dideoxiforskolina, NPPB, y ácido araquidónico (Chan et al., 1992; Kubo y Okada, 1992; McCann et al., 1989; Rasola et al., 1992; Rugolo et al., 1992; Yantorno et al., 1992).

Como se muestra en el trabajo 6 (Figura 2), en astrocitos la liberación de taurina y ácido aspártico sensible a volumen se inhibe, de manera dependiente de la concentración, en presencia de los inhibidores de canales de cloro ácido niflúmico, dipiridamol y DIDS. Al examinar los flujos de taurina como entrada del aminoácido en condiciones hiposmóticas se observa que el DIDS y el dipiridamol también inhibieron significativamente este proceso (Trabajo 5, figura 6). Estos resultados sugieren que los distintos aminoácidos

podrían ser movilizados a través del canal de cloro que se activa como consecuencia del aumento en el volumen celular. Esta hipótesis fue propuesta por primera vez por Banderali y Roy (1992), quienes a través del uso de técnicas electrofisiológicas, demostraron en células MDCK la presencia de un canal aniónico que se activa en condiciones hiposmóticas, y que presenta una gran permeabilidad para el ácido aspártico, el ácido glutámico y la taurina. Recientemente se describió, en una línea celular glial (C6), que la taurina es capaz de permear a través del canal de cloro que se activa como consecuencia del aumento de volumen (Jackson y Strange, 1993).

En trabajos previos de nuestro laboratorio se ha sugerido que los distintos aminoácidos que son movilizados en condiciones hiposmóticas utilizan una vía común, debido a que en células MDCK la liberación de los diversos aminoácidos sensible a volumen se inhibió en presencia de DIDS o quinidina (Sánchez-Olea et al., 1991). Interesantemente la salida de sorbitol sensible a volumen también se reduce marcadamente en presencia de DIDS o quinidina (Siebens y Spring, 1989), lo cual sugiere la existencia de una vía común de transporte para osmolitos orgánicos tan distintos como los aminoácidos y los polialcoholes. En eritrocitos de lenguado el mecanismo de transporte sensible a volumen es capaz de movilizar taurina, glucosa y uridina, y se inhibe en presencia de los inhibidores de canales de cloro furosemida, ácido niflúmico, MK-196, DIDS y NPPB (Kirk et al., 1992). Recientemente se demostró en

células C6, que la liberación de cloro, taurina e inositol se inhiben en presencia de diversos inhibidores de canales de cloro (Jackson y Strange, 1993), lo cual apoya la hipótesis de que los aminoácidos, polialcoholes y cloro son movilizados a través de una vía común que se activa como consecuencia de un incremento en el volumen celular.

Se han descrito distintas clases de canales de cloro, clasificados polr su dependencia a activadores: a) Activados por AMP cíclico, b) activados por calcio y c) sensibles al potencial de membrana (Chan et al., 1992). Se han realizado investigaciones con el fin de examinar si el canal de cloro sensible a volumen corresponde con alguno de estos canales de cloro y los resultados señalan que se trata de un canal distinto, que no corresponde a ninguno de los mencionados previamente. Chan et al., (1992) reportaron que un anticuerpo contra un péptido sintético del canal de cloro causante de la fibrosis cística, es capaz de inhibir por completo la actividad del canal de cloro activado por AMP cíclico y solo parcialmente al canal de cloro sensible a volumen. Un reporte previo señala que las corrientes de cloro inducidas por un incremento de volumen presentan exactamente las mismas características en células epiteliales normales, que expresan el canal de cloro regulado por AMP cíclico, y en células que muestran el fenotipo de la fibrosis cística, es decir carecen del canal sensible a AMP cíclico (Solc y Wine, 1991). Además, la exposición a condiciones de hiposmolaridad produce un aumento en las

corrientes de cloro máximas producidas por calcio o AMP cíclico, lo que refuerza la hipótesis de que el canal de cloro sensible a volumen es diferente al activado por alguna de estas condiciones (Chan et al., 1992).

La investigación en el área de los canales de cloro se impulsará con la reciente clonación de un canal de cloro de células MDCK, ya que por técnicas de biología molecular se pueden aislar genes desconocidos que presenten una cierta homología con algún gene de secuencia conocida y, es muy probable que el canal de cloro sensible a volumen resultara ser una proteína muy similar a los otros tipos de canales de cloro. La comprobación final de la hipótesis de que una misma proteína se encuentra involucrada en la movilización de cloro y de los distintos osmolitos orgánicos tendrá que esperar hasta el momento en el que se purifique esa proteína o a que se clone el gen y se exprese en la preparación adecuada. Si esta teoría resulta verdadera entonces es posible que se presente un problema semántico con la denominación de "canal de cloro" y se plantee un nuevo término como "canal de osmoefectores" o "permeasa de osmolitos".

5) MECANISMOS DE MOVILIZACION DE TAURINA EN CONDICIONES DE HIPEROSMOLARIDAD.

Hasta el momento se han discutido los eventos asociados con los flujos de osmolitos que ocurren durante el proceso de regulación de volumen observado en condiciones hiposmóticas. En el presente trabajo también se exploraron los mecanismos que operan durante la movilización de osmolitos orgánicos que resulta de la exposición de las células nerviosas a soluciones hiperosmóticas. Thurston et al. (1980) reportaron que, en el cerebro de ratón, una condición de hipernatremia (aumento en la concentración extracelular de cloruro de sodio) producía un aumento en la concentración de algunos de los aminoácidos libres, principalmente de la taurina.

Posteriormente se ha descrito que en el cerebro de mamíferos una condición de hiperosmolaridad produce una acumulación intracelular de diversos osmolitos orgánicos como inositol, betaína, glicerofosforilcolina y varios aminoácidos libres (Lohr et al., 1988; Heilig et al., 1989; Thurston et al., 1989; Lien et al., 1990). Olson y Goldfinger (1990) demostraron que los astrocitos en cultivo crecidos en condiciones de hiperosmolaridad también responden con una acumulación intracelular de taurina, sin embargo no se exploró el mecanismo involucrado en este proceso. En el trabajo 7 (tabla 1) se muestra que los astrocitos no solo acumulan taurina sino también otros aminoácidos libres al ser crecidos en un exceso de cloruro de sodio en el medio de cultivo. La acumulación de estos aminoácidos podría resultar de un incremento en la

actividad de los acarreadores dependientes de sodio, específicos para cada aminoácido (Trabajo 7, figura 3) y no necesariamente del incremento en la osmolaridad del medio externo. Sin embargo, al crecer los astrocitos en un medio en el que la osmolaridad se aumentó con sacarosa se mantiene la mayor parte de los incrementos en los niveles celulares de aminoácidos libres (Trabajo 7, tabla 1), lo que demuestra que el incremento en la osmolaridad es la señal responsable de este proceso.

La acumulación de taurina en los astrocitos se asocia con una disminución de la concentración extracelular del aminoácido en el medio de cultivo (Trabajo 7), lo que sugiere que el proceso de acumulación involucra un incremento en la captura y/o una disminución en la liberación del aminoácido.

Con el fin de determinar si un aumento en la actividad del transportador dependiente de sodio podría ser el mecanismo responsable de la acumulación de taurina en condiciones hiperosmóticas, se determinó el efecto de un tratamiento crónico con soluciones hiperosmóticas sobre los parámetros cinéticos de la acumulación de taurina. La captura de taurina en astrocitos ocurre a través de un sistema saturable, dependiente de sodio y, a concentraciones elevadas, de un componente independiente de sodio y no saturable (Schousboe et al., 1976). La condición de hiperosmolaridad aumentó la Vmax y no afectó la Km del transportador no saturable (Trabajo 7, figura 4 y tabla II), lo que sugiere que posiblemente el número de transportadores para la

taurina se ha incrementado por síntesis de novo como consecuencia de la exposición a condiciones hiperosmóticas.

El curso temporal del aumento en los niveles celulares del aminoácido muestra que la taurina se incrementa significativamente sólo después de 6 h y el valor máximo se alcanza hasta las 12 h de crecimiento en el medio hiperosmótico (Trabajo 7, figura 2), lo cual es un tiempo suficiente durante el cual se podría inducir la síntesis de proteínas. Este mecanismo de adaptación se ha descrito en células renales, en las que una condición de hiperosmolaridad resulta en un aumento en los niveles de otros osmolitos orgánicos como inositol, sorbitol y betaína, que se asocia con un incremento en la Vmax para el acarreador de estos compuestos (Nakanishi et al., 1989). En células MDCK se ha descrito que una condición de hiperosmolaridad estimula notablemente los niveles del RNA mensajero del transportador de betaína (Yamauchi et al., 1991) e inositol (Yamauchi et al., 1993) a través de un aumento en la velocidad de transcripción del gen (Yamauchi et al., 1993). Al parecer la acumulación de los distintos osmolitos orgánicos observada en condiciones de hiperosmolaridad resulta de un aumento en los sistemas de captura, a través de un aumento en la síntesis de los acarreadores dependientes de sodio.

En los astrocitos la condición de hiperosmolaridad produjo, además de un incremento en la Vmax del componente saturable, una disminución en la magnitud del componente difusional de la captura de taurina (Trabajo 7, figura 4 y tabla II). Como se observa en la

Figura 1 (trabajo 7), un aumento en la magnitud de este componente difusional se asocia con un incremento en la liberación de taurina, por lo que se decidió examinar la liberación espontánea de este aminoácido en condiciones hiperosmóticas. La liberación de taurina, tanto radiactiva (Trabajo 7, figura 5) como endógena (Trabajo 7, figura 6), se encontró marcadamente disminuída, lo que corrobora la relación entre la magnitud del componente difusional de la captura de taurina y la de la liberación espontánea del aminoácido.

En conclusión se puede mencionar que los astrocitos expuestos a condiciones hiperosmóticas responden con mecanismos adaptativos que resultan en la acumulación de taurina y otros aminoácidos libres. Para la taurina estos mecanismos adaptativos involucran tanto un aumento en la captura como una disminución en la liberación del aminoácido.

CONCLUSIONES

1) Los astrocitos utilizan preferentemente osmolitos orgánicos (aminoácidos libres) sobre los osmolitos inorgánicos (potasio) durante los procesos de regulación de volumen mostrados por estas células en condiciones hiposmóticas.

2) El mecanismo de salida de potasio en respuesta a reducciones extremas en la osmolaridad del medio externo (reducciones de 50% o mayores) involucra la participación de un canal iónico, posiblemente activado por la distensión de la membrana que ocurre durante el incremento de volumen celular. La liberación de potasio en estas condiciones requiere de la salida simultánea de un anión (fisiológicamente del cloruro) que permita una electroneutralidad en el movimiento de los iones.

3) En neuronas granulares en cultivo, un incremento en el volumen celular producido por soluciones hiposmóticas se encuentra asociado con un aumento en los niveles intracelulares de calcio. Permanece por determinar la relación de este catión con los flujos de osmolitos observados en estas condiciones.

4) En astrocitos y neuronas granulares, los movimientos transmembranales de taurina sensibles a volumen se encuentran dirigidos únicamente por el gradiente de concentración del

aminoácido y no parecen involucrar el transportador dependiente de sodio.

5) El mecanismo de transporte que se activa por un incremento en el volumen celular permite la movilización de aminoácidos neutros y aminoácidos ácidos, pero no reconoce a aminoácidos básicos.

6) La vía de transporte de aminoácidos activada por hiposmolaridad se inhibe en presencia de fármacos que interfieren con la actividad de canales de cloro, lo que sugiere una mecanismo de transporte común para estos osmolitos.

7) El incremento en los niveles celulares de taurina (y posiblemente de otros aminoácidos) observado en condiciones hiperosmóticas, involucra tanto la activación del transportador dependiente de sodio (a través de un aumento en la Vmax) como una disminución en la liberación basal del aminoácido.

REFERENCIAS

Amende, L.M., Pierce, S.K. 1980. Free amino acid mediated volume regulation of isolated Noetia ponderosa red blood cells: Control by Ca^{2+} and ATP. J. Comp. Physiol. 138:291-298.

Atlas, M., Bahl, J.J., Roeske, W., Bressler, R. 1984. In vitro osmoregulation of taurine in fetal mouse hearts. J. Mol Cell. Cardiol 16:311-320.

Awapara, J. 1955. Taurine content of some animal organs. Fed. Pro. 14:175.

Bagnasco, S.M., Uchida, S., Balaban, R.S., Kador, P.F. 1987. Induction of aldose reductase and sorbitol in renal inner medullary cells by elevated extracellular NaCl. Proc. Natl. Acad. Sci. 84:1718-1720.

Bagnasco, S.M., Murphy, H.R., Bedford, J.J., Burg, M.B. 1988. Osmoregulation by slow changes in aldose reductase and rapid changes in sorbitol flux. Am. J. Physiol. 254:C788-C792.

Balaban, R.S., Burg, B. 1987. Osmotically active solutes in the renal inner medulla. Kidney Int. 31:562-564.

Ballatory, N., Boyer, J.L. 1992. Taurine transport in skate hepatocytes II. Volume activation, energy, and sulfhydryl dependence. Am. J. Physiol. 262:G451-G460.

Banderali, U., Roy, G. 1992. Anion channels for amino acids in MDCK cells. Am. J. Physiol. 263:C1200-C1207.

Beck, F.K., Dörge, A., Thurau, K. 1988. Renal Physiol. Biochem.

Borgese, F., García-Romeu, F., Motais, R. 1987. Control of cell volume and ion transport by B-adrenergic catecholamines in erythrocytes of rainbow trout, Salmo gairdneri. J. Physiol. 382:123-144.

Bourke, R.S., Tower, D.B., 1966. Fluid compartmentation and electrolytes of cat cerebral cortex in vitro I. Swelling and solute distribution in mature cerebral cortex. J. Neurochem. 13:1071-1097.

Bourke, R.S., 1969. Studies of the development and subsequent reduction of swelling of mammalian cerebral cortex under isosmotic conditions in vitro. Exp. Brain Res. 8:232-248.

Bourke, R.S., Kimelberg, H.K., Daze, M., Church, G. 1983. Swelling and ion uptake in cat cerebrocortical slices: Control by neurotransmitters and ion transport mechanisms. Neurochem. Res. 8:5-24.

Bourne, P.K., Cossins, A.R. 1984. Sodium and potassium transport in trout (Salmo gairdneri) erythrocytes. J. Physiol. 347:361-375.

Boyle, P.J., Conway, E.J. 1941. Potassium accumulation in muscle and associated changes. J. Physiol. 100:1-63.

Bricteux-Grégoire, S., Duchteau-Bosson, Gh., Jeuniaux, Ch., Florkin, M. 1962. Constituants osmotiquement actifs des muscles de crabe chinois Eriocheir sinensis. Arch. Int. Physiol. Biochim. 70:273-286.

Cala, P.M. 1980. Volume regulation by Amphiuma red blood cells. The membrane potential and its implications regarding the nature of the ion-flux pathways. J. Gen. Physiol. 76:683-708.

Cala, P.M. 1983. Cell volume regulation by Amphiuma red blood cells. The role of Ca^{2+} as a modulator of alkali metal/H^+ exchange. J. Gen. Physiol. 82:761-784.

Cala, P.M. 1985a. Volume regulation by Amphiuma red blood cells: characteristics of volume-sensitive K/H and Na/H exchange. Mol. Physiol. 8:199-214.

Cala, P.M. 1985b. Volume regulation by Amphiuma red blood cells: strategies for identifying alkali metal/H^+ transport. Federation Proc. 44:2500-2507.

Cala, P.M. 1986. Volume-sensitive alkali metal-H transport in Amphiuma red blood cells. Curr. Top. Membr. Transp. 26:79-99.

Cala, P.M., Mandel, L.J., Murphy, E. 1986. Volume regulation by Amphiuma red blood cells: cytosolic free Ca and alkali metal-H exchange. Am. J. Physiol. 250:C423-C429.

Cala, P.M. 1990. Principles of cell volume regulation, ion flux pathways and the roles of anions. In: "Chloride channels and carriers in nerve, muscle, and glial cells. Plenum Press, New York, pp. 67-83.

Chan, H-Ch., Goldstein, J., Nelson, D. 1992. Alternate pathways for chloride conductance activation in normal and cystic airway epithelial cells. Am. J. Physiol. 262: C1273-C1283.

Choi, D.W. 1987. Ionic dependence of glutamate neurotoxicity in cortical cell culture. J. Neurosci. 7:369-379.

Christensen, O. 1987. Mediation of cell volume regulation by Ca^{2+} influx through strech-activated channels. Nature 330:66-68

Cooke, K.R., Macnight, A.D.C. 1984. Effects of medium acetate on cellular volume in rabbit renal cortical slices. J. Physiol. 349:135-156.

Costa, C.J., Pierce, S.K. 1983. Volume regulation in the red coelomocytes of Glycera dibranchiata: An interaction of amino acids and K+ effluxes. J. Comp. Physiol. 151:133-144.

Davis, C.W., Finn, A.L. 1985. Cell volume regulation in frog urinary bladder. Fed. Proc. 44:2520-2525.

Dépêche, J., Schoffeniels, E. 1975. Changes in electrolytes, urea and free amino acids of Poecilia reticulata embryos following high salinity adaptation of the viviparous female. Biochm. System. Ecol. 3:111-118.

Domínguez, L., Montenegro, J., Pasantes-Morales, H. 1989. A volume-dependent chloride sensitive component of taurine release stimulated by potassium from retina. J. Neurosci. Res. 22:356-361.

Dunham, P.B., Ellory, J.C. 1981. Passive potassium transport in low potassium sheep red cells: dependence upon cell volume and chloride. J. Physiol. 318:511-530.

Ellory, J.C., Hall, A.C., Stewart, G.W. 1985. Volume-sensitive passive potassium fluxes in red cells. En: Gilles, R., Gilles-Baillien, M. (eds). Transport Processes, Iono- and Osmoregulation. Current Comparative Approaches. Springer-Verlag, pp. 401-410.

Eveloff, S.L., Warnock, D.G. 1987. Am. J. Physiol. 252:F1-F10.

Falke, L.C., Misler, S. 1989. Activity of ion channels during volume regulation by clonal N1E115 neuroblastoma cells. Proc. Natl. Acad. Sci. 86:3919-3923.

Fincham, D.A., Wolowyk, M.W., Young, J.D. 1987. J. Membr. Biol. 96:45-56.

Fugelli, K., Zachariassen, K.E. 1976. The distribution of taurine, gamma-aminobutyric acid and inorganic ions between plasma and erythrocytes in flounder (Platichthys flesus) at different plasma osmolalities. Comp. Biochem. Physiol. 55A:173-177

Fugelli, K., Reiersen, L.O. 1978. Volume regulation in flounder erythrocytes. The effect of osmolality on taurine influx. En: Barker Jorgensen, C., Skadhauge, E. (eds). Osmotic and Volume Regulation. Munksgaard, pp. 418-428.

Fugelli, K., Thoroed, S.M. 1986. J. Physiol. 374:245-261

Fyhn, H.J. 1976. Holeuryhalinity and its mechanisms in a cirriped crustacean, Balanus improvisus. Comp. Biochem. Physiol. 53A:19-30
Gardner-Medwing, A.R., Coles, J.A., Tsacopoulos, M. 1981. Clearence of extracellular potassium: evidence for spatial buffering by glial cells in the retina of the drone. Brain Res. 290:452-457.

Gerard, J.F. 1975. Volume regulation and alanine transport. Response of isolated axons of Callinectes sapidus Rathbun to hypo-osmotic conditions. Comp. Biochem. Physiol. 51A:225-229.

Gilles, R. 1975. Mechanisms of iono and osmoregulation. En: Kinne, O (ed). Wiley-Liss, pp. 259-347.

Gilles, R. 1977. Effects of osmotic stress on the proteins concentration and pattern of Eriocheir sinensis blood. Comp. Biochem. Physiol. 56A:109-114.

Gilles, R. 1979. Intracellular organic effector. En: Gilles R. (ed). Mechanisms of Osmoregulation in Animals: Maintenance of Cell Volume. New York: Johm Wiley, pp. 111-154.

Grinstein, S., Dupré, A., Rothstein, A. 1982. Volume regulation by human lymphocytes: Role of calcium. J. Gen. Physiol. 79:849-868.

Grinstein, S., Clarke, C.A., Rothstein, A. 1983. Activation of Na^+/H^+ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. J. Gen. Physiol. 82:619-638

Grinstein, S., Rothstein, A., Sarkadi, B., Gelfand, E.W. 1984. Responses of lymphocytes to anisotonic media: Volume-regulating behavior. Am. J. Physiol. 246:C204-C215.

Grinstein, S., Furuya, W., Cragoe, E.J. Jr. 1986. Volume changes in activated human neutrophils: The role of Na^+/H^+ exchange. J. Cell. Physiol. 128:33-40.

Grinstein, S., Clarke, C.A., Dupre, A., Rothstein, A. 1987. Volume-induced increase of anion permeability in human lymphocytes. J. Gen. Physiol. 80:801-823

Grinstein, S., Foskett, J.K. 1990. Ionic mechanisms of cell volume regulation in leukocytes. Annu. Rev. Physiol. 52:399-414.

Guggino, W.B., Oberleithner, H., Giebisch, G. 1985. Relationship between cell volume and ion transport in the early distal tubule of the Amphiuma kidney. J. Gen. Physiol. 86:31-58.

Gullans, S.R., Blumenfeld, J.D., Balschi, J.A., Kaleta, M., Brenner, R.M. 1988. Accumulation of major organic osmolytes in rat renal inner medulla in dehydration. Am. J. Physiol. 255:F626-F634.

Hazama, A., Okada, Y. 1988. Ca^{2+} sensitivity of volume regulatory K^+ and Cl^- channels in cultured human epithelial cells. J. Physiol. 402:687-702.

Heilig, C.W., Stromski, M.E., Blumenfeld, J.D., Lee, J.P., Gullans, S.R. 1989. Characterization of the major brain osmolytes that accumulate in salt-loaded rats. Am. J. Physiol. 257:F1108-F1116.

Hempling, H.G., Thompson, S., DuPre, A. 1977. Osmotic properties of human lymphocyte. J. Cell. Physiol. 93:293-302.

Hendil, K.B., Hoffmann, E.K. 1974. Cell volume regulation in Ehrlich ascites tumor cells. J. Cell. Physiol. 84:115-125.

Hertz, L. 1978. An intense potassium uptake into astrocytes, its further enhancement by high concentrations of potassium and its possible involvement in potassium homoestasis at the cellular level. Brain Res. 145:202-208.

Hoffmann, E.K., Handel, K. B. 1976. J. Comp. Physiol. 108:279-286.

Hoffmann, E.K. 1978. Regulation of cell volume by selective changes in the leak permeabilities of Ehrlich ascites tumor cells. En: Jorgensen, C.B., Skadhauge, E. (eds). Osmotic and Volume Regulation. Munksgaard, pp. 397-417

Hoffmann, E.K. Lambert, I.H. 1983. Amino acid transport and cell volume regulation in Ehrlich ascites tumour cells. J. Physiol. 338:613-625.

Hoffmann, E.K., Sjohlm, C., Simonsen, L.O. 1983. Na^+, Cl^- co-transport in Ehrlich ascites tumor cells activated during volume regulation (regulatory volume increase). J. Membr. Biol. 76:269-280

Hoffmann, E.K., Simonsen, L.O., Lambert, I.H. 1984. Volume-induced increase of K^+ and Cl^- permeabilities in Erlich ascites tumor cells. Role of internal $Ca2^+$. J. Membr. Biol. 78:211-222.

Hoffmann, E.K., Lambert, I.H., Simonsen, L.O. 1986. Separate, Ca^{2+}-activated K^+ and Cl^- transport pathways in Ehrlich ascites tumor cells. J. Membr. Biol. 91:227-244.

Hoffmann, E.K. 1987. Volume regulation in cultured cells. Curr. Top. Membr. Transp. 30:125-180

Hoffmann, E.K., Simonsen, L.O. 1989. Membrane mechanisms in volume and pH regulation in vertebrate cells. Physiol. Rev. 69:315-382.

Islas, L., Pasantes-Morales, H., Sánchez, J.A. 1993. Characterization of Stretch-activated ion channels in cultured astrocytes. Glia 8:87-96.

Jackson, P., Strange, K. 1993. Role of volume-sensitive anion channels as a pathway for swelling-activated inosito and taurine efflux. Am. J. Physiol, en prensa.

Jacobsen, J.G., Smith, L.H. 1968. Biochemistry and physiology of taurine and taurine derivatives. Physiol. Rev. 48:424-511.

Kamino, K., Inouye, K., and Inouye, A., 1973. Potassium ion-induced

swelling of nerve-ending particles by light-scattering measurement. Biochim. Biophys. Acta 330:39-52.

Kimelberg, H.K., Frangakis, M.V. 1985. Furosemide- and bumetanide-sensitive ion transport and volume control in primary astrocyte cultures from rat brain. Brain Res. 361:125-134.

Kimelberg, H.K., Ransom, B.R. 1986. Physiological and pathological aspects of astrocytic swelling. En: Astrocytes. Vol. 3. S. Fedoroff y A. Vernadakis (eds). Academic Press, New York, pp. 129-166.

Kimelberg,, H.K., Kettenmann, H. 1990. Swelling-induced changes in electrophysiological propierties of cultured astrocytes and oligodendrocytes. I. Effect on membrane potentials, input impedance and cell-cell coupling. Brain Res. 529:255-261.

Kregenow, F.M. 1971. The response of duck erythrocytes to hypertonic media. Further evidence for a volume-controlling mechanism. J. Gen. Physiol. 58:396-412

Kregenow, F.M. 1981. Annu. Rev. Physiol. 43:493-505.

Kregenow, F.M., Caryk, T., Siebens, A.W. 1985. Further studies of the volume-regulatory response of Amphiuma red cells in hypertonic media. Evidence for amiloride-sensitive Na/H exchange. J. Gen. Physiol. 86:565-584.

Kristensen, L. O. 1980. Energization of alanine transport in isolated rat hepatocytes. Electrogenic Na^+-alanine co-transport leading to increased K^+ permeability. J. Biol. Chem. 255:5236-4243.

Kristensen, L.O., Folke, M. 1984. Volume-regulatory K^+ efflux during concentrative uptake of alanine in isolated rat hepatocytes. Biochem. J. 221:265-268.

Kristensen, L.O. 1986. Association between transports of alanine and cations across cell membrane in rat hepatocytes. Am. J. Physiol. 251:G575-G584.

Kubo, M., Okada, Y. 1992. Volume-regulatory Cl^- channel currents in cultured human epithelial cells. J. Physiol. 456:351-371.

Lang, F., Paulmichl, M., Voelkl, H., Gstrein, E., Friedrich, F. 1987. Electrophysiology of cell volume regulation. In: Molecular Nephrology. Biochemical Aspects of Kidney Function. Kovacevic, Z., Guder, W.G. (eds.). Berlin: de Gruyter, pp. 133-139

Lange, R. 1963. The osmotic functions of amino acids and taurine in the mussel Mytilus edulis. Comp. Biochem. Physiol 10:173-179.

Larson, M., Spring, K. R. 1984. Volume regulation by Necturus

gallbladder: basolateral KCl exit. J. Membr. Biol. 81:219-232

Larson, M., Spring, K.R. 1987. Volume regulation in epithelia. Curr. Top. Membr. Transp. 30:105-123.

Lau, K.R., Hudson, R.L., Schultz, S.G. 1984. Cell swelling increases a barium-inhibitable potassium conductance in thebasolateral membrane of Necturus small intestine. Proc. Natl. Acad. Sci. 81:3591-3594.

Lauf, P.K. 1982. Evidence for chloride dependent potassium and water transport induced by hyposmotic stress in erythrocytes of the marine teleost, Opsanus tau. J. Comp. Physiol. 146:9-16.

Lauf, P.K. 1985. On the relationship between volume- and thiol-stimulated K^+ Cl^- fluxes in red cells membranes. Mol. Physiol. 8:215-234

Lehmann, A., 1989. Effect of microdialysis-perfusion with anisoosmotic media on extracellular amino acids in the rat hippocampus and skeletal muscle. J. Neurochem 54:525-535.

Lien, Y-H., Shapiro, J.L., Chan, L. 1990. Effects of hypernatremia on organic brain osmoles. J. Clin. Invest. 85:1427-1435.

Lipton, P. 1973. Effects of membrane depolarization on light scattering by cerebral cortical slices. J. Physiol. 231:365-383.

Lohr, J.W., McReynolds, J., Grimaldi, T., Acara, M. 1988. Effect of acute and chronic hypernatremia on *myo*-inositol and sorbitol concentration in rat brain and kidney. Life Sci. 43:271-278.

MacKnight, A.D.C. 1983. Volume regulation in mammalian kidney cells. Mol. Physiol. 4:17-31.

MacKnight, A.D.C. 1985. Cellular responses to extracellular osmolality. En: Seldin, D.W., Giebisch, G. (eds). The Kidney Physiology and Pathophysiology. New York: Raven, pp. 117-132.

Macknight, A.D.C. 1988. Renal Physiol. Biochem. 3-5:114-141.

MacLeod, R.J., Hamilton, J.R. 1991. Volume regulation initiated by Na^+-nutrient cotransport in isolated mammalian villus enterocytes. Am. J. Physiol. 260:G26-G33.

Mayer, M.L.A. 1985. A calcium-activated chloride current generates the after-depolarization of rat sensory neurones in culture. J. Physiol. 364:217-239.

McEwan, G.T.A., Brown, C.D.A., Hirst, B.H., Simmons, N.L. 1992. Hypo-osmolar stimulation of transepithelial Cl^- secretion in cultured human T_{84} intestinal epithelial layers. Biochim. Biophys

Acta 1135:180-183.

McManus, T.J., Schmidt, W.F., III. 1978. Ion and co-ion transport in avian red cells. En: Hoffman, E.K. (ed). Membrane Transport Processes. Raven, vol. 1. pp. 79-106.

Mills, J.W., Skiest, D.J. 1985. Role of cyclic AMP and the cytoskeleton in volume control in MDCK cells. Mol. Physiol. 8:247-62.

Moller, M., Mollgard, K., Lund-Andersen, H., and Hertz, L., 1974. Concordance between morphological and biochemical estimates of fluid sapaces in rat brain cortex slices. Exp. Brain Res. 22:299-334.

Moran, W.M., Pierce, S.K. 1984. The mechanism of crustacean salinity tolerance: cell volume regulation by K^+ and glycine fluxes. Mar. Biol. 81:41-46.

Morris, C.E., Sigurdson, W.J. 1989. Stretch-inactived ion channels coexist. Science 243:807-809

Nakanishi, T., Balaban, R.S., Burg, M.B. 1988. Survey of osmolytes in renal cell lines. Am. J. Physiol. 255:C181-C191.

Nieminen. M.L., Tuomisto, L., Solatunturi, E., Eriksson, L., Paasonen, M.K. 1988. Taurine in the osmoregulation of the Battleboro rat. Life Sci. 42:2137-2143.

Olson, J.E., Goldfinger, M.D. 1990. Amino acid content of rat cerebral astrocytes adapted to hyperosmotic medium in vitro. J. Neurosci. Res. 27:241-246.

O'Neill, W.C. 1989. Cl-dependent K transport in a pure population of volume-regulating human erythrocytes. Am. J. Physiol. 256:C858-C864.

Orkand, R.K., Dietzel, I., Coles, J.A. 1984. Light-induced changes in extracellular volume in the retina of the drone, Apis mellifera. Neurosci. Lett. 45:273-278.

Orskov, S.L. 1954. The potassium absorption by pigeon blood cells. A considerable potassium absorption by pigeon- and hen blood cells is observed when a hypertonic sodium chloride solution is added. Acta Physiol. Scand. 31:221-229.

Pappius, H.M., and Elliot, K.A.C., 1956. Factors affecting the potassium content of incubated brain slices. Can. J. Biochem. Physiol. 34:1053-1067.

Parker, J.C. 1977. Solute and water transport in dog and cat red blood cells. En: Ellory, J.C., Lew, V.L. (eds.). Membrane transport

in red cells. London: Academic, pp. 427-465

Parker, J.C. 1983. Volume-responsive sodium movements in dog red blood cells: anion effects. Am. J. Physiol. 244:C313-C317.

Parker, J.C. 1986. Na-proton exchange in dog red blood cells. Curr. Top. Membr. Transp. 26:101-114.

Parker, J.C. 1988. Volume-activated transport systems in dog red blood cells. Comp. Biochem. Physiol. 90:539-542.

Pasantes-Morales, H., Domínguez, L., Montenegro, J., and Morán, J., 1988. A chloride-dependent component of the release of labeled GABA and taurine from the chick retina. Brain Res. 459:120-130.

Pasantes-Morales, H., Schousboe, A. 1988. Volume regulation in astrocytes: a role for taurine as an osmoeffector. J. Neurosci. Res. 20:505-509.

Pasantes-Morales, H., Morán, J., Schousboe, A. 1990. Volume-sensitive release of taurine from cultured astrocytes: properties and mechanism. Glia 3:427-432.

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

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

Pierce, S.K., Greenberg M.J. 1972. The nature of cellular volume regulation in marine bivalves. J. Exp. Biol. 57:681-692.

Pierce, S.K., Politis, A.D., Smith, L.H. Jr., Rowland, L.M. 1988. A Ca^{2+} influx in response to hypo-osmotic stress may alter osmolyte permeability by a phenothiazine-sensitive mechanism. Cell Calcium 9:129-140.

Pierce, S.K., Politis, A.D. 1990. Ca^{2+}-Activated cell volume recovery mechanisms. Annu. Rev. Physiol. 52:27-42.

Rasola, A., Galietta, L.J.V., Gruenert, D.C., Romeo, G. 1992. Ionic selectivity of volume-sensitive currents in human epithelial cells. Biochim. Biophys Acta 1139:319-323.

Rassin, D.K., Sturman, J.A., Gaull, G.E. 1977. Taurine in developing rat brain: Subcellular distribution and association with synaptic vesicles of (^{35}S) taurine in maternal, fetal and neonatal rat brain. J. Neurochem. 28:41-50.

Roy, G., Sauvé, R. 1987. Effect of anisotonic media on volume, ion and amino acid content and membrane potential of kidney cells (MDCK) in culture. J. Membr. Biol. 100:83-96.

Roy, G., Malo, C. 1992. Activation of amino acid diffusion by a volume increase in cultured kidney (MDCK) cells. J. Membr. Biol. 130:83-90.

Rugolo, M., Mastrocola, T., De Luca, M., Romeo, G., Galietta, L.J.V. 1992. A volume-sensitive chloride conductance revealed in cultured keratinocytes by $^{36}Cl^-$ efflux and whole-cell patch clamp recording. Biochim. Biophys. Acta 1112:39-44.

Sackin, H. 1989. A stretch-activated K^+ channel sensitive to cell volume. Proc. Natl. Acad. Sci. 86:1731-1735

Sachs, F. 1991. Mechanical transduction by membrane ion channels: A mini review. Molec. Cell. Biochem., 104:57-60.

Saltin, B., Sjogaard, G., Strange, S., Juel, C. 1987. Redistribution of K^+ in the human body during muscular exercise; ist role to maintain whole body homeostasis.

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

Sarkadi, B., Mack, E., Rothstein, A. 1984. Ionic events during the volume response of human peripheral blood lymphocytes to hypotonic media. II. Volume-and time-dependent activation and inactivation of ion transport pathways. J. Gen. Physiol. 83:513-527.

Sarkadi, B., Cheung, R., Mack, E., Grinstein, S., Gelfand, E.W., Rothstein, A. 1985. Cation and anion transport pathways in volume regulatory response of human lymphocytes to hyposmotic media. Am. J. Physiol. 248:C480-C487.

Sarkadi, B., Parker, J.C. 1991. Activation of ion transport pathways by changes in cell volume. Biochim. Biophys. Acta 1071:407-427.

Schmidt, W.F., III, McManus, T.J. 1977a. Ouabain-insensitive salt and water movements in duck red cells. I.Kinetics of cation transport under hypertonic conditions. J. Gen. Physiol. 70:59-79.
Schmidt, W.E., III, McManus, T.J. 1977b. Ouabain-insensitive salt and water movements in duck red cells. II.Norepinephrine stimulation of sodium plus potassium cotransport. J. Gen. Physiol. 70:81-97.

Schoffeniels, E., Gilles, R. 1970. Osmoregulation in aquatic arthropods. En: Florkin, M., Scheer, B. (eds.). Chemical Zoology. Academic Press, vol. V. pp. 255-286.

Schoffeniels, E., 1976. Biochemical approaches to osmoregulatory processes in crustacea. En: Davies PS (ed.). Perspectives in Experimental Biology. Pergamon Press. pp. 107-124.

Schultz, S.G., Hudson, R.L., Lapointe, J.-Y. 1985. Electrophysiological studies of sodium cotransport in epithelia: toward a cellular model. Ann. NY Acad. Sci. 456:127-135

Schultze, E., Neuhoff, V., 1983. Uptake, autoradiography and release of taurine and homotaurine from retinal tissue. Neuroscience 18:253-268.

Siebens, A.W., Kregenow, F.M. 1985. Volume-regulatory responses of Amphiuma red cells in anisotonic media. The effect of amiloride. J. Gen. Physiol. 86:527-564.

Siebens, A.W., Spring, K.R. 1989. A novel sorbitol transport mechanism in cultured renal papillary epithelial cells. Am. J. Physiol. 257:F937-F946.

Smith, H.E. 1992. Cloning and expression of a high affinity taurine transporter from rat brain. Mol. Pharmacol. 42:563-569.

Smith, L.H. Jr., Pierce, S.K. 1987. Cell volume regulation by molluscan erythrocytes during hypoosmotic stress: Ca^{2+} effects on ionic and organic osmolyte effluxes. Biol. Bull. 172:407-418

Solc, Ch., Wine, J.J. 1991. Swelling-induced and depolarization-induced Cl^- channels in normal and cystic fibrosis epithelial cells. Am. J. Physiol. 261:C658-C674.

Spring, K.R., Ericson, A.C. 1982. J. Membr. Biol. 69:167-176.

Suzuki, M., Kawahara, K., Ogawa, A., Morita, T., Kawaguchi, Y., Kurihara, S., Sakai, O. 1990. $[Ca^{2+}]i$ rises via G protein during regulatory volume decrease in rabbit proximal tubule cells. Am. J. Physiol. 258:F690-F696.

Taniguchi, J., Guggino, W.B. 1989. Membrane stretch: a physiological stimulator of Ca^{2+}-activated K^+ channels in medullary thick ascending limb cells. Am. J. Physiol. 257:F347-F352

Thurston, J.H., Hauhart, R.E., Dirco, J.A. 1980. Taurine: A role in osmotic regulation of mammalian brain and possible clinical significance. Life Sci. 26:1561-1568.

Thurston, J.H., Hauhart, R.E., Naccarato, E.G. 1981. Taurine: Possible role in osmotic regulation of the mammalian heart. Science 214:1373-1374.

Thurston, J.H., Sherman, W.R., Hauhart, R.E., Kloepper, R.F. 1989. *Myo*-inositol: a newly identified nonnitrogenous osmoregulatory

molecule in mammalian brain. Pediatr. Res. 26:482-485.

Tiedemann, F., Gmelin, L.H. 1827. Einige neue bestandtheile der galle des ochsen. Ann. Physik. Chem. 9:326-337.

Ubl, J., Murer, H. Kolb, H.A. 1988. Ion channels activated by osmotic and mechanical stress in membranes of opossum kidney cells. J. Membr. Biol. 104:223-232.

Ussing, H.H. 1986. Epithelial cell volume regulation illustrated by experiments in frog skin. Renal Physiol. 9:38-46.

Vislie, T. 1983. Cell volume regulation in fish heart ventricles with special reference to taurine. Comp. Biochem. Physiol. 76A:507-514.

Volkl, H., Lang, F. 1988. Ionic requirement for regulatory cell volume decrease in renal straight proximal tubules. Pfluegers Arch. 412:1-7.

Walz, W., Hinks, E.C., 1985. Carrier-mediated KCl accumulation accompanied by water movements is involved in the control of physiological K^+ levels by astrocytes. Brain Res. 343:44-51.

Walz, W., Kimelberg, H. 1985. Differences in cation transport properties of primary astrocytes cultures from mouse and rat brain. Brain Res. 340:333-340.

Walz, W. 1987. Swelling and potassium uptake in cultured astrocytes. Can. J. Physiol. Pharmacol. 65:1051-1057.

Warren, M.K., Pierce, S.K. 1982. Two cell volume regulatory systems in the Limulus myocardium: An interaction of ions and quaternary ammonium compounds. Biol. Bull. 163:504-506.

Watson, P.A. 1990. Direct stimulation of adenylate cyclase by mechanical forces in S49 mouse limphoma cells durin hyposmotic swelling. J. Biol. Chem. 265:6569-6575.

Wirthensohn, G.S., Lefrank, S., Schmolke, M., Guder, W.G. 1989. Regulation of organic osmolayte concentration in tubules from rat renal inner medulla. Am. J. Physiol. 256:F128-F135.

Wong, S.M.E., Chase, H.S. 1986. Role of intracellular calcium in cellular volume regulation. Am. J. Physiol. 250:C841-C852.

Worrell, R.T., Butt, A.G., Cliff, W.H., Frizzell, R.A. 1989. A volume-sensitive chloride conductance in human colonic cell line T84. Am. J. Physiol. 256:C1111-C1119.

Yancey, P.H. 1988. Osmotic effectors in kidneys of xeric and mesic rodents: corticomedullary distributions and changes with water

availability. J. Comp. Physiol. B 158:369-380.

Yamauchi, A., Uchida, S., Kwon, H.M., Preston, A.S., Robey, R.B., Garcia-Perez, A., Burg, M.B., Handler, J.S. 1992. Cloning of a Na^+- and Cl^--dependent betaine transporter that is regulated by hypertonicity. J. Biol. Chem. 267:649-652.

Yamauchi, A., Uchida, S., Preston, A.S., Kwon, H.M., Handler, J.S. 1993. Hypertonicity stimulates transcription of gene for Na^+-*myo*-inositol cotransporter in MDCK cells. Am. J. Physiol. 264:F20-F23.

Yantorno, R.E., Carré, D.A., Coca-Prados, M., Krupin, T., Civan, M.M. 1992. Whole cell patch clamping of ciliary epithelial cells during anisosmotic swelling. Am. J. Physiol. 262:C501-C509.