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REGULACIÓN DEL VOLUMEN CELULAR EN ASTROCITOS: MODULACIÓN POR TROMBINA

TESIS

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PRESENTA:

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ABREVIATURAS

BAPTA	ácido 1,2-bis (2-aminofenoxi) etano-N, N, N', N'-tetra-acético.
BAPTA-AM	ácido 1,2-bis (2-aminofenoxi) etano-N, N, N', N'-tetra-acético (acetoximetil éster).
BK	canal de potasio de conductancia amplia
$[Ca^{2+}]_i$	concentración de calcio intracelular
CaCCs	canales de cloro activados por calcio
Cl^-_{vol}	canal de cloro activado por volumen
DAG	diacilglicerol
DCPIB	ácido 4-[(2-butil-6,7-dicloro-2-ciclopentil-1-oxo-3H-inden-5-yl) oxi] butanoico
DIDS	4,4’ diisotiocianatoestilbeno-2,2’- ácido disulfónico
DL-TBOA	DL-treo-beta-benziloxiaspartato
DRV	decremento regulador del volumen
EGFR	(*epidermal growth factor receptor*), receptor al factor de crecimiento epidérmico
ERK	(extracellular-signal regulated kinase) cinasa regulada por señalización extracelular
FAK	(*focal adhesion kinase*) cinasa de adhesión focal
GPCR	(*G-protein coupled receptor*) receptor acoplado a proteínas G
GTP	trifosfato de guanosina
HEPES	ácido 4-(2-hidroxietil) piperazina-1-etanosulfónico
IP_3	inositol 1, 4, 5 trifosfato
$[K^+]_e$	concentración extracelular de potasio
NMDA	N-metil-D-aspartato
NPPB	5-nitro-2-(3-fenilpropilamino) ácido benzoico
MAPK	(*mitogen-activated protein kinase*) proteina cinasa activada por mitógenos
PAR	(*protease activated receptor*) Receptor activado por proteasas
PI3K	fosfatidilinositol 3-cinasa
PIP_2	fosfatidilinositol 4, 5-bifosfato
PKC	proteína cinasa C
PLC_β	isoforma β de la fosfolipasa C
PPACK	fenilalanil-prolil-arginina-clorometil cetona
SITS	4-acetamido-4'-isotiocianato-estilbeno-2,2'- ácido disulfónico
SNC	sistema nervioso central
SCN^-	tiocianato
$TFLLR\text{-}NH_2$	H-Thr-Phe-Leu-Leu-Arg-NH2 (péptido sintético agonista del receptor PAR-1)
TRP	receptor de potencial transitorio
VSOAC	(*volume sensitive organic anion channel*) canal aniónico de movilización de osmolitos orgánicos activado por volumen

RESUMEN

Los astrocitos son el tipo celular del sistema nervioso que exhibe un aumento en su volumen bajo diversas condiciones patológicas en las que se presenta edema celular cerebral. Como respuesta a este hinchamiento, los astrocitos movilizan activamente osmolitos intracelulares para contrarrestar el desbalance osmótico y lograr la recuperación del volumen original; uno de estos osmolitos es el glutamato, el principal neurotransmisor excitador. El aumento en los niveles de glutamato en el medio extracelular, puede llevar a la sobreactivación de sus receptores ionotrópicos neuronales y conduce a la muerte neuronal por excitotoxicidad. Bajo este escenario, un factor capaz de exacerbar el daño, es el incremento de los niveles de trombina en el tejido cerebral que ocurre cuando la patología asociada a edema transcurre junto con una alteración en la barrera hematoencefálica. La trombina ejerce efectos a nivel celular mediante la activación de los receptores PAR (Receptores Activados por Proteasas) acoplados a proteínas G. El daño por trombina en el cerebro bajo patologías asociadas a edema cerebral como la isquemia, se reporta consistentemente aunque los mecanismos que subyacen a este efecto lesivo no han sido aclarados.

La hipótesis planteada en esta investigación propone a la trombina como un elemento capaz de incrementar la movilización de glutamato que ocurre en respuesta al aumento en volumen. Los resultados de nuestra investigación en astrocitos en cultivo muestran que la trombina incrementa notablemente la salida de glutamato en condiciones de edema cerebral hiposmótico e isosmótico in vitro. Este efecto está mediado por la activación del receptor PAR-1 y participan la vía de señalización que conduce a la elevación Ca^{2+} intracelular iniciada por la fosfolipasa C, así como activación de PI3K. En el modelo de hinchamiento isosmótico, consistente en la presencia de una elevada concentración de K^{+} extracelular como ocurre durante la isquemia o el trauma craneoencefálico, se identificó a la trombina como un agente potenciador de la salida de glutamato en astrocitos únicamente a través de la vía de liberación activada por el aumento en volumen, sin afectar otros mecanismos de salida como son la exocitosis o la operación reversa del transportador. Este efecto requiere la previa apertura de la vía sensible al volumen. Las acciones de la trombina afectan igualmente la salida de taurina, considerada como representativo de la vía de liberación de osmolitos orgánicos. En conjunto estos resultados postulan a la potenciación de glutamato a través de la vía activada por volumen como un posible mecanismo de generación de daño causado por trombina en patologías que transcurren asociadas a edema cerebral y a una pérdida en la integridad de la barrera hematoencefálica.

ABSTRACT

In pathological conditions associated to brain edema, cell swelling is mainly seen in astrocytes. These glial cells respond to volume changes with the extrusion of intracellular solutes or osmolytes together with osmotically obligated water to recover their original cell volume. Glutamate, the major excitatory neurotransmitter, also play a role in water homeostasis; this represents a risk for brain excitability and viability since an increase in extracellular glutamate concentration due to cerebral edema may lead to neuronal ionotropic receptors overactivation, which triggers an auto-propagated chain of damage resulting in excitotoxic neuronal death. An exacerbation of this damage can occur when a rise in thrombin concentration takes place in cerebral tissue due to a disturbance in the blood brain barrier as occurs in many pathologies concurrent with cerebral edema. Thrombin exerts multiple cellular responses through the activation of the G-protein coupled receptors: PARs (Protease-Activated Receptors). Toxic effects associated with high concentrations of thrombin have been widely reported although the precise mechanisms underlying these effects are still unclear.

The hypothesis for the present research is that thrombin can increase the volume activated release of glutamate which could then be responsible for the damage observed in the presence high concentrations of thrombin. Results of our research in cultured astrocytes confirmed that thrombin leads to a remarkable increase of glutamate release under conditions of isosmotic and hyposmotic cerebral edema *in vitro*. This effect was mediated by the activation of PAR-1 and the subsequent signal transduction pathways that included intracellular calcium mobilization, elicited by PLC and the activation of PI3K. In the isosmotic swelling model, consisting in cell exposure to high extracellular potassium concentration as occurs in ischemia or head trauma, thrombin was shown to potentiate glutamate release through the volume-activated pathway and without affecting other release mechanisms as exocitosis or the reverse operating transporter. This effect of thrombin required a prior activation of the volume sensitive pathway. Thrombin actions influenced also the release taurine, largely considered as the representative for the organic osmolyte pathway. Taken together, these findings suggest that thrombin potentiation of glutamate release through the volume-activated pathway may be a mechanism involved in its toxic effects, observed in pathologies that concur with cerebral edema and a disruption of the blood brain barrier.

1. INTRODUCCIÓN

La preservación del mecanismo de regulación del volumen celular, a través de las distintas ramas de la evolución biológica, expone la relevancia fisiológica intrínseca de este proceso homeostático. El volumen de una célula es una característica determinada genéticamente y su alteración súbita repercute, sin lugar a dudas, sobre la compleja arquitectura de los compartimentos intracelulares, así como en las redes metabólicas y de señalización intracelular que dependen en gran medida de la concentración y distribución espacial de iones y biomoléculas.

La membrana plasmática se describe como semipermeable debido a su casi nula permeabilidad a las proteínas, su alta permeabilidad al agua y en menor grado y de manera selectiva a varios solutos de bajo peso molecular; esto origina que en ausencia de gradientes de presión hidrostática significativos, el flujo neto de agua a través de la membrana plasmática esté en función de la generación de gradientes de presión osmótica por concentraciones diferenciales de solutos a ambos lados de la membrana (Lang, 2011).

La ausencia de pared celular en las células animales y la naturaleza semipermeable de la membrana plasmática, son características que favorecen un estado proclive a modificaciones en el volumen. La estrategia de los organismos animales terrestres para evitar estas modificaciones continuas y mantener su homeostasis, consiste en regular finamente la osmolaridad del líquido intersticial que baña las células que los conforman; asegurando así, variaciones muy estrechas mediante el sistema renal en coordinación con el sistema neuroendocrino (Pedersen *et al.*, 2011). Aún bajo estas condiciones fisiológicas controladas, las células no están exentas de pequeñas modificaciones, ya que la generación de microgradientes osmóticos locales y transitorios es una consecuencia común de las actividades celulares básicas, en las que constantemente se forman y disipan elementos osmóticamente activos, como ocurre durante la síntesis y degradación de macromoléculas, procesos de dinámica del citoesqueleto o de captación de nutrientes y exocitosis, entre muchos otros (Hoffmann *et al.*, 2009).

El volumen celular puede presentar alteraciones más considerables bajo diversas condiciones patológicas en las que se pierde el control sobre la osmolaridad del plasma. El término edema proviene del griego οἴδημα, "hinchazón" y hace referencia al exceso de líquido en los tejidos que ocurre como consecuencia de una dinámica anormal de los fluidos. La causa más común de hinchamiento celular bajo condiciones anisosmóticas es la hiponatremia, los cambios en volumen que ocurren bajo estas situaciones no son permanentes ya que las células inician rápidamente mecanismos activos de recuperación del volumen.

Distintas patologías transcurren con aumentos en volumen bajo condiciones en las

que no se presentan cambios en la osmolaridad externa y son consecuencia más bien, de cambios en la redistribución iónica o de un aumento en el contenido intracelular de moléculas osmóticamente activas acompañadas por agua. Este aumento en volumen bajo condiciones de isosmolaridad ocurre en patologías como hipoxia, isquemia, epilepsias, trauma craneano o encefalopatía hepática (Lambert *et al.*, 2008).

La descripción más detallada del origen de las alteraciones en el volumen celular bajo condiciones patológicas, se abordará en secciones posteriores bajo el marco de las patologías que se asocian a el tipo celular de interés para este estudio.

1.1. El decremento regulador del volumen

Para contrarrestar un aumento en volumen, las células ponen en marcha el decremento regulador del volumen (**DRV**), un proceso complejo y dinámico que dirige una serie de adaptaciones celulares necesarias para enfrentar y revertir la alteración en volumen; entre éstas figuran la reorganización del citoesqueleto, modificaciones en la adhesión celular y la salida activa de solutos osmóticamente activos u osmolitos que al generar flujos de agua conducen al equilibrio osmótico y a la recuperación del volumen (Pasantes-Morales *et al.*, 2006).

El DRV puede entenderse como un proceso de tres etapas interconectadas; la cuales de manera muy general consisten en: la detección del cambio en volumen, su amplificación a través de una red de transducción de señales y la activación de mecanismos efectores. A continuación, se describirán en mayor detalle. El proceso general del DRV se encuentra esquematizado en la figura 1, al final de este apartado.

1.1.1. La detección del cambio en volumen

Las células son capaces de sensar y responder ante cambios en su volumen del orden de 3% (Wehner *et al.*, 2003; Hoffmann *et al.*, 2009). El elemento que tiene la función de sensar el cambio en volumen no está claramente identificado; sin embargo, hay varias propuestas con evidencia substancial que permanecen bajo discusión, las cuales se pueden englobar dentro de aquellas que plantean elementos que tienen que ver con cambios a nivel bioquímico y las que lo hacen a nivel mecánico.

Los cambios de carácter bioquímico, que se presentan durante el aumento en volumen, y a los que podía estar respondiendo el sensor de volumen ocurren principalmente sobre la fuerza iónica o el agrupamiento de las macromoléculas (Minton *et al.*, 1992; Burg, 2000; Sabirov *et al.*, 2000). El aumento en volumen, induce variaciones en el estado de asociación de las proteínas citosólicas lo que altera substancialmente sus

propiedades bioquímicas y en consecuencia su función, pudiendo llevar a un aumento o a una disminución de su actividad.

Las alteraciones celulares de tipo mecánico, se pueden detectar a nivel de la membrana plasmática o en la estructura del citoesqueleto. La remodelación del citoesqueleto generada por el aumento en volumen, se traduce también en cambios en la morfología membranal ya que ambas guardan una estrecha relación. (Hoffmann et al. 2009). Una gran variedad de moléculas insertadas en la membrana pueden responder a estos fenómenos, entre las cuales figuran el receptor EGFR, integrinas, así como canales activados por estiramiento. Sin embargo, a pesar de la existencia de varios casos documentados sobre esta activación de proteínas de membrana y dado que el DRV activa una diversidad de moléculas en una estrecha ventana espacio-temporal, es difícil establecer con claridad la señal inicial de aquellas que pertenecen a la red de transducción (Pasantes-Morales *et al.*, 2004; Lezama *et al.*, 2005; Pasantes-Morales *et al.*, 2006).

Ya que ninguna de las propuestas discutidas, abordada de manera individual, presenta evidencia suficiente para ser considerada la identidad encargada de sensar el volumen, se ha planteado que esta tarea está a cargo más bien, de un sistema conformado por varios de los elementos descritos anteriormente, que responden dependiendo de la magnitud y la naturaleza del cambio en volumen (Hoffmann *et al.*, 2009).

1.1.2. Osmotransducción. El papel de calcio

La detección del cambio en volumen pone en marcha la segunda etapa del DRV que engloba la señalización intracelular bajo la que se amplifica la señal del cambio en volumen y se conecta hacia los distintos efectores. El hinchamiento celular tiene profundos efectos en la señalización intracelular e involucra, además de la activación de los mecanismos de recuperación del volumen, moléculas relacionadas con las respuestas a estrés, sobrevivencia y reorganización del citoesqueleto, entre otras (Pasantes-Morales *et al.*, 2000a; Hoffmann y Pedersen, 2006).

Las vías de señalización activadas por el DRV, parecen ser en su mayoría, específicas para los distintos tipos celulares que han sido estudiados; sin embargo, dos tipos de señalización son constantes a través de los diversos tipos celulares: la activación de proteínas cinasa de tirosinas y el incremento en los niveles de Ca^{2+}_{i}. Con respecto a estas no se ha determinado por completo su función específica y jerarquía dentro del DRV, ni se ha establecido claramente la existencia de alguna interacción entre ellas (Pasantes-Morales *et al.*, 2006).

Dentro de la variedad de proteínas cinasas que modifican su actividad durante variaciones en el volumen celular se encuentran PI3K, PKC, los miembros de la familia de

las MAPK (JNK, ERK1/2 y p38) y diversas cinasas con actividad tirosina cinasa como miembros de la familia src, FAK y algunos receptores membranales (de La Paz *et al.*, 2002; Liu *et al.*, 2003; Lezama *et al.*, 2005). Las MAPK incrementan, en la mayoría de los casos, su actividad bajo condiciones hiposmóticas (principalmente ERK 1/2); sin embargo, parecen estar mayormente relacionadas con la activación de mecanismos adaptativos ante cambios en volumen a largo plazo, que al involucrar la participación de factores de transcripción, modulan la expresión de genes relacionados con el transporte y metabolismo de los osmolitos (Bettinger y Amberg, 2007). Las demás cinasas, con perfiles de activación a tiempos cortos que varían de acuerdo al tipo celular, se han relacionado más estrechamente con algunas vías de salida de osmolitos durante el DRV; en este sentido, se ha demostrado que los inhibidores de la familia src tienen un efecto sobre la corriente de Cl^- activada por volumen y canales de K^+ del tipo BK y K_v (Cohen, 2005).

La reducción en la osmolaridad extracelular va acompañada en un gran número de tipos celulares de un incremento en $[Ca_{2+}]_i$; sin embargo, solo en algunos sistemas se ha demostrado que el calcio participa directamente sobre la movilización de osmolitos. La intervención del calcio en el DRV es especialmente evidente en aquellos sistemas celulares, principalmente de origen epitelial, que utilizan vías de movilización de osmolitos activadas directamente por calcio; como son, la activación de K^+ del tipo BK. Además de los canales de K^+ también se ha demostrado que algunas conductancias de Cl^- y de taurina pueden ser reguladas por procesos dependientes de calcio y calcio-calmodulina (MacLeod y Hamilton, 1999; Li *et al.*, 2002)

La fuente del aumento en Ca^{2+}_i depende del tipo celular evaluado, aunque la mayoría de los estudios enfocados a dilucidar el papel del calcio en la regulación del volumen celular, describen que el aumento en calcio tiene un componente de entrada extracelular y otro de liberación de pozas intracelulares. Los canales de calcio que se han descrito como involucrados en la entrada de calcio durante la hiposmolaridad incluyen canales activados por estiramiento de membrana, canales de calcio activados por voltaje de tipo L, y canales TRP (Wehner *et al.*, 2003; Liedtke y Kim, 2005; Gees *et al.*, 2010)

1.1.3. Mecanismos efectores: osmolitos y su movilización

Los osmolitos que la célula moviliza durante el DRV son esencialmente de dos tipos: iones inorgánicos, principalmente los iones de mayor abundancia intracelular el Cl^- y K^+ y compuestos orgánicos de bajo peso molecular. Los osmolitos orgánicos son un grupo heterogéneo de pequeñas moléculas entre las que se encuentran compuestos metilados (metilaminas, glicerofosforilcolina y betaina), polialcoholes (sorbitol o inositol), aminoácidos

libres y sus derivados como glutamina, glutamato, glicina, GABA y taurina (Junankar y Kirk, 2000; Khan *et al.*, 2010; Hoffmann y Pedersen, 2011).

Algunos de los osmolitos orgánicos se conocen como osmolitos compatibles, un término que indica que llevan a cabo su función osmoreguladora sin comprometer otras funciones en la célula; entre estos la taurina es considerada como el osmolito representativo, debido a sus diversas propiedades entre las que sobresale su presencia en un gran número de tipos celulares a muy elevadas concentraciones (1-40 mM) y de forma soluble en el citosol. Además, la taurina no forma parte de la estructura de las proteínas, y no participa en reacciones metabólicas, con excepción de la síntesis del ácido taurocólico, lo que le permite desplazarse dentro y fuera de la célula, sin afectar el metabolismo celular. La rápida movilización de la taurina en respuesta al aumento en volumen está ampliamente documentada y se ha descrito su salida frente a cambios tan pequeños en osmolaridad como 2-3 mOsmolas (Pasantes-Morales *et al.*, 2000b).

Las vías de movilización de iones y osmolitos orgánicos durante el DRV han sido estudiadas en detalle y se ha llevado a cabo la caracterización de sus propiedades a nivel farmacológico y para el caso de los canales que movilizan a los iones sus características electrofisiológicas; sin embargo, a excepción del K^+ y a pesar de la extensa investigación dedicada a este campo, no se ha logrado identificar de manera específica la identidad molecular encargada de la movilización del Cl^- ni de los osmolitos orgánicos (Pasantes-Morales *et al.*, 2006).

La movilización del K^+ ocurre a través de vías activadas por fenómenos coincidentes con el aumento en volumen celular como son la despolarización por la salida inicial de Cl^-, que activa canales de K^+ activados por voltaje (K_v), o el aumento en calcio que puede activar canales de K^+ del tipo BK. Se ha reportado que distintos canales de K^+ pueden activarse durante el aumento en volumen en un mismo tipo celular; ya que mientras cambios pequeños en la osmolaridad, activan un canal presumiblemente del tipo de maxi-canales de K^+, dependiente de Ca^{2+} y sensible a caribdotoxina; cambios más pronunciados, activan una corriente independiente de Ca^{2+} que parece corresponder a algún canal de la familia 4M2P. (MacLeod y Hamilton, 1999; Ordaz *et al.*, 2004).

El Cl^- se moviliza a través del denominado canal de cloro regulado por volumen (Cl^-_{vol}); este canal aniónico no selectivo, muestra entre sus características biofísicas una rectificación saliente e inactivación a potenciales positivos y dependiente de tiempo, presenta una secuencia de permeabilidad Einsenman I: : $SCN^->I^->Br^->Cl^->F^->$gluconato y depende de la presencia de ATP intracelular más no de su hidrólisis (d'Anglemont de Tassigny *et al.*, 2003; Okada, 2006). Esta corriente es sensible a inhibidores generales de canales de Cl^- como DIDS, SITS, 9-AC, NPPB, DDFK, ácido niflúmico y ácido flufenámico;

recientemente, el compuesto DCPIB, se describió como un inhibidor selectivo para el canal y sin efectos visibles sobre otros canales de Cl^- examinados (Decher *et al.*, 2001). A pesar de que se ha reportado en numerosas ocasiones la clonación del canal que produce esta corriente, con entidades moleculares pertenecientes a de la familias ClC y CaCCs, entre otros, con el paso del tiempo todas estas propuestas han sido refutadas o permanecen altamente cuestionadas (Nilius *et al.*, 1996; Almaca *et al.*, 2009; Okada *et al.*, 2009).

Los osmolitos orgánicos se movilizan a través de una vía difusional presumiblemente a través de un poro parecido a un canal que se ha denominado canal de movilización de osmolitos orgánicos sensible a volumen (VSOAC). La movilización de osmolitos orgánicos muestra una dirección de flujo determinada por el gradiente de concentración, sin la contribución de transportadores dependientes de Na^+ y exhibe un perfil farmacológico notablemente similar al del Cl^-_{vol}; sin embargo, existen múltiples evidencias que las identifican como vías independientes por lo que las similitudes que llegan a presentar sugieren una interdependencia entre ambas vías (Sanchez-Olea *et al.*, 1991; Sanchez-Olea *et al.*, 1996; Abdullaev *et al.*, 2006).

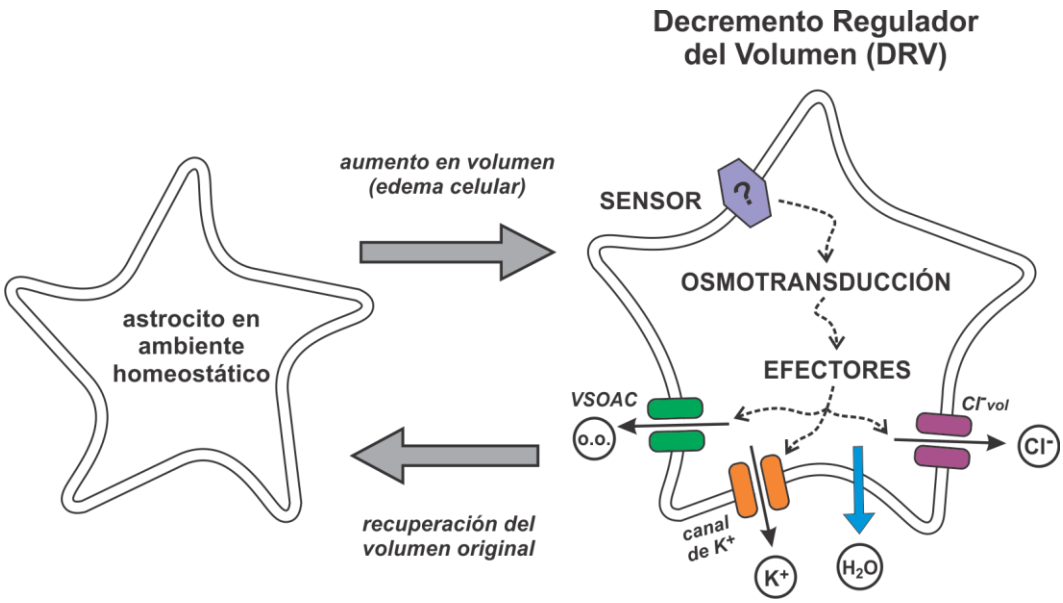

Figura 1. El Decremento Regulador del Volumen (DRV). Las células se encuentran en un ambiente de osmolaridad extracelular finamente regulada. Un cambio en la osmolaridad externa o una redistribución de iones o moléculas puede originar un aumento en el volumen celular por entrada de agua osmóticamente obligada. El DRV, la respuesta celular activa ante el hinchamiento, consta de tres etapas: el "sensor" de volumen, cuya identidad permanece desconocida, se encarga de la detección del cambio en volumen y la activación de las vías de osmotransducción que finalmente ponen en marcha la movilización de osmolitos. Los osmolitos más comúnmente usados por las células son los iones con mayor presencia intracelular: cloro y potasio, que se movilizan a través de canales; así como el grupo heterogéneo de los osmolitos orgánicos (o.o.) que se moviliza a través del denominado canal aniónico de osmolitos orgánicos activados por volumen (VSOAC).

1.2. Edema cerebral. Relevancia de los astrocitos

La alteración del volumen celular adquiere una relevancia especial en el cerebro debido a las restricciones físicas que impone el cráneo a la expansión del tejido cerebral. La condición de edema cerebral, ejerce inicialmente una fuerte presión sobre los pequeños capilares originando una disminución en la irrigación de oxigeno y nutrientes, posteriormente puede presentarse ruptura de vasos y en casos muy extremos, si la condición persiste, el tejido cerebral se desplaza hacia la zona de menor resistencia el *foramen magnum*; originando la compresión del tallo cerebral con consecuencias extremadamente delicadas debido a que esta estructura es la vía de comunicación del cuerpo con el encéfalo y ubica centros nerviosos implicados en la generación y regulación de los ritmos respiratorio y cardiaco del organismo (Pasantes-Morales, 1996; Sterns y Silver, 2006; Verbalis, 2010).

Aunado a esto, el edema cerebral tiene importantes implicaciones a nivel celular, ya que la liberación hacia el espacio extracelular de los iones Cl^- y K^+, debida a la propia activación del proceso de DRV, así como de algunos osmolitos orgánicos pueden alterar la excitabilidad neuronal; tal es el caso del glutamato, el neurotransmisor excitador más importante del sistema nervioso que al estar presente en altas concentraciones a nivel intracelular es empleado por la célula como un osmolito orgánico durante el DRV (Pasantes-Morales *et al.*, 2002a).

El edema cerebral puede ser vasogénico o celular; el primero ocurre cuando se genera un daño en la barrera hematoencefálica y origina la expansión del espacio intersticial por la entrada de agua y proteínas provenientes del plasma. El de tipo celular ocurre cuando hay una acumulación de agua en el citosol debida a perturbaciones en la distribución normal de osmolitos en el espacio intersticial. El desarrollo de edema vasogénico suele llevar a la generación de edema celular y viceversa, ya que la expansión del volumen citosólico puede producir perturbaciones de la barrera hematoencefálica (Pasantes-Morales y Cruz-Rangel, 2009).

1.2.1. El papel de la astrocitos en el edema celular cerebral

A nivel celular son los astrocitos los que presentan hinchamiento bajo condiciones de edema cerebral. Los astrocitos son la población glial más abundante en el cerebro y tienen un papel fundamental en la preservación de la homeostasis, ya que entre otras cosas, aportan de nutrientes y factores de crecimiento a las neuronas, juegan un papel central en el ciclo del glutamato y mantienen los niveles extracelulares neurotransmisores y de K^+ adecuados para la excitabilidad neuronal; además constituyen la parte celular de la

barrera hematoencefálica y conforman la matriz sobre la que se distribuyen y migran las neuronas (Panickar y Norenberg, 2005).

El hinchamiento preferente de los astrocitos sobre las neuronas puede ser el resultado de un manejo distinto de los osmolitos o de una redistribución de osmolitos entre neuronas y células gliales como parte de su función como salvaguardas de la homeostasis neuronal. En este sentido, un estudio *in vivo* de Nagelhus y colaboradores en 1993 mostraron que en el cerebelo de ratas, la aplicación de una condición experimental de hiponatremia por reducción en osmolaridad del plasma, genera una inmediata redistribución del alto contenido celular de taurina de las células Purkinje hacia los astrocitos cercanos; el resultado de este fenómeno es que los astrocitos aumentan su volumen, mientras que las neuronas permanecen sin cambios (Nagelhus *et al.*, 1994).

Otro factor podría ser la presencia de un mecanismo intrínseco que prevenga la entrada de agua a las neuronas, como una reducida expresión de aquaporinas. La AQP-4 es la más expresada en el cerebro y se encuentra principalmente en astrocitos; exhibe una alta presencia en los pies de los astrocitos que conforman la barrera hematoencefálica y en los astrocitos subependimales, aunque también se expresa en otras regiones cerebrales (Venero *et al.*, 2001; Badaut *et al.*, 2002; Papadopoulos y Verkman, 2007).

Uno de los mecanismos homeostáticos a cargo de los astrocitos y que puede estar implicado en el hinchamiento observado bajo algunas condiciones patológicas es el "amortiguamiento del K^+"; a través de este mecanismo se regula la concentración de K^+ extracelular ($[K^+]_e$) y se mantiene en niveles constantes cercanos a 3 mM (Somjen, 1979). Los procesos neuronales como la señalización eléctrica rápida involucran la entrada de Na^+ y la salida de K^+ de las neuronas y dado a que el volumen del espacio extracelular es muy limitado en el SNC, aún pequeños flujos de K^+ hacia el exterior celular pueden producir cambios considerables en la $[K^+]_e$ (Sykova y Nicholson, 2008). Las modificaciones en la $[K^+]_e$ tienen un profundo impacto sobre la fisiología de las células del CNS ya que alteran características tan importantes como el potencial de reposo de la membrana, la activación e inactivación de canales sensibles a voltaje, la transmisión sináptica y el transporte electrogénico de los neurotransmisores. Es por esto, que el exceso de K^+ en el espacio intersticial es removido y acumulado temporalmente por los astrocitos, desde donde circula a través de las uniones comunicantes del sincicio astroglial, hacia zonas de menor concentración (Kofuji y Newman, 2004).

Existen diversos elementos involucrados en la remoción y temporal acumulación de K^+ en los astrocitos; entre ellos se han descrito algunos canales y transportadores. El principal canal de K^+ presente en astrocitos es el canal de rectificación entrante de K^+ (Kir), su isoforma más expresada es el Kir 4.1. Este canal presenta propiedades únicas que lo

postulan como un elemento importante del mecanismo de amortiguamiento del K^+, entre estas se encuentran la facilitación de un flujo entrante de iones K^+ sobre su salida, su alta probabilidad de apertura al potencial de reposo de la membrana y el incremento en su conductancia conforme aumenta la $[K^+]_e$ (Butt y Kalsi, 2006). Se ha demostrado que ratones *knockout* para el Kir 4.1 exhiben una captación de K^+ deficiente (Kucheryavykh *et al.*, 2007; Olsen y Sontheimer, 2008).

La acumulación de K^+ en astrocitos también ha sido ligada a la actividad del NKCC. Este cotransportador electroneutro pertenece a la familia de los SLC12, su isoforma NKCC1 está ampliamente distribuida en diversos tejidos y tipos celulares incluyendo a los astrocitos. El transportador opera acoplando la energía de los gradientes transmembranales y bajo la estequiometria $1Na^+:1K^+:2Cl^-$ (Isenring y Forbush, 2001) En astrocitos la acumulación intracelular de K^+ debida a un aumento en su concentración extracelular, puede ser parcialmente prevenida mediante los inhibidores de NKCC furosemida o bumetanida o por la remoción experimental del Na^+ y Cl^- extracelular (Walz, 1992; Ransom *et al.*, 1996).

En condiciones patológicas como la hipoxia, la isquemia y la depresión cortical propagada, la $[K^+]_e$ puede alcanzar niveles tan altos como 60-80 mM, este aumento extracelular en K^+ puede llevar a que los sistemas de amortiguamiento del K^+ se vean sobrepasados y los astrocitos acumulen un exceso de K^+ generando hinchamiento celular (Kofuji y Newman, 2004; Leis *et al.*, 2005).

1.2.2. La hiponatremia y la generación de edema celular cerebral hiposmótico

El edema cerebral es una condición asociada a numerosas patologías, con consecuencias clínicas frecuentemente más graves que la propia patología de origen; el edema cerebral hiposmótico ocurre principalmente durante el curso de hiponatremia aguda. La hiponatremia se define como una reducción en la concentración de Na^+ en el suero de un valor normal de 145 a 136 mEq/litro y dado que el Na^+ es el elemento más abundante en el plasma de los animales terrestres, una disminución en su concentración se traduce inmediatamente en un decremento de la osmolaridad plasmática. La disminución en la concentración de Na^+ puede deberse a una retención o exceso de agua en el plasma que origine su dilución, o porque se reduzcan directamente los niveles de sodio sérico. La retención de agua ocurre bajo diversas patologías como insuficiencia renal o cardiaca, así como por una secreción inadecuada de la hormona antidiurética; un exceso de agua por su parte, puede deberse a una ingesta rápida y excesiva de agua que sobrepase la capacidad renal, tal como ocurre durante la polidipsia psicótica. Por otra parte, un decremento en el Na^+ sérico ocurre generalmente debido a una pérdida excesiva

de Na^+, en respuesta a una deficiencia en mineralocorticoides, durante episodios de diarrea o vómito severo o de quemaduras extensas, así como durante pruebas de resistencia física extrema. El síndrome nefrótico, una severa disfunción renal, lleva también a hiponatremia; se presenta en el contexto de una variedad de enfermedades primarias y sistémicas, tales como diabetes, lupus eritematoso, y en ciertas neoplasias (Pasantes-Morales *et al.*, 2002b).

1.2.3. La isquemia y la generación de edema celular cerebral isosmótico

La isquemia cerebral es una patología que induce hinchamiento celular en condiciones isosmóticas, es decir, ocurre sin la alteración de la osmolaridad plasmática; se presenta cuando hay una reducción o interrupción en el flujo sanguíneo que impide a las células nerviosas recibir un correcto suministro de oxigeno y glucosa, sus principales elementos metabólicos. Esto se traduce inmediatamente en una falla energética celular, ya que la alta tasa metabólica del cerebro y la baja capacidad de almacenamiento y síntesis de sustratos, requiere de su aporte constante a través de un elevado flujo sanguíneo. La disminución en los niveles de ATP origina el arresto de la ATPasa Na^+/K^+ y el colapso progresivo de los gradientes iónicos transmembranales; esta situación, provoca la despolarización generalizada de las células que eleva los niveles de K^+_e. Como ya se describió anteriormente, el aumento en los niveles de K^+ extracelular propicia su acumulación excesiva en astrocitos induciendo un aumento en su volumen; se ha reportado el incremento de la actividad funcional del NKCC1, así como, su expresión bajo condiciones de isquemia. Además, la magnitud del edema celular disminuye en presencia de su bloqueador bumetanida o en ratones *knockout* para el NKCC1 (Su *et al.*, 2002a; Chen y Swanson, 2003; Jayakumar y Norenberg, 2010).

Paralelo al incremento de K^+ extracelular, durante la isquemia se presenta un aumento en la concentración de glutamato debido a que su remoción del espacio extracelular, a cargo principalmente de los astrocitos, depende del funcionamiento de transportadores acoplados al gradiente de Na^+. Y dado que la isquemia propicia la inversión del gradiente de Na^+ por la alta despolarización y el arresto de la ATPasa Na^+/K^+, bajo esta situación, el transportador no solo falla en remover el glutamato extracelular sino que contribuye a su salida desde dentro de la célula por su operación en reversa (Gamba, 2005). Otra vía que colabora a la elevación nociva de la concentración extracelular de glutamato es la activada por el incremento en el volumen celular, ya que el glutamato es uno de los osmolitos que la célula moviliza como parte del proceso activo de la regulación del volumen celular. Estas elevadas concentraciones de glutamato eventualmente disparan la cascada excitotóxica que consiste en la sobreexcitación de los receptores ionotrópicos a glutamato, principalmente el receptor de tipo NMDA, con la consecuente entrada masiva

de calcio a la célula y la activación de diversas rutas enzimáticas que participan en la muerte neuronal como proteasas, lipasas, endonucleasas y especies reactivas de oxígeno (Camacho y Massieu, 2006; Rossi *et al.*, 2007). A este nivel cualquier otro elemento que promueva la liberación de glutamato será responsable de una exacerbación del daño excitotóxico.

1.3. Modulación del decremento regulador del volumen por Receptores Acoplados a Proteínas G (GPCRs)

Los receptores acoplados a proteínas G (GPCRs) constituyen la más grande y diversa superfamilia de receptores membranales, su mecanismo de transducción clásico se inicia con el cambio conformacional que induce la unión extracelular de su ligando y promueve la activación de las proteínas G heterotriméricas a las que se encuentra asociado en la parte intracelular. Las proteínas G son un complejo de tres subunidades α, β y γ que al activarse se disocian en α y el complejo βγ, pudiendo cada uno activar a su vez moléculas efectoras específicas. Existen varias isoformas de proteínas Gα y dependiendo del subtipo al que se encuentre asociado el receptor, es la vía de señalización que desencadena (Marinissen y Gutkind, 2001).

La primera evidencia que mostró un papel para los GPCRs dentro de la osmoregulación fue reportada por Bender y colaboradores (1993) quienes describieron, que cultivos primarios de astrocitos expuestos a una condición hiposmótica, conseguían recuperar su volumen más rápidamente en presencia de ligandos de GPCRs como endotelina o norepinefrina (Bender *et al.*, 1993). Algunas evidencias indirectas, posteriormente descritas, apuntaron fuertemente a que la participación de GPCRs podía estar mediada en parte, a través de su conocida capacidad de inducir la movilización intracelular de calcio; entre estas, Cardin y colaboradores (2003) mostraron que, al generar un aumento en los niveles de calcio intracelular mediante el uso del ionóforo de Ca^{2+} ionomicina durante la hiposmolaridad, se observa un aumento prominente de la salida osmosensible de taurina y la tasa de recuperación de su volumen original también se ve significativamente incrementada. El trabajo de Cardin, postuló al Ca^{2+} como un elemento potenciador de la respuesta dirigida por los mecanismos del DRV ante la hiposmolaridad, esto debido a que el efecto es únicamente observable cuando el incremento en calcio coexiste con un estímulo hiposmótico, no así cuando es aplicado en condiciones isosmóticas (Cardin *et al.*, 2003). Aunado a esto, el hecho de que la mayor parte de las células lleven a cabo el DRV de manera independiente de Ca^{2+} (como se menciona en la sección de Osmotransducción) y que por otra parte el aumento en calcio mimetizado por el ionóforo tenga un efecto potenciador sobre la salida de osmolitos activada por volumen

planteó la posibilidad de que es la elevación de calcio, por encima de los niveles alcanzados durante hiposmolaridad, la que adquiere un papel modulador de las respuestas del DRV.

Estudios posteriores mostraron la habilidad de distintos agonistas de GPCR para inducir el fenómeno de potenciación en cultivos de la línea de fibroblastos Swiss 3T3. Franco y colaboradores (2004) mostraron que la adición de ATP al estímulo hiposmótico induce una potenciación de la salida de taurina mediada por los receptores purinérgicos metabotrópicos P2Y; la respuesta, parcialmente dependiente de la liberación intracelular de Ca^{2+}, está mediada por las moléculas de señalización PI3K y CaMKII (Franco *et al.*, 2004b). En una investigación, que marca el antecedente directo del presente estudio, Vázquez-Juárez y colaboradores mostramos que la trombina ejerce un efecto similar, de potenciación de la liberación de taurina, así como de la corriente de Cl^- activada por volumen y acelera la tasa a la que las células recuperan su volumen; la vía se señalización caracterizada en este estudio involucra la activación del receptor PAR-1 por la trombina y la participación de una vía mediada por calcio y una vía independiente de calcio que incluye la transactivación del receptor EGFR mediada por la cinasa citosolica c-src (Vazquez-Juarez *et al.*, 2008b).

1.4. Trombina y receptores activados por proteasas (PARs)

1.4.1. Estructura de la trombina

La trombina (EC 3.4.21.5) es una enzima proteolítica de la familia de las serina proteasas, denominadas así por la presencia de una serina en el sitio activo que resulta esencial para la hidrólisis del enlace peptídico. Se sintetiza en el hígado en forma de su zimógeno protrombina y desde ahí es liberada hacia el plasma sanguíneo; su forma activa se genera en respuesta al daño del tejido vascular y en presencia de los factores Xa, Va, calcio y fosfolípidos de membrana.

A nivel de estructura esta proteasa de 37 kDa está constituida por dos cadenas peptídicas, la ligera A de 49 aa y la pesada B de 259 aa, unidas a través de un puente disulfuro; esta última aloja el sitio catalítico y los dos exositios que modulan su interacción con el sustrato. Los estudios cristalográficos indican que la trombina tiene una estructura globular elipsoidal y que el sitio activo se encuentra dentro de una hendidura angosta y pronunciada lo que le brinda especificidad (Stubbs y Bode, 1995; Grand *et al.*, 1996).

Tradicionalmente se ha conocido a la trombina por su papel central en el proceso de coagulación sanguínea, ya que cataliza la conversión del fibrinógeno circulante en monómeros de fibrina insoluble que al polimerizarse conforman la matriz fibrosa del coágulo sanguíneo (Coughlin, 2000); sin embargo, actualmente se sabe que es una

enzima multifuncional que además de participar en la hemostasis, es capaz de inducir una diversidad de respuestas celulares mediante la señalización a través de los receptores activados por proteasas (PARs; **P**roteinase-**A**ctivated **R**eceptors) (Grand *et al.*, 1996; Coughlin, 2005).

1.4.2. Receptores activados por proteasas

Los PARs son una familia de receptores perteneciente a la superfamilia de GPCRs. Hasta el momento se han descrito cuatro miembros de la familia: del PAR-1 al 4, denominados así de acuerdo al orden en que fueron clonados (Macfarlane *et al.*, 2001).

El mecanismo de activación de los receptores PAR es peculiar, ya que a diferencia del común de receptores G que transducen en respuesta a la unión de un ligando soluble, los PARs se activan por la unión intramolecular de una secuencia peptídica que se encuentra encriptada en la región amino-terminal extracelular del propio receptor, la cual se expone mediante el corte proteolítico (Fig. 2); la trombina induce la activación de los miembros PAR-1 y -4, la activación del PAR-3 se encuentra bajo discusión y el PAR-2 se activa mediante el corte por otras serina proteasas, principalmente tripsina. (Coughlin, 2000; Macfarlane *et al.*, 2001; Hollenberg y Compton, 2002; Coughlin, 2005). El sitio de corte N-terminal y las secuencias de unión intramolecular son específicas para cada uno de los cuatro receptores; con base en esta característica se han desarrollado péptidos sintéticos que mimetizan las secuencias-ligando, lo cual ha sido una herramienta muy útil para el estudio específico de cada receptor PAR y para descartar otras múltiples acciones que las enzimas proteolíticas pudieran tener a nivel celular (Vu *et al.*, 1991).

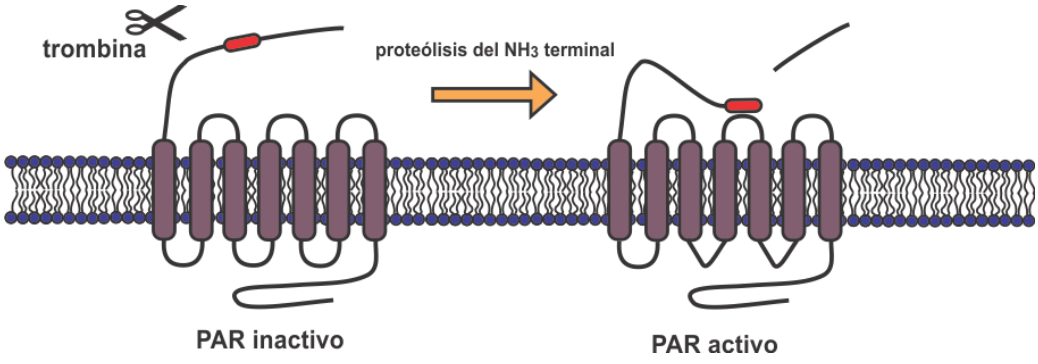

Figura 2. Mecanismo único de activación de los receptores de la familia PAR. La activación se lleva a cabo mediante el corte proteolítico por la trombina que desenmascara una nueva secuencia amino-terminal. La unión intramolecular de esta secuencia a la segunda asa intracelular genera el cambio conformacional que inicia la señalización. Modificada de Coughlin, 2005.

El prototipo de la familia PAR y más estudiado es el receptor PAR1. La isoforma humana está constituida por 425 aminoácidos y presenta en el extremo amino-terminal,

dos regiones de interacción con trombina, altamente conservadas entre los receptores de mamíferos. La primera región de interacción, interviene en el reconocimiento del sitio de corte del receptor humano por el dominio catalítico de la trombina (LDPR41↓S42FLLRN) y entre los residuos 50 al 55 se sitúa el dominio rico en residuos cargados (D50KYEPF55) que interacciona fuertemente con el exositio I de unión a aniones de la trombina; la presencia de este dominio explica la activación del receptor PAR-1 a bajas concentraciones de trombina (Nanevicz *et al.*, 1995).

El PAR-3 presenta al igual que PAR-1, la región de interacción con el exositio de unión a aniones de la trombina; sin embargo, su corte proteolítico no inicia cascadas de señalización en murinos y parece insensible a la activación por el péptido sintético que mimetiza la secuencia amino-terminal expuesta tras del corte. Estas evidencias han llevado a proponer que el PAR-3 funciona más bien como un cofactor para la activación del receptor de baja afinidad PAR-4 el cual carece de sitios de interacción a exositios de la trombina. La hipótesis planteada sostiene que el PAR-3 interaccionaría con el dominio de unión a aniones de la trombina, estabilizando la enzima, lo que facilitaría la activación del PAR-4 (Nakanishi-Matsui *et al.*, 2000; Hollenberg y Compton, 2002).

1.4.3. Vías de señalización del PAR-1

El receptor PAR-1 induce una gran diversidad de respuestas celulares mediante su asociación con los subtipos de proteínas G heterotriméricas G_q (α_q), G_i (α_i), G_{12} (α_{12}) y G_{13} (α_{13}). La vía clásica de cada subtipo se describe brevemente a continuación: la asociación del receptor a la proteína $G_{q/11}$ activa a la fosfolipasa C β (PLCβ) que conduce la catálisis de fosfatidilinositol-bifosfato (PIP_2) de membrana en inositol-trifosfato (IP_3) y diacilglicerol (DAG), el IP_3 induce el aumento de los niveles de $[Ca^{2+}]_i$ mediante la apertura de canales específicos en el retículo endoplásmico, el Ca^{2+} liberado junto con el DAG activan a las isoformas clásicas (α, β y γ) de la proteína cinasa C (PKC) (Babich *et al.*, 1990; Hung *et al.*, 1992). Por su parte la asociación de G_i, desencadena la inhibición de la producción de AMPc y con de G_{12}/ G_{13} regula señales hacia citoesqueleto mediante la estimulación de la polimerización de actina y la formación de fibras de estrés (Hung *et al.*, 1992; Buhl *et al.*, 1995; Kanthou *et al.*, 1996; Gohla *et al.*, 1999) (Fig. 3).

La señalización de los PAR se ve rápidamente concluida por mecanismos como la fosforilación en la región carboxilo terminal citoplásmica, mediada por la cinasa del receptor acoplado a proteínas G (GRK), la unión de proteínas adaptadoras como la β-arrestina y la internalización del receptor dirigida predominantemente hacia lisosomas para su posterior degradación ya que dada su activación de naturaleza proteolítica, no puede ser reciclado (Hoxie *et al.*, 1993; Ishii *et al.*, 1994; Trejo, 2003). La rápida inactivación que muestran los receptores PARs induce la formación de una cantidad finita de segundos

mensajeros; de esta manera, la concentración que alcanzan los mensajeros dentro de la célula en un momento determinado, es proporcional a la tasa en que los receptores se proteolizan y activan, lo que les permite dirigir respuestas proporcionales a la concentración de trombina (Ishii *et al.*, 1993; Coughlin, 2005).

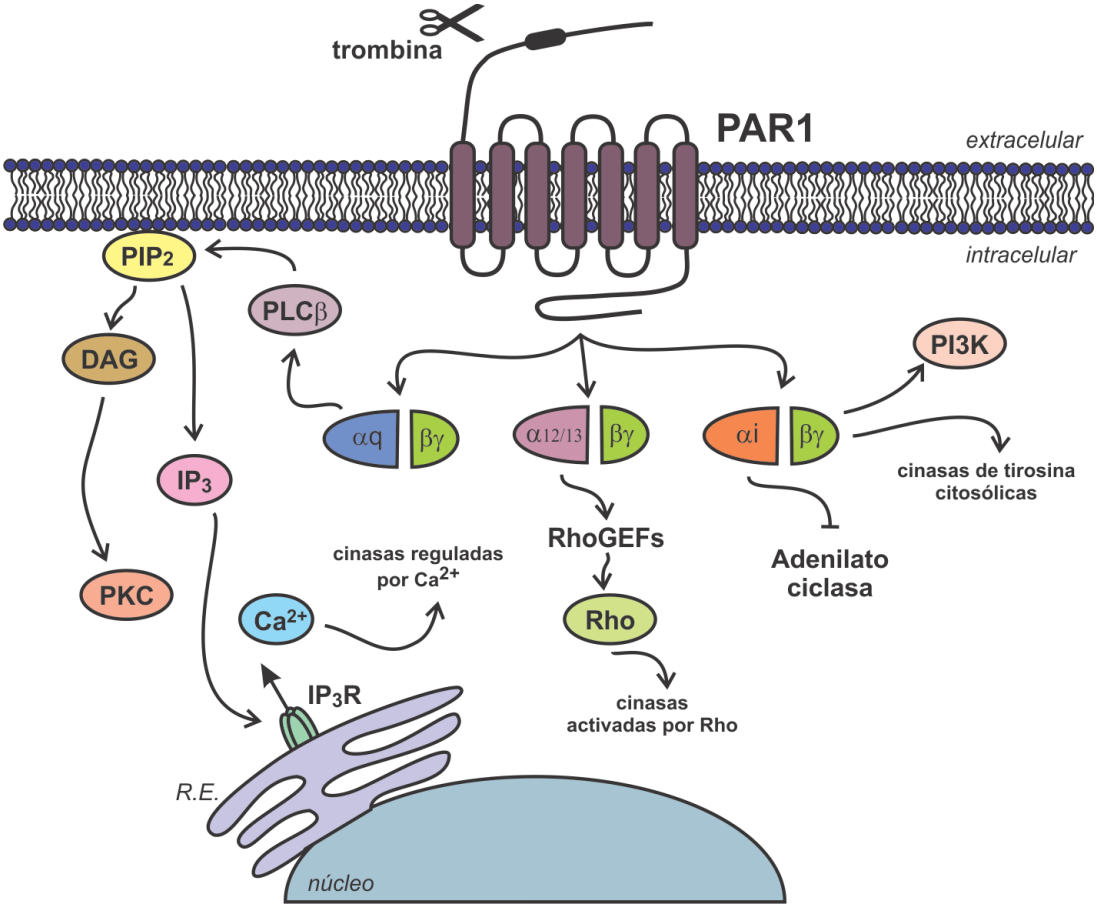

Figura 3. **Vías de señalización activadas por el PAR1**. Cascada abajo de la activación del receptor se encuentran las vías asociadas a Gαq, Gα12/13 y Gαi. Abreviaturas: PAR1, receptor activado por proteasas 1; PLCβ, fosfolipasa C β, PIP2, fosfatidilinositol 4,5-bifosfato; DAG, diacilglicerol; IP3, inositol trifosfato; PKC, proteína cinasa C; RhoGEFs, factores intercambiadores de nucleótidos de guanina para Rho; PI3K, fosfoinosítido-3 cinasa; R.E., retículo endoplásmico. Modificada de Coughlin, 2005.

1.5. Trombina y activación de PARs en el cerebro

La protrombina y su forma activa, al igual que muchas de las macromoléculas circulantes en la sangre, no atraviesan la barrera hematoencefálica (BHE) en condiciones fisiológicas; sin embargo, el cerebro posee los elementos necesarios para producir, activar y regular la función de la trombina endógenamente (Sokolova y Reiser, 2008).

Se ha detectado la presencia de protrombina y su RNAm en el bulbo olfatorio, la corteza, el cerebelo y en cultivos primarios de astroglía y de líneas celulares de sistema

nervioso de humano y rata (Deschepper *et al.*, 1991; Dihanich *et al.*, 1991; Weinstein *et al.*, 1995).

La amplia distribución tanto de la trombina como de los receptores PAR, sugiere abiertamente su papel como molécula local de señalización en el cerebro. Numerosos estudios han demostrado que los receptores PARs inducen una gran variedad de respuestas en el cerebro, aunque aún no es claro cuáles de estás ocurren de manera fisiológica. En diversos tipos neuronales y en astrocitos, se ha observado que la trombina modula cambios en la morfología celular, induce la retracción de neuritas y revierte la forma estrellada de los astrocitos a una morfología plana de tipo epitelial. En astrocitos induce además, la secreción del factor de crecimiento nervioso NGF y del vasoconstrictor endotelina-1 e interviene en señales de proliferación (Grand *et al.*, 1996; Suidan *et al.*, 1996).

1.5.1. Trombina y PARs en condiciones patológicas asociadas a edema cerebral

Las respuestas inducidas por trombina en el cerebro se mantienen altamente controladas a diferentes escalas, ya que existe una regulación tanto de los elementos responsables de la conversión de pro-trombina a trombina así como de la expresión de los receptores PAR; también, se ha detectado la presencia de inhibidores endógenos que mantienen los niveles de trombina activa en un estrecho margen. Sin embargo, se ha demostrado que este balance puede verse seriamente alterado en condiciones patológicas.

Está ampliamente documentada la participación de la trombina en situaciones de daño por isquemia, trauma craneano y episodios hemorrágicos. La trombina en estas patologías se encuentra en elevadas concentraciones debido al aumento en su expresión endógena (Riek-Burchardt *et al.*, 2002; Hua *et al.*, 2003; de Castro Ribeiro *et al.*, 2006), mientras que los niveles de su proteína inhibidora PN-1 se mantienen normales (Riek-Burchardt *et al.*, 2002). La protrombina circulante en la sangre se encuentra en altas concentraciones (~1μM) y durante el transcurso de patologías como trauma craneano o isquemia, puede generarse una cantidad suficiente de trombina sistémica que potencialmente ingresará en el parénquima cerebral si se presentan alteraciones en la permeabilidad de la barrera hematoencefálica (BHE) (Ossovskaya y Bunnett, 2004; Keep *et al.*, 2008). El aumento en la cantidad de trombina en el cerebro bajo condiciones patológicas puede generar la activación anormal de los receptores PAR con consecuencias lesivas, a este respecto, existe amplia evidencia del papel tóxico de la trombina el cerebro en patologías asociadas a el edema celular pero aún se desconocen los mecanismos de daño (Smith-Swintosky *et al.*, 1995; Riek-Burchardt *et al.*, 2002; Hua *et al.*, 2003; de Castro

Ribeiro *et al.*, 2006).

En estudios con modelos *in vivo* de isquemia global e isquemia focal transitoria y permanente (Ohyama *et al.*, 2001; Hua *et al.*, 2003; Cuomo *et al.*, 2007) y también en modelos *in vitro* que reproducen algunas de las características presentes en isquemia, como privación de glucosa y oxigeno (Smith-Swintosky *et al.*, 1995; de Castro Ribeiro *et al.*, 2006), se observó que la administración de inhibidores de trombina como el argatroban, la hirudina, la PN-1 y la antitrombina tiene efectos protectores, que desaparecen al agregar una cantidad equimolar de trombina; la administración exógena de trombina en estas condiciones tiene como consecuencia la exacerbación del daño.

A nivel de receptores PAR, se ha descrito que bajo condiciones experimentales de isquemia severa *in vitro* (privación de oxígeno y glucosa) e isquemia focal transitoria o permanente *in vivo* se induce una sobreexpresión de los receptores PAR-1 y PAR-3 (Striggow *et al.*, 2001). La participación de los receptores PAR fue posteriormente confirmada mediante estudios con ratones *knockout* para el receptor PAR-1, ya que estos ratones presentan una disminución en volumen de infarto y en la muerte celular, con respecto a ratones control, tras la aplicación de un modelo de isquemia focal transitoria o un modelo de hipoxia/isquemia; los efectos observados en los ratones *knockout* fueron corroborados mediante el uso de fármacos antagonistas del PAR-1 (Junge *et al.*, 2003; Olson *et al.*, 2004).

2. PLANTEAMIENTO DEL PROBLEMA

La regulación del volumen celular es el proceso que se activa en respuesta a una condición de aumento en volumen e involucra la salida activa de osmolitos intracelulares con la finalidad de recuperar el volumen original. Este proceso tiene una relevancia singular en el cerebro debido a que su fisiología es especialmente susceptible a las alteraciones que conlleva el edema, como son la disminución del espacio extracelular y la modificación de los niveles extracelulares de iones y neurotransmisores. Durante el edema cerebral son los astrocitos las células que aumentan su volumen y como parte del proceso de recuperación de volumen celular, liberan al espacio extracelular osmolitos potencialmente dañinos como el aminoácido glutamato, que al ser el principal neurotransmisor excitador debe mantenerse en niveles controlados en el espacio extracelular, ya que su presencia en exceso induce la sobreactivación de sus receptores ionotrópicos y da origen a la cascada excitotóxica que conduce a la muerte neuronal. A este nivel cualquier otro elemento que promueva la liberación de glutamato será responsable de una exacerbación del daño excitotóxico.

Se ha demostrado que la adición de trombina conjunta a un estímulo hiposmótico en fibroblastos Swiss 3T3 tiene un efecto potenciador sobre los mecanismos efectores del DRV como la liberación de osmolitos orgánicos. El papel tóxico que presenta una elevada concentración de trombina en el cerebro ha sido descrito en varios estudios pero aún se desconoce el mecanismo responsable del daño; la trombina puede aumentar su concentración en el cerebro durante el curso de patologías en las que se compromete el correcto funcionamiento de la barrera hematoencefálica, algunas de estas patologías se encuentran asociadas a edema cerebral. Este conjunto de elementos conducen a plantear si ¿La trombina tiene un efecto potenciador de la liberación de glutamato como osmolito en condiciones de aumento en volumen? y ¿Cuáles son los elementos involucrados en este efecto?

3. HIPÓTESIS

La trombina tiene un efecto potenciador sobre la movilización de los osmolitos glutamato y taurina que ocurre en astrocitos como respuesta a condiciones de aumento en volumen celular. El efecto por trombina estaría mediado a través de la señalización intracelular de calcio, posterior a la activación de algún miembro de la familia de receptores activados por proteasas PAR, y posiblemente también involucra la activación de proteínas con actividad de cinasa de residuos de tirosina.

4. OBJETIVO GENERAL

Investigar si en astrocitos bajo condiciones de aumento en volumen, la trombina mediante la activación de receptores PAR y la movilización intracelular de calcio genera una potenciación de la movilización de los osmolitos orgánicos glutamato y taurina.

Objetivos específicos

4.1. Trombina y edema hiposmótico

- Examinar el efecto de la trombina sobre la movilización del glutamato y taurina inducida bajo condiciones de hinchamiento hiposmótico en astrocitos.
- Identificar la vía de movilización que modula la trombina.
- Examinar posibles moléculas de señalización involucradas en el efecto de la trombina incluyendo inicialmente:
  1. Participación de receptores PAR
  2. Activación de vías de movilización de Ca^{2+}_i,
- Determinar la interacción de estas vías con otras moléculas de señalización activadas por hiposmolaridad como proteínas con actividad cinasa de tirosina.

4.2. Trombina y edema isosmótico

Investigar la potenciación de la liberación del glutamato y taurina por trombina bajo un modelo de aumento en volumen en condiciones isosmóticas por perturbación de la homeostasis del K^+ extracelular en astrocitos.

- Examinar las características de la potenciación, haciendo un análisis comparativo con la condición hiposmótica.
- Identificar la participación de receptores PAR y el papel de la movilización de calcio intracelular.

5. RESULTADOS

Los resultados de esta tesis doctoral se presentan en tres partes; la primera parte muestra los datos del efecto que induce la presencia trombina en la salida de glutamato estimulada por un aumento en volumen de carácter hiposmótico en astrocitos. Estos resultados están publicados en el artículo: Ramos-Mandujano G, Vazquez-Juarez E, Hernandez-Benitez R, Pasantes-Morales H. 2007. Thrombin potently enhances swelling-sensitive glutamate efflux from cultured astrocytes. Glia 55(9):917-925.

La segunda parte abarca la investigación enfocada a esclarecer las vías de señalización que participan durante la potenciación por trombina de la movilización de taurina y glutamato estimulada por hiposmolaridad; así como la posible participación de proteínas con actividad de cinasa de tirosinas. Estos resultados se encuentran publicados en el artículo: Cruz-Rangel S, Hernandez-Benitez R, Vazquez-Juarez E, Lopez-Dominguez A, Pasantes-Morales H. (2008). Potentiation by thrombin of hyposmotic glutamate and taurine efflux from cultured astrocytes: signalling chains. Neurochem Res 33(8):1518-1524.

La tercera parte comprende el estudio del efecto de la trombina sobre la movilización de osmolitos inducida por un modelo de aumento en volumen isosmótico; este modelo se basa en la presencia de una elevada concentración de K^+ extracelular, una característica presente en patologías como la isquemia. Los resultados correspondientes a esta parte se encuentran publicados en el artículo: Vazquez-Juarez E, Hernandez-Benitez R, Lopez-Dominguez A, Pasantes-Morales H. (2009). Thrombin potentiates d-aspartate efflux from cultured astrocytes under conditions of K homeostasis disruption. J Neurochem. 111(6):1398-1408.

La cuarta parte incluye algunos resultados no publicados acerca de los elementos de señalización involucrados en la potenciación por trombina bajo el modelo de aumento en volumen isosmótico. Estos resultados se corresponden con la vía de señalización hallada para la potenciación por trombina bajo condiciones de aumento en volumen hiposmótico.

GLIA 55:917–925 (2007)

Thrombin Potently Enhances Swelling-Sensitive Glutamate Efflux from Cultured Astrocytes

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KEY WORDS

intracerebral hemorrhage; PAR-1; volume-sensitive anion channel; DCPIB; tamoxifen; astrocyte swelling

ABSTRACT

High concentrations of thrombin (Thr) have been linked to neuronal damage in cerebral ischemia and traumatic brain injury. In the present study we found that Thr markedly enhanced swelling-activated efflux of ^3H-glutamate from cultured astrocytes exposed to hyposmotic medium. Thr (0.5–5 U/mL) elicited small ^3H-glutamate efflux under isosmotic conditions and increased the hyposmotic glutamate efflux by 5- to 10-fold, the maximum effect being observed at 15% osmolarity reduction. These Thr effects involve its protease activity and are fully mimicked by SFFLRN, the synthetic peptide activating protease-activated receptor-1. Thr potentiation of ^3H-glutamate efflux was largely dependent on a Thr-elicited increases in cytosolic Ca^{2+} ($Ca^{2+}{}_i$) concentration ($[Ca^{2+}]_i$). Preventing $Ca^{2+}{}_i$ rise by treatment with EGTA-AM or with the phospholipase C blocker U73122 reduced the Thr-increased glutamate efflux by 68%. The protein kinase C blockers Go6976 or chelerythrine reduced the Thr effect by 19%–22%, while Ca/calmodulin blocker W7 caused a 63% inhibition. In addition to this Ca^{2+}-sensitive pathway, Thr effect on glutamate efflux also involved activation of phosphoinositide-3 kinase (PI3K), since it was reduced by the PI3K inhibitor wortmannin (51% inhibition). Treating cells with EGTA-AM plus wortmannin essentially abolished Thr-dependent glutamate efflux. Thr-activated glutamate release was potently inhibited by the blockers of the volume-sensitive anion permeability pathway, NPPB (IC_{50} 15.8 μM), DCPIB (IC_{50} 4.2 μM), and tamoxifen (IC_{50} 6.6 μM). These results suggest that Thr may contribute to the excitotoxic neuronal injury by elevating extracellular glutamate release from glial cells. Therefore, this work may aid in search of neuroprotective strategies for treating cerebral ischemia and brain trauma.

INTRODUCTION

Excitotoxicity is linked to a number of brain injuring situations, including ischemia and hemorrhagic or traumatic episodes (Won et al., 2002; Xi et al., 2002). In ischemia, neuronal death in the perifocal area occurs by a sustained increase in extracellular glutamate levels, generated by the combined effects of swelling-evoked release, excessive depolarization, and the impaired operation of the Na^+-dependent carrier, this later resulting from the energy failure and dissipation of the transmembrane Na^+ gradient (Won et al., 2002). Under these conditions, any additional factor enhancing glutamate efflux from brain cells will exacerbate the excitotoxic damage. The purpose of the present study is to investigate whether thrombin (Thr) might be one of these factors. Thr is present in brain during primary cerebral hemorrhages, or after the blood brain barrier disruption in trauma episodes. Increasing evidence relates Thr to brain injury after cerebral ischemia, as later discussed in detail (De Castro Ribeiro et al., 2006; Xi et al., 2003). Thr also seems to be involved in the neurological complications in HIV (Boven et al., 2003) and it is found accumulated in the senile plaques of the Alzheimer brain (Akiyama et al., 1992). Cell responses to Thr occur via the protease-activated receptors (PAR) 1,3, and 4 (Junge et al., 2004). These receptors are coupled to members of the G-protein families particularly Gq, Gi, and G12/13, and through this interaction Thr elicits a variety of downstream signaling chains (Coughlin, 2000). The link of Thr with glutamate efflux here investigated, is based on recent reports showing that ligand activation of G-protein coupled receptors (GPCR) potentiates the swelling-evoked efflux of amino acids such as taurine and glutamate, which in a variety of cells including brain cells, are acting as osmolytes. Activation of purinergic receptors, cholinergic muscarinic receptors, and lysophosphatidic acid receptors enhances the volume-sensitive efflux of glutamate (D-aspartate) and taurine (Cheema et al., 2005; Franco et al., 2004; Heacock et al., 2006; Mongin and Kimelberg, 2005).

Depending upon its levels in brain, Thr may exert neuroprotective properties at low concentrations (Pike et al., 1996; Striggow et al., 2000), or an injuring effect at high concentration (Xi et al., 2003). Subnanomolar concentrations of Thr potentiates the swelling-induced taurine release and this mechanism may contribute to Thr neuroprotection (Cheema et al., 2005). In contrast, high levels of Thr in brain are recognized as a condition contributing to neuronal death in ischemia, intracerebral

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hemorrhage, and trauma (De Castro Ribeiro et al., 2006; Xi et al., 2003). A common feature of these pathologies is astrocyte swelling, derived from multiple factors such as lactacidosis, arachidonic acid accumulation, K^+ transport, and glutamate uptake, all of them activated in ischemia and trauma (Kimelberg, 2005). Consequently, if Thr potentiates glutamate efflux from swollen astrocytes, it will contribute to exacerbate excitotoxicity and may be a key factor aggravating brain injury. In the present study we found a strong effect of Thr, at concentrations present in injuring conditions in brain, inducing glutamate efflux and increasing its release from astrocytes swollen by exposure to hyposmolarity. The Thr effects on glutamate efflux are suppressed by blockers of the volume-sensitive anion channel (VSAC).

MATERIALS AND METHODS

Materials

Basal medium Eagle, fetal bovine serum and Fura-2 were from Invitrogen, Eugene, OR. Thrombin (Thr) was from Vital Products Boynton Beach, FL. W7-HCl, chelerythrine chloride, Go6976, EGTA-AM, were from Calbiochem-Novabiochem Corp. (San Diego, CA). DCPIB (4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid) was from TOCRIS. D-[2,3-^{3}H] aspartic acid and L-[G-^{3}H] glutamic acid were from New England Nuclear (Boston, MA). Thr from human plasma, NPPB (5-Nitro-2-(3-phenylpropyl-amino)-benzoic acid), tamoxifen, niflumic acid, DIDS (4,4′-Diisothiocyanato-stilbene-2,2′-disulfonic acid), U73122, PPACK and the synthetic peptides SFFLRN and GYPGKF were from Sigma Chemical (St. Louis, MO). All salts for preparation of medium solutions were from Merck (Darmstadt, Germany).

Cell Cultures and Solutions

Primary cultures of astrocytes were obtained from 8-day-old rat cerebella. Dissociated cell suspensions were plated at 2.1×10^4 cells/cm^2 density in plastic dishes, with Basal medium Eagle, 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/mL streptomycin, and incubated at 37°C in humidified 5% CO_2/95% air atmosphere. Cells were used after 2–3 weeks in culture. Isosmotic medium contained (in mM): 135 NaCl, 5 KCl, 1.7 KH_2PO_4, 1.17 $MgSO_4$, 1 $CaCl_2$, 10 glucose and 10 HEPES, pH 7.4 (300 mOsm). Hyposmotic solutions (7.5, 15, and 30% reduction) were prepared reducing NaCl. Osmolarity was verified in a freezing point osmometer.

^{3}*H*-Glutamate Release Experiments

Astrocytes preloaded 1 h with ^{3}*H*-glutamic acid (0.075 μCi/mL) in isosmotic medium were washed, and superfused at 1 mL/min for 5 min to reach a stable efflux baseline. Then the isosmotic medium was replaced with the experimental medium and samples were collected for 8 min. Radioactivity collected every min and that remaining in cells was measured in a liquid scintillation counter. Results are expressed as radioactivity released per minute as percentage of the total radioactivity incorporated during loading.

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

$[Ca^{2+}]_i$ was determined using fura-2/AM in astrocytes cultured on rectangular cover-glasses in plastic dishes (60 mm), as previously described (Franco et al., 2004). Fluorescence was estimated in a Fluoromax-3 Horiba luminescence spectrometer. Excitation wavelength was alternated between 340 and 380 nm and fluorescence intensity was monitored at 510 nm. Fluorescence was measured under isosmotic conditions until the baseline was stable and then medium was made hyposmolar by addition of the appropriate volume of distilled water. Values were used to calculate the ratio of fluorescence intensity (fluorescence at 340 nm/fluorescence at 380 nm).

Statistical Analysis

All data are given as means ± SEM. Statistical significance was determined using analysis of variance (ANOVA) followed by Tukey's test. Statistically significant differences were considered at $^*P < 0.01$, $\zeta P < 0.05$.

RESULTS

Thrombin Markedly Increases the Hyposmotic Efflux of ^{3}*H*-Glutamate from Cultured Astrocytes

A 30% reduction in osmolarity increased ^{3}*H*-glutamate efflux from cultured astrocytes from 0.5% (of total accumulated radioactivity) in isosmotic conditions, to 1.86% at the peak release, reached 2–3 min after the stimulus. The net release of labeled glutamate, i.e. hyposmotic release minus isosmotic release was 4.6% (Figs. 1A,B). Addition of Thr (5 U/mL) markedly stimulated the hyposmotic glutamate efflux, increasing the peak release to 8.6% and the net release to 23.3%, i.e., a 5-fold increase over release in the absence of Thr (Figs. 1A,B). Thr-induced increase was observed in a range of 7.5% to 30% hyposmotic conditions, with maximal potentiation at 15% (Fig. 1B). Thr also slightly increased glutamate efflux in isosmotic conditions (Fig. 1B). Thr effect was concentration-dependent, with maximal effect at 20 nM (0.1U/mL). No further increase was observed at higher concentrations, up to 1 μM (5 U/mL) (Fig. 1C). All these experiments were made using ^{3}*H*-glutamate as cell glutamate tracer, but this efflux may be underestimated if glutamate is metabolized to glutamine and other compounds not responding as osmolytes. Thr effect was then examined in cells loaded with ^{3}*H*-D-aspartate, a non

metabolizable analogue of glutamate. D-aspartate efflux in 30% hyposmotic medium was 5.3% (±0.68; $n = 6$) and 32.8% (±38; $n = 6$) in 30% hyposmotic plus Thr, i.e. 6-fold higher than the control in the absence of Thr.

Commercial-grade bovine plasma-derived Thr (166 U/mg protein) was used throughout the present study but since a recent report (Weinstein et al., 2005) showed differences in microglial cell responses between bovine-derived or low activity Thr and the recombinant human Thr of high activity (3490 U/mg), we compared the effects of bovine-plasma derived Thr of 166 U/mg and the human plasma-derived Thr of ≥2000 U/mg. At the same activity, the effect of the two Thr preparations on glutamate efflux was identical (results not shown).

Thr effect increasing hyposmotic glutamate efflux may result from a receptor-mediated protease action. This was confirmed by the abolishment of Thr effect by pretreatment with the protease inhibitor PPACK (Fig. 2). Thr effects may occur through the PAR. The subtypes PAR-1, PAR-3, and PAR-4 are Thr receptors, and their activation initiates Thr signaling cascades. Using the PAR-activating synthetic peptides SFFLRN and GYPGKF for PAR-1 and PAR-4, respectively, we aimed to investigate whether Thr effect increasing hyposmotic glutamate efflux is a receptor-mediated action. Figure 3A shows a concentration-response curve of SFFLRN effects. At 2-μM the peptide significantly increased glutamate efflux and at 5–10 μM the agonist fully mimicked the effect of Thr. The PAR-4-activating peptide GYPGKF at much higher concentration (50 μM) only increased glutamate efflux to a level corresponding to 3% of the Thr effect (Fig. 3B). These results suggest an effect of Thr increasing glutamate efflux by protease-mediated action essentially via the PAR-1 subtype.

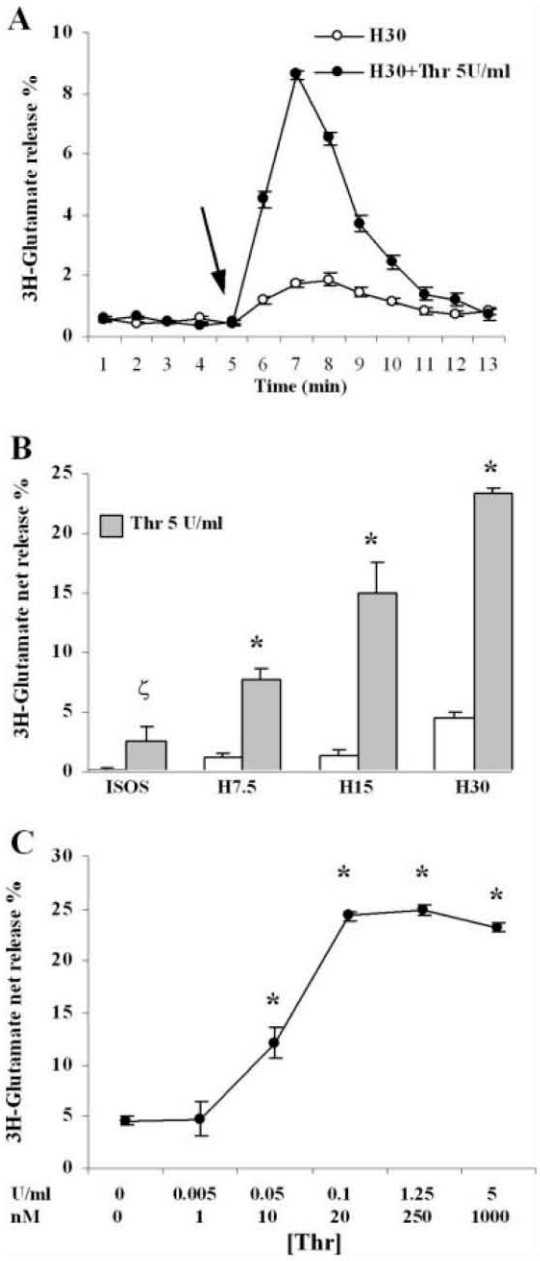

Fig. 1. Effect of thrombin (Thr) increasing the hyposmotic efflux of ^3H-glutamate from cultured cerebellar astrocytes. **A**: Time course of ^3H-glutamate efflux evoked by a 30% reduction of external osmolarity and the potentiation by Thr (5 U/mL). Astrocytes preloaded with ^3H-glutamate were superfused (1 mL/min) with isosmotic medium during 5 min, and then (arrow) 8 min with 30% hyposmotic medium (○) or with hyposmotic medium plus Thr (●). Points represent the radioactivity released at each fraction, expressed as percentage of total radioactivity accumulated during loading. **B**: Effect of Thr increasing glutamate efflux in isosmotic or hyposmotic (7.5, 15, and 30%) conditions. Bars represent ^3H-glutamate released in the five main fractions of the peak minus five fractions of the release in isosmotic conditions (net release). Dashed bars: Thr 5 U/mL, empty bars: no Thr added. **C**: Effect of increasing concentrations of Thr (0.005–5 U/mL) on hyposmotic (30%) ^3H-glutamate efflux. Points represent net ^3H-glutamate release as above described. Results are means ± SE of 3–8 experiments. Significantly different from controls in the absence of Thr $^*P < 0.01$; $P < 0.05$.

Ca^{2+} Signaling in the Effect of Thr on Glutamate Efflux

Hyposmolarity leads consistently to $[Ca^{2+}]_i$, increase whose magnitude and source are cell specific (Pasantes-Morales and Morales-Mulia, 2000). In cerebellar astrocytes, 30% hyposmolarity elicited only a small increase in $[Ca^{2+}]_i$ (Fig. 4). Thr in contrast, confirming its effect in numerous cell types, elicited a much higher $[Ca^{2+}]_i$ rise. The source of this $[Ca^{2+}]_i$ rise was investigated treating cells with Ca^{2+}-free medium (plus 0.1 mM EGTA) to evaluate the extracellular Ca^{2+} contribution, or with ionomicin to deplete all internal stores. Both, external and internal sources contribute with about 50% to the Thr effect elevating Ca^{2+} (Fig. 4). Thr-induced $[Ca^{2+}]_i$ rise seems mediated by PAR-1 as shown by the effect of SFFLRN reproducing fully the Thr effect (Fig. 4).

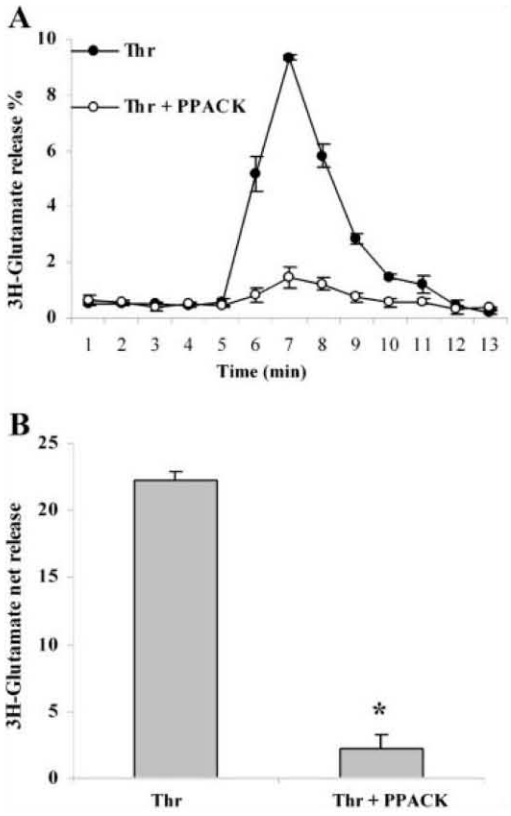

Fig. 2. Effect of Thr treatment with the protease inhibitor PPACK on hyposmotic ^3H-glutamate efflux. **A**: Thr was preicubated (30 min) with the protease inhibitor PPACK (1 μM) and the assay proceeded as in Fig. 1. **B**: Bars represent net ^3H-glutamate release as described in Fig. 1. Results are means ± SE of 4 experiments. Significantly different from the control in the absence of the protease blocker $^*P < 0.01$.

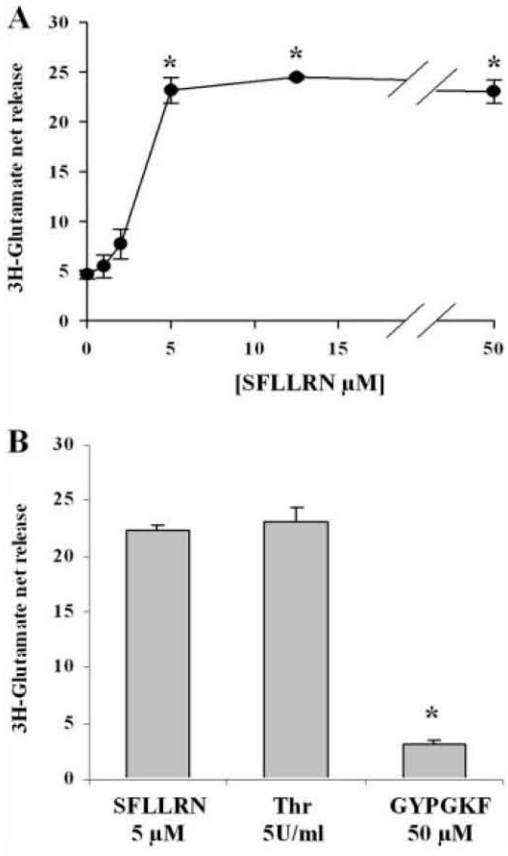

Fig. 3. Effect of the PAR-1 and PAR-4-activating synthetic peptides SFFLRN and GYPGKF increasing hyposmotic ^3H-glutamate efflux. **A**: Effect of increasing concentrations of SFFLRN (1–50 μM) on ^3H-glutamate efflux. **B**: Comparison of the effects of Thr and of SFFLRN (5 μM) or GYPGKF (50 μM) enhancing ^3H-glutamate efflux. Experimental assay and results as in Fig. 1. Means ± SE of 3–5 experiments. Significantly different from controls in the absence of the agonists $^*P < 0.01$.

The Thr-increased hyposmotic ^3H-glutamate efflux from astrocytes was strongly Ca^{2+}-dependent. Figure 5 shows a 68% glutamate efflux reduction in cells treated with 25 μM EGTA-AM in Ca^{2+}-free medium. Removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus 0.1 mM EGTA) decreased 32% glutamate efflux. The $[Ca^{2+}]_i$ dependent fraction of Thr-increased glutamate efflux importantly involved phospholipase C (PLC), since its blockade with U73122 inhibited the release by 64% (Figs. 5A,B) (Table 1). These results stress the participation of a PLC-dependent $Ca^{2+}{}_i$ pool in the effect of Thr on the hyposmotic glutamate efflux. In the absence of Thr glutamate efflux was also markedly Ca^{2+}-and PLC-dependent (Table 1).

The influence on glutamate efflux of signaling elements activated downstream $[Ca^{2+}]_i$ increase such as protein kinase C (PKC) and calmodulin protein (CaM) were next examined. The general PKC blocker chelerythrine (2.5 μM) 22% reduced the Thr-increased glutamate efflux and the Ca^{2+}-dependent PKC isoform blocker Gö6976 (1–10 μM) reduced it 19% (Fig. 5C). Inhibition of CaM with W7 reduced glutamate efflux in the presence of Thr by 66% (Fig. 5C). The hyposmotic glutamate efflux in the absence of Thr was similarly affected by these blockers (Table 1).

Involvement of Phosphoinositide-3 Kinase

Stimulation of GPCR, particularly of the G12/13 types, is known to activate phosphoinositide-3 kinase (PI3K), likely via the dissociated G-protein βγ subunits. (Brock et al., 2003). The hyposmotic efflux of taurine (Cardin et al., 2003) is sensitive to PI3K inhibition. Similarly, Thr-increased glutamate efflux was 51% reduced by the PI3K blocker wortmannin (Fig. 6). PI3K influences the Thr-enhanced glutamate efflux by a Ca^{2+}-independent

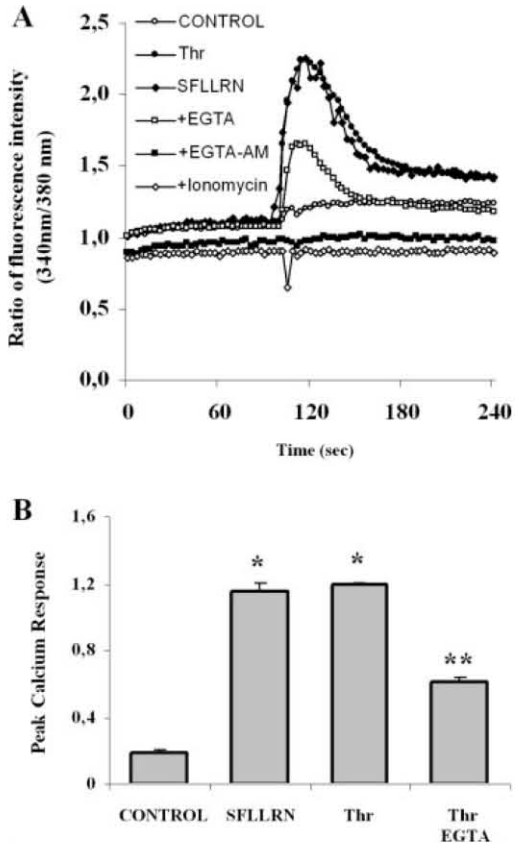

Fig. 4. Changes in $[Ca^{2+}]_i$ elicited by hyposmolarity and by hyposmolarity plus thrombin or SFFLRN. $[Ca^{2+}]_i$ was measured with fura-2/AM, as detailed in Methods. **A**: Increase in $[Ca^{2+}]_i$ evoked by hyposmotic conditions in absence (○) or presence (●) of thrombin 5 U/mL or (▲) SFFLRN 5 μM. Effects of: (□) Ca^{2+}-free medium plus 0.1 mM EGTA, (■) 25 μM EGTA-AM, or (◇) treatment with 1 μM ionomycin (5 min) in external Ca^{2+}-free conditions. Results expressed as ratio of fluorescence intensity (340/360 nm). **B**: Quantification of the peak Ca^{2+} response in the indicated conditions. Results correspond to the peak value minus the basal value for each condition. Significantly different from the 30% hyposmotic control condition $^*P < 0.01$. Significantly different from the Thr condition $^{**}P < 0.01$.

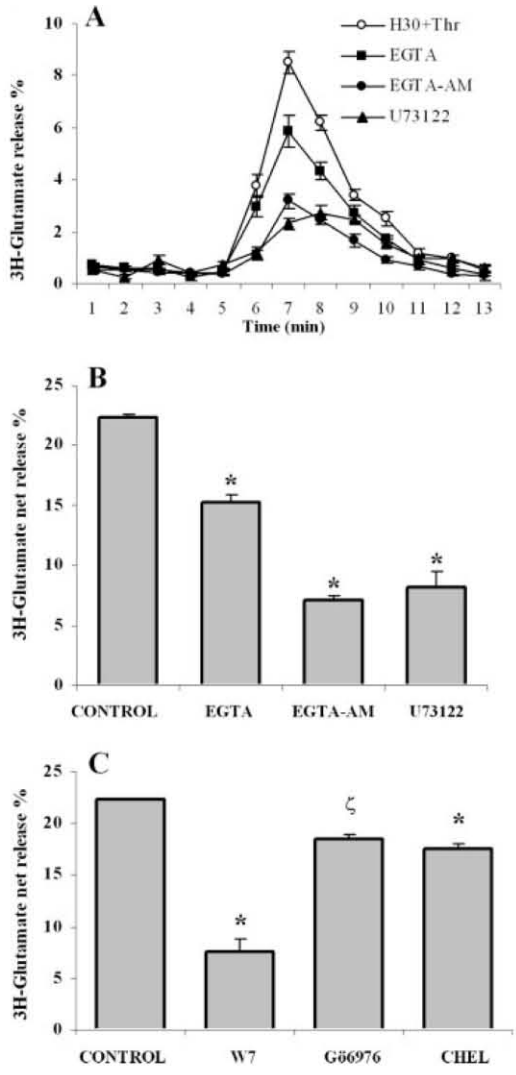

Fig. 5. Ca^{2+} signaling in the hyposmotic plus Thr ^3H-glutamate efflux. **A**: Effect of external Ca^{2+} omission (■) or of treatment with EGTA-AM (25 μM) (●) or U73122 (5 μM) (▲). Astrocyte loading and superfusion assay as described in Fig. 1. The Ca^{2+}-free-medium contained 0.1 mM EGTA. **B**: Bars represent data from A expressed as net release as in Fig. 1. **C**: Effect of W7 (50 μM), Gö6976 (1 μM), or chelerythrine (2.5 μM), on ^3H-glutamate efflux. Bars are net release as defined in Fig. 1. Cells were preincubated with the inhibitors during 20 min and the drugs were present in the media throughout the experiment. Control bar corresponds to the hyposmotic plus Thr net glutamate release without any treatment. Means ± SE of 3–8 experiments. $^*P < 0.01$; $P < 0.05$.

mechanism, since when wortmannin and a Ca^{2+}-free condition were tested together, showed mostly additive effects and this treatment essentially suppressed glutamate efflux (93% inhibition) (Fig. 6).

Thr-Increased Glutamate Efflux Occurs Through the Volume-Sensitive Anion Pathway

Thr-increased glutamate efflux from astrocytes occurs through the efflux pathway typically sensitive to the VSAC blockers. NPPB, tamoxifen, and DCPIB showed a strong effect reducing the Thr-stimulated glutamate efflux, with IC_{50} of 15.8, 4.2, and 6.6 μM, for NPPB, DPCIB, and tamoxifen, respectively (Fig. 7). DIDS 50 μM and niflumic acid (500 μM) reduced it 80%–84% (Table 1). As expected, the hyposmotic glutamate efflux in the absence of Thr was also markedly sensitive to all these blockers (70%–100% inhibition) (Table 1). The Thr-elicited glutamate efflux in isosmotic conditions was

TABLE 1. Effect of Ca^{2+}-Free Conditions and of PLC, PKC, PI3K, and VSAC Blockers on the ^3H-Glutamate Efflux Stimulated by 30% Hyposmotic Condition in the Presence or Absence of Thrombin (Thr)

	Percentage inhibition from control release	
	H30	H30+THR
EGTA-AM 25 μM	73.7	68.5
U73122 5 μM	66.7	63.87
Ca^{2+}_e-free + EGTA 0.1 mM	69.9	31.9
Gö6976 1μM	21.4	18.5
Chelerythrine 2.5 μM	31.8	21.7
Wortmannin 100 nM	48.3	50.8
DCPIB 10 μM	97.4	92.8
NPPB 50 μM	99.7	97.1
DIDS 50 μM	85.1	79.9
Niflumic acid 500 μM	100	84.1
Tamoxifen 10 μM	70	94.7

Astrocytes were exposed to the various conditions and blockers as described in Results. The numbers represent the percentage inhibition from the control release. Data are means of 3–8 experiments, with SEM always lower than 10%.

essentially suppressed by NPPB, DCPIB, or tamoxifen (Fig. 8).

DISCUSSION

The present results showed, to our knowledge for the first time, a dramatic effect of Thr increasing the volume-sensitive release of glutamate from astrocytes, swollen by exposure to hyposmotic conditions. This effect of Thr was observed at low and mild reductions in osmolarity, but also a significant increase of glutamate efflux was evoked by Thr in isosmotic conditions. Activation of a variety of GPCR by their ligands, enhances the efflux of osmolytes from a number of cell types. ATP, oxotremorin-M, Thr, norepinephrine, sphingosin-1-phosphate, and lysophosphatidic acid, all potentiate the swelling-activated release of taurine (Cheema et al., 2005; Franco et al., 2004; Heacock et al., 2006; Manolopoulus et al., 1997; Thoroed et al., 1995). The volume-sensitive efflux of D-aspartate is also increased by ATP and oxotremorin-M (Heacock et al., 2004; Mongin and Kimelberg, 2005). All these studies, including the present work, show that the GPCR-mediated increase of taurine and glutamate (or D-aspartate) is sensitive to Cl^- channel blockers, suggesting a volume-activated organic anion channel as the translocation pathway involved.

Thr effect enhancing glutamate efflux from astrocytes is mediated by its protease activity, being suppressed by the protease blocker PPACK. The effect of Thr is likely occurring through the protease activated receptor PAR-1. Four members of PARs have been identified (PAR 1-4) and all of them are expressed in astrocytes (Junge et al., 2003; Wang et al., 2002). Although Thr can activate PAR-1, PAR-3, and PAR-4, most of its known actions in astrocytes occur via the prototype member of the family PAR-1. This subtype is also that primarily involved in the effect of Thr increasing glutamate efflux, since the PAR-1 agonist SFFLRN reproduced it fully, while the PAR-4 agonist was essentially ineffective. Thr increased taurine efflux in astrocytoma cells is also mediated by PAR-1 (Cheema et al., 2005).

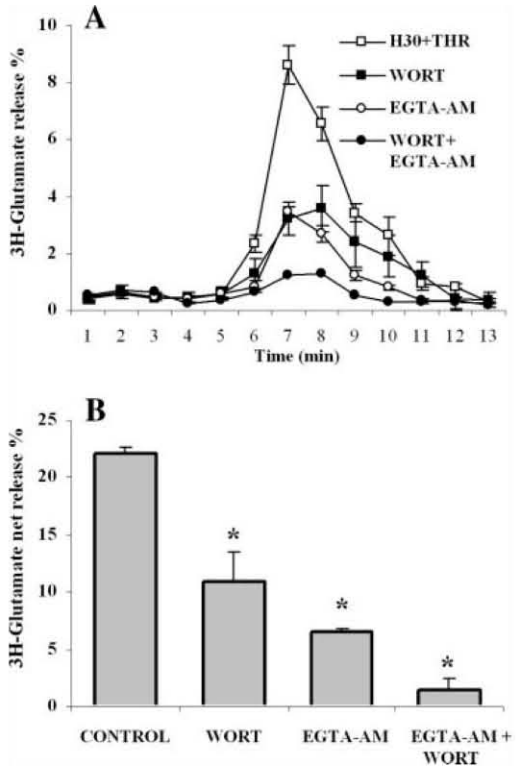

Fig. 6. Effect of PI3K blockade on the hyposmotic plus Thr ^3H-glutamate efflux. **A**: Time course of hyposmolarity plus thrombin ^3H-glutamate release (□) treated with wortmannin (100 nM) (■), EGTA-AM (25 μM) (○) or EGTA-AM plus wortmannin (●). Superfusion assay and treatment with blockers as in Fig. 3. **B**: Bars represent data from A expressed as net release as in Fig. 1. Control bar corresponds to the hyposmotic plus Thr net glutamate release without any treatment. Means ± SE of 3–8 experiments. $^*P < 0.01$.

A common feature of the GPCR activation is an increase of Ca^{2+}_i levels and the consequent set in motion of Ca^{2+}-mediated signaling pathways. The influence of the Ca^{2+}-associated signaling elements on GPCR effects increasing osmolyte fluxes has been examined in detail, and certain differences have been found between the different receptors and the various cell types. Glutamate efflux stimulated by Thr in astrocytes is markedly Ca^{2+}-dependent, being similar in this respect to the taurine and glutamate fluxes elicited via purinergic and muscarinic receptors in a variety of cell types. Preventing the $[Ca^{2+}]_i$ rise evoked by these receptors reduced by 68%–90% the osmolyte fluxes (Franco et al., 2004; Heacock et al., 2006; Mongin and Kimelberg, 2005 and present results). In contrast, stimulation of taurine efflux occurring via lysophospholipids receptors is only partially (30–40%) attenuated after suppression of the receptor-elicited $[Ca^{2+}]_i$ increase (Heacock et al., 2006). Noteworthy, this difference in Ca^{2+} sensitivity is found in spite of the robust rise in $[Ca^{2+}]_i$ evoked by the GCPR agonists in all cases (Heacock et al., 2006). Some differences have been found also on the PLC influence.

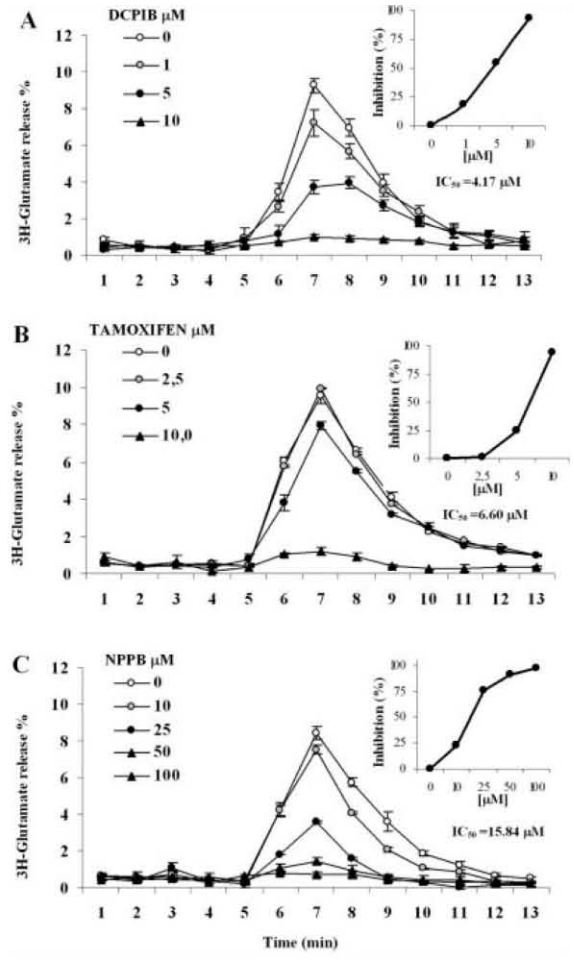

Fig. 7. Dose-dependent effect of DCPIB (A), tamoxifen (B) and NPPB (C) on the hyposmotic plus Thr ^3H-glutamate efflux. Astrocytes were preincubated during 30 min with the indicated concentration of the blockers and the drugs were present in media throughout the experiment. Superfusion assays as in Fig. 1. Insets are plots of net glutamate release versus drug concentration. Net release calculated as in Fig. 1. IC_{50} values for the blockers are indicated. Means ± SE of 4–8 experiments.

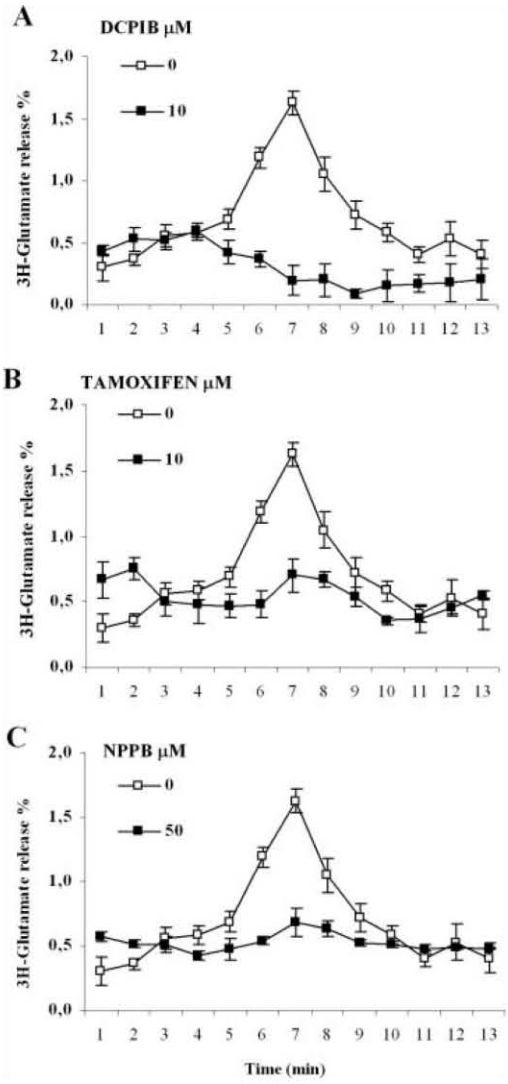

Fig. 8. Effect of NPPB, DCPIB, and tamoxifen DCPIB (A), tamoxifen (B) and NPPB (C) on the Thr elicited ^3H-glutamate efflux in isosmotic conditions. The superfusion assay was as described in Fig. 1 except that the stimulus was a Thr-containing isosmotic medium. To test the effects of the volume-sensitive anion channel- blockers, astrocytes were preincubated during 30 min with the indicated amount of the drugs and then superfused with media containing the same concentration of the blockers. Means ± SE of 3–8 experiments.

PLC blockade markedly attenuated the Thr-stimulation of glutamate efflux in astrocytes, to about the same magnitude as the Ca^{2+}-free condition, stressing the importance of PLC on the Thr effect. PLC is also involved in taurine efflux enhanced by oxotremorine-M in SH-SY5Y neuroblastoma cells, but is not participating in the effect mediated by lysophospholipid receptors in the same cells (Heacock et al., 2006). The role of PKC on taurine or glutamate efflux increased by GPCR is also different depending on the type of receptor involved. Thus, while PKC contributes to taurine efflux enhanced by agonists of muscarinic and lysophospholipid receptors in SH-SY5Y cells (Heacock et al., 2006; Loveday et al., 2003) it has little influence when the potentiation is mediated by purinergic receptors (Franco et al., 2004; Mongin and Kimelberg, 2005). Similarly, we found here that the PKC blockers chelerythrine and Gö6976, this later specific for Ca^{2+}-dependent PKC isoforms, only mildly reduced the Thr-stimulated glutamate efflux. The Ca^{2+}/CaM, in contrast, is an important downstream signaling element in the Thr enhanced glutamate efflux from astrocytes as well as in the effect of ATP on taurine and D-aspartate efflux (Franco et al., 2004; Mongin and

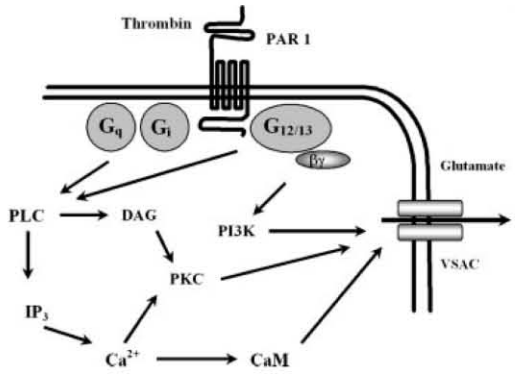

Fig. 9. Schematic representation of suggested signaling pathways involved in the Thr-stimulated ^{3}H-glutamate efflux from cultured astrocytes, based on the present results. Two pathways are contributing, one is the Ca^{2+}-PLC-dependent pathway, possibly resulting from the PAR-1-mediated Gq and Gi activation and the other one involves PI3K activated possibly by G12/13 βγ subunits. CaM and PKC act as downstream effectors. The final interaction of these signals with the glutamate efflux pathway remains to be clarified. The molecular identity of the pathway is so far unknown.

Kimelberg, 2005). All these results show a wide range of variation between the contributions of Ca^{2+}-related signaling pathways on the GPCR effect stimulating osmolyte fluxes. The reason for these differences is so far unclear.

PI3K is involved in the Thr action increasing glutamate efflux from astrocytes, which was 30% reduced by the PI3K blocker wortmannin. PI3K can be activated by Thr via a Ca^{2+}/ras pathway or by a Ca^{2+}-independent mechanism, involving G-protein ($Gi_{12/13}$) βγ subunits (Hawes et al., 1996; Coughlin, 2000). This later possibility is supported by our results showing that Ca^{2+} omission and PI3K blockade have an additive effect, suppressing glutamate efflux when tested together. From all these observations, it is concluded that the effect of Thr on glutamate efflux here described, results from the combined operation of a PLC-mediated Ca^{2+}-dependent pathway (70%) and a PI3K-dependent signaling (30%) (Fig. 9).

The present results provide elements to consider a potential link between the injuring effect of Thr and excitotoxic damage. Excitotoxicity is the main process related to secondary neuronal injury and death in ischemia and other pathologies (Yi and Hazell, 2006). Several mechanisms concur to generate the extracellular glutamate increase in ischemia. Excessive depolarizacion, and the reversal operation of the glutamate carriers are main sources of extracellular glutamate, but release through the swelling-activated pathway, particularly from astrocytes, is an important additional mechanism, particularly in regions of incomplete ischemia (Feustel et al., 2004). These observations stress the interest of our present results showing a prominent increase of glutamate efflux by Thr, which can be then considered as an important contributor to excitotoxicity.

There is evidence relating Thr and brain damage during ischemia. At short term, Thr activity is increased in the ischemic core and Thr receptors are upregulated (Xi et al., 2002). Consistent with these observations, inhibitors of the Thr actions such as argatroban and hirudin increase neuronal survival (Kawai et al., 1996; Karabiyikoglu et al., 2004) and in the same line, transgenic mice lacking PAR-1 exhibit less ischemic damage than their wild type littermates (Striggow et al., 2001). An early necrotic neurotoxicity elicited by Thr has been recently documented in the rat striatum in vivo (Fujimoto et al., 2007). The mechanisms responsible for Thr-related neuronal injury are still poorly understood. In this respect, our results showing a marked increase of glutamate efflux from astrocytes raise the question of whether excitotoxicity contributes in part to the Thr induced neuronal damage. A connection between Thr neuronal injury and excitotoxicity is suggested by the increase in the NMDA receptor response observed in hippocampal CA1 pyramidal cells following activation of PAR-1 (Gingrich et al., 2000). The Thr-evoked stimulation of glutamate release here described was notably strong in astrocytes showing some degree of swelling, but it was also observed in not-swollen cells, a result of interest in evaluating Thr injuring effect at more distant ischemic perifocal areas where cell swelling is not prominent.

Glutamate efflux potentiated by Thr was suppressed by the anion channel blockers tamoxifen, DCPIB, and NPPB. DCPIB, a selective inhibitor of the VSAC (Decher et al., 2001) showed the most potent effect, with an IC_{50} of 4.7 μM. This blocker also markedly reduced the hyposmotic D-aspartate release in cultured astrocytes (Abdullaev et al., 2006). These results point to the VSAC as the pathway involved in the Thr-evoked increase in hyposmotic glutamate efflux. Accordingly, neuroprotective strategies may consider the use of blockers of this pathway, combined with Thr receptor inhibitors, under conditions such as ischemia and head trauma in which high Thr brain levels and astrocyte swelling are coincident.

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ORIGINAL PAPER

Potentiation by Thrombin of Hyposmotic Glutamate and Taurine Efflux from Cultured Astrocytes: Signalling Chains

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Abstract Activation of protein-activated receptor (PAR-1) by thrombin potentiates the hyposmotic efflux of ^{3}H-D-aspartate and ^{3}H-taurine from cultured cerebellar astrocytes. This effect is mediated by a thrombin-elicited increase in cytosolic Ca^{2+} levels $[Ca^{2+}]_i$ and the activation of phosphoinositide-3-kinase (PI3K). These signalling pathways operate independently showing additive effects if prevented simultaneously. The contribution of the Ca^{2+}-mediated pathway to thrombin-increased D-aspartate or taurine efflux, evaluated by the inhibitory effect of preventing $[Ca^{2+}]_i$ rise, was higher for D-aspartate (64% efflux decrease) than for taurine (40% decrease). The PI3K blocker decreased 48% and 36% D-aspartate and taurine efflux, respectively. Hyposmolarity increases phosphorylation of EGFR and c-src, but thrombin did not enhance this effect. Blockade of EGFR/src phosphorylation marginally reduced (11–14%) the hyposmolarity plus thrombin efflux of D-aspartate; taurine efflux was more sensitive to these blockers (18–26%). Since thrombin has no effect increasing EGFR/src phosphorylation in astrocytes, the contribution of this transactivation pathway may represent the inhibition of the hyposmotic efflux solely.

Keywords Thrombin transactivation · Epidermal growth factor receptor · Volume regulation · Swelling

Special issue article in honor of Dr. Ricardo Tapia.

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Introduction

Animal cells have a characteristic volume defined for each cell lineage. Preserving this volume is necessary to maintain the cytoarchitecture and the proper concentration of signalling molecules in the cytosol. Although in physiological conditions the extracellular fluids have a highly controlled osmolarity, the water content in intracellular compartments can be transiently and continuously altered during the normal function of the cell. Nutrient uptake, cell motility, secretion, synaptic ion gradients, synthesis and degradation of macromolecules are, among others, situations creating local osmotic gradients. Adaptive mechanisms to counteract these changes are necessary to maintain the cytoarchitecture necessary for the correct spatial assembly of transduction networks. Cell volume is also disturbed in pathological conditions. Cell volume gain occurs when the osmolarity of the fluids is perturbed by failure in the mechanisms of osmotic control. Pathologies leading to hyponatremia generate hyposmotic swelling [1]. This occurs in acute or chronic renal failure, hepatic cirrhosis, congestive heart failure, or nephrotic syndrome, psichogenic polydipsia or during endurance athletic events. Hyponatremia occurs also in the elderly and during pregnancy [1]. Cell swelling occurs also without a change in external osmolarity. This type of swelling known as cytotoxic swelling is due to ion redistribution or accumulation of osmotically active solutes, followed by obligated water. It is commonly associated with hypoxic or ischemic episode and with hepatic encephalopathy [2, 3, 4]. Changes in cell volume are potentially harmful in all cells but it is particularly detrimental in brain, due to the restrictions to expansion imposed by the rigid cranium. As expansion occurs, the constraining of small vessels generates episodes of ischemia, infarct, excitotoxicity and neuronal death. In

extreme conditions, caudal herniation of the brain parenchyma through the foramen magnum affects brain stem nuclei resulting in death by respiratory and cardiac arrest.

Astrocytes are the brain cells preferentially swelling in both, hyposmotic or isosmotic conditions. In hyposmolarity, the higher swelling observed in astrocytes with respect to neurons may be due either to the preferential expression of aquaporins or to a more efficient operation of volume regulation in neurons as compared to astrocytes. This later notion is supported by a study showing that upon a hyposmotic condition in vivo, the Purkinje cells in the cerebellum release an abundant pool of taurine which is transferred to neighbour astrocytes [5]. As consequence of this taurine translocation, neurons are preserved while astrocytes swell. In isosmotic conditions generating high levels of extracellular K^+, the role of astrocytes protecting neurons by siphoning K^+ results in astrocyte swelling [6, 7].

The cell response to hyposmotic swelling has been extensively examined in brain cells in culture, neurons as well as astrocytes. Upon exposure to reduced external osmolarity, cells first swell and then activate a mechanism of volume regulatory decrease (RVD) consistent in the extrusion of osmotically active solutes followed by water. This is an active mechanism occurring in spite of the persistence of the hyposmotic condition [8, 9]. The osmolytes involved in RVD are the main intracellular ions K^+ and Cl^- and a large number of heterogeneous small organic molecules, grouped on the term of organic osmolytes [10]. Amino acids are part of this pool of organic osmolytes. Taurine, GABA and glutamate are released from neurons and astrocytes in response to hyposmotic swelling [11]. This might affect neuronal excitability, GABA and glutamate being major neurotransmitters.

Besides the activation of osmolyte efflux pathways, other effectors are also linked to swelling and RVD. Membrane remodelling, reorganization of the cytoskeleton, activation of stress-detecting molecules and reactions to alert the cell of situations threatening survival. All these are reactions necessary for the cell to adapt to the complex and varied changes imposed by the change in cell volume, during swelling first and during the subsequent volume recovery. It is not surprising then, that changes in cell volume activate a variety of signalling chains, which are now being explored in detail. Furthermore, from the number of signalling molecules activated it is necessary to assign each of them to the variety of responses evoked by swelling. A further complication may result if the signalling chains are not the same in all cells. An example of this possible variation is the influence of Ca^{2+} on RVD. An increase in $[Ca^{2+}]_i$ is a most consistent response of cells to swelling. However, RVD is Ca^{2+}-dependent in some cells and Ca^{2+}-independent in other cells [12]. In the first case, $[Ca^{2+}]_i$ increase influence Ca^{2+}-activated K^+ channels, mainly of the large-conductance Ca^{2+}-dependent K^+ channels, and elicit K^+ efflux, which is an early event in RVD. In contrast, in another large number of cells, these channels are not involved in RVD but the corrective K^+ efflux occurs through other types of K^+ channels such as voltage-dependent or swelling activated K^+ channels [13, 14], which are not responding to Ca^{2+} increase. In these cells, consequently, RVD is Ca^{2+}-independent. Other osmolyte pathways such as the volume-sensitive channel and the taurine (organic osmolyte) efflux pathway are largely Ca^{2+}-independent [12, 13].

An interesting observation is that even in those cells showing Ca^{2+}-independent RVD, increasing $[Ca^{2+}]_i$ over the levels elicited by the hyposmotic stimulus markedly potentiates some of the osmolyte efflux pathways, notably the organic osmolyte efflux pathway. This effect of $[Ca^{2+}]_i$ increasing organic osmolyte fluxes has been observed when $[Ca^{2+}]_i$ rise is evoked by a Ca^{2+} ionophore (ionomycin) [15] or by increasing $[Ca^{2+}]_i$ by activation of G-protein coupled receptors (GPCRs) of the subfamily activating a PLC-Ca^{2+} pathway [Rev. in 16]. At present, a variety of these receptors have been shown to stimulate the hyposmotic taurine efflux in various cell types. Purinergic, PAR-1, M3 muscarinic, H1 histamine and lysophospholipid receptors increase the hyposmotic taurine efflux in a large variety of cell types [Rev. in 16]. This effect of GPCRs is also exerted on other osmolytes. Glutamate (D-aspartate) efflux is potentiated by agonists of purinergic and PAR-1 receptors in cultured astrocytes [17, 18] and by the muscarinic cholinergic receptor agonist Oxo-M in SH-SY5Y cells [19], this receptor also increases the swelling sensitive efflux of myo-inositol from SH-SY5Y cells [20].

These effects of GPCRs agonists increasing organic osmolyte fluxes appear to be essentially mediated by the PLC-Ca^{2+} pathway, being all of them Ca^{2+}-dependent to some extent. The degree of dependence, though, varies according to the agonists examined, the osmolyte traced and the magnitude of osmolarity reduction [16].

Besides the Ca^{2+}-dependent signalling chain, a variety of GPCR activate other signalling cascades via their interaction with growth factor receptors. These form an important family of transmembrane molecules with intrinsic tyrosine kinase activity, which activate a plethora of intracellular signalling elements. Hyposmotic swelling activates some of these receptors. EGFR activates by hyposmolarity in fibroblasts and keratinocytes [21, 22] and phosphorylation of ErbB4 is increased by swelling in cerebellar granule neurons [23]. There is evidence now available about an active cross-talk between GPCRs and tyrosine kinases, by a mechanism known as transactivation. EGFR and members of the src family are the molecules more directly involved in this interplay [24, 25, 26].

GPCR/tyrosine kinase crosstalk is only starting to be examined in reference to cell volume regulation. A recent study from our group in 3T3 fibroblasts [27] shows that activation of PAR-1 by thrombin potentiates the hyposmotic phosphorylation of EGFR and src and that these two molecules have an important contribution to the hyposmotic efflux of taurine activated by thrombin. In contrast, the increase of the hyposmotic glutamate efflux by activation of two GPCRs, the purinergic P2Y receptor and the PAR-1 receptor, appear to be largely dependent on the PLC-Ca^{2+} pathway [17, 18]. This is a marked difference with the response in fibroblasts in which inhibition of the PLC-Ca^{2+} pathway by EGTA-AM or U73122 reduced the efflux of taurine by 40–50% [27]. This may be due to a cell difference or to an osmolyte difference. This was investigated in the present study in which we examined (i) the effect of thrombin on EGFR or src phosphorylation increased by hyposmolarity, (ii) the effect of src and EGFR blockers on the thrombin increased glutamate efflux and (iii) the effect of the same treatment on the potentiation by thrombin of the hyposmotic taurine efflux.

Results and Discussion

Thrombin Markedly Enhanced $[Ca^{2+}]_i$ in Astrocytes Which Contributes to the Potentiation of Glutamate and Taurine Efflux

Cultured astrocytes in isosmotic conditions have a $[Ca^{2+}]_i$ of about 60 nM. Reduction in external osmolarity (30%) induced a small and sustained increase in $[Ca^{2+}]_i$ which in average is 64.3% higher than in isosmotic conditions, reaching 100 nM (Fig. 1). Addition of thrombin markedly enhances $[Ca^{2+}]_i$. At the peak, $[Ca^{2+}]_i$ is more than 5-fold that found in isosmotic conditions and 4.5-fold that elicited by hyposmolarity (Fig. 1). This is a well known effect of thrombin, documented in a large variety of cells. In a previous study in cultured cerebellar astrocytes [17] we investigated the source of this thrombin-elicited $[Ca^{2+}]_i$ rise, by treating cells with Ca^{2+}-free medium (plus 0.1 mM EGTA) to evaluate the contribution of extracellular Ca^{2+}, or with ionomycin to deplete all internal stores. It was found that the contribution of external and internal sources is similar, of about 50% each. We now examined the influence of preventing $[Ca^{2+}]_i$ rise on the efflux of taurine potentiated by thrombin, and compared to that previously observed for glutamate.

Exposure to 30% hyposmotic solution increased ^{3}H-D-aspartate efflux from cultured astrocytes. The efflux of ^{3}H-D-aspartate in isosmotic conditions which was in average 0.6% (percentage of total accumulated radioactivity), increased to about 3% at the peak release reached 2–3 min after the stimulus. Thereafter the efflux slowly inactivated towards basal levels. The net release in the four largest fractions i.e., the hyposmotic release minus the release in isosmotic conditions reached 7% (Fig. 2a). Addition of thrombin (5 U/ml) markedly potentiated the hyposmotic glutamate release. In the presence of thrombin, the net release of the four main fractions was 38%, corresponding to 5.4-fold increase over the hyposmotic release (Fig. 2a). These results agree with those previously reported in cerebellar astrocytes measuring ^{3}H-glutamate efflux [17]. The effect of thrombin increasing ^{3}H-D-aspartate efflux was largely dependent on the thrombin-induced increase in $[Ca^{2+}]_i$. This is a well known signalling pathway evoked by the thrombin-induced PAR-1 activation. In the present study, preventing $[Ca^{2+}]_i$ rise by EGTA-AM (Fig. 2b)

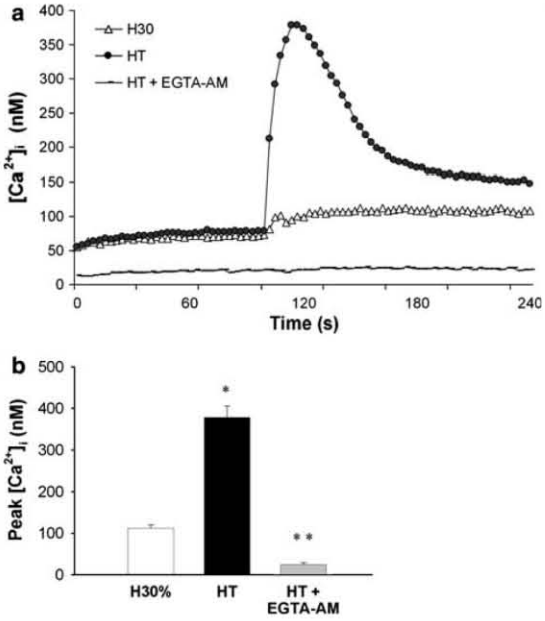

Fig. 1 Hyposmolarity and hyposmolarity plus thrombin-induced increase in $[Ca^{2+}]_i$ in cultured cerebellar astrocytes. Cells plated on rectangular glass coverslips (10 × 50 mm) in plastic dishes were loaded by incubation with fura-2-AM (2.5 μM) for 60 min. Digital images of the cells were obtained in a Fluoromax-3 Horiba luminescence spectrometer at an emission wavelength of 510 nm using paired exposures to 340 and 380 nm excitation wavelength sampled every 3 s. The change in $[Ca^{2+}]_i$ was monitored by fluorescence under isosmotic conditions until the baseline was stable and then medium was made hyposmolar (30%) by addition of the appropriate volume of distilled water. (**a**) Representative traces of the increase in $[Ca^{2+}]_i$ determined from the ratiometric changes in fluorescence. (**b**) Peak transient levels in $[Ca^{2+}]_i$ reached at the indicated conditions: H30%, medium with 30% reduced osmolarity; HT: hyposmotic plus thrombin medium. Data are means ± SE ($n = 4$) analyzed using analysis of variance (ANOVA) followed by Tukey test. Significantly different from the hyposmotic condition $^*P < 0.01$; significantly different from the HT condition $^{**}P < 0.01$

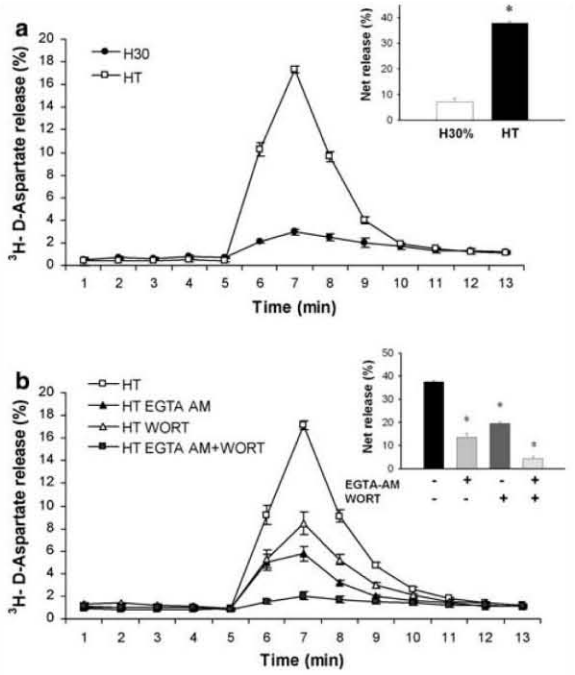

Fig. 2 Effect of thrombin increasing the hyposmotic efflux of ^{3}H-D-aspartate from cultured cerebellar astrocytes. (**a**) Time course of ^{3}H-D-aspartate release by hyposmotic 30% condition (H30%) (●) and its potentiation by thrombin (5 U/ml; HT) (□). Cells preloaded with ^{3}H-D-aspartate were superfused (1 ml/min) with isosmotic medium, and then (fraction No. 5) with 30% hyposmotic medium. Isosmotic medium, 300 mOsm; 135 mM NaCl; H30% made by equiosmolar reduction of NaCl. Thrombin was added to the hyposmotic solutions. Points represent the radioactivity released at each fraction, expressed as percentage of total radioactivity accumulated during loading. Bars represent the release of ^{3}H-D-aspartate released in the four main fractions of the peak after the stimulus minus four fractions of the release in isosmotic conditions (net release). Dashed bars: thrombin 5 U/ml (HT), empty bars: no thrombin added (H30%). (**b**) Effects of PI3K blockade by wortmannin and of prevention of Ca^{2+} rise by treatment with EGTA-AM on hyposmolarity plus thrombin-elicited ^{3}H-D-aspartate efflux. Cells stimulated with hyposmolarity plus thrombin (□), pretreated with wortmannin 250 nM (△) or with EGTA-AM 25 μM in external Ca^{2+}-free medium (▲) or with the two conditions simultaneously (■). Bars represent net release as above described. Results are means ± SE of 3–8 experiments and analyzed using analysis of variance (ANOVA) followed by Tukey test. Significantly different from the control condition HT, $^*P < 0.05$

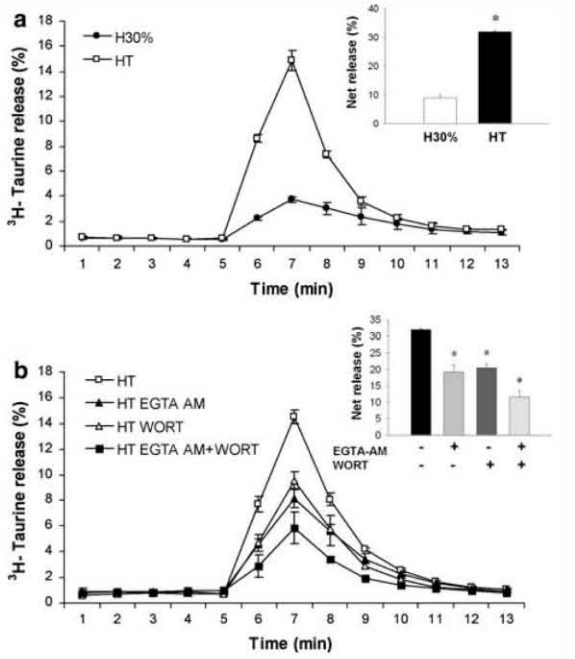

Fig. 3 Effect of thrombin increasing the hyposmotic efflux of ^{3}H-taurine from cultured cerebellar astrocytes. (**a**) Time course of ^{3}H-taurine release by exposure to osmolarity reduced medium (H30%) (●) and its potentiation by addition of thrombin (5 U/ml; HT) (□). Experimental procedure details as in Fig. 2. (**b**) Effects of PI3K blockade by wortmannin and of prevention of Ca^{2+} rise by treatment with EGTA-AM on hyposmolarity plus thrombin-elicited ^{3}H-taurine efflux from cultured cerebellar astrocytes. Cells stimulated with hyposmolarity plus thrombin (□), pretreated with wortmannin 250 nM (△), with EGTA-AM 25 μM in external Ca^{2+}-free medium (▲) or with the two conditions simultaneously (■). Points represent the radioactivity released at each fraction, expressed as percentage of total radioactivity accumulated during loading. Bars represent net release of ^{3}H-taurine as described in Fig. 2. Results are means ± SE of 3–8 experiments and analyzed using analysis of variance (ANOVA) followed by Tukey test. Significantly different from HT, $^*P < 0.05$

reduced D-aspartate efflux by 64%, stressing the importance of the Ca^{2+}-dependent signalling pathway on the effect of thrombin.

Thrombin action on the hyposmotic taurine efflux was next examined. Figure 3a shows the time course of ^{3}H-taurine efflux evoked by hyposmolarity. This response has been described in a large variety of cells including cultured astrocytes. In most cell types, the hyposmotic taurine efflux is only marginally Ca^{2+}-dependent [12]. However, increasing $[Ca^{2+}]_i$ by ionomycin markedly potentiates this efflux [15]. As shown in Fig. 3a, a 3.6-fold increase of hyposmotic taurine efflux was observed in the presence of thrombin. Thrombin potentiates taurine efflux also in astrocytoma and neuroblastoma cells [28, 29] and in 3T3 fibroblasts [27]. The hyposmotic efflux of taurine enhanced by thrombin was 40% decreased by cell treatment with EGTA-AM (Fig. 3b). This inhibition is lower than that found for glutamate efflux, and more close to the 45% found in a previous study in fibroblasts [27].

The Contribution of PI3K

An additional element in the signalling pathway by which thrombin potentiates the hyposmotic D-aspartate and taurine efflux is PI3K. Inhibition of PI3K by wortmannin

inhibited the thrombin-increased hyposmotic D-aspartate efflux by 48%. The simultaneous treatment with EGTA-AM, to prevent the thrombin-elicited $[Ca^{2+}]_i$ rise and of wortmannin to inhibit PI3K, essentially abolished D-aspartate efflux (almost 90% inhibition), showing that the two signalling pathways operate independently and almost fully account for the hyposmotic efflux of D-aspartate in the presence of thrombin (Fig. 2b). Blockade of PI3K also reduced the hyposmotic plus thrombin efflux of taurine by 36% (Fig. 3b), a less potent effect than that observed for D-aspartate. When cells are treated with EGTA-AM and wortmannin together, the hyposmotic plus thrombin taurine efflux decreased 63% (Fig. 3b), also significantly less than the inhibition of D-aspartate in the same condition. This may suggest the involvement of another signalling element for taurine efflux.

Thrombin is not Increasing the Hyposmolarity-Evoked EGFR or src Phosphorylation in Astrocytes

Transactivation of growth factor receptors by GPCRs is well documented [24–26]. A link between PAR-1 activation by thrombin and EGFR has been described in various cell types, although the nature and prevalence of this transactivation in connection with RVD or osmolyte fluxes has not been explored in detail. A study in 3T3 fibroblasts showed the potentiation by thrombin of the hyposmotic increase in EGFR phosphorylation [27]. This was not observed in cultured astrocytes, as shown in Fig. 4. The hyposmotic condition increased EGFR phosphorylation by about 40% over phosphorylation in isosmotic medium, but at variance to that found in fibroblasts, the presence of thrombin did not further enhance this effect (Fig. 4). The EGFR phosphorylation blocker AG1478 markedly reduced the increase elicited by the hyposmotic condition.

The phosphorylation of c-src was also enhanced by hyposmolarity in 3T3 fibroblasts [27], but similar to EGFR, thrombin did not increase but rather reduced c-src phosphorylation (Fig. 5). The src blocker PP2 has a potent inhibitory effect and essentially abolished the hyposmolarity-elicited phosphorylation in the presence or absence of thrombin. These results show that, in contrast to the effect of thrombin in fibroblasts increasing c-src phosphorylation, this interaction is not observed in cultured astrocytes. These results show that in these cells the EGFR/src pathway is not a prominent signalling chain in the process of thrombin potentiation of the hyposmotic glutamate efflux.

Contribution of the EGFR/src Pathway to the Thrombin-Elicited Efflux of Glutamate and Taurine

We next examined the effect of blockade of the EGFR/src pathway on the thrombin potentiated efflux of D-aspartate and taurine from cultured astrocytes. The thrombin-potentiated hyposmotic D-aspartate release was reduced 11–14% upon blockade of EGFR or c-scr phosphorylation by AG1478 or PP2 (Fig. 6a). The release of taurine was more sensitive to these blockers, being inhibited 18–26% (Fig. 6b). This suggests a higher contribution of this pathway to taurine efflux as compared to D-aspartate. However, the contribution of EGFR/src in astrocytes is lower than that found in a previous study in fibroblasts. At variance to that found in astrocytes, in these cells we found an effect of thrombin increasing EGFR and src phosphorylation induced by hyposmolarity and a reduction of over 40% of hyposmolarity plus thrombin-induced taurine efflux [27]. Since in astrocytes, thrombin had no effect increasing the hyposmolarity-evoked EGFR or c-src phosphorylation, the reduction in D-aspartate and taurine efflux by its blockade may correspond to the inhibition of the hyposmotic release in the absence of thrombin. The contribution of the three signaling elements, i.e. $[Ca^{2+}]_i$ rise, PI3K and EGFR on taurine efflux is demonstrated by results in Fig. 7

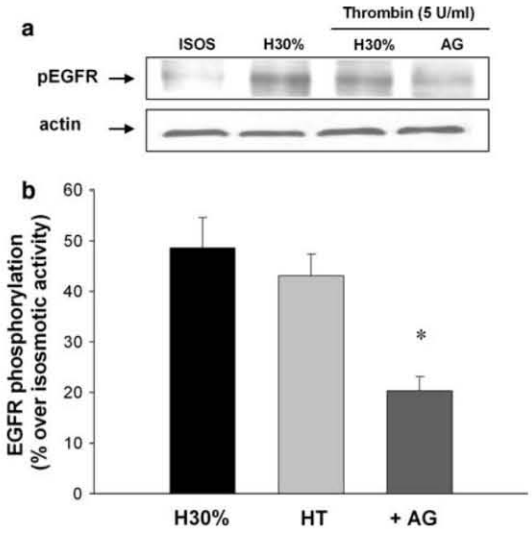

Fig. 4 Effects of hyposmolarity and of hyposmolarity plus thrombin on EGFR phosphorylation. Serum starved cultured cerebellar astrocytes were exposed to the indicated conditions for 3 min and lysates were assayed for EGFR phosphorylation by Western blotting with anti-phospho EGFR (Tyr 1173) antibody. (**a**) Representative blot showing the phosphorylation of EGFR induced by hyposmolarity (H30%) and hyposmolarity plus thrombin (5 U/ml, HT) in astrocytes pre-treated or not with the EGFR inhibitor AG1478 (10 μM) for 20–30 min; lower blot: anti-actin antibody used as an internal standard to normalize protein concentration in each lane. (**b**) Bands were quantified by densitometer and EGFR phosphorylation is expressed in bars as percentage of the phosphorylation increase over the activity in the isosmotic condition. Data are means ± SE ($n = 3$–5) and analyzed using analysis of variance (ANOVA) followed by Tukey test. Significantly different from HT condition $^*P < 0.01$

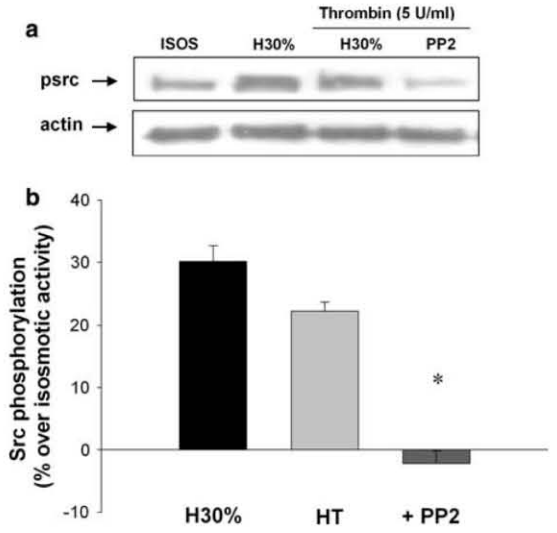

Fig. 5 Effects of hyposmolarity and of hyposmolarity plus thrombin on c-src phosphorylation. Cell lysates obtained as in Fig. 4 were assayed for c-src phosphorylation by Western blotting with anti-phospho c-src (Tyr 418) antibody. (**a**) Representative blot showing the phosphorylation of c-src induced by hyposmolarity (H30%) and hyposmolarity plus thrombin (5 U/ml, HT) in astrocytes pre-treated or not with the src family inhibitor PP2 (10 μM) for 20–30 min; lower blot: anti-actin antibody used as an internal standard to normalize protein concentration in each lane. (**b**) Bands were quantified by densitometer, and c-src phosphorylation is expressed as in Fig. 4. Significantly different form the HT condition $^{*}P < 0.01$

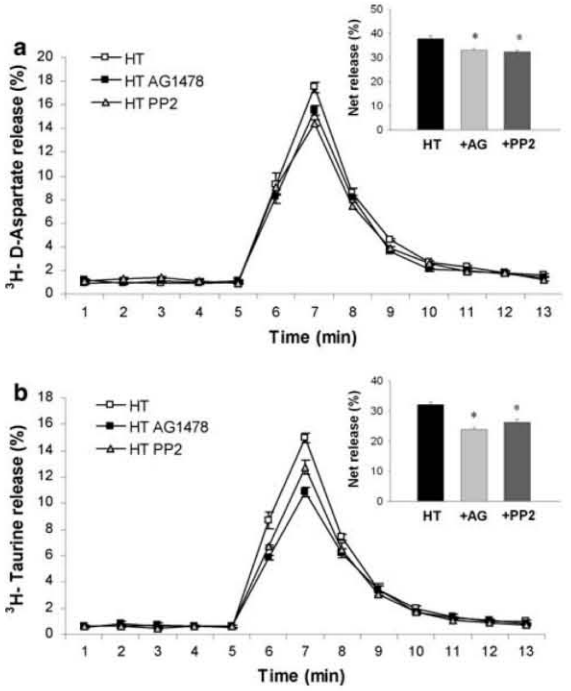

Fig. 6 Effects of EGFR and c-src phosphorylation blockade on (**a**) ^{3}H-D-aspartate and (**b**) ^{3}H-taurine efflux elicited by hyposmolarity plus thrombin (HT), in cells pretreated with AG1478 (10 μM, 30 min) or with PP2 (10 μM, 30 min). Bars represent net release as in Fig. 2. Means ± SE of 3–8 experiments. Significantly different from the control (HT) condition, $^{*}P < 0.05$

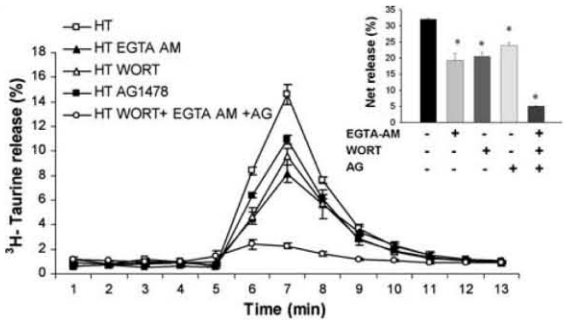

Fig. 7 Additive inhibitory effects of simultaneous blockade of the Ca^{2+}-dependent, PI3K and EGFR pathways on ^{3}H-taurine efflux. ^{3}H-taurine efflux in cells stimulated with HT (□), treated with EGTA-AM (25 μM) in external Ca^{2+}-free medium (▲), wortmannin (250 nM, 1 h) (Δ) and AG1478 (10 μM, 30 min) (■), or the three conditions simultaneously (○). Bars represent net ^{3}H-taurine release as in Fig. 2. Means ± SE of 3–8 experiments. Significantly different from HT, $^{*}P < 0.05$

showing the additive effect of blockade of each one of these pathways, which essentially abolished taurine efflux elicited by hyposmolarity plus thrombin.

Results of the present study showed that in cultured astrocytes, D-aspartate efflux evoked by hyposmolarity plus thrombin is importantly mediated by a Ca^{2+}-dependent pathway, plus a contribution of PI3K, and that blockade of these two pathways reduces its release over 90%. The src/EGFR signalling pathway may account for the remaining 10%, and may reflect the marginal contribution of this pathway of the hyposmotic efflux in the absence of thrombin. In the case of taurine, the same signalling elements contribute to the hyposmotic plus thrombin efflux, but the Ca^{2+}- and PI3K-dependent pathways are less influential and the EGFR/src pathway is more influential than for D-aspartate efflux. These results indicate a difference between taurine and D-aspartate efflux regarding the importance of Ca^{2+}/PI3K, as well as a difference between cultured astrocytes and fibroblasts regarding the importance of the thrombin transactivation of the EGFR/src pathway, which in astrocytes is clearly lower than in fibroblasts. The reason for this difference is so far unclear but may be due either to cell differences or to a lower expression of EGFR in astrocytes at 15 days in vitro, as used in this study, since it is known that EGFR expression is reduced in differentiated astrocytes [30]. Studies on other cell types as well as a study in astrocytes at earlier stages of in vitro differentiation may contribute to clarify these points.

A significant contribution of PI3K in the thrombin increased hyposmotic efflux of D-aspartate and taurine efflux was found in the present study. The participation of PI3K might result from thrombin/EGFR transactivation, in turn influencing PI3K. This is not supported though, by the relatively minor influence of the EGFR/src pathway on D-aspartate and taurine fluxes, lower than that of PI3K, as well as by the additive contribution of the PI3K and the EGFR/src pathways here demonstrated. Another possible mechanism of PI3K activation is through the $\beta\gamma$ subunits of the G-protein coupled to the PAR receptor. This possibility remains to be investigated.

It is worthy to notice that the increase in taurine and glutamate efflux elicited by thrombin as well as by other GPCR agonists occurs essentially through the volume-sensitive organic osmolyte efflux pathway, being essentially abolished by blockers of this pathway.

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Thrombin potentiates D-aspartate efflux from cultured astrocytes under conditions of K^+ homeostasis disruption

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Abstract

Thrombin levels increase in brain during ischemia and hemorrhagic episodes, and may contribute to excitotoxic neural damage. This study examined the effect of thrombin on glutamate efflux from rat cortical cultured astrocytes using ^{3}H-D-aspartate as radiotracer. The glutamate efflux was initiated by addition of 100 mM K^+ plus 1 mM ouabain (K/O) to replicate extracellular and intracellular ionic changes that occur during cerebral ischemia. Upon exposure to K/O, astrocytes swelled slowly and progressively with no evidence of volume regulation. The K/O-induced swelling was inhibited by 65% with bumetanide and 25% with $BaCl_2$, suggesting contribution of $Na^+/K^+/Cl^-$ co-transporter and Kir channels. K/O-elicited ^{3}H-D-aspartate that consisted of two phases. The first transient component of the release corresponded to 13.5% of total ^{3}H-D-aspartate loaded. It was markedly reduced (61%) by the glutamate transporter blocker DL-threo-b-Benzyloxyaspartic acid and weakly inhibited (21%) by the volume-sensitive anion channel blocker 4-[(2-Butyl-6,7dichloro-2-cyclopentyl-2,3-dihidro-1oxo-1H-inden-5-yl)oxy] butanoic acid (DCPIB). During the second sustained phase of release, cells lost 45% of loaded of ^{3}H-D-aspartate via a mechanism that was insensitive to DL-threo-b-Benzyloxyaspartic acid but nearly completely suppressed by DCPIB. Thrombin (5 U/mL) had only marginal effects on the first phase but strongly potentiated (more than two-fold) ^{3}H-D-aspartate efflux in the second phase. The effect of thrombin effect was proportional to cell swelling and completely suppressed by DCPIB. Overall our data showed that under K/O swelling conditions, thrombin potently enhance glutamate release via volume-sensitive anion channel. Similar mechanisms may contribute to brain damage in neural pathologies which are associated with cell swelling, glutamate efflux and increased thrombin levels.

Keywords: cytotoxic swelling, volume regulation, excitoxicity, protease-activated receptors.

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Swelling of brain cells, predominantly astrocytes, occurs either by a decrease in external osmolarity, or under isosmotic conditions by redistribution of ions and organic osmolytes which accumulate into the cells, generating the driving force for water influx. Isosmotic swelling occurs in brain associated with pathologies such as epilepsies, ischemia, hepatic encephalopathy and cranial trauma (Mongin and Kimelberg 2004; Pasantes-Morales and Franco 2005). Astrocytes are the brain cells which predominantly swell under these conditions, as consequence of their crucial role of clearance from the extracellular space, of potential injuring molecules such as K^+, ammonium, or lactate, thus maintaining an optimal environment for neuronal function (Leis *et al.* 2005; Norenberg *et al.* 2005; Syková and Nicholson 2008). Mechanisms of uptake and/or metabolism operate specifically in astrocytes to accomplish this homeostatic function (Chen and Swanson 2003). However, during the progress of pathologies, the clearance capacities of astrocytes may be exceeded or forced to operate at maximal rate, a situation in which astrocytes not only fail to restore homeostasis, but may trigger responses that exacerbate and spread the original damage (Mongin and Kimelberg 2004; Pasantes-Morales and Franco 2005). Swelling is an early expression of this exceeded buffering capacity of astrocytes. Astrocyte swelling occurs in ischemia due to K^+ and Cl^- accumulation

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Abbreviations used: K/O, 100 mM K^+ plus 1 mM ouabain; DCPIB, 4-[(2-Butyl-6,7dichloro-2-cyclopentyl-2,3-dihidro-1oxo-1H-inden-5-yl)oxy] butanoic acid; TBOA, DL-threo-b-Benzyloxyaspartic acid; NKCC, $N^+/K^+/Cl^-$ co-transporter; Kir, inwardly rectifying K^+ channel; PAR, proteinase activated receptor; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.

followed by osmotically-driven water. The enhanced extracellular K^+ levels, which may reach concentrations of up to 80 mM, generate an ionic imbalance harmful for neuronal excitability (Walz 2000; Rossi *et al.* 2007; Doyle *et al.* 2008). Also involved in astrocytic K^+ clearance is the Na^+/K^+ ATPase (Leis *et al.* 2005). If, as in ischemia, the ATPase activity is reduced or impaired, the dissipation of Na^+ and K^+ transmembrane gradients may further contribute to swelling and to disturb in addition, the normal operation of transporters which use the driving force of these gradients for the uptake of a variety of molecules, including the highly neurotoxic excitatory amino acid glutamate (Camacho and Massieu 2006; Doyle *et al.* 2008; Malarkey and Parpura 2008). This situation contributes to neuronal death by excitotoxicity, particularly at the perifocal areas of global ischemia (Won *et al.* 2002; Rossi *et al.* 2007). Under these conditions, any additional factor enhancing glutamate efflux from brain cells will aggravate the excitotoxic damage. Thrombin may be one of such factors.

Besides the role of thrombin in blood coagulation, this molecule exerts a variety of effects on brain cells, which depending on thrombin concentration may be either cytoprotective or cytotoxic (Wang and Reiser 2003). Thrombin is present in brain in low concentrations, which dramatically increase in ischemia as well as in other hemorrhagic or traumatic episodes (Xi *et al.* 2003; De Castro Ribeiro *et al.* 2006; Hua *et al.* 2007). Thrombin effects occur through PAR-1, PAR-3 and PAR-4 receptors, activated by a proteolytic cleavage mechanism via G protein-coupled signaling pathways (Coughlin 2000). The PAR receptors are present in astrocytes (Junge *et al.* 2004). The link of thrombin with glutamate efflux here investigated is based on recent reports showing that ligand activation of G protein-coupled receptors, including PAR receptors, potentiates the swelling-evoked efflux of amino acids such as taurine and glutamate, which in a variety of cells are acting as osmolytes and in brain may have the dual role of osmolytes and neurotransmitters (Fisher *et al.* 2008; Vázquez-Juárez *et al.* 2008).

In a previous study, we showed a marked effect of thrombin increasing hyposmotic-swelling induced glutamate efflux from cultured astrocytes (Ramos-Mandujano *et al.* 2007). The purpose of the present study is to investigate whether thrombin potentiates glutamate efflux evoked by isosmotic swelling under conditions disturbing the astrocytic capacity for K^+ clearance, i.e. high extracellular K^+ levels and ATPase blockade by ouabain (Leis *et al.* 2005). If this occurs, thrombin may exacerbate neurotoxicity and brain damage in pathologies concurrent with a disturbed K^+ homeostasis. Due to the time required for the experiments, the non-metabolizable analogue of glutamate, D-aspartate, was used in this study as tracer for glutamate.

Materials and methods

Materials

Basal medium Eagle, fetal bovine serum and fura-2AM were purchased from Invitrogen. Ouabain octahydrate, L-glutamine (non-animal source), bumetanide, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and the synthetic peptide SFLLRN were from Sigma–Aldrich Chemical (St Louis, MO, USA). FSLLRN, TFLLR and AYPGKF were from Bachem Americas (Torrance, CA, USA). Pen Strep (penicillin streptomycin) was from GIBCO, Invitrogen's brand, Invitrogen (Carlsbad, CA, USA). DCPIB (4-[(2-Butyl-6, 7dichloro-2-cyclopentyl-2,3-dihidro-1oxo-1H-inden-5-yl)oxy] butanoic acid) and DL-TBOA were from TOCRIS Bioscience (Ellisville, MO, USA), and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and PPACK from Calbiochem (San Diego, CA, USA). Thrombin was from Vital Products (bovine plasma origin, specific activity > 2000 U/mg protein). Radiactive molecules [2,3-^{3}H]-Taurine and D-[2,3-^{3}H]-aspartic acid were from American Radiolabeled Chemicals Inc. (St Louis, MO, USA) and Amersham Biosciences (Buckinghamshire, UK) respectively. Salts for preparation of medium solutions were from J.T. Baker (NaCl, KCl, KH_2PO_4, $MgSO_4$, $CaCl_2$, and $BaCl_2$), Sigma Chemicals (D-(+)-Glucose) and Roche (Indianapolis, IN, USA) (Hepes).

Cell cultures

Cortical astrocyte cultures were obtained from Wistar 1 day-old rat pups. Dissociated cell suspensions were plated at 3×10^6 cells in 35 mm Petri dishes, with basal medium Eagle, 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. For cell volume measurements astrocytes were grown on coverslips set over 60 mm Petri dishes with 1.5×10^6 cells. Cultures were incubated at 37°C in humidified 5% CO_2/95% air atmosphere, until confluence.

Solutions

Isosmotic control medium contained (in mM) 135 NaCl, 5 KCl, 1.7 KH_2PO_4, 1.17 $MgSO_4$, 1 $CaCl_2$, 5 glucose, 10 HEPES (300 mOsm, with pH 7.4). Isosmotic K/O solutions were prepared replacing 100 mM NaCl with 100 mM KCl, plus 1 mM ouabain. Hyposmotic solution (210 mOsm) was prepared correspondingly reducing NaCl. Osmolarity was verified by a freezing point osmometer from Precision Systems Inc. (Natick, MA, USA).

Cell volume measurements

Volume measurements were performed by estimating the changes in relative cell volume with a large-angle light-scattering system (McManus *et al.* 1993; Pedersen *et al.* 2002). Astrocytes cultured on coverslips were placed at 50° angle relative to the excitation light in a cuvette filled with isosmotic K/O. To test the effect of hyposmolarity the cuvette was filled with isosmotic medium during 2 min and then distilled water was added to attain the osmolarity required. Cells were excited at 585 nm with an argon arc lamp (emission was detected at 585 nm). Data are expressed as the inverse of the emission signal as light intensity inversely correlates with cell volume, according to the equation I_o/I_t (where I_o = the emission signal average when basal signal has been reached just before the stimulus; I_t = emission signal at time t). It should be noticed that absolute volume values cannot be obtained with this

method, but that it is useful mainly to comparatively evaluate changes in cell volume evoked by different conditions or in the presence of inhibitors. This has to be considered for all mentions to cell volume made through the manuscript.

Release experiments

Astrocytes preloaded 1 h with ^{3}H-D-aspartate or ^{3}H-taurine (0.3 μCi/mL) were washed and superfused at 1 mL/min with isosmotic control medium up to reach a stable efflux baseline. Then, medium was replaced by isosmotic K/O solution superfused continued during 40 min. To test the effect of blockers DL-threo-b-Benzyloxyaspartic acid (TBOA) and DCPIB, cells were 30 min pre-incubated with the inhibitor or the corresponding vehicle. Thrombin (5 U/mL) was added as indicated in the figures. At the end of the experiment, radioactivity in samples (collected during 45 min) and that remaining in cells was measured in a liquid scintillation counter. Results are expressed as radioactivity released per minute as percentage of the total radioactivity incorporated during loading.

Ca^{2+} measurements

To estimate changes in $[Ca^{2+}]_i$ astrocytes cultured on rectangular coverslips in plastic dishes (60 mm), were incubated with fura-2/AM (2 μM) for 40 min. The coverslips were then gently washed in control medium to remove the extracellular dye and were placed at a 50° angle relative to the excitation light path in a cuvette filled with control medium or with K/O solution in a Fluoromax-3, Horiba luminescence spectrometer. Excitation wavelength was alternated between 340 and 380 nm and fluorescence intensity was monitored at 510 nm. The values obtained through this procedure were used to calculate the ratio of fluorescence intensity (fluorescence at 340 nm/fluorescence at 380 nm).

Data analysis

Statistical differences between experimental groups were determined by Student's *t*-test and analysis of variance (ANOVA) followed by Tukey test, statistically significant differences were considered at $^*p < 0.05$. All data are given as mean ± SEM.

Results

High extracellular K^+ concentration and ouabain (K/O) increased astrocyte volume under isosmotic conditions

Cultured astrocytes exposed to 100 mM KCl and 1 mM ouabain (isosmotic solution made by equiosmolar reduction of NaCl) (K/O) showed a continuous increase in cell volume. Swelling started immediately after the treatment and progressively increased to reach a maximum after 27 min. No significant further swelling was observed up to 50 min. Maximal K/O-induced astrocyte swelling (evaluated with the limitations inherent to the light-scattering method) was 16% in average over cell volume in controls (Fig. 1a). K/O-elicited swelling was markedly reduced (66%) when astrocytes were treated with 10 μM bumetanide, a known blocker of the $Na^+/K^+/Cl^-$ co-transporter (NKCC1). Swelling was also decreased (27%) by 200 μM $BaCl_2$, (barium) which at this concentration acts as blocker of inwardly rectifying K^+ channels. Treatment with the two blockers simultaneously, abolished astrocyte swelling (Fig. 1a). Astrocytes exposed to 30% hyposmotic solution show immediate swelling, with maximal peak of 22% over the basal value, attained almost immediately after the stimulus. Thereafter, a progressive reduction in cell volume was observed (Fig. 1b). This result, included in the present study with comparative purposes, confirms other reports showing the typical regulatory volume decrease in cultured astrocytes (Pasantes-Morales *et al.*

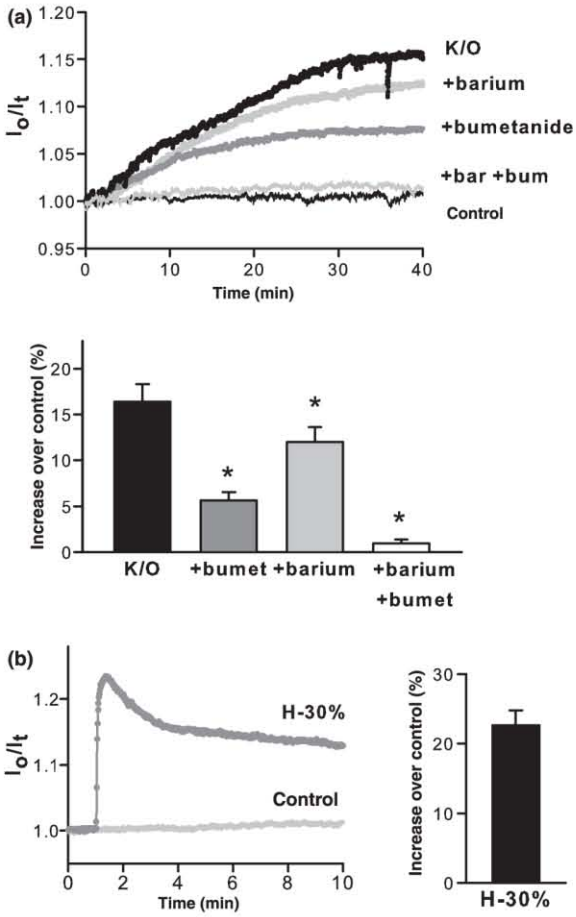

Fig. 1 Swelling of cortical astrocytes exposed to isosmotic K/O and effects of the NKCC cotransporter blocker bumetanide and the Kir channel blocker barium. **(a)** Representative traces of volume changes in cells exposed to KCl (100 mM), replacing equiosmolar NaCl, plus 1 mM ouabain (K/O), and the effect of bumetanide (10 μM) barium (200 μM) and both. Bumetanide (10 μM) was preincubated 30 min in control medium before treatment with K/O, and was present throughout the experiment. Barium (200 μM) was added at the time of exposure to K/O medium. Results in bars (means ± SE of 6–10 experiments) correspond to maximal swelling expressed as percentage over volume under control conditions. (b) Astrocyte swelling and volume regulation in 30% hyposmotic medium (H-30%). *Significantly different from control K/O $p < 0.05$.

1994; Olson *et al.* 1995; Cardin *et al.* 1999). Thrombin added to the control medium elicited a marginal increase of 2.1 ± 0.84% (n = 6) of astrocyte volume.

Treatment with K/O increased D-aspartate via two different routes

Astrocytes loaded with ^{3}H-D-aspartate and superfused with control medium, released labeled D-aspartate at a rate of 0.5% per min. Treatment with K/O increased D-aspartate release with a biphasic pattern. In the first phase, a fast and large increase in D-aspartate was observed, reaching a peak release of 1.7%, 3 min after the stimulus (Fig. 2a). Then, efflux slowly decreased showing release fractions of 1.6–1.2% per min during about 8 min. Thereafter, a second phase of release was observed, in which D-aspartate efflux increased slowly and progressively, without showing any decline during the time of the experiment (40 min). (Fig. 2a). The amount of D-aspartate released during the first phase corresponds to 13.5% of loaded D-aspartate, and the amount released in the second phase is 31% resulting in a total release of 45% (Fig. 2a).

The mechanism of D-aspartate efflux during the two identified phases was investigated using the Na^+-dependent glutamate carrier blocker TBOA, and DCPIB, a specific and potent blocker of the volume-sensitive anion channel. Cells were incubated with the blockers during 20 min before

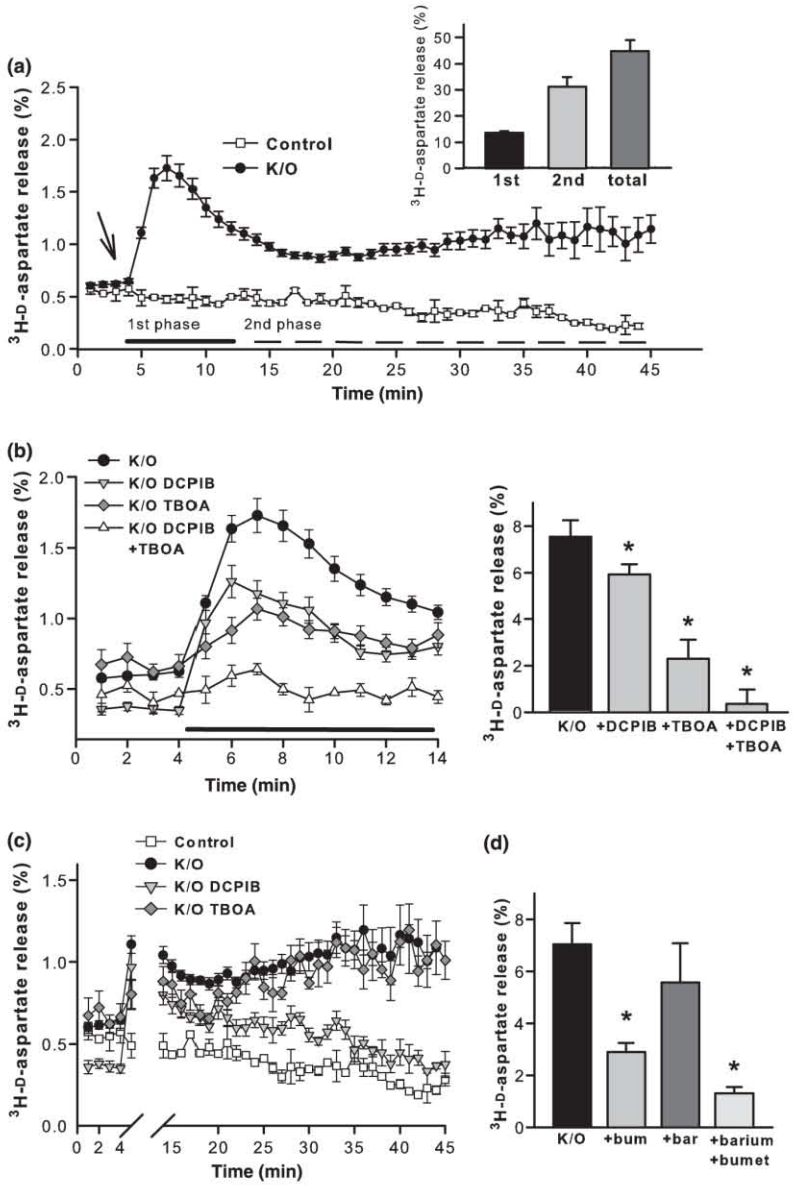

Fig. 2 D-Aspartate efflux from cortical astrocytes evoked by K/O and effect of swelling and carrier blockers. Astrocytes preloaded with ^{3}H-D-aspartate were superfused (1 mL/min) with control medium during 4 min, and then (arrow) with the same control medium (□) or the isosmotic K/O medium (100 mM KCl replacing the equivalent NaCl), and 1 mM ouabain (●). Points represent the radioactivity released at each fraction, expressed as percentage of total radioactivity accumulated during loading. (a) The time-course of ^{3}H-D-aspartate showing two phases, a first phase (solid bottom line), activated and inactivated within 10 min after exposure to K/O, and a progressively increasing second phase of release (bottom dashed line) during the next 30 min. In bars is the amount of ^{3}H-D-aspartate release at each phase, expressed as percentage of the total label accumulated in loading. (b) ^{3}H-D-aspartate release in K/O during the first phase (10 min after K/O, solid bottom line) and effects of 5 μM TBOA and 10 μM DCPIB and both. (c) Efflux and effect of blockers TBOA, DCPIB during the second phase (min 15–40 after K/O). (□) Control; (●) K/O; (▽) K/O plus DCPIB; (◆) K/O plus TBOA. (d). Effect of cell swelling blockers bumetanide (10 μM) and barium (200 μM) on D-aspartate release in K/O during the second phase (30–40 min after K/O). Bars represent net efflux, i.e. release under the K/O condition minus release without K/O, in the presence of the blockers. Results are means ± SE of 6–8 experiments. *Significantly different from control K/O $p < 0.05$.

treatment with K/O. Fig. 2b shows a marked inhibitory effect of TBOA on the first phase of D-aspartate efflux, which is reduced up to 61%. DCPIB showed also an inhibitory effect, decreasing D-aspartate efflux by 21%. Simultaneous treatment with the two blockers reduced the efflux up to 84% (Fig. 2b). An opposite inhibition pattern was found for the second release phase of D-aspartate, which was essentially insensitive to TBOA but markedly reduced by DCPIB (Fig. 2c). These results suggest that swelling is the stimulus to evoke the second phase of D-aspartate release. This was confirmed by the marked reduction of D-aspartate efflux when cell swelling was inhibited by bumetanide (59%), barium (19%) or bumetanide plus barium (81%) (Fig. 2d). Results on the time-course and mechanisms of D-aspartate efflux confirm those reported by Rutledge and Kimelberg (1996).

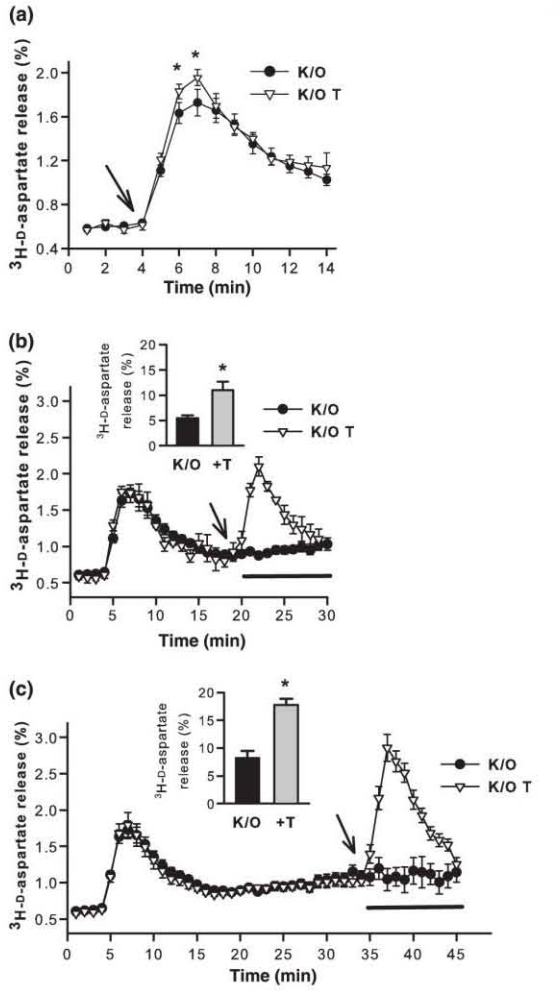

Fig. 3 Effect of thrombin on K/O-induced efflux of D-aspartate from cultured cortical astrocytes (a) ^{3}H-D-aspartate efflux during 10 min after K/O exposure (first phase) and the potentiation by 5 U/mL thrombin, added at the arrow. (b) ^{3}H-D-aspartate efflux during 30 min after K/O and the potentiation by thrombin added at the arrow. (c) K/O-evoked ^{3}H-D-aspartate efflux during 40 min and the potentiation by thrombin added at the arrow. Points represent the radioactivity released at each fraction, expressed as percentage of total accumulated under the following conditions: (●) K/O; (▼) K/O plus thrombin. Bars in b and c show ^{3}H-D-aspartate released in 10 fractions (bottom line) in the presence or absence of thrombin and represent net efflux (K/O minus control release) under the indicated conditions, Points in the curves and results in bars, are means ± SE of six experiments. *Significantly different from controls in the absence of thrombin $p < 0.05$.

D-Aspartate efflux evoked by K/O is potentiated by thrombin

Thrombin added at the same time as K/O evoked a small but significant enhancement of D-aspartate efflux (Fig. 3a), but when added 15 min after exposure to K/O, once the first release phase has almost inactivated, a large potentiation of D-aspartate efflux was observed, increasing by 101% the K/O-evoked efflux (Fig. 3b). An even higher potentiation of D-aspartate efflux of about 117% was observed when thrombin was applied when the second phase has progressed, 30 min after the stimulus (Fig. 3c). Thrombin evoked release of D-aspartate was unaffected by TBOA and essentially abolished by DCPIB, NPPB or DIDS (Fig. 4a). These results suggest that the effect of thrombin occurs primarily on D-aspartate fluxes across the volume-activated anion channel. In further support to this notion, the thrombin potentiated D-aspartate efflux was essentially suppressed by preventing astrocyte swelling with bumetanide and barium (Fig. 4b).

K/O evoked taurine efflux is potentiated by thrombin

Taurine efflux examined in the same conditions exhibited only a small increase as result of K/O exposure in the initial minutes, corresponding to the first phase of release (Fig. 5a). Thereafter, taurine efflux increased continuously during the next 30 min with no sign of inactivation. Taurine released was 8% and 35% in the first and second phase, respectively, resulting in a total release of 43% (Fig. 5a). Taurine efflux evoked by K/O was abolished by DCPIB (Fig. 5a). Thrombin increased taurine efflux, by a DCPIB-sensitive mechanism (Fig. 5b).

The mechanisms of thrombin effect increasing taurine and D-aspartate efflux

The mechanism mediating thrombin effects on K/O efflux of D-aspartate likely involves its action as a protease on a PAR receptor. This is supported by results in Fig. 6a, showing how thrombin pretreatment with the protease inhibitor PPACK, prevented the thrombin potentiation of K/O-evoked D-aspartate efflux. The PAR-1 isoform seems that predominantly involved in thrombin effect, since the PAR-1 agonists SFLLRN and TFLLR (5 μM), fully replicate the effect of thrombin on D-aspartate efflux, while the scrambled peptide,

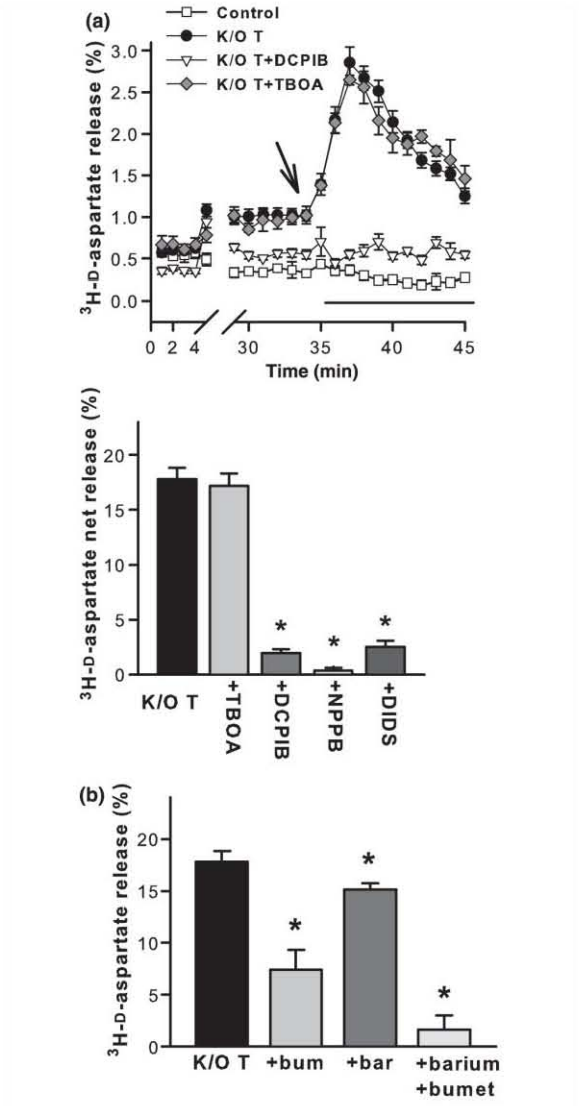

Fig. 4 Effects of swelling-, carrier- and volume-sensitive pathway-blockers on K/O-evoked D-aspartate potentiated by thrombin. (a) Effects of TBOA, DCPIB, NPPB and DIDS on D-aspartate released during the last 10 min of perfusion with K/O plus thrombin (bottom line). (b) Effects of the K/O induced swelling blockers bumetanide and barium on the D-aspartate release as in (a). Astrocytes were pre-incubated with the blockers, 5 μM TBOA, 10 μM DCPIB, 50 μM NPPB or DIDS, 10 μM bumetanide, during 30 min, and then the experiment was carried out as in Fig. 2. Barium (200 μM) was added at the time of K/O treatment. Bars represent net efflux (K/O minus control release) under the indicated conditions, and are means ± SE of 4–6 experiments. *Significantly different from K/O plus thrombin condition $p < 0.05$.

FSLLRN was without effect (Fig. 6b). The PAR-4 agonist AYPGKF (50 μM) was essentially ineffective (Fig. 6b).

Thrombin interaction with PAR receptors is known to elicit a marked increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). This effect is consistently observed in a large variety of cells and was also found in the present study in cortical cultured astrocytes treated with K/O. The magnitude of thrombin effect increasing K/O-induced D-aspartate release was markedly different during the time of the experiment as above described. To exclude a variation in the extent of thrombin-evoked $[Ca^{2+}]_i$ rise during the experiment as the reason for this difference, the change in $[Ca^{2+}]_i$ was examined when thrombin was applied 2 min (Fig. 7a), 15 or 30 min after K/O stimulus (Fig. 7b and c). Figure 7b shows no difference in $[Ca^{2+}]_i$ elevation at any of the times examined.

Discussion

The present results showed swelling in cultured astrocytes under isosmotic conditions, evoked by treatment with high extracellular K^+ concentrations and ouabain (K/O). The swelling time-course observed contrasts notably with that induced by 30% reduction in osmolarity in the same preparation (cultured astrocytes) (Pasantes-Morales *et al.* 1994; Olson *et al.* 1995; Cardin *et al.* 2003 and present results). Whereas maximal volume under the hyposmotic condition was attained almost immediately after the stimulus, it required about 30 min to be reached in K/O-treated cells. Another remarkable difference is that while hyposmotic swelling is followed by an active process of volume recovery, in the K/O-treated cells there is no evidence of volume regulation, though a plateau is reached at a certain time.

Astrocyte swelling elicited by K/O treatment is the consequence of K^+ and Cl^- accumulation, followed by osmotically obligated water (Ransom *et al.* 1996; Walz 2000). A small proportion may come also from intracellular Na^+ raised by Na^+/K^+ ATPase blockade, which is though, counteracted by the suppressed K^+ accumulation via the ATPase. Under this condition, net K^+ uptake is accomplished primarily by activation of the electroneutral co-transporter NKCC. NKCC1 is the isoform expressed in cultured astrocytes and there is evidence in support of the substantial contribution of this transporter to the uptake phase of K^+ clearance by astrocytes (Walz 1987; Su *et al.* 2002a, b; Mongin 2007). The present results showing a marked reduction in K/O-induced swelling when NKCC1 is blocked by bumetanide are in line with these previous observations. The glial inwardly rectifying K^+ channels of the Kir family channels are proposed as an additional pathway for K^+ accumulation and K^+ buffering. The Kir4.1 isoform is expressed in cultured and *in situ* astrocytes and constitute the major part of the astrocytic Kir conductance (Olsen and Sontheimer 2008; Benesova *et al.* 2009). We found that blockade of Kir channels with barium led to a mild decrease of astrocyte swelling, suggesting a modest contribution of this mechanism to K^+ uptake in cultured cortical astrocytes.

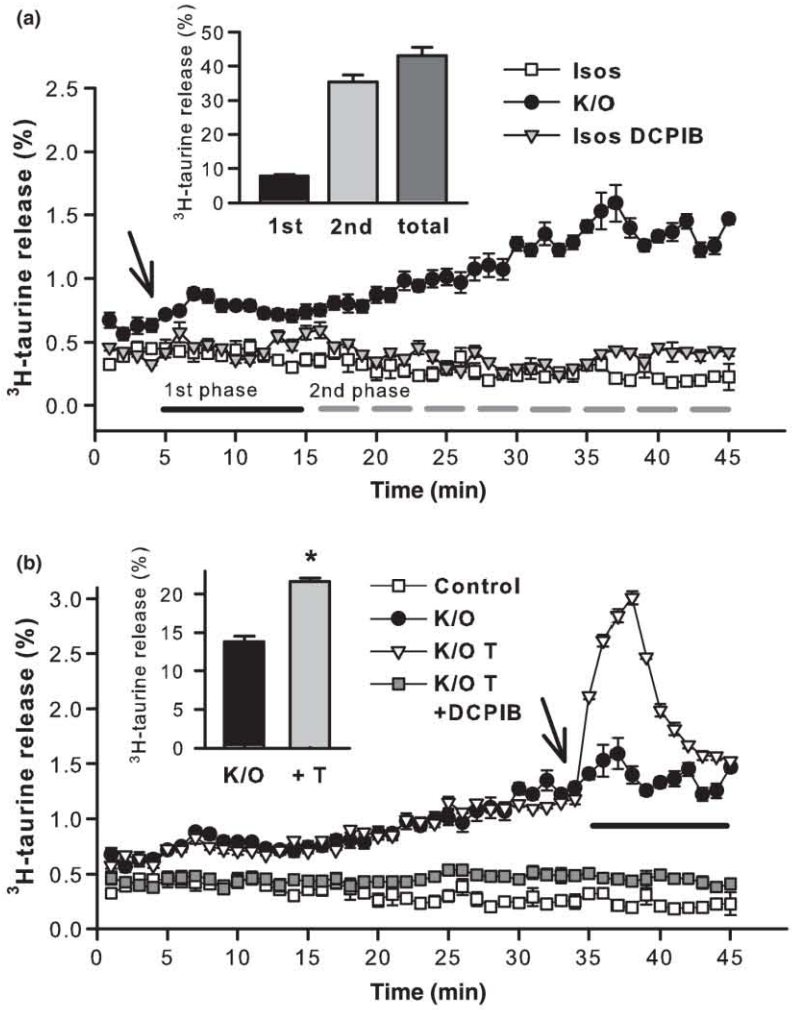

Fig. 5 Effect of K/O on ^{3}H-taurine release, its potentiation by thrombin and the effect of DCPIB. (a) Astrocytes were preloaded with ^{3}H-taurine and treated as in Fig. 2. (b) Time-course of K/O-evoked taurine efflux and the effect of thrombin (5 U/mL) added 30 min after K/O exposure (bottom line) and the effect of DCPIB (10 μM) on thrombin potentiation. DCPIB treatment as described in Fig. 2. Points represent the radioactivity released at each fraction, expressed as percentage of total ^{3}H-taurine accumulated under the following conditions: (□) Control; (●) K/O; (▽)) K/O plus DCPIB; (▽) K/O plus thrombin (■) K/O plus thrombin plus DCPIB. Bars in (a) represent net efflux (K/O minus control release) in the two phases of release. Bars in b show taurine released in 10 fractions (bottom line) in the presence or absence of thrombin. *Significantly different at $p < 0.05$.

The time-course of K/O-induced swelling in cortical cultured astrocytes showed no evidence of an efficient volume regulation, which contrasts with the fast volume recovery observed after hyposmotic swelling. This is a predictable result since the typical regulatory volume decrease observed under hyposmotic conditions is accomplished to a large extent, by K^+ and Cl^- extrusion (Wehner 1998; Stutzin and Hoffmann 2006) which cannot occur in high extracellular K^+ concentrations. The pool of organic osmolytes, including glutamate, taurine and myo-inositol (Rutledge and Kimelberg 1996; Cardin *et al.* 1999; Isaacks *et al.* 1999), is mobilized, attenuating the magnitude of swelling, and is presumably responsible for the observed swelling plateau, but appears insufficient to accomplish cell volume recovery when the K/O condition persists.

Taurine and D-aspartate efflux elicited by K/O was comparatively examined in the present study, and marked differences were found in the release pattern between the two amino acids. In contrast to the fast and large release of D-aspartate observed immediately after K/O exposure, only a marginal increase in taurine efflux was observed. Differences in the carrier properties may contribute to the difference observed, since while glutamate transporter is Na^+ and K^+-dependent, taurine carrier is only Na^+-dependent and consequently is less influenced by changes in external K^+. Results showing that prevention of taurine efflux by DCPIB, a specific blocker of the volume-sensitive anion channel (Decher *et al.* 2001), points to this pathway as the main route for taurine translocation.

The release of D-aspartate from astrocytes was also increased by K/O treatment, as previously reported (Rutledge and Kimelberg 1996). The efflux time-course shows two different phases: an initial phase, of fast activation and inactivation, and a second phase, of delayed and progressive efflux, detectable as long as the K/O condition persists. The pharmacological profile of these two phases revealed two different mechanisms for release. The initial phase, markedly reduced (60%) by the carrier blocker TBOA, is

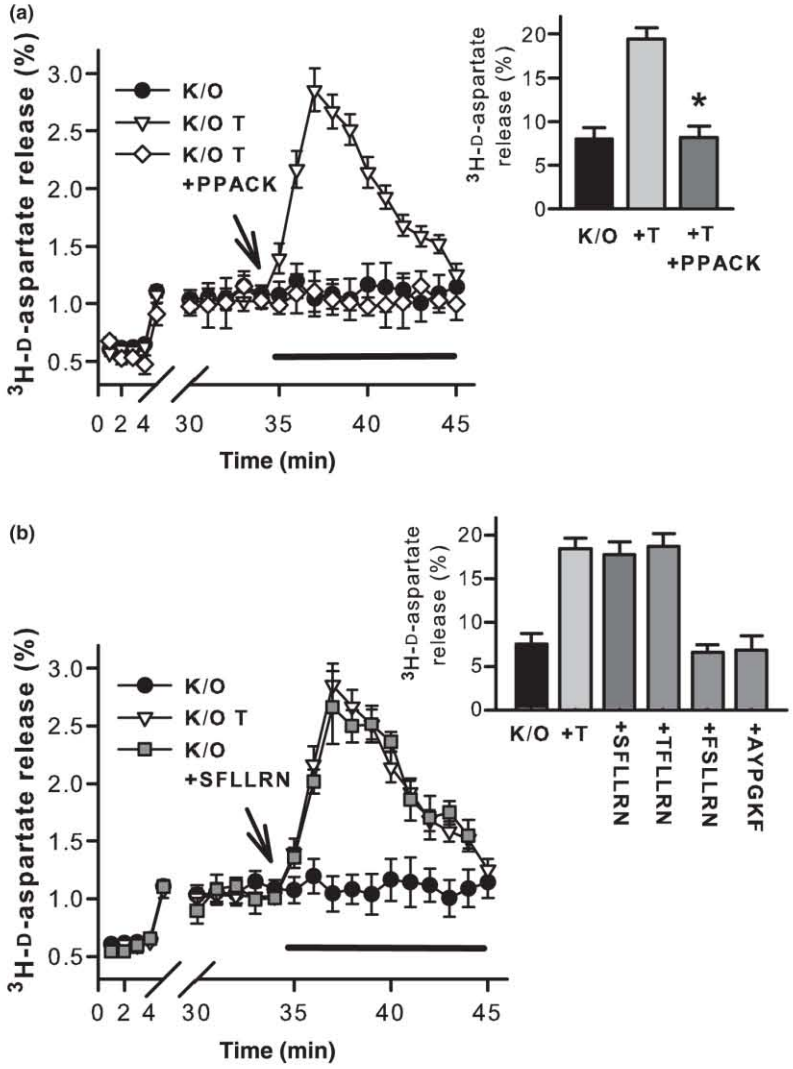

Fig. 6 Effect of PPACK and PAR agonists on K/O-evoked D-aspartate release. (a) Thrombin pre-incubated 30 min with PPACK (1 μM) was applied to cells exposed to K/O during 30 min as in Fig. 4c. (b) Effect of the synthetic peptides, PAR-1 agonists, SFLLRN or TFLLR (5 μM), FSLLRN (scrambled peptide), the PAR-4 agonist, AYPGKF (50 μM), were added to K/O-treated cells replacing thrombin, 30 min after K/O as in Fig. 3c. Bars in (a) represent D-aspartate released in the last 10 fractions (bottom line) by K/O, K/O plus thrombin and K/O plus PPACK-treated thrombin. In (b) bars illustrate D-aspartate release in the presence of the peptides as in (a). Results are means ± SE of four experiments. *Significantly different from K/O plus thrombin, by $p < 0.05$.

then likely occurring via the transport reversal, a condition favored by the dissipation of ionic gradients and depolarization. Interestingly, a fraction of 21% of D-aspartate release in this first phase was reduced by DCPIB, the volume-sensitive pathway blocker, suggesting that even small changes in cell volume as occurring in the first minutes after treatment with K/O, enhance glutamate efflux via this pathway. The simultaneous presence of TBOA and DCPIB reduced 95% the D-aspartate efflux from this first fraction, excluding mechanisms other than swelling and carrier-mediated efflux as contributors to D-aspartate release. The second phase of D-aspartate efflux showed a markedly different time-course as compared with the initial phase, and a different pattern of sensitivity to TBOA and DCPIB. While the carrier blocker had no effect, D-aspartate efflux was abolished by DCPIB, NPPB or DIDS, a result that points to swelling as the main stimulus for this release. The swelling-dependent phase of D-aspartate efflux is also evident by the effect preventing this efflux when cell swelling is reduced by treatment with bumetanide and barium. A previous study has shown a strong inhibitory effect of bumetanide on D-aspartate release elicited by high K^+ concentrations (Su *et al.* 2002a, b), a result confirmed in the present results. All these observations clearly establish that D-aspartate efflux is elicited by both, depolarization/dissipation of the ionic gradients and cell swelling, and proceeds via two different routes, as has been previously demonstrated by Rutledge and Kimelberg (1996). The same conclusion has been reached after substantial evidence regarding glutamate efflux in a variety of experimental models of ischemia, *in vitro* and *in vivo* (Nelson *et al.* 2003; Phillis and O'Regan 2003; Mongin and Kimelberg 2004; Swanson *et al.* 2004; Kimelberg 2005). This similarity is expected since the experimental paradigm of the

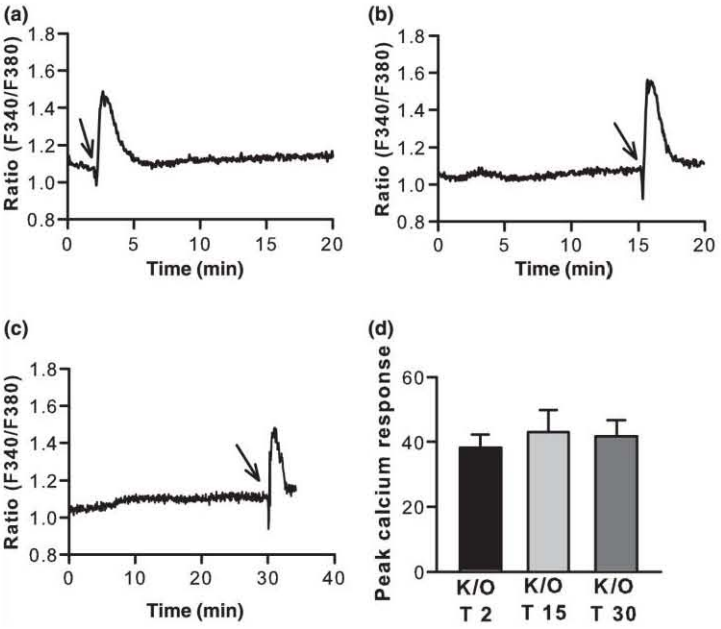

Fig. 7 Changes in $[Ca^{2+}]_i$ elicited by thrombin in astrocytes at different times of exposure to K/O: (a). Thrombin added 2 min after K/O exposure (b). 15 min after K/O. (c) 30 min after K/O. Results are expressed as ratio of fluorescence intensity (340/380 nm) (d). Quantification of the peak of $[Ca^{2+}]_i$ response for each condition (bars were obtained from the peak value minus the basal K/O value). $[Ca^{2+}]_i$ was measured with fura-2AM (2 μM) as detailed in Methods. Means ± SE from 6 to 10 experiments.

present study replicates intracellular and extracellular ionic changes that occur during cerebral ischemia, and has been often considered as an ischemic-like model (Rutledge and Kimelberg 1996). In contrast to glutamate, taurine efflux, which is also reported to be released in ischemia models (Phillis and O'Regan 2003; Mongin and Kimelberg 2004), seems to respond largely to swelling.

The main interest of the present study was to investigate whether thrombin potentiates glutamate efflux under ischemic-like conditions, thus potentially aggravating the risk of excitotoxicity. It should be noticed that in all experiments, D-aspartate was used as tracer for glutamate. Glutamate participates in multiple reactions related to brain energetic demands, and excitability (Dienel and Hertz 2005; Rossi *et al.* 2007) and in astrocytes particularly, glutamate is actively metabolized via glutamine synthetase (Isaacks *et al.* 1999). Therefore, the amount of glutamate released by K/O and K/O plus thrombin may be lower than that of D-aspartate. If this is too low to promote excitotoxicity remains to be demonstrated.

We showed in a previous report a marked effect of thrombin increasing glutamate efflux from cultured astrocytes swollen by hyposmolarity (Ramos-Mandujano *et al.* 2007) and the present study demonstrates a similar effect of thrombin in a model of isosmotic swelling, in this case elicited by intracellular K^+, Na^+ and Cl^- intracellular accumulation. As above mentioned, in contrast to the immediate and fast increase in cell volume after a hyposmotic stimulus, swelling under the K/O condition has a temporal pattern allowing us to demonstrate that thrombin potentiation of glutamate efflux, D-aspartate in this case, occurs with a magnitude proportional to the degree of swelling. A small but significant effect of thrombin increasing D-aspartate release was observed within the first minutes after the stimulus, when only minute changes in cell volume occur. Later, the potentiation by thrombin is much higher, with a magnitude related to the extent of swelling. In full accordance with this conclusion, when swelling is prevented by bumetanide and barium, the thrombin-potentiated D-aspartate efflux was essentially suppressed.

The effect of thrombin found in the present study, in agreement with that observed on hyposmotic glutamate efflux, involved a protease-activated receptor (PAR), mainly the PAR-1 isoform, which is present in astrocytes (Wang *et al.* 2002; Wang and Reiser 2003; Junge *et al.* 2004). Thrombin activation of PAR receptors elicits a signaling pathway resulting in $[Ca^{2+}]_i$ increase in astrocytes confirming its effect in numerous cell types. Thrombin increased $[Ca^{2+}]_i$ in astrocytes from two main sources, extracellular Ca^{2+} and Ca^{2+} from the endoplasmic reticulum stores (Ramos-Mandujano *et al.* 2007). As above mentioned, thrombin potentiation of D-aspartate and taurine efflux was higher when thrombin was applied after longer times after the K/O treatment. This pattern is not due to any difference in the extent of thrombin-elicited $[Ca^{2+}]_i$ elevation, which was found to be the same all along the experiment. It seems, in contrast, related to the degree of cell swelling which is progressively increasing. Altogether, these results show that glutamate (D-aspartate) efflux can be enhanced in swollen cells under isosmotic conditions including those replicating ischemia, provided that a threshold swelling is attained.

The thrombin-elicited increase in glutamate efflux from astrocytes might contribute to ischemic-induced neuronal death by excitotoxicity (Feustel *et al.* 2004; Mongin 2007) particularly since brain thrombin levels notably increase in ischemia. Other observations relate thrombin with excitotoxicity, such as the ischemia-induced up-regulation of PAR receptors (Xi *et al.* 2003) for which thrombin is the main substrate, or reported thrombin action increasing the efficiency of the glutamate NMDA-type receptor, which may exacerbate glutamate potential damage (Gingrich *et al.* 2000; Lee *et al.* 2007; Sharp *et al.* 2008). Altogether, these observations point to a possible effect of thrombin aggravating the excitotoxic damage known to occur in ischemia. In support to this possibility is the resistance to ischemic damage observed in transgenic mice defective in PAR-1, and the increased neuronal survival by treatment with PAR-1 blockers argatroban and hirudin (Kawai *et al.* 1996; Striggow *et al.* 2000, 2001; Karabiyikoglu *et al.* 2004).

The effects of thrombin increasing D-aspartate efflux were found abolished by preventing swelling with bumetanide and barium. Also taurine and D-aspartate release were suppressed by DCPIB. Altogether, these results point to the swelling-activated permeability pathway as the site of thrombin influence. DCPIB is a specific and potent blocker of the volume-sensitive glutamate efflux from astrocytes as shown by previous results from us and others (Abdullaev *et al.* 2006; Ramos-Mandujano *et al.* 2007). DCPIB and other Cl^- channel blockers also inhibit the swelling-induced efflux of organic osmolytes (Abdullaev *et al.* 2006; Shennan 2008). There is still controversy on whether the swelling-sensitive Cl^- channel itself is the permeability pathway for the organic osmolytes, including glutamate, a controversy raised by consistent observation of an inhibitory effect of essentially all the volume-sensitive Cl^- channel blockers on the volume-sensitive efflux of organic osmolytes. If this means that the same pathways carries both Cl^- and organic osmolytes or that they are so closely interconnected that blockade of one, blocks also the other one, is still uncertain. In any event, there is evidence of a strong effect of DCPIB reducing swelling-induced glutamate efflux in astrocytes and more recently DCPIB was also shown to prevent glutamate efflux evoked by middle cerebral artery occlusion-induced ischemia in adult rat. In support to the critical role played by cell swelling as a route for glutamate efflux leading to excitotoxic damage in ischemia, DCPIB showed a significant reduction of the infarct volume in this *in vivo* ischemia model (Zhang *et al.* 2008).

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5.4. CUARTA PARTE (RESULTADOS NO PUBLICADOS)

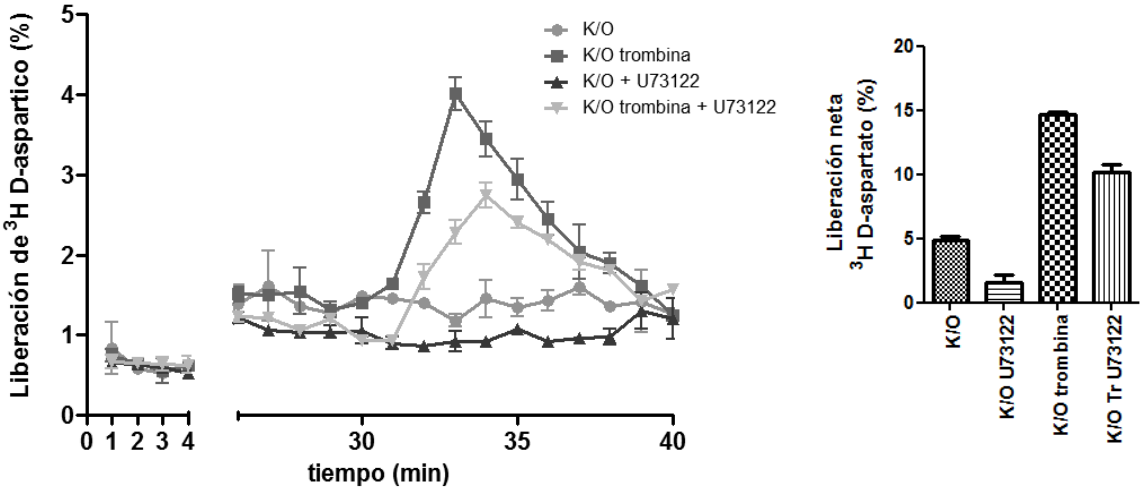
En esta parte se presentan los resultados hallados respecto a algunos elementos de señalización que participan en la cascada activada por el corte proteolítico del receptor a trombina bajo condiciones de aumento en volumen isosmótico por una alteración en la homeostasis del potasio.

En la tabla 1 se resumen los fármacos utilizados y su correspondiente inhibición de la liberación evocada solamente por el estímulo de una alta concentración extracelular de potasio, así como sobre la potenciada por trombina.

Blanco	**Fármaco**	**% de inhibición bajo la condición de $\uparrow[K^+]_e$**	
		sin estímulo	**+ trombina**
PLCβ	*U73122 2.5 µM*	67%	31%
Ca^{2+} intracelular	*BAPTA-AM 25 µM*	27%	38%
Vaciamiento poza de Ca^{2+} del R.E.	*Tapsigargina 0.5 µM*	40%	36%
Ca^{2+} extracelular	*BAPTA 0.1 mM*	21%	14%
PI3K	*Wortmannin 100 nM*	79%	55%
CaMKII	*KN-93 5 µM*	-	48%
Ca^{2+}_i + PI3K	*BAPTA-AM + Wortmannin*	-	93%

Tabla 1. Efecto de la inhibición de elementos de la vía de señalización que conduce a un aumento en Ca^{2+} intracelular así como de la actividad de PI3K sobre la liberación de glutamato bajo condiciones de alteración en la homeostasis de potasio en ausencia o presencia de trombina. Los fármacos (a excepción de la tapsigargina) fueron preincubados 15 minutos antes del tratamiento con una alta concentración de K^+ extracelular y estuvieron presentes durante todo el experimento. La tapsigarguina se agregó 10 minutos previos al tratamiento con una alta concentración de K^+ extracelular durante 5 minutos y el experimento se realizó en medio libre de Ca^{2+} para evitar el relleno de la poza intracelular de Ca^{2+} ubicada en el R.E. Los datos se basan en el promedio los curso-temporales de las figuras 4 y 5.

a.



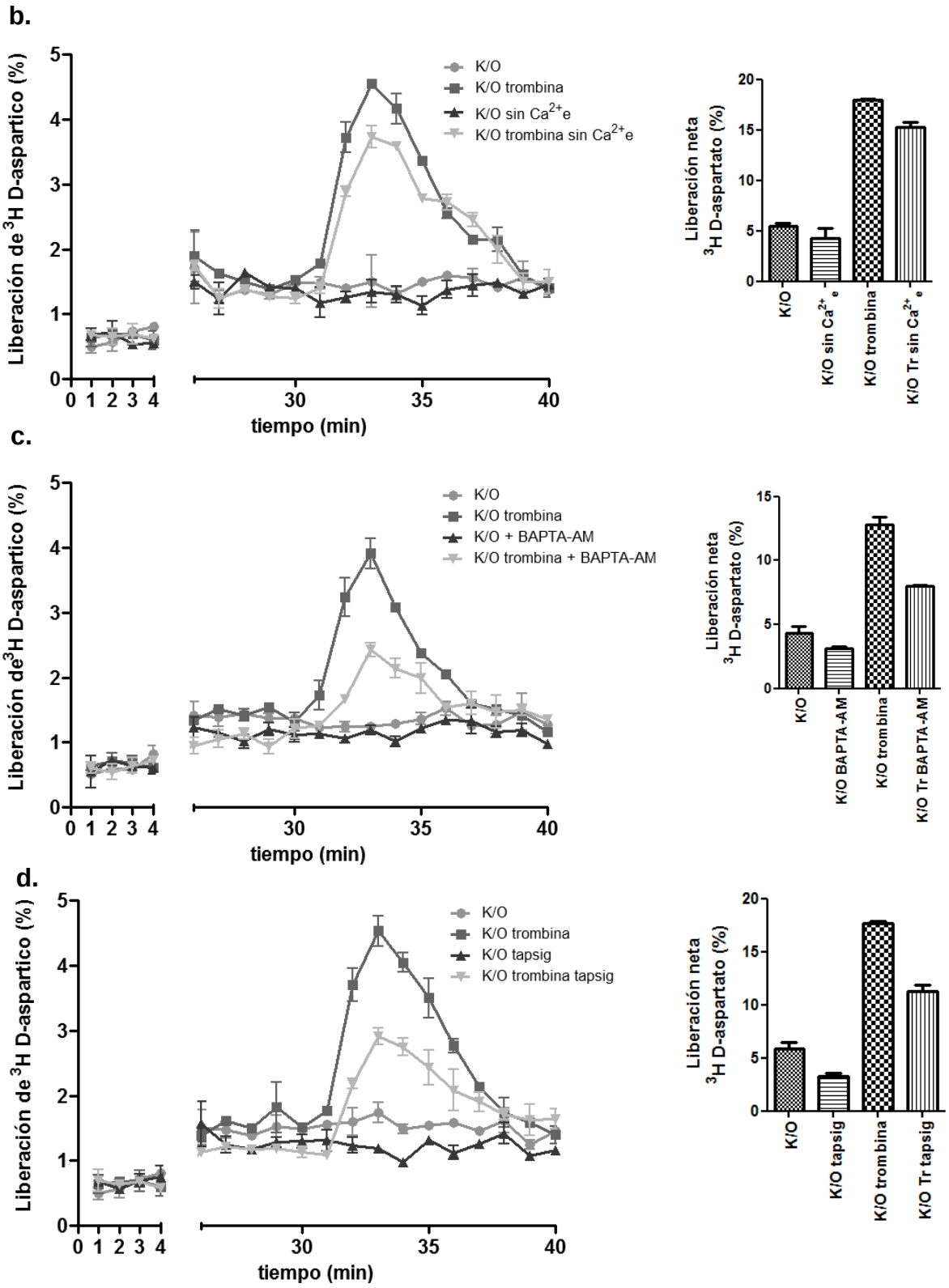


Figura 4. Curso temporal y cuantificación de la liberación de ^{3}H-D-aspartato bajo la condición de aumento en volumen por alteración de la homeostasis del potasio en presencia o ausencia de trombina y de diferentes inhibidores/quelantes: U73122 (**a**), sin calcio extracelular + BAPTA (**b**), BAPTA-AM (**c**) y pretratado con tapsigargina (**d**). En las gráficas de la izquierda se muestra la liberación basal (4 min), a partir del minuto 5 y hasta el final del experimento, los astrocitos fueron sometidos a una solución isosmolar (300 mOsm) de [100 mM] de KCl. La trombina se agrega patir del minuto 31. En barras, se muestra la cuantificación de la liberación acumulada entre el

minuto 31 y el 36. Los resultados se muestran como los promedios ± E.E. de 4 a 6 experimentos.

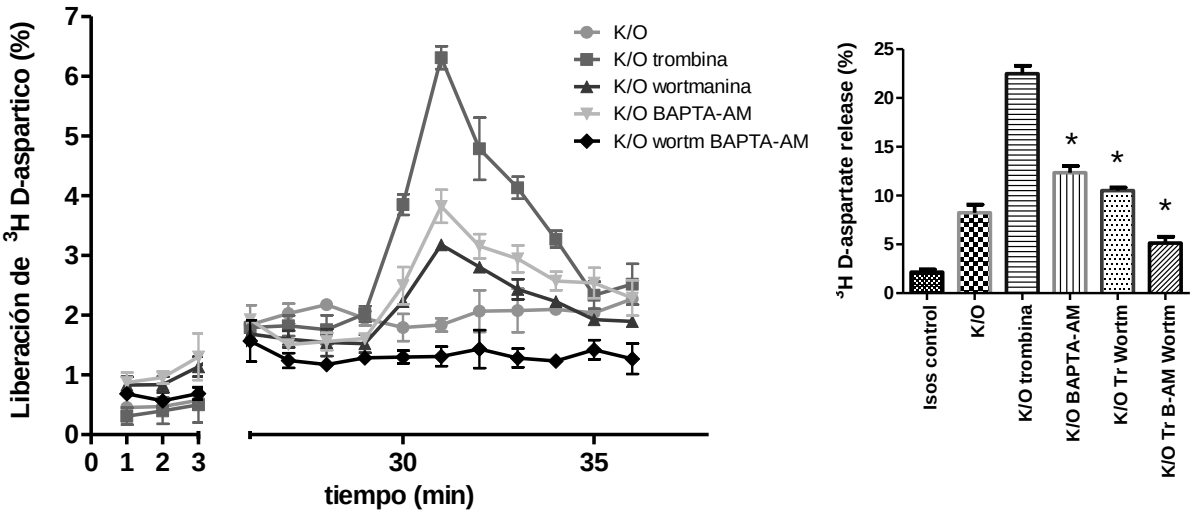


Figura 5. Curso temporal y cuantificación de la liberación de ^{3}H-D-aspartato bajo la condición de aumento en volumen por alteración de la homeostasis del potasio en presencia de trombina y del inhibidor wortmanina, el quelante BAPTA-AM o ambos agregados conjuntamente. Los resultados se muestran como en la Fig. 4 y son los promedios ± E.E. de 4 a 6 experimentos.

6. DISCUSIÓN

En este trabajo de tesis doctoral se abordó el estudio del efecto de la trombina sobre el decremento regulador de volumen en astrocitos, demostrando su participación como un agente potenciador de la salida de osmolitos en respuesta al edema celular de astrocitos. La potenciación por trombina de taurina y glutamato, bajo los modelos de hiponatremia y de perturbación en la homeostasis del K^+, es orquestada por la activación del receptor perteneciente a la superfamilia de receptores acoplados a proteínas G PAR-1 principalmente asociado a una señalización intracelular de calcio. Los resultados hallados respecto a la movilización del glutamato, junto a otras evidencias actuales, apoyan una conexión entre la presencia de altas concentraciones de trombina en el cerebro y la exacerbación del daño por excitotoxicidad bajo condiciones de edema cerebral.

A pesar de que estos resultados ya fueron discutidos en sus publicaciones correspondientes, en este apartado se rediscutirán ampliamente algunos de los aspectos más relevantes con el fin de integrar los resultados obtenidos y mostrar algunas propuestas para el avance de este campo de estudio. La página 70 contiene un modelo esquemático que engloba los resultados más significativos de esta tesis y al cual se hará referencia en varias secciones de la discusión.

6.1. Edema hiposmótico en astrocitos y su modulación por trombina

Las alteraciones en el volumen celular que se presentan bajo diversas condiciones patológicas representan sin duda un desafío para cualquier tipo celular; sin embargo, en el cerebro el reto es aún mayor debido al estrecho margen que impone el cráneo a la expansión del tejido. El edema cerebral compromete severamente la sobrevivencia y función normal del tejido debido al aumento en la presión intracraneal que origina la compresión vascular y herniación hacia el tallo cerebral por ser la zona de menor resistencia. A nivel celular se ha identificado desde hace tiempo, que bajo condiciones de edema, los astrocitos son el tipo celular que exhibe hinchamiento; esta característica está relacionada en parte con su papel en el mantenimiento de la homeostasis iónica, ya que los eficientes mecanismos de remoción de solutos extracelulares y la alta expresión de acuaporinas los convierte fácilmente en el blanco de desbalances osmóticos (Simard y Nedergaard, 2004; Pasantes-Morales y Vazquez-Juarez, 2012). También se ha descrito que esté fenómeno puede ser el resultado de un mecanismo más eficiente de regulación de volumen presente en neuronas, ya que de acuerdo con observaciones bajo una condición hiposmótica *in vivo* en neuronas de Purkinje del cerebelo, estas células liberan

una gran cantidad de taurina que es capturada por los astrocitos adyacentes; este hecho mantiene a las neuronas exentas de alteraciones en su volumen mientras que los astrocitos presentan hinchamiento (Nagelhus *et al.*, 1994).

La respuesta de los astrocitos ante el aumento el volumen y en particular la movilización de osmolitos orgánicos se ha estudiado desde hace varios años. (Pasantes-Morales *et al.*, 1994; Cardin *et al.*, 1999). El interés que motivó la primera parte de esta investigación, enfocada al edema hiposmótico en astrocitos y su modulación por trombina, provino de nuestro estudio previo realizado en la línea celular de fibroblastos Swiss 3T3, el cual mostró por primera vez la capacidad modulatoria de la trombina sobre la movilización de osmolitos orgánicos inducida bajo una condición de hinchamiento por reducción en la osmolaridad externa (Vazquez-Juarez *et al.*, 2008b). Este estudio reveló que la trombina es capaz de potenciar la salida de taurina, el aminoácido considerado como representante del grupo de osmolitos orgánicos. La característica más sobresaliente de la potenciación por trombina en los fibroblastos Swiss 3T3 fue la magnitud alcanzada en comparación con la que exhiben otros agonistas de receptores acoplados a proteínas G, entre los que destacan el ATP, la bradicinina, la vasopresina, la endotelina y el LPS (Falktoft y Lambert, 2004; Franco *et al.*, 2004b; Heacock *et al.*, 2006; Vazquez-Juarez *et al.*, 2008a). Con este antecedente y dado que los estudios en hiposmolaridad experimental han demostrado que los aminoácidos preferentemente empleados como osmolitos en el cerebro son la taurina y el glutamato (Pasantes-Morales *et al.*, 1993; Massieu *et al.*, 2004), la primera parte de este trabajo se enfocó en averiguar si la trombina era capaz de modular la movilización del glutamato en astrocitos debida a una condición de hiposmolaridad.

El interés de estudiar la movilización de glutamato en este trabajo proviene de la relevancia que adquiere este aminoácido, con múltiples funciones a nivel biológico, en el SNC; ya que en el tejido cerebral de los animales, además de estar involucrado en el metabolismo y la síntesis de proteínas, se desempeña como el neurotransmisor excitador más importante. Esta característica da por resultado que en el cerebro los niveles extracelulares de glutamato se encuentren estrechamente regulados y que su gradiente de concentración a través de la membrana alcance magnitudes del orden de 10^{-3} (Erecinska y Silver, 1990). Esta elevada concentración intracelular y su disponibilidad para movilizarse hacia afuera de la célula favorecen su participación como osmolito durante el proceso de regulación de volumen. La distribución fisiológica del glutamato es altamente dinámica ya que continuamente es liberado de las terminaciones nerviosas, pero al mismo tiempo, su señalización se mantiene altamente restringida gracias a su rápida recaptura principalmente por el funcionamiento de los trasportadores de glutamato en astrocitos el

GLT-1 y GLAST; esta recaptura previene que la concentración extracelular de glutamato alcance altos niveles que generen la sobreactivación de sus receptores ionotrópicos, una situación que induce muerte neuronal denominada excitotoxicidad (Kostandy, 2012; Marmiroli y Cavaletti, 2012). El estudio de la movilización exacerbada hacia el medio extracelular de glutamato provocada por la presencia de trombina en condiciones de aumento en volumen, fue uno de nuestros principales intereses debido a estas consecuencias lesivas que conlleva la elevada presencia extracelular de este neurotransmisor en el cerebro.

La hipótesis propuesta acerca del efecto de la trombina sobre la movilización de glutamato en astrocitos se comprobó en los estudios que conforman el primer artículo de la sección de resultados de esta tesis. Los resultados obtenidos rebasaron por mucho las expectativas, ya que aunque la trombina había mostrado en el estudio en Swiss 3T3 un efecto notable que amplificaba la salida de taurina debida a hiposmolaridad a más del doble, su capacidad de amplificar la movilización de ^{3}H-glutamato en astrocitos alcanzó una magnitud de cinco veces la exhibida al aplicarse el estímulo hiposmótico aislado. Un segundo resultado obtenido al utilizar el análogo no metabolizable de glutamato ^{3}H-D-aspártato con la finalidad de evitar la subestimación de la magnitud de la movilización de glutamato, debido a una dilución de la marca radioactiva en vías metabólicas que podría ocurrir con el empleo de ^{3}H-glutamato, arrojó una movilización de 6 veces la magnitud observada por el estímulo hiposmolaridad aislado (Ramos-Mandujano *et al.*, 2007).

La cantidad de trombina utilizada en el estudio para obtener tal potenciación corresponde a la magnitud que se presenta en el cerebro bajo condiciones patológicas en las que se ha demostrado que tiene un efecto tóxico (Xi *et al.*, 2003). Como se mencionó en la introducción, en el cerebro la trombina y sus receptores PAR se encuentran presentes de manera fisiológica pero se encuentran estrechamente regulados a nivel de expresión y por la presencia de proteínas inhibitorias de la enzima. Al parecer, su función fisiológica está relacionada con procesos de crecimiento celular, de cambios en morfología y de liberación de mediadores inflamatorios; sin embargo, bajo condiciones patológicas en las que se presentan alteraciones en los componentes de la barrera hematoencefálica, se detectan altas concentraciones de trombina que han sido asociadas principalmente a la activación anormal de receptores PAR con manifestaciones lesivas (Rohatgi *et al.*, 2004).

La trombina es una enzima con múltiples sitios de unión a proteínas desde donde puede ejercer una amplia variedad de efectos celulares (Di Cera, 2008); en nuestro estudio se demostró que su efecto ocurre a través de su actividad de proteasa ya que un

tratamiento previo con el compuesto sintético PPACK que se une covalentemente a este sitio inhibió completamente su efecto sobre la movilización del ^{3}H-glutamato. Los astrocitos expresan los tres subtipos de receptores PAR activados por la actividad proteasa de la trombina PAR-1, -3 y -4 (Hollenberg, 2002; Junge *et al.*, 2004). En nuestro trabajo en el modelo de liberación hiposmótica de osmolitos se encontró que el efecto sobre de la movilización de ^{3}H-glutamato por trombina en astrocitos está mediado a través de la activación del receptor PAR-1, ya que la adición del péptido agonista específico del receptor logró mimetizar por completo el efecto de la trombina.

Cascada abajo del receptor PAR-1 se han descrito las vías canónicas que acompañan a la activación de las proteínas G heterotriméricas $G\alpha_q$, $G\alpha_i$, $G\alpha_{12/13}$. En este estudio se caracterizó en primer lugar la participación de la vía que involucra la activación de $G\alpha_q$ -PLC y que continúa hacia la salida de calcio de la poza intracelular ubicada en el retículo endoplásmico. La prioridad del estudio de esta vía está sustentada en el trabajo de Cardín y colaboradores de 2003 en el que se describe como el aumento en los niveles de calcio intracelular mediante el uso del ionóforo de Ca^{2+} ionomicina, de manera concurrente con el estímulo de hiposmolaridad, potencia la salida osmosensible de taurina. Para este propósito se inhibió el aumento en calcio intracelular mediante la utilización del quelante EGTA-AM, la reducción de poco más de la mitad del efecto de la trombina ante la presencia del agente quelante mostró ser además muy similar al efecto obtenido mediante el uso del inhibidor de PLC U73122; este hecho apoya fuertemente la hipótesis de que la vía intracelular iniciada por $G\alpha_q$ hacia PLC y la movilización de Ca^{2+} intracelular juega un papel importante en el fenómeno de la potenciación. Sin embargo, aún cuando la elevación del calcio intracelular parece estar detrás de una buena parte del efecto, deja en claro que existen más elementos de señalización involucrados, lo cual es de esperarse debido a que el efecto de la ionomicina en el estudio previo de Cardin, alcanza solo a duplicar la movilización debida a hiposmolaridad, mientras que el efecto de trombina como se mencionó anteriormente alcanza magnitudes de potenciación de 5 a 6 veces. Una aproximación farmacológica empleada para caracterizar más detalladamente la vía de calcio intracelular sugiere la participación de la Ca^{2+}-calmodulina (CaM) y descarta la intervención de la proteína cinasa C (PKC) en la cascada de señalización generada por la activación del receptor PAR-1 (Ramos-Mandujano *et al.*, 2007) Figura 6, c (pág. 70).

La vía de señalización independiente de Ca^{2+} que opera en el mecanismo de movilización de ^{3}H-glutamato por trombina parece involucrar la participación de la cinasa de fosfoinosítidos PI3K como demuestra la inhibición aditiva que ejerce la presencia de wortmanina en la condición ausente de calcio intracelular (EGTA-AM). La activación de la

PI3K puede deberse a la activación del dímero βγ de las proteínas G o de la transactivación de un receptor de la familia de cinasas de tirosina, como fue mostrado anteriormente para el caso de la señalización bajo la potenciación por trombina en fibroblastos 3T3 (Stoyanov *et al.*, 1995; Vazquez-Juarez *et al.*, 2008b). Este aspecto se abordó directamente en el segundo artículo que se presenta en la sección de resultados y se discutirá más adelante.

Respecto a la vía a través de la cual se moviliza el glutamato en respuesta a la presencia de trombina la evidencia actual sostiene que los astrocitos pueden movilizar glutamato a través de por lo menos tres mecanismos diferentes: (1) la exocitosis dependiente de Ca^{2+}, (2) la operación en reversa de los transportadores de glutamato dependientes de energía y (3) la salida activada por hinchamiento (Kimelberg *et al.*, 1990; Junankar y Kirk, 2000; Rossi *et al.*, 2000; Parpura *et al.*, 2004). El primer acercamiento estuvo enfocado en determinar si el ^{3}H-glutamato movilizado por trombina ocurría a través de la vía que se encontraba en funcionamiento debido al estímulo de hiposmolaridad: la vía de movilización de osmolitos orgánicos activada por volumen (VSOAC) que fue descrita en la introducción. Para este propósito se emplearon los inhibidores tamoxifen y NPPB, dos agentes inespecíficos empleados clásicamente para inhibir de manera exitosa la vía activada por volumen; así como el inhibidor específico de la vía, el DCPIB (Decher *et al.*, 2001; Abdullaev *et al.*, 2006). Los tres fármacos previnieron la salida de glutamato estimulada por hiposmolaridad así como la observada tras la adición de trombina conjunta a la condición hiposmótica, indicando que el osmolito se mueve a través de la misma vía y que existe un fenómeno de amplificación por trombina de la salida de glutamato debida a hiposmolaridad. Esta amplificación corresponde a una potenciación ya que bajo condiciones isosmóticas la trombina no ejerce más que un incipiente efecto, lo que indica que no es un elemento capaz de activar como tal la vía regulada por volumen y requiere de la apertura previa, en este caso debida a la presencia del estímulo de hiposmolaridad, para que tener un efecto sobre la movilización de osmolitos.

Como se mencionó anteriormente, en el segundo artículo se investigó la participación de una intercomunicación entre el receptor PAR-1 y el receptor con actividad cinasa de tirosina EGFR, ya que fue una vía novedosa descrita para el efecto mediado por trombina de la potenciación de taurina en fibroblastos Swiss 3T3 y con amplio sustento, ya que durante la década pasada se exploró y caracterizó ampliamente la activación y participación de receptores de la familia con actividad cinasa de tirosinas principalmente el EGFR durante la hiposmolaridad, así como de proteínas citosólicas con actividad cinasa de tirosinas como la cinasa src. Tanto los receptores como las proteínas citosólicas participan

como elementos de señalización en una gran variedad de respuestas adaptativas al cambio en volumen que incluyen procesos de adhesión, reorganización del citoesqueleto, mecanismos de estrés y la activación y operación de la salida de osmolitos, esta participación de proteínas con actividad de cinasa de tirosinas está ampliamente descrita por trabajos previos del grupo de la Dra. Pasantes y sustentada en varios tipos celulares (Tilly *et al.*, 1993; Haussinger y Schliess, 1999; de La Paz *et al.*, 2002; Franco *et al.*, 2004a; Lezama *et al.*, 2005; Pasantes-Morales *et al.*, 2006). En la actualidad existen varios trabajos que abordan la posibilidad de la activación de proteínas con actividad cinasa de tirosinas bajo la cascada de señalización activada por GPCRs como resultado de una intercomunicación entre receptores de la superfamilia de GPCRs con el EGFR (Gschwind *et al.*, 2001; Little *et al.*, 2011; Burch *et al.*, 2012). Los principales intermediarios, propuestos para la intercomunicación desde el receptor PAR hacia EGFR, han sido la proteína cinasa citosólica src que puede fosforilar directamente al receptor EGFR y activarlo, y las metaloproteasas que al activarse liberan al ligando de EGFR que se encuentra de manera endógena en la membrana plasmática. Sin embargo, en este estudio se demostró que en astrocitos la participación de las cinasas de tirosina es muy baja y no contribuye más que en un escaso 10% a la señalización bajo el efecto de la trombina en la movilización de taurina y D-aspartato. Las razones de esta señalización diferencial entre lo encontrado en el estudio con fibroblastos Swiss 3T3 y los astrocitos, podría deberse a una expresión diferencial de proteínas entre cultivos primarios y líneas celulares (Cruz-Rangel *et al.*, 2008). La baja participación del EGFR en la señalización por trombina hacia la potenciación de la liberación de glutamato, descarta la hipótesis de la participación de la cinasa de fosfoinosítidos PI3K como subsecuente a la activación de un receptor del tipo EGFR, por lo que es necesario ampliar el estudio experimental con otros enfoques para aclarar cuál es su posición cascada abajo de la activación del receptor PAR-1.

6.2. Edema isosmótico en astrocitos y su modulación por trombina

La segunda parte de la investigación se centró en estudiar los efectos de la trombina en un modelo de hinchamiento isosmótico. El abordaje experimental para alcanzar este objetivo consistió en el modelo de hinchamiento por perturbación de la homeostasis del potasio; este modelo, que se ha empleado anteriormente para investigar la biología de estas células del SNC bajo condiciones patológicas, reproduce una de las características presentes en isquemia o trauma craneano directamente involucrada en el hinchamiento de astrocitos: una elevada concentración de K^+ extracelular (Walz, 1987; Olson *et al.*, 1995; Rutledge y Kimelberg, 1996; Cardin *et al.*, 1999).

Los astrocitos se encargan de la remoción de los iones K^+ que se liberan al espacio extracelular por la actividad normal de las neuronas y durante patologías como la isquemia, la falla energética que trae consigo el colapso de los gradientes transmembranales, induce la salida masiva de K^+ al espacio extracelular alcanzando magnitudes que desafían la capacidad de los astrocitos para mantener la homeostasis (Pasantes-Morales y Vazquez-Juarez, 2012). Algunas mediciones realizadas en estudios *in vivo* reportan concentraciones de K^+ extracelular de hasta 80 mM; estos altos niveles se traducen en una acumulación neta de KCl en los astrocitos generando un gradiente osmótico para la entrada de agua y el consecuente aumento en volumen celular (Walz, 1987; Kimelberg, 2005; Rossi *et al.*, 2007). Nuestro estudio describe como una población de astrocitos *in vitro* sometida a la condición de una sustitución isosmótica del medio extracelular de 100 mM de NaCl por KCl, produce un patrón curso-temporal de aumento en el volumen lento y progresivo que alcanza un máximo a los 30 minutos y posteriormente se mantiene estacionario. Este patrón es notablemente distinto al hinchamiento súbito producido por la condición hiposmótica que se abordó en la primera parte del estudio. Otra diferencia muy importante entre los dos modelos estudiados fue la capacidad de los astrocitos para recuperar su volumen, ya que mientras bajo el estímulo hiposmótico la célula es capaz de alcanzar una pronta recuperación, en el estímulo con una elevada concentración de K^+ extracelular no se observa un ajuste en el volumen. La falta de regulación activa del volumen celular en el modelo de hinchamiento por una elevada concentración de K^+ extracelular se debe muy probablemente a que en esta condición son los iones K^+ y Cl^- los que originan la condición de hinchamiento y está ampliamente descrito que estos dos iones tienen una contribución muy importante al DRV (Hoffmann y Pedersen, 2011). La fase estacionaria que adquiere el cambio en volumen tras alcanzar el máximo, posiblemente corresponde a la contribución de la liberación de la poza de osmolitos orgánicos; pero resulta insuficiente para lograr un decremento significativo del cambio en volumen (Vazquez-Juarez *et al.*, 2009).

Existen diversos mecanismos involucrados en la acumulación de K^+ en los astrocitos que conduce al aumento en volumen; sin embargo, el aporte más importante parece corresponder al cotransportador electroneutro Na^+-K^+-Cl^- 1 (NKCC1) (Pasantes-Morales y Vazquez-Juarez, 2012). La prueba más contundente de la participación de este cotransportador está plasmada en trabajos publicados por el grupo de Sun en 2002, que demuestran una notable disminución en el aumento en volumen inducido por una elevada concentración de K^+ extracelular en ratones deficientes de NKCC1. Consistente con este y otros reportes previos (Su *et al.*, 2002a; Su *et al.*, 2002b), nuestros resultados postulan al NKCC1 como el mecanismo dominante de entrada de KCl dada la sensibilidad a bumetanida de un componente importante, más no de todo, el aumento en volumen por

una elevada concentración de K^+ extracelular. Además de la substancial y bien estudiada participación del NKCC1 en la remoción del K^+ durante condiciones de una elevada concentración de K^+ extracelular, existe evidencia que describe a los canales Kir (canales de K^+ de rectificación entrante), como uno de los mecanismos involucrados en la remoción fisiológica del K^+ y que pudiera sobreactivarse bajo condiciones de perturbación en la homeostasis de K^+. Los canales Kir, en especial la isoforma 4.1, son el tipo de canal de K^+ más abundante de los astrocitos y a diferencia de los otros canales, permiten preferentemente el flujo de iones K^+ en la dirección entrante (Kofuji y Newman, 2004). En nuestro modelo la disminución significativa del aumento en volumen en presencia de bario, un bloqueador general de los canales de K^+, pero que a bajas concentraciones (100 µM) se emplea como inhibidor selectivo de canales Kir, confirma la participación de estos canales en la remoción extracelular de K^+. En sustento de los dos mecanismos aquí propuestos para el transporte de K+, la eliminación del aumento en volumen tras la adición conjunta de bario y bumetanida, postulan al NKCC1 y los canales Kir como los dos mecanismos que de manera independiente, inducen el aumento en volumen a través de la remoción del K^+ extracelular y su acumulación intracelular (Vazquez-Juarez *et al.*, 2009) Fig. 6, b (pág. 70).

La movilización de los osmolitos taurina y glutamato en respuesta al aumento en volumen por una elevada concentración de K^+ extracelular, ha sido reportada previamente en otros estudios aunque no fue explorada con tanto detalle como en nuestro estudio (Rutledge y Kimelberg, 1996; Cardin *et al.*, 1999). La salida de taurina fue explorada debido la estrecha relación que mantiene su movilización con el cambio en volumen, lo que aporta información muy significativa sobre el comportamiento de la poza de osmolitos orgánicos que se moviliza a través de la vía activada por volumen bajo el modelo de hinchamiento por una elevada concentración de K^+ extracelular. El curso temporal de la liberación de taurina, como era de esperarse, exhibe un patrón muy similar al trazo que describe el aumento en volumen bajo este modelo, aunque no muestra una inactivación dentro del tiempo que abarca el experimento (40 minutos); estos resultados apoyan la hipótesis anteriormente propuesta de que movilización de osmolitos orgánicos es posiblemente la responsable de la fase estacionaria que exhibe el trazo de cambio en volumen bajo nuestro modelo.

Por su parte, el curso temporal de la movilización de ^{3}H-D-aspartato exhibe un trazo ligeramente distinto al descrito para la taurina, lo que sugiere la participación de distintas vías de movilización involucradas. En nuestro estudio, la salida de ^{3}H-D-aspartato sigue un patrón bifásico que consiste en una salida temprana transitoria y una segunda fase que

presenta un pequeño incremento de carácter sostenido. La primer fase es sensible en su mayoría al pretratamiento con el inhibidor TBOA lo cual apunta hacia la participación del trasportador de glutamato operando en reversa y tiene un pequeño componente sensible al inhibidor de la vía activada por volumen (Vazquez-Juarez *et al.*, 2009). Estos resultados se corresponden parcialmente con un modelo similar explorado por Kimelberg y colaboradores en 1996, ya que la primera fase de liberación en este estudio es prevenida por el inhibidor del transportador TBHA pero no reportan la participación la vía activada por volumen; sin embargo, cabe destacar que el fármaco empleado para comprobar la participación de la vía activada por volumen en ese estudio fue el inhibidor de canales aniónicos L-644,711, el cual no es un inhibidor selectivo de esta vía a diferencia del DCPIB empleado en nuestro estudio (Olson *et al.*, 1995; Rutledge y Kimelberg, 1996; Decher *et al.*, 2001; Vazquez-Juarez *et al.*, 2009).

La participación de la operación en reversa del transportador como mecanismo de liberación de glutamato al espacio extracelular durante periodos de isquemia o inhibición metabólica ha sido demostrada consistentemente (Zeevalk *et al.*, 1998; Li *et al.*, 1999; Longuemare *et al.*, 1999; Seki *et al.*, 1999; Rossi *et al.*, 2000). Los trasportadores transmembranales acoplan el transporte de Na^+ y K^+ a favor de su gradiente de concentración para movilizar glutamato; en el ambiente fisiológico normal del cerebro los gradientes de concentración favorecen el transporte de glutamato hacia el interior de la célula; sin embargo, en situaciones patológicas como isquemia, las condiciones iónicas perturbadas pueden favorecer el funcionamiento en reversa. En nuestro estudio en el modelo de una elevada concentración de K^+ extracelular, la operación del transporte en reversa durante la primera fase de la liberación de glutamato, comprobado mediante el empleo del inhibidor TBOA, fue el mecanismo que tuvo la mayor contribución (Vazquez-Juarez *et al.*, 2009) Fig. 6, d (pág. 70).

La segunda fase de la movilización de glutamato en respuesta a la condición de una elevada concentración de K^+ extracelular, de aumento lento y sostenido está mediada en su totalidad por el canal aniónico regulado por volumen (VSOAC) ya que fue prevenida en nuestro estudio mediante la utilización del DCPIB, el inhibidor específico de la vía (Decher *et al.*, 2001; Abdullaev *et al.*, 2006). Estos datos confirman el resultado del estudio de Kimelberg y colaboradores de 1996 en el que obtuvieron una disminución significativa de la segunda fase mediante el uso del inhibidor general de canales aniónicos L-644,711 (Rutledge y Kimelberg, 1996) Fig. 6, e (pág. 70).

Tomando en cuenta el patrón curso temporal del aumento en volumen y la participación de las dos vías en la salida de glutamato en el modelo de perturbación de la homeostasis del K^+ extracelular, se realizó la adición de trombina en distintos puntos de la curva de movilización de glutamato. Estos experimentos mostraron que el efecto máximo de potenciación se alcanza cuando la trombina se agrega en la fase del curso temporal en la que el aumento en volumen es mayor. En este sentido, los resultados en el modelo de altas concentraciones de K^+ extracelular confirman los que obtuvimos en el modelo hiposmótico, en el que mostramos que la movilización potenciada por trombina es en su totalidad sensible a los inhibidores comunes de la vía de salida de osmolitos activada por trombina DIDS, NPPB y DCPIB, mientras que el inhibidor del transportador no mostró ningún efecto. Las investigaciones de esta tesis sustentan la hipótesis de que la trombina ejerce una potenciación sobre la liberación de glutamato sobre la vía sensible a volumen y no en aquella presentada por la operación reversa del transportador.

El modelo de perturbación en la homeostasis del K^+ aporta información relevante sobre las características de la potenciación por trombina de la movilización de osmolitos debida a hinchamiento, algunas de las cuales se discuten a continuación:

- Los estudios previos a este trabajo que demuestran algún tipo de modulación por agonistas de receptores acoplados a proteínas G del DRV, emplean modelos de hinchamiento por reducción en la osmolaridad extracelular (Vazquez-Juarez *et al.*, 2008a), por lo que existían bases para plantear la posibilidad de que el factor involucrado en la potenciación fuera un elemento concurrente a la disminución de la osmolaridad extracelular (como la reducción de la fuerza iónica intracelular) y no el aumento en volumen *per se*. A partir de nuestros resultados, en los que la trombina es capaz de potenciar la movilización de osmolitos en dos modelos distintos de hinchamiento, es posible generalizar el efecto de la trombina como un agente modulador de la respuesta celular de movilización de osmolitos ante un aumento en volumen.

- El patrón de aumento en volumen, notablemente distinto, aporta información sobre el trazo que describe la salida potenciada por trombina que bajo la condición de hiposmolaridad exhibe una cinética muy similar a la del curso temporal transitorio del aumento en volumen. En el modelo de una elevada concentración de K^+ extracelular el patrón de potenciación es también transitorio lo que es indicativo de la presencia de un mecanismo de inactivación de la señalización iniciada por el receptores PAR-1, que detiene el efecto aún cuando la salida de osmolitos debida a aumento en volumen persiste (la vía activada por volumen permanece abierta). A este respecto, es necesario aclarar si

esta inactivación ocurre a nivel de internalización/inactivación del receptor o de mecanismos operando sobre la señalización de segundos mensajeros.

- Desde hace algunos años se ha descrito en forma consistente que la movilización de los aminoácidos glutamato y taurina bajo condiciones de hinchamiento hiposmótico ocurre a través de la vía activada por volumen, el VSOAC, ya que es totalmente abatida por compuestos caracterizados como bloqueadores generales de canales de Cl^-, usados comúnmente para inhibir la vía y más recientemente por DCPIB su inhibidor específico (Pasantes-Morales *et al.*, 2006). Por su parte la movilización de glutamato bajo condiciones de hinchamiento por una elevada concentración de K^+ extracelular ocurre a través de distintas vías y no solo a través de la vía activada por volumen (Rutledge y Kimelberg, 1996; Vazquez-Juarez *et al.*, 2009). Esta posibilidad de observar varias vías de movilización de aminoácidos activadas bajo un mismo modelo permitió responder la pregunta de sí la trombina ejercía su efecto únicamente sobre la vía de movilización de glutamato activada por volumen o podía inducir la salida a través de cualquier vía abierta.

- Las características del aumento en volumen inducido por la condición de una elevada concentración de K^+ extracelular, también nos permitieron demostrar claramente la relación de proporcionalidad directa entre la potenciación de la liberación de glutamato y el grado de aumento en volumen; ya que a medida que el volumen de los astrocitos aumenta y existe una mayor contribución de la vía activada por volumen en la liberación de glutamato es posible inducir una mayor potenciación con la adición de trombina. Esta relación es menos evidente bajo la condición hiposmótica debido a la cinética que describe la salida de osmolitos al aumentar la magnitud de la reducción de la osmolaridad; esta cinética es una curva sigmoide que muestra la saturación de la vía conforme es mayor la reducción en la osmolaridad, por esta razón aún cuando se pueden inducir cambios mayores en volumen, la potenciación nunca rebasa la asíntota de saturación que alcanza de manera aislada una condición de marcada hiposmolaridad. Otra observación que apoya la relación entre aumento volumen y potenciación y descarta el efecto de algún epifenómeno asociado al desbalance iónico en el modelo de una elevada concentración de K^+ extracelular, es la inhibición de la potenciación de glutamato obtenida al bloquear el mecanismo que induce el aumento en volumen (transportador NKCC1 y en menor grado canales Kir) mediante la aplicación bario-bumetanida; de esta manera, aún cuando la condición de una elevada concentración de K^+ extracelular se encuentra presente se demuestra que si no existen los mecanismos que generan el aumento en volumen, la presencia de trombina permanece sin efecto.

- Debido a que la movilización de calcio intracelular parece jugar un papel importante en el fenómeno de potenciación por trombina de la movilización de osmolitos por aumento en volumen celular, se exploró la posibilidad de que fuera este el elemento relacionado con la magnitud de la potenciación por trombina y no el componente de aumento en volumen. Durante el estímulo hiposmótico está ampliamente descrito que existe un aumento en los niveles intracelulares de calcio y tras la adición del ionóforo ionomicina o la trombina estos niveles se ven ampliamente rebasados y coinciden con la presencia del efecto potenciador siempre y cuando exista la apertura previa de la vía como se ha mencionado en esta discusión. Durante los experimentos en el modelo de perturbación de la homeostasis del K^+, se realizaron mediciones de los pulsos intracelulares de calcio generados tras la adición de trombina a diferentes tiempos del curso temporal de aumento en volumen y no se encontraron diferencias significativas entre el pico de calcio generado por trombina a los diferentes tiempos en que fue agregada. Este resultado apoya nuevamente la caracterización como potenciación del efecto por trombina sobre la salida de osmolitos y su dependencia del aumento en volumen y la apertura de la vía para llevar a cabo su efecto.

En nuestras investigaciones, se identificaron algunos de los elementos dentro de la cascada de señalización que se desencadena después de la activación del PAR-1. Sin embargo, aún hay varios aspectos del mecanismo de potenciación por trombina que permanecen sin respuesta; entre ellos figura el elemento clave entre volumen y receptor a trombina responsable de la potenciación. Así como la etapa del proceso de DRV con el que interactúa la trombina para ejercer su efecto sobre la movilización a través de la vía activada por volumen. A este respecto, es poco probable que la trombina participe a nivel del sensor de volumen ya que por si sola no es capaz de inducir una liberación significativa de osmolitos orgánicos, también quedó descartada la posibilidad de que la trombina propiciara un incremento en volumen *per se* que amplificara la respuesta celular ante el hinchamiento. Las opciones más viables sugieren que algunos elementos de la cascada de señalización desencadenada por la trombina pueden estar sobreactivando directamente elementos de la cascada de osmotransducción o que exista una cooperación de elementos activados por trombina y los inducidos solamente por hiposmolaridad sobre la vía abierta de translocación de osmolitos orgánicos. La principal limitante que existe para probar alguna hipótesis que ayude a esclarecer a estas incógnitas es el desconocimiento de la identidad molecular de la vía de movilización de osmolitos orgánicos activada por cambios en volumen.

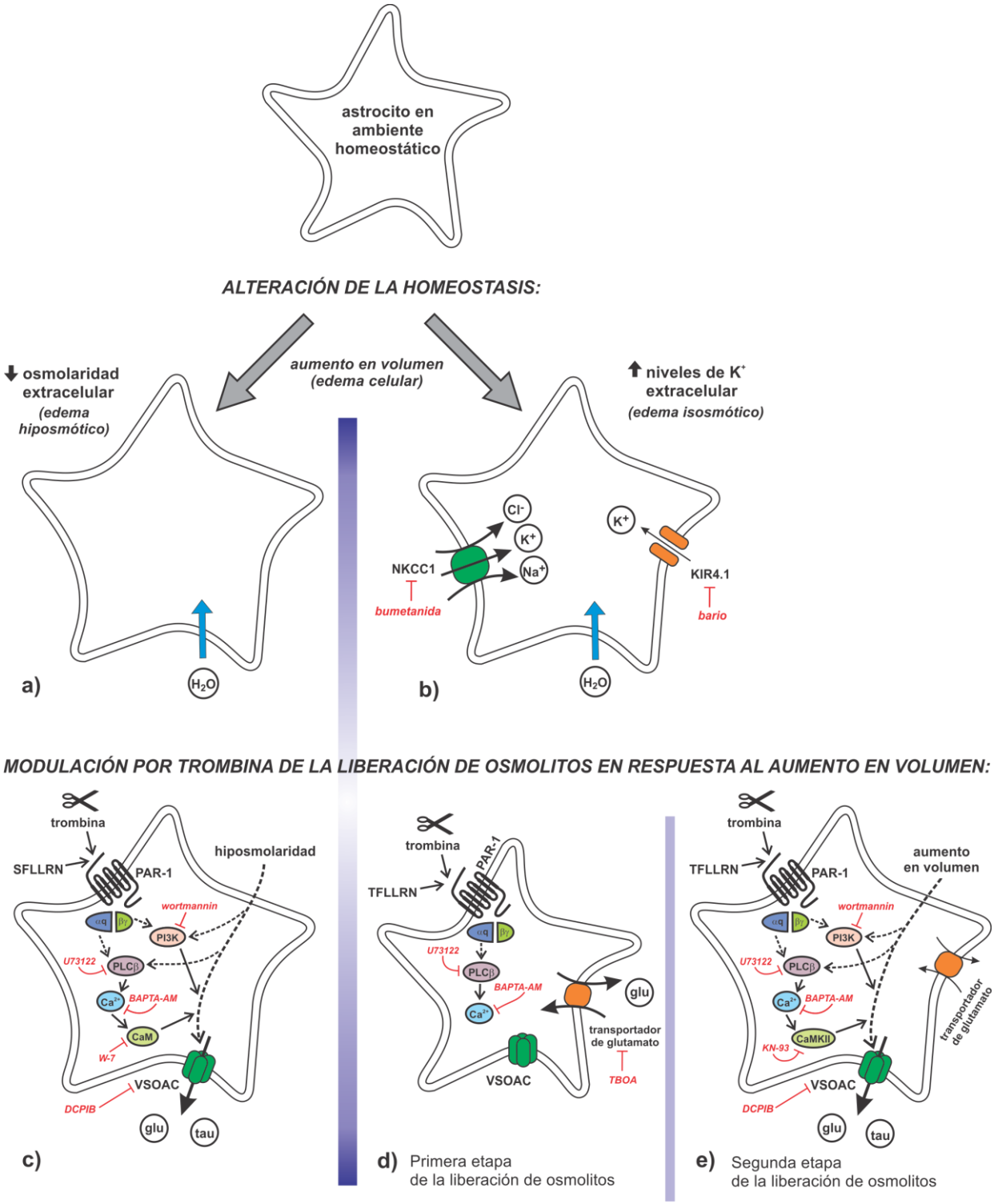


Figura 6. Modelo representativo de la modulación por trombina sobre la salida de osmolitos inducida por el aumento en volumen de astrocitos bajo los modelos experimentales de hiposmolaridad y alteración de los niveles extracelulares de potasio. La alteración de la homeostasis bajo una condición de reducción en la osmolaridad extracelular origina el aumento en volumen de carácter hiposmótico por entrada de agua osmóticamente obligada (**a**), mientras que bajo condiciones isosmóticas, una redistribución iónica producto de la alteración en la homeostasis del potasio, origina la sobreactivación de los mecanismos de remoción del potasio: el cotransportador NKCC y el canal KIR, que conduce a la acumulación intracelular

de potasio y agua (**b**). Bajo estas condiciones de hinchamiento se activan los mecanismos del DRV que llevan a la salida de los osmolitos orgánicos, glutamato y taurina, a través de la vía activada por volumen el VSOAC; la adición de trombina en esta fase de hinchamiento induce la potenciación de esta liberación de glutamato y taurina, mediante la activación del receptor PAR-1 y la participación independiente de la PI3K y la vía que involucra el aumento en calcio intracelular mediado por PLC (**c y e**). La liberación de glutamato bajo el modelo de alteración en la homeostasis del potasio presenta dos fases (**d y e**), la primera transcurre durante los primeros 10 minutos de tratamiento experimental bajo los que se presenta un incipiente aumento en volumen y la liberación de glutamato ocurre a través de la operación reversa del transportador; durante esta fase la trombina no tiene efecto sobre la salida de glutamato, aún cuando se activa la señalización cascada abajo del receptor, registrada mediante la respuesta de liberación intracelular de calcio (**d**). Durante la segunda fase, el aumento en volumen ya significativo, genera la apertura de la vía sensible a volumen VSOAC y la adición de trombina ejerce entonces un efecto potenciador (**e**).

6.3. La potenciación de la movilización de glutamato por trombina y su asociación a situaciones de muerte neuronal en el cerebro (Perspectivas)

Los resultados obtenidos en el presente estudio proveen elementos suficientes para considerar un vínculo potencial entre el efecto tóxico que se ha descrito debido a la presencia de altas concentraciones de trombina en el cerebro y el daño excitotóxico. La excitotoxicidad, un factor de daño característico subsecuente a episodios de isquemia y otras patologías, se genera por la sobreactivación de receptores ionotrópicos en las neuronas (Wang y Qin, 2010; Kostandy, 2012). Bajo condiciones patológicas existen diversos mecanismos a través de los cuales puede movilizarse glutamato hacia el medio extracelular, entre ellas la despolarización excesiva, la operación en reversa de los transportadores de glutamato y la salida a través de la vía activada por aumento en volumen. Nuestros resultados indican que la presencia de trombina es capaz de aumentar en una magnitud por demás significativa la salida de glutamato debida a hinchamiento, la cual puede ser considerada como un factor importante de contribución a la muerte por excitotoxicidad. Esta contribución es mucho más tangible bajo el modelo de hinchamiento por una elevada concentración de K^+ ya que los gradientes alterados impiden a los astrocitos recapturar eficientemente el glutamato extracelular y, como se comprobó en nuestras investigaciones, este mecanismo no solo está interrumpido sino que funciona en sentido inverso convirtiéndose en otra vía de liberación de glutamato hacia el medio extracelular. Es bajo esta condición que una potenciación como la exhibida por trombina puede tener severas consecuencias sobre la función neuronal.

En apoyo a esta hipótesis, existe una amplia evidencia que demuestra la relación entre la presencia de una elevada concentración de trombina en modelos *in vivo* de isquemia global e isquemia focal y la magnitud del daño encontrado en el tejido (Striggow *et al.*, 2000; Ohyama *et al.*, 2001; Hua *et al.*, 2003; Cuomo *et al.*, 2007). En estos estudios se observa directamente que la administración de inhibidores de trombina como el argatroban, la hirudina, la PN-1 y la antitrombina muestran efectos protectores, que desaparecen al agregar una cantidad equimolar de trombina; así como, que la administración exógena de la proteasa genera la exacerbación del daño. Los receptores PAR activados por trombina, especialmente el receptor PAR-1 también se encuentran relacionados a estos episodios de daño (Junge *et al.*, 2003; Xi *et al.*, 2003), este vínculo se demuestra por la disminución en el daño por isquemia que presenta el ratón mutante carente del receptor PAR-1 en comparación al ratón silvestre (Striggow *et al.*, 2001; Wang *et al.*, 2012).

Existen otras evidencias que apoyan nuestro planteamiento sobre el posible daño de la trombina mediado por glutamato, en patologías que conllevan edema celular cerebral. Por ejemplo, se ha demostrado un incremento en la actividad del receptor NMDA y la excitabilidad neuronal observada en la región CA1 de las células piramidales del hipocampo tras la activación del receptor PAR-1 (Gingrich *et al.*, 2000; Han *et al.*, 2011). Y en esta misma línea, en un estudio *in vivo* de isquemia por oclusión de la arteria cerebral media, la administración de DCPIB el inhibidor específico de la vía de movilización de glutamato activada por volumen, reduce en 75% el volumen de tejido dañado y los niveles de glutamato cuantificados en el fluido extracelular (Zhang *et al.*, 2008).

Los mecanismos a través de los cuales la trombina ejerce sus efectos lesivos aún no se conocen con detalle; a este respecto nuestros resultados exponen un mecanismo viable que permite explicar la muerte asociada a la presencia de trombina bajo patologías asociadas a edema cerebral como la isquemia. Bajo este supuesto sería importante someter a experimentación esta hipótesis y de ser confirmada considerar diferentes elementos dentro del proceso de movilización de osmolitos orgánicos, como un posible blanco terapéutico para contener el daño generado en patologías en las que coexista la presencia de edema cerebral y una alta concentración de trombina.

7. CONCLUSIONES

Los hallazgos más notables de este trabajo de investigación fueron:

1. La trombina genera la potenciación de la salida de taurina y glutamato, inducida por un aumento en volumen ya sea de carácter hiposmótico o isosmótico, a través de la activación del receptor PAR-1.
2. La vía de señalización desencadenada por el PAR-1, requiere de la elevación del calcio intracelular y la activación de PI3K para ejercer su efecto potenciador.
3. La trombina potencia únicamente la salida de glutamato y taurina que ocurre a través de la vía de movilización de osmolitos orgánicos activada por volumen sensible a DCPIB.
4. La trombina ejerce su efecto potenciador siempre que la vía de movilización de osmolitos se encuentre activada debido a cambios significativos en el volumen celular, sin importar la naturaleza isosmótica o hiposmótica de este.

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Review

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On the Role of G-Protein Coupled Receptors in Cell Volume Regulation

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RVD • Thrombin • ATP and volume • Taurine

Abstract
Cell volume is determined genetically for each cell lineage, but it is not a static feature of the cell. Intracellular volume is continuously challenged by metabolic reactions, uptake of nutrients, intracellular displacement of molecules and organelles and generation of ionic gradients. Moreover, recent evidence raises the intriguing possibility that changes in cell volume act as signals for basic cell functions such as proliferation, migration, secretion and apoptosis. Cells adapt to volume increase by a complex, dynamic process resulting from the concerted action of volume sensing mechanisms and intricate signaling chains, directed to initiate the multiple adaptations demanded by a change in cell volume, among others adhesion reactions, membrane and cytoskeleton remodeling, and activation of the osmolyte pathways leading to restablish the water balance between extracellular/intracellular or intracellular/intracellular compartments. In multicellular organisms, a continuous interaction with the external milieu is fundamental for the dynamics of the cell. It is in this sense that the recent surge of interest about the influence on cell volume control by the most extended family of signaling elements, the G proteins, acquires particular importance. As here reviewed, a large variety of G-protein coupled receptors (GPCRs) are involved in this interplay with cell volume regulatory mechanisms, which amplifies and diversifies the volume-elicited signaling chains, providing a variety of routes towards the multiple effectors related to cell volume changes.

Introduction

The ability to regulate volume is an ancient trait conserved throughout evolution in animal cells from essentially all species. This reflects the importance for cell homeostasis of maintaining a strict volume control, including that of intracellular compartments, to preserve the cytoarchitecture required for the correct assembly of signaling complexes. Although systemic osmolarity in physiological conditions is highly controlled, intracellular osmolarity is continuously compromised by the generation of local and transient osmotic microgradients derived from

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uptake of nutrients, secretion, synthesis and degradation of macromolecules, remodeling of the cytoskeleton and extracellular matrix and transynaptic ionic gradients [1, 2]. Cell volume disturbances, particularly cell volume increase, occur in numerous pathologies. Hyponatremia associated with a large number of pathological situations characteristically results in cell swelling. Cell volume gain also occurs without a change in external osmolarity, as consequence of ion redistribution or increased cellular content of molecules acting as organic osmolytes. This isosmotic swelling, also known as cytotoxic swelling, occurs in hypoxia, ischemia, epilepsies, cranial trauma or hepatic encephalopathy. Cell volume changes have been considered for long time as a challenge for animal cells, compromising their homeostasis. Recent evidence, however, points to the intriguing concept of cell volume as a signaling element for basic cell functions such as migration, proliferation or apoptotic death.

Regulatory volume decrease

The high permeability to water characteristic of most animal cells requires the presence of mechanisms allowing the cell to face contingencies derived from water entry. Uncontrolled changes in cell water, by disturbing the intracellular concentration of signal molecules being at a certain point free in intracellular compartments, may send equivocal signals, disturbing the cell function and the intercellular communication. When water enters the cell as result of an external osmolarity reduction, a complex mechanism for volume recovery is activated. This basic mechanism has been preserved in essentially all species throughout evolution. This adaptive mechanism termed regulatory volume decrease (RVD) is accomplished by the extrusion of intracellular osmotically active solutes up to reach the water balance imposed by the new conditions. The osmolytes involved are the main intracellular ions K^+ and Cl^- and a number of small organic molecules, including amino acids, polylcohols, and amines [2, 3]. RVD initiates by sensing the cell volume change, followed by activation of transduction signaling chains allowing the cell to organize the multiple and concerted responses necessary to face the volume gain challenge (Fig. 1). The mechanism(s) or molecules acting as volume sensors are not fully identified. Integrins, growth factor receptors, changes in macromolecular crowding, ionic strength or $[Ca^{2+}]_i$ and phospholipases activity, are all considered as related to volume sensing mechanisms [4]. Detection of the cell volume change is followed by the activation of signaling networks, in which $[Ca^{2+}]_i$ rise and phosphorylation of protein tyrosine kinases and their targets, have a prominent role (Fig. 1). Several of these kinases activate by the volume increase but the specific role for each of them has yet to be ascribed to the variety of adaptive cell responses to swelling first, and to volume correction later. These include among others, construction and deconstruction of cell adhesions, reorganization of the cytoskeleton, temporal deformation and remodeling of the membrane. Also, swelling is detected by the cell as a stressful situation, and consequently alerts the mechanisms for survival. Part of the volume transduction chains are directed to activate the osmolyte efflux pathways ultimately leading to cell recovery (Fig. 1). The hierarchy, interplay and connection of the signaling molecules activated by cell volume changes are not yet fully established.

RVD activated by hyposmotic swelling has been extensively investigated but less is known about cell volume control in cytotoxic swelling. The extent of volume recovery may vary, depending primarily on the mechanisms responsible for swelling. When swelling is driven by redistribution of ions, the main mechanisms for volume recovery involving ion extrusion are not working. Organic osmolyte efflux pathways still operate but their contribution may not be sufficient to accomplish an efficient volume regulation.

Modulation by GPCRs of the regulatory volume decrease

Activation of GPCRs initiates a broad spectrum of intracellular signaling chains influencing a diversity of cellular responses. Elicited signals are determined by the receptor interaction with the various families of G proteins, which in turn, transduce signals through both α and $\beta\gamma$ subunits. Proteins from the G_q family mediate PLC-dependent generation of IP3 and DAG, mobilization of Ca^{2+} from intracellular stores and PKC activation. $G_{12/13}$ family provide a pathway to Rho-ROK and the G_i proteins control the cyclic AMP pathway by inhibition of the adenylyl cyclase. The effects of GPCR agonists increasing osmolyte fluxes and RVD here discussed, suggest a prominent role of the G_q family, since in most cases are mediated by increases in $[Ca^{2+}]_i$, which add to that elicited by swelling. This $[Ca^{2+}]_i$ increase is one of the most consistent cell responses to hyposmotic swelling, although osmolyte fluxes and RVD are Ca^{2+}-independent in a number of cells [5]. An intriguing observation from

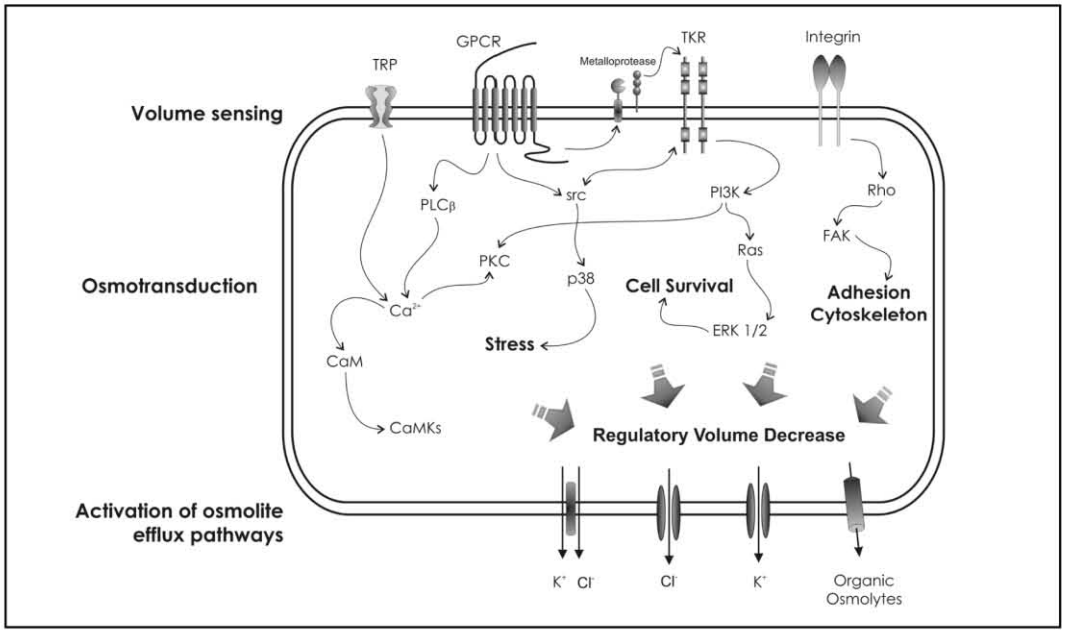

Fig. 1. Schematic model of the molecular mechanisms involved in the three main steps necessary for the cell volume recovery after hyposmotic swelling: 1. volume sensing, 2. osmotransduction and 3. osmolyte efflux pathway activation. The scheme illustrates the elements involved in each one of the steps required for cell volume adjustment and their suggested interplay. In addition to the reactions leading to the corrective osmolyte fluxes, other signaling chains are evoked related to adaptive responses of the cell to the volume changes occurring during swelling first and during RVD. These include adhesion processes, cytoskeleton remodeling, stress-sensing mechanisms and cell survival and protecting signals. EGFR: Epidermal growth factor receptor; GPCR: G protein coupled receptors; TRP: Transient receptor potential channel; PI3K: phosphoinositide-3 kinase; src: src for sarcoma oncogene; PLC: phospholipase C; PKC: protein kinase C; Ras, Rho: members of the Ras family of small GTPases; FAK: focal adhesion kinase; ERK 1/2: extracellular signal-regulated kinases 1 and 2; CaM: calmodulin; CaMK II: calcium/calmodulin-dependent protein kinase II; TRK: tyrosine kinase receptor.

our laboratory was that even in these cells, $[Ca^{2+}]_i$ rise over levels induced by hyposmotic swelling as that generated by ionomycin, results in a dramatic enhancement of taurine efflux and a faster and more efficient RVD [6]. Interestingly, the ionomycin-induced increase in taurine efflux was only evident in swollen cells and not in those with unchanged volume, in spite of the large increases in $[Ca^{2+}]_i$ elicited by the ionophore in the two conditions. In this sense, what $[Ca^{2+}]_i$ rise propitiates is a potentiation of the taurine efflux and not only the linear increase of a Ca^{2+}-dependent mechanism. Results on the effect of the ionophores on taurine fluxes and RVD raised the question of whether increasing $[Ca^{2+}]_i$ by physiological mechanisms such as the activation of GPCRs, is able to evoke the same response, i.e. increasing osmolyte fluxes and RVD rate. This review documents the early studies on the topic and the surge of recent reports supporting a modulatory role of GPCRs on cell volume regulation.

GPCRs and organic osmolyte efflux

Organic osmolytes involved in RVD are a group of small organic molecules with heterogeneous molecular structure: amino acids (taurine, glutamate, glycine, GABA), polyalcohols (sorbitol, myo-inositol), amines (creatine, phosphoethanolamine, glycero-phosphorylcholine) and N-acetyl-aspartate. Despite their difference in structure it has been suggested that they permeate through the same pathway. Taurine is often considered as representative of organic osmolytes and

Receptor	Agonist	Transduction mechanisms	Cell Type	Ref.
TAURINE				
Purinergic P2Y	ATP	PLC-Ca^{2+}- CaM, CaMK II, TK	Swiss 3T3 fibroblasts	19
	ATP	Ca^{2+}	Human breast cancer cells	10
	ATP	NI	NIH3T3 fibroblasts	7
	ATP	Ca^{2+}	Human tracheal cell line	9,13
	ATP	Ca^{2+}, CaM, nPKC	HeLa cells	14
H1 histamine	Histamine	Ca^{2+}, CaM, nPKC	HeLa cells	14
PAR-1	Thrombin	NI	Myoblastic cell lines	16
	Thrombin	Ca^{2+}_i, PKC	Astrocytoma cell line	17,18
	Thrombin	NI	HeLa cells	14
	Thrombin	PLC-Ca^{2+}, src/EGFR	Swiss 3T3 fibroblasts	19
Bradykinin	Bradykinin	Ca^{2+}, CaM, nPKC	HeLa cells	14
M_3 muscarinic	Oxo-M	Ca^{2+}_i, Ca^{2+}_e, PLC, PKC	SH-SY5Y neuroblastoma	20,21
Lysophospholipid	LPA, SP1	Ca^{2+}_i, PKC	SH-SY5Y neuroblastoma	21
Prostanoid	PGE1	cAMP, PKA	L-murine fibroblasts	27
β-adrenergic	NE	cAMP	Flounder erythrocytes	26
GLUTAMATE				
Purinergic P2Y	ATP	Ca^{2+}_i, CaM, CaMKII, PKC	Cultured astrocytes	22,23
PAR-1	Thrombin	Ca^{2+}_i, PLC	Cultured astrocytes	24
M3 muscarinic	Oxo-M	TK	SH-SY5Y neuroblastoma	20
MYO-INOSITOL				
M3 muscarinic	Oxo-M	Ca^{2+}, PKC	SH-SY5Y neuroblastoma	25

Table 1. Potentiation by GPCR agonists of swelling-induced organic osmolyte fluxes. PLC: phospholipase C; CaM: calmodulin; CaMK II: calcium/calmodulin-dependent protein kinase II; TK: tyrosine kinase; NI: not identified; nPKC: novel protein kinase C; Ca^{2+}_i: intracellular calcium; EGFR: epidermal growth factor; Oxo-M: oxotremorine-M; Ca^{2+}_e: extracellular calcium; LPA: lysophosphatidic acid; SP1: sphingosine 1-phosphate; PGE1: prostaglandin E1; PKA: protein kinase A.

has particular features which are very convenient to trace its role as an osmolyte. Taurine is present in high concentrations in most animal cells, largely free in the cytosol. It is not a protein constituent and is essentially inert in terms of metabolism. Taurine is the most sensitive among organic molecules, to volume perturbations, with the lowest release threshold and the largest amount released [7, 8]. Swelling-induced taurine efflux occurs through a leak pathway, presumably an anion channel-like pore. This pathway is known as volume-sensitive organic anion channel (VSOAC).

Swelling-evoked taurine release is partly Ca^{2+} dependent in some cell types but in many of them is largely Ca^{2+}-independent. In all cases, however, increasing $[Ca^{2+}]_i$ over levels raised by hyposmolarity by treatment with Ca^{2+} ionophores, markedly potentiates the swelling-induced taurine efflux. Interestingly, there is recent evidence showing a similar potentiation when $[Ca^{2+}]_i$ is raised by agonists of GPCRs in a variety of cell types. As shown in Table 1, the volume-sensitive taurine efflux is increased by metabotropic purinergic receptors, likely of the P2Y2 or P2Y4 subtype, in airway epithelial cells [9], human breast cancer cells [10] 3T3 fibroblasts [11], hepatoma HTC cells [12], tracheal cells [13], HeLa cells [14] and hippocampal neurons [15]. Taurine efflux is also increased by PAR-1 activation in myoblasts [16], HeLa cells [14], astrocytoma [17], and neuroblastoma SH-SY5Y cells [18] and in 3T3 fibroblasts [19], and by M3 muscarinic, H1 histamine and lysophospholipid receptors [14, 20, 21]. Glutamate (D-aspartate) efflux is potentiated by agonists of purinergic and PAR-1 receptors in cultured astrocytes [22-24] and by the muscarinic cholinergic receptor agonist Oxo-M in SH-SY5Y cells [20]. This condition also increases the swelling sensitive efflux of myo-inositol from SH-SY5Y cells [25].

These effects of GPCR agonists increasing organic osmolyte fluxes appear to be essentially mediated by the PLC-Ca^{2+} pathway, being all of them Ca^{2+}-dependent to some extent. The degree of dependence, though, varies according to the agonists examined, the osmolyte traced

Receptor	Agonist	Cell Type	Method	Transduction mechanisms	Ref.
Purinergic	ATP	Cultured astrocytes	PC-WC	NI	74
PAR-1	Thrombin	CPAE cells	PC-WC	Ca^{2+}-independent	34
	Thrombin	Intestine 407 cells	^{125}I efflux	NI	33
	Thrombin	SH-SY5Y cells	^{125}I efflux	Ca^{2+}-PKC independent	18
	Thrombin	Ehrlich-Lettre cells	PC-WC	NI	35
		Swiss3T3 cells	PC-WC	PLC-Ca^{2+}, src/EGFR	19
Endothelin	ET1, ET2	Atrial cells	PC-WC	MEK-dependent	36
		Intestine 407 cells	^{125}I efflux	NI	33
Bradykinin		Intestine 407 cells	^{125}I efflux	Ca^{2+}-dependent, TK	33
M3 muscarinic	Oxo-M	SH-SY5Y cells	^{125}I efflux	$Ca^{2+}{}_i$-PKC	18

Table 2. Potentiation by GPCR agonists of ICl^-_{swell}. PC-WC: patch-clamp, whole cell; NI: not identified; PAR-1: protease-activated receptor 1; MEK: MAPK/ERK-kinase; PLC: phospholipase C; PKC: protein kinase C; EGFR: epidermal growth factor receptor; ET: endothelin; M3: muscarinic cholinergic; TK: tyrosine kinase; Oxo-M: oxotremorine M.

and the magnitude of osmolarity reduction. In 20-30% hyposmotic solutions, the effect of purinergic or M3 muscarinic receptor activation increasing and glutamate efflux is largely Ca^{2+}-dependent (86-90%), regardless of the cell type [20, 23]. A PLC-dependent, thapsigargin-sensitive increase in $[Ca^{2+}]_i$, accounts for most of the ATP effects increasing taurine efflux. CaM and CaMKII are also part of this signaling cascade [11]. The increased efflux of taurine by thrombin-induced PAR-1 activation is 50% Ca^{2+}-dependent in 3T3 fibroblasts [19] and more dependent (80%) in astrocytes (unpublished). A role for PKC on the stimulatory effect of GPCR activation on taurine and glutamate efflux is suggested by its attenuation by PKC blockers or down-regulation of the enzyme. This is observed for the thrombin, LPA and Oxo-M-stimulated taurine efflux in astrocytoma and neuroblastoma cells [17, 20, 21] and for the ATP increased D-aspartate efflux in cultured astrocytes [23]. The fact that PKC and $[Ca^{2+}]_i$ depletion are additive in some systems suggests the operation of two different pathways and the participation of Ca^{2+}-independent PKC isoforms [23]. Taurine efflux increased by SP1 or LPA is less dependent on the Ca^{2+} pathway, being only 34-38% reduced when $[Ca^{2+}]_i$ elevation is prevented by treatment with BAPTA-AM or thapsigargin [21]. This feature may reflect the unique ability of these agonists to concurrently stimulate G_i, G_q and $G_{12/13}$ proteins. A more complex signaling mechanism for the PAR-1 potentiation of taurine efflux is found in 3T3 fibroblasts. An EGFR/src signaling pathway accounts for about 50% of the taurine efflux evoked by thrombin [19]. The two signaling chains, i.e. the Ca^{2+}-dependent and the EGFR/src-mediated pathways operate independently. This is not observed in astrocytes in which similar to that found for the P2Y potentiation depends primarily on Ca^{2+}-mediated pathways.

The modulatory influence of GPCRs on organic osmolyte efflux may not be restricted to the Ca^{2+}-signaling mediated receptors. In flounder erythrocytes, norepinephrine markedly increases the swelling-activated taurine efflux via a cAMP-mediated pathway [26]. A recent study in murine fibroblasts shows that receptors acting via the G_i family, activating cyclic AMP-dependent mechanisms also potentiate the volume-sensitive taurine efflux, with a magnitude similar to that of the PLC-Ca^{2+}-signaling pathway [27] (Table 1).

It is important to remark that the increase in organic osmolyte efflux elicited by GPCR agonists occurs essentially through the VSOAC, being abolished by blockers of this pathway. It is also worthy to mention that all studies here reviewed have been performed in cells swollen by hyposmolarity. It is still unknown whether the effects of GPCR activation are reproduced in conditions of isosmotic swelling.

GPCRs and Cl^-_{swell}

The swelling-activated Cl^- channels (Cl^-_{swell}) are present in a large variety of cells and in most of them exhibit similar properties. The ICl^-_{swell} current is outwardly rectifying, of intermediate conductance, with inactivation kinetics and a requirement for activation of free intracellular ATP. Cl^-_{swell} have a broad permeability to anions, including large anions as benzoate or glutamate, and are sensitive to general Cl^- channel blockers [28].

The effect of intracellular perfusion of GTPγ activating a Cl^- current with the features of ICl^-_{swell} first suggested a role for G proteins on the volume-sensitive Cl^- efflux pathway [29-32]. This was supported by a study in Intestine 407 cells showing that a variety of GPCR

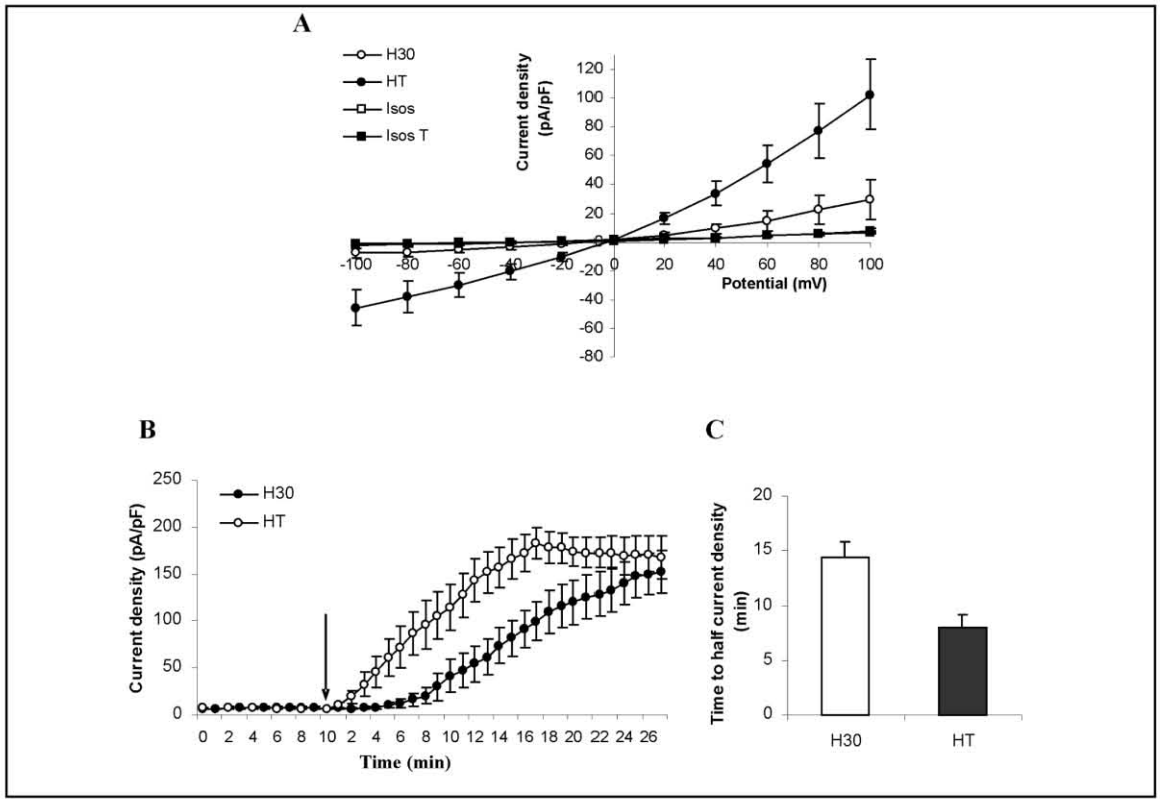

Fig. 2. Thrombin increases the amplitude and reduces the time for activation of the hyposmolarity-induced Cl^- current in Swiss 3T3 fibroblasts. A. Current density vs. voltage curves from cells in isosmotic medium (□), isosmotic medium + thrombin (■), 30% hyposmotic medium (H30) (○) and 30% hyposmotic + thrombin (HT) (●). B. Time course of ICl^-_{swell} activation by hyposmolarity or by hyposmolarity + thrombin. Traces show the current density at 100 mV in the indicated experimental conditions. C. Time to half activation in 30 % hyposmotic medium (clear bar) and 30 % hyposmotic medium + thrombin (dark bar). Results are means ± SE of 6-8 experiments. Experimental procedure details in [17].

agonists, ATP, thrombin, bradykinin and endothelin, all increase the swelling-induced efflux of ^{125}I [33]. It was later shown that thrombin increases the hyposmotic ^{125}I efflux from neuroblastoma cells [18] and that thrombin and endothelin increase ICl^-_{swell} amplitude in pulmonary artery endothelial, Ehrlich-Lettre ascites and atrial cells [34-36]. Noradrenaline exhibit a complex effect on ICl^-_{swell} in portal vein myocytes, inducing an increase in ICl^-_{swell} amplitude by acting on α-1 adrenoceptors and a decrease by stimulating β-adrenoceptors [37] (Table 2).

A detailed description of the effect of thrombin in ICl^-_{swell} in 3T3 fibroblasts is shown in Fig. 2. This Cl^- current exhibits the features characteristic of ICl^-_{swell}. It is an outwardly rectifying current, with inactivation kinetics at large positive voltages and a reversal potential of -4 mV, close to the predicted Cl^- equilibrium potential in the condition of the experiment. Thrombin influenced ICl^-_{swell} in fibroblasts by markedly enhancing the current density, and decreasing the time to half activation. Thrombin effect is exerted apparently on the same level of current density, as would be expected if it is not affecting the number of channels in the membrane. As shown in Fig. 2, thrombin alone did not induce by itself large currents, as it has been previously reported in endothelial cells [34].

In contrast to the primarily Ca^{2+}-dependent effect of GPCR activation on the organic osmolyte fluxes, the effect on Cl^- efflux pathways is described as Ca^{2+}-dependent or Ca^{2+}-independent. The hyposmotic ^{125}I potentiation by bradykinin in Intestine 407 cells depends completely on $[Ca^{2+}]_i$ rise and is PKC independent [33]. In contrast, ^{125}I efflux increased by thrombin from SH-SY5Y neuroblastoma cells is basically independent of Ca^{2+} and PKC [18]. In pulmonary artery endothelial cells the thrombin-induced increase of ICl^-_{swell} is unaffected

when the thrombin-induced $[Ca^{2+}]_i$ rise is prevented and cells maintain a basal $[Ca^{2+}]_i$ of 50-100 nM [34] but in 3T3 fibroblasts, the effects of thrombin are reduced in essentially Ca^{2+}-free conditions [19]. The effect of endothelin in atrial cells is insensitive to PKC down-regulation [36]. These differences in the mechanism of the modulatory effect of GPCR agonists might be attributed either to the cell type, to differences in the cell swelling degree, or to the magnitude of Ca^{2+} buffering.

A common feature of the GPCR agonists potentiation of ICl^-_{swell} is that no effect is observed in isomotic conditions. It is only when swelling has activated the efflux pathway that the modulatory effect can be observed. However, in cultured corneal keratocytes, a chloride current identified as ICl^-_{swell} is activated by LPA and SP1 in isosmotic conditions [38]. Since as previously mentioned, these agonists may interact with other G-protein families and their effects on RVD-related events have to be examined in more detail.

GPCRs and volume-activated K^+ channels

Understanding the influence of GPCRs on volume corrective K^+ fluxes is complicated by the fact that events concurrent with cell swelling, such as membrane depolarization, $[Ca^{2+}]_i$ rise or membrane stretching, activate a variety of K^+ channels which are potential contributors to K^+ efflux from swollen cells. It is actually documented that voltage-dependent, stretch-activated, Ca^{2+}-activated K^+ channels of small, intermediate or large conductance and 2P-4TM channels, contribute to the K^+ efflux elicited by swelling in different cell types [39, 40]. Particularly the Ca^{2+}-activated K^+ channels of large conductance (BK) are primarily involved in the swelling-evoked K^+ efflux and RVD [39, 40]. Most of these channels are modulated by GPCR ligands, but the consequence of this effect on the volume-sensitive K^+ efflux and cell volume regulation has been examined in only few cases. BK channels are stimulated by endothelin, bradykinin, histamine, and isoproterenol in a variety of cells, but this effect is analyzed regarding mainly their action on vasoconstriction, or on other cell responses and not in connection with RVD. In any event, it is expected that when BK channels are responsible for the volume-sensitive K^+ efflux, GPCR activation in swollen cells will certainly influence volume regulation. Thrombin and ionomycin stimulate K_v channels in human platelets, but similarly, no connection is established with volume regulation [41].

Studies directly related to GPCR agonist effects on K^+ channels activated by volume changes are so far limited. In cultured astrocytes, the volume-sensitive ^{86}Rb efflux is Ca^{2+}-independent, but is markedly potentiated by the ionomycin-induced $[Ca^{2+}]_i$ increase [42]. A K^+ channel directly activated by swelling has been described in Ehrlich ascites cells, which is insensitive to the classical K^+ channel blockers but is inhibited by clofilium [43]. It is proposed that this channel is tonically inhibited by a pertussis-sensitive G protein [43]. Activation of purinergic P2Y receptors potentiates BK channels or ^{86}Rb efflux in a kidney cell line (Vero cells), in Ehrlich ascites [44, 45], and Intestine 407 cells [33] and in cultured human cancer cells [46]. Bradykinin, thrombin or histamine activate a charybdotoxin-sensitive ^{86}Rb efflux in Ehrlich ascites cells [45, 47]. These results altogether, show that, in contrast to the universality of the volume-sensitive Cl^- and organic osmolyte efflux pathways, the variety of K^+ channel types involved in volume regulation difficults the analysis of the potential effect of GPCR activation. An additional factor further complicating the interpretation of the effect of GPCR agonists is that, in contrast to that found for organic osmolytes or Cl^-_{swell}, the GPCR ligands activate K^+ channels in non swollen cells.

GPCRs and RVD

An early study by Bender and coworkers in cultured astrocytes [48] demonstrated, to our knowledge for the first time, the involvement of GPCRs in RVD. This study showed that endothelin, thrombin, bradykinin, ATP and carbachol, all accelerate RVD via a PLC-dependent mechanism. Further support to a G-protein role in RVD came from a study in in cervical cancer cells showing facilitation of RVD by GTPγS [49]. Subsequent studies showed that ATP facilitates RVD in airway epithelial and in Intestine 407 cells [50, 51]. Thrombin has this same effect accelerating RVD in 3T3 fibroblasts [19]. RVD facilitation is also promoted by the $[Ca^{2+}]_i$ rise elicited by ionomycin or A23187 [6], suggesting that Ca^{2+}-dependent events mediate the effect of GPCR agonists. This is confirmed by the effect reducing RVD of inhibitors of the PLC-Ca^{2+} signaling pathway [19], an expected result in view of the key role of $[Ca^{2+}]_i$ rise on the effect of GPCR activation increasing osmolyte fluxes, as above discussed. A more complex signaling mediates the effect of thrombin accelerating RVD in 3T3 fibroblasts, which besides the PLC-Ca^{2+}- CaM-CaMKII pathway, involves src and EGFR as a parallel signaling route [19].

This will be further discussed below.

The monomeric G-proteins of the Rho family

The low-molecu lar weight G-proteins of the Rho subfamily control multiple cellular processes, many of them related to their action as regulators of the actin cytoskeleton organization. Changes in cell volume during swelling and the subsequent RVD, involve dramatic changes in the cytoskeleton, which are necessary for the cell to adapt successfully to the dynamics of these events [52]. Rho (isoforms A,B,C) and their targets, as well as isoforms from the Cdc42 and Rac subfamilies participate in this process, interacting in a complex dynamic balance [53-55]. Either through this effect or independently of cytoskeleton changes, Rho-signaling participates on Cl^-_{swell} activation in various cell types, as shown by the effects of bacterial toxins which specifically block Rho GTPases, or by the influence of a constitutively active Rho mutant expression [54-56]. Evidence at present suggests the requirement of a functional Rho pathway for Cl^-_{swell} operation but not for its activation, postulating a permissive role for this pathway. On this respect, an active Rho pathway may facilitate Cl^-_{swell} operation by promoting the formation of an actin-based platform for the proper assembly of elements involved in Cl^-_{swell} trigger.

The involvement of Rho GTPases in K^+ and taurine efflux pathways is supported by a study in NIH3T3 cells expressing constitutively active Rho. A marked increase in the hyposmolarity-evoked taurine and ^{86}Rb efflux is observed in these cells [56]. Not only the efflux rate constant was enhanced but the osmolarity threshold was reduced as well. Osmolyte fluxes in isosmotic conditions are comparable in the wild type and the Rho activated cells, being in this respect, similar to the effect of receptors linked to heterotrimeric G-proteins. In congruence with the effect of active Rho increasing two important osmolyte efflux pathways, volume regulation after swelling was markedly increased in cells expressing the active Rho pathway [56]. Consistent with the notion of a permissive role for the Rho signaling pathway, the hyposmotic stimulus does not directly activate the Rho pathway [54] but a number of molecules responding to swelling are known to activate them including integrins, growth factor receptors and guanine nucleotide exchange factors. In contrast to the heterometric G-proteins, monomeric G-proteins of the Rho subfamily are not directly activated through ligand binding, though a subset of GPCRs including those activated by lysophosphatidic acid and thrombin participate in cytoskeleton remodeling and other cell responses associated with volume changes, via Rho-dependent pathways.

Is the permissive pathway mediated by tyrosine kinases?

A remarkable and consistent feature of the effect of GPCR agonists on organic osmolyte fluxes and ICl^-_{swell} is that it is exerted only when the efflux pathway is already in operation. The precise nature of this permissive pathway is not yet fully established, but there is evidence of a prominent role for tyrosine kinases (TK). The involvement of TK in volume regulation activated after hyposmotic swelling has been recognized since the early report by Tilly *et al.* [57] showing inhibition by general TK blockers of the osmosensitive fluxes of K^+ and Cl^- in an epithelial cell line. Subsequent studies confirmed these results for specific TK and for organic osmolytes in a variety of cell types [58]. TK such as FAK, and members of the Src family, activate in response to hyposmotic swelling [58-61]. Another important family of signaling molecules, the growth factor receptors, which are transmembrane molecules with intrinsic TK activity, are also responsive to this stimulus. EGFR phosphorylation is increased by hyposmotic cell swelling in fibroblasts and keratinocytes [61, 62] and phosphorylation of ErbB4 increases by swelling in cerebellar granule neurons [63]. The phosphorylation state of these TK modulates osmolyte efflux pathways and RVD, which are inhibited by TK blockers, and correspondingly potentiated by blockade of tyrosine phosphatases [58]. The TK signaling chain can be linked to volume sensing molecules, such as integrins or the growth factor receptors themselves. A sequence of integrin - FAK - src activation by swelling is well established [59, 60]. A src-EGFR connection as swelling signal is also documented [19]. The extensive signaling network formed by these kinases may be part of the permissive pathway on which GPCR agonists exert their influence.

Tyrosine kinases and GPCR cross-talk

The involvement of tyrosine kinases in osmolyte efflux pathways and volume regulation is relevant to the topic of this review considering the evidence now available about a cross talk between GPCRs and TK, a mechanism known as transactivation. EGFR and members of the Src

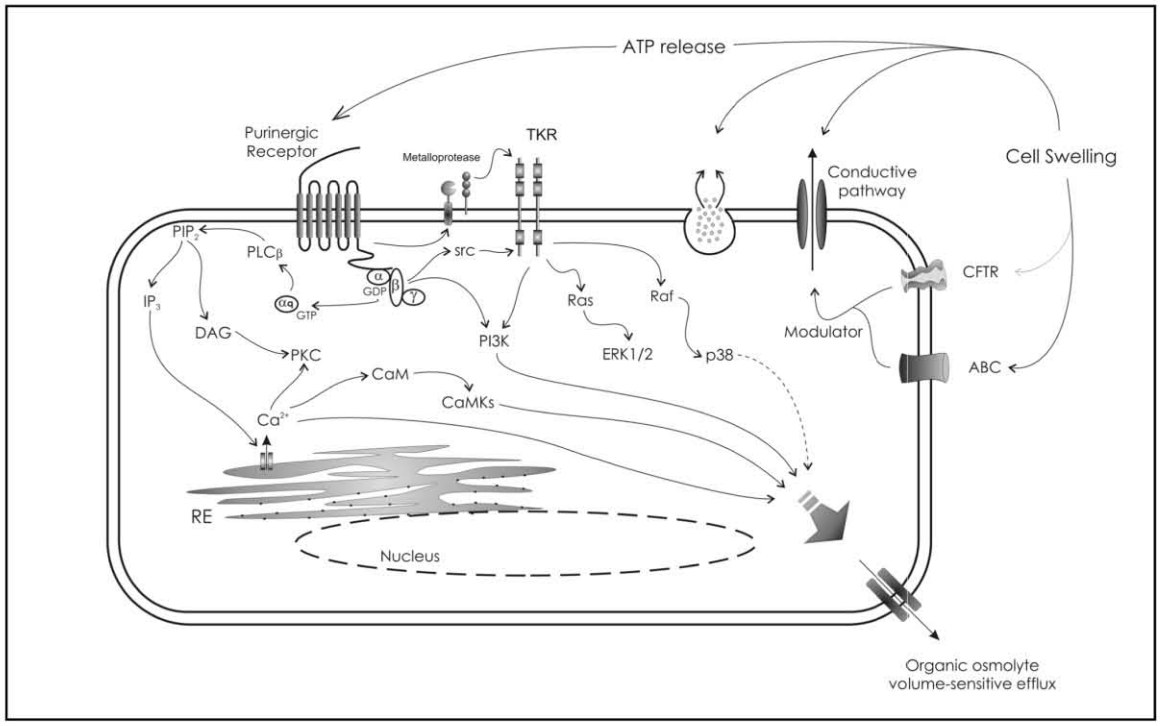

Fig. 3. Hyposmotic swelling evokes ATP release by exocytosis, or a conductive, anion channel-like pathway modulated possibly by CFTR or ATP-binding cassette members. Released ATP attains levels sufficient to activate purinergic receptors of the P2Y subfamily. The signaling chain evoked by these receptors has an influence on the hyposmotic $[Ca^{2+}]_i$ rise, on osmolyte fluxes and RVD. There is still controversy of whether the sole activation of these receptors by external ATP is able to elicit ICl^-_{swell} or organic osmolyte fluxes in non swollen cells.

family are the molecules more directly involved in this interplay [64-67]. GPCR/tyrosine kinase crosstalk is only starting to be examined in reference to cell volume regulation. A recent study from our group in 3T3 fibroblasts [19] shows that activation of PAR-1 by thrombin potentiates the hyposmotic phosphorylation of EGFR and src. The contribution of this transactivation pathway to volume regulation is evaluated by the effect attenuating thrombin effects on taurine efflux, ICl^-_{swell} and RVD by the blockers AG1478 (EGFR) or PP2 (src). This transactivation pathway may be linked to thrombin activated PLC-Ca^{2+}-mediated signaling chain, or may independently influence the volume regulatory mechanisms. In the study in fibroblasts, the two pathways operate separately but this may not be the case for other cell types. The proposed GPCR/tyrosine kinase interaction is an avenue for diversifying the signaling cascades activated by cell volume changes. This diversification may be most convenient considering that cell volume is now known to be involved in cell functions as diverse as proliferation, migration and apoptosis, which may require different signaling routes.

GPCR modulation of RVD in physiology and pathology

Autocrine GPCR agonists

The stimulatory effect of GPCR agonists on osmolyte fluxes and RVD is at present firmly established. A question is then raised as whether these receptors are part of the volume recovery signaling chain or act essentially as modulators, adding new signals to the chain activated after swelling. Purinergic and leukotriene receptors have been proposed to operate as receptors for autocrine factors released by swelling. Derived from eicosanoids, the cysteinil leukotrienes in particular LTD4, are released to the extracellular space, bind to a leukotriene receptor and via trimeric G proteins initiate a signaling pathway resulting in modulation of the taurine efflux or

Cell type	ATP_e	ATP scavenger, receptor blockers*	Ref
ICl^-_{swell}			
HTC rat hepatoma	-	Inhibition	38
Tracheal epithelial cells	-	Kinetics modulation	13
Biliary epithelial cells	-	Inhibition	77
Intestine 407 cells	Potentiation	Inhibition	79
Human hepatocytes	-	Prevent activation	71
Cultured astrocytes	Activation	Inhibition	74
Hippocampal neurons	Activation	Inhibition	15
Taurine and glutamate efflux			
Glutamate			
Cultured astrocytes	Potentiation	No effect	22
Taurine			
Swiss 3T3 fibroblasts	Potentiation	Inhibition	11
Tracheal epithelial cells	Potentiation	–	9
Tracheal epithelial cells	Potentiation	Inhibition	13
Hippocampal neurons	Potentiation	Inhibition	15
Human breast cell line	Potentiation	Inhibition	10
RVD			
HTC rat hepatoma	-	Inhibition	38
Biliary epithelial cells	-	Inhibition	77
Intestine 407 cells	Facilitation	Inhibition	51
Human hepatocytes	-	Inhibition	71
Turbot hepatocytes	-	Inhibition	76
Airway epithelial cells	Facilitation	Inhibition	50

Table 3. Influence of ATP and P2Y receptors on volume regulation events *Apyrase, suramine, reactive Blue and pyridoxal phosphate.

activation of a K^+ current. This proposed signaling cascade is supported by the effect reducing taurine efflux and RVD of lipoxygenase blockers or by inhibition of the synthesis of arachidonic acid and leukotrienes [7, 36, 68].

An autocrine/paracrine role for ATP on volume regulation is proposed based on the numerous reports on a swelling-induced efflux of ATP [50, 69-74] (Fig. 3). Since the early study by Wang *et al.* [69] the swelling-induced ATP release has been consistently observed in various cell types including carcinoma and hepatoma cells, airway epithelial cells and cultured astrocytes [69-74]. Quantification of the amount of ATP released has shown that the levels attained in the immediate vicinity of the cell surface are sufficient to activate P2Y purinergic receptors [50, 74]. The volume-sensitive ATP release may occur via exocytosis or more commonly through a conductive, anion channel-like pathway, possibly regulated by CFTR or ATP-binding cassette proteins [69, 70, 73] (Fig. 3). While there is general agreement about the effect of swelling inducing ATP release, the key question of whether P2Y receptor activation is a trigger or a modulator of osmolyte fluxes and RVD is still controversial. In favor to the autocrine role is the effect reducing osmolyte fluxes and volume recovery of agents or conditions impairing the purinergic-mediated transmission shown in a variety of cell types [50, 69-76] (Table 3). Against this notion is the fact that external ATP in some cases [22, 23, 77, 78] but not always [74], is *per se* unable to raise osmolyte fluxes in non swollen cells. In this respect, however, the consideration raised about other GPCR agonists on the requirement of a prior activation of the permissive pathway are valid for ATP. Furthermore, in support to the autocrine role for ATP is the fact that, in contrast to other GPCR agonists, swelling is the trigger for its release. The volume-sensitive ATP release is of particular interest in brain due to the critical role played by ATP in astrocyte signaling and in the astrocyte-neuron interaction. ATP participates importantly in glia-glia communication via Ca^{2+}-wave propagation, which is mediated by ATP release in various preparations [79]. Widespread propagation of Ca^{2+} waves through astrocytes is important in pathological states such as seizure and brain trauma, two conditions associated with cell swelling.

Potentially harmful effects of GPCR agonists

The GPCR interaction with signaling molecules activated during RVD diversifies and amplifies its signaling network and makes the process faster and more efficient. In certain situations, however, the potentiation of osmolyte fluxes induced by GPCR agonists represents a risk for potential harm. The marked potentiation by thrombin of glutamate efflux from astrocytes is an example of such situation [24]. Besides acting as an osmolyte, glutamate is a major excitatory neurotransmitter, and if released in excess to the extracellular space induces overfunction of excitatory neuronal receptors resulting in cell death. In pathological situations such as head trauma, hemorrhagic episodes and ischemia, there is a marked increase of brain thrombin levels, which together with the astrocyte swelling characteristic of these conditions, precipitates the excessive glutamate efflux and excitotoxicity. Hyperexcitability may also occur by a thrombin-induced enhancement of glutamate release from swollen nerve endings. In addition, the widespread propagation of Ca^{2+} waves through astrocytes evoked by ATP and possibly by other GPCR agonists as well, increases in pathological states such as seizure and brain trauma [79], two conditions concurrent with cell swelling.

Volume-related cell functions potentially modulated by GPCRs

Synaptic transmission

Swelling and GPCR activation may importantly modulate the synaptic function. In isolated nerve endings, hyposmotic swelling increases the efflux of amino acids such as GABA and glutamate, which serve the double role of osmolytes and neurotransmitters [80]. Moreover, swelling evokes a series of events such as depolarization and Ca^{2+} rise, via exocytosis, which also increase the synaptic efflux of molecules like norepinephrine which are essentially neurotransmitters and do not participate in volume regulation [81]. Among the GPCR ligands known to increase osmolyte efflux are those which activate neurotransmitter metabotropic receptors, such as cholinergic and noradrenergic receptors. Thus, swelling-induced neurotransmitter release and its potentiation by metabotropic receptors may have a potent modulatory role of synaptic transmission, in both physiological and pathological conditions.

Cell proliferation, migration and apoptosis

Cell migration involves numerous reactions closely interconnected with changes in cell volume. Extensive remodeling of the cytoskeleton, construction and deconstruction of cell adhesions are indispensable for a coordinated cell migration. Furthermore, changes associated to cell migration generate intracellular local osmotic gradients creating volume changes in the microenvironment, which demand an immediate adjustment to guarantee the polarity and direction for the proper displacement of the migrating cell [82, 83].

Volume control has been related to cell proliferation. Cell volume is determined genetically and is an attribute for each cell type. Before dividing, cells must attain the characteristic volume of the parent cells. A phenomenological correlation between cell volume and proliferation is suggested by results showing that swelling elicited by either hyposmolarity or blockade of volume-sensitive K^+ channels decrease proliferation [84]. Diverse types of K^+ channels, including a swelling-activated channel, have been implicated in tumour cell proliferation [85] and Cl^-_{swell} channels have been also considered to participate in the cell cycle progression based on evidence showing that Cl^-_{swell} are activated during cell proliferation, and conversely, Cl^-_{swell} blockers interfere with proliferation [86-89]. Therefore, GPCR ligands known to participate in cell proliferation in a variety of cell types, such as thrombin, angiotensin, bradykinin, lysophosphatidic acid and sphingosine 1 phosphate may interact with volume-sensitive pathways. Another site of GPCR influence is the stimulation of growth factor receptors, a reaction known as transactivation. Hyposmotic swelling in some cells activate EGFR and this effect is potentiated by GPCR agonists [11, 19]. This concerted action may result in a more effective activation of the various EGFR downstream signaling pathways eventually influencing cell survival and proliferation. The interplay between GPCRs and volume-activated mechanisms, so far largely unexplored, warrants future investigation.

Cell volume decrease has been always recognized as a hallmark of apoptosis, but it is only recently that this volume reduction, termed apoptotic volume decrease, is considered as part of the apoptotic signaling. Apototic volume decrease is an early event in the program, occurring before other characteristic traits such as caspase 3 cleavage, cytochrome c release and translocation, endonuclease activation and DNA

fragmentation [90], Apoptotic volume decrease relies on the same mechanisms of RVD, i.e. the active translocation of intracellular osmolytes, K^+, Cl^- and taurine [90-95], which noteworthy, activate in non swollen or even shrunk cells. Modulation by GPCRs on these mechanisms has not been examined in detail, but represent a most intriguing venue for future research.

Conclusion and perspectives

Cell volume is determined genetically for each cell lineage, but it not a static feature of the cell. It is of extreme importance that volume is kept unchanged to maintain cell homeostasis, but when changes occur, they affect basic aspects of the biology of the cell and may represent a risk for the whole organism. The recent surge of interest about a modulation of the basic mechanisms for cell volume control by the most important and extended family of signaling elements, the G proteins, opens a plethora of possibilities for diversifying the signaling routes for the variety of cell functions involving volume changes. Synaptic transmission, intercellular communication, proliferation, migration, secretion and apoptosis, all may be influenced by this diverse and intricate interplay between GPCRs and the mechanisms for cell volume control. This is an intriguing avenue for future research.

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OVERVIEW

Transporters and Channels in Cytotoxic Astrocyte Swelling

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Abstract Brain edema is a severe clinical complication in a number of pathologies and is a major cause of increased morbidity and death. The swelling of astrocytes caused by a disruption of water and ion homeostasis, is the primary event contributing to the cytotoxic form of brain edema. Astrocyte cytotoxic swelling ultimately leads to transcapillary fluxes of ions and water into the brain parenchyma. This review focuses on the implication of transporters and channels in cytotoxic astrocyte swelling in hyponatremia, ischemia, trauma and hepatic encephalopathy. Emphasis is put on some salient features of the astrocyte physiology, all related to cell swelling, i.e. predominance of aquaporins, control of K^+ homeostasis and ammonia accumulation during the brain ammonia-detoxifying process.

Keywords Brain ischemia · Aquaporins · Brain edema · NKCC1 · $NC_{Ca\text{-}ATP}$ channel

Introduction

Brain edema and the resulting increase in intracranial pressure is a severe complication of numerous pathologies and is often a major cause of death. Astrocytes are the cells primarily contributing to brain edema. This is first, because astrocytes are more abundant than any other brain cell type, and second, because of the highly selective set of channels and transporters at the astrocyte plasma membrane implicated in ion and water fluxes. As components of the brain-blood barrier, astrocytes are involved in the fluid dynamics between blood and interstitial and intracellular brain compartments. Astrocyte swelling known also as cytotoxic swelling, occurs as result of changes in extracellular osmolarity or in isotonic conditions by ion redistribution between the intracellular and extracellular brain compartments. Disruption of ion homeostasis at these compartments activates transcapillary ion and water fluxes, leading to ionic brain edema. This may occur without physical or functional disruption of the tight junctions at the blood–brain barrier. If this barrier is affected, fluid leakage and proteins and cells intrude the brain parenchyma and establish the vasogenic edema. This type of edema appears in late stages of head trauma and ischemia, in brain tumors, multiple sclerosis, vascular or metabolic diseases or infections such as meningitis or septic encephalitis. Cytotoxic swelling occurs in hyponatremia, ischemia, brain trauma and hepatic encephalopathy. Astrocyte cytotoxic swelling in these pathologies is the subject of this review. Emphasis is put on some attributes of the astrocyte physiology, all related to cell swelling, i.e. predominance of AQPs, K^+ uptake during the spatial buffering, and ammonia accumulation during the brain ammonia-detoxifying process.

Transporters and Channels in Ischemia and Trauma

NKCC Cotransporter

Astrocyte cytotoxic swelling is an early event in brain ischemia. Hypoxic or ischemic episodes interrupt ATP synthesis affecting the operation of the Na^+/K^+ ATPase.

Special Issue: In honor of Leif Hertz.

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The reduced activity of the pump, by failing to perform the adequate transmembrane Na^+/K^+ exchanges, initiates the ion gradient dissipation, increasing intracellular Na^+ $[Na^+]_i$ and extracellular K^+ $[K^+]_o$. This is the first step in a chain of membrane permeability changes resulting in large increases in $[Na^+]_i$ and high $[K]_o$. Passive Cl^- and water fluxes follow the ion redistribution, and astrocytes swell. To reduce the excess of $[K^+]_o$, astrocytes set in motion mechanisms as the spatial buffering, furthermore contributing to cytotoxic swelling [1–3].

K^+ is critical for brain excitability as the ion primarily responsible for setting the resting membrane potential values of astrocytes and neurons, as well as in repolarization after the nerve impulse transmission. $[K^+]_o$ in brain are tightly regulated through a dynamic equilibrium in which astrocytes play a key role. Excess of $[K^+]_o$ is cleared by the spatial K^+ buffering, a process consisting in the efficient accumulation of K^+ at regions of high extracellular levels, and its subsequent spreading through the astrocyte syncytium, to be then released at remote locations with normal $[K^+]_o$ [3]. Mild increases in $[K^+]_o$ are cleared by K^+ entry, in part via the inwardly rectifying K^+ channels, particularly the Kir 4.1 [3]. The two-pore domain K^+ channels may also contribute to the K^+ uptake in some types of glial cells [4]. Large increases in $[K^+]_o$ as occur in ischemia or trauma activate another mechanism for K^+ clearance: the cotransporter NKCC.

NKCC is part of the SLC12 gene family of electroneutral cotransporters, energetically sustained by the transmembrane ion gradients. Two isoforms of NKCC have been identified: NKCC1 which is broadly distributed in animal tissues and cells, including astrocytes, neurons and oligodendrocytes, and NKCC2, present only in kidney [5]. The transporter operates with a stoichiometry of $1Na^+$ $1K^+$ $2Cl^-$. NKCC1 is activated by high $[K^+]_o$ a salient feature of ischemia [rev in 6]. It is blocked by bumetanide and furosemide, and is regulated by the phosphorylation status of the molecule, established by the concerted actions of kinases and phosphatases. NKCC1 phosphorylation via the signalling chain formed by SPAK, OSR1 and WNKs 1 and 4 has been demonstrated in some cell types [5], but this has not yet been explored in astrocytes. Other kinase pathways proposed to participate in NKCC1 activation in ischemia and trauma are the MAP kinases ERK1/2, cJun and p38, Ca^{++}-mediated signal transduction pathways, oxidative/nitrosative stress and inflammatory cytokines [6–8].

K^+ contributes to cytotoxic astrocyte swelling in ischemia by a sequence of reactions initiated by $[K^+]_o$, activation of NKCC1 and increased K^+ uptake by astrocytes. This chain of events certainly contributes to correct $[K^+]_o$ levels, but with the consequence of increasing astrocyte ion content and driving osmotically directed water influx, all this resulting in cell swelling. This sequence of events and their interplay have been demonstrated either in cultured astrocytes or in vivo in ischemia experimental models. Ischemia activates NKCC1 in brain by increasing both the transporter protein expression and its functional activity [6]. The causal link of these events with brain edema is proved by the effect of the transporter blocker bumetanide reducing edema [9]. Genetic ablation of NKCC1 gives similar results, as ischemic brain edema is considerably less in NKCC1 knockout mice than in the wild type animals [10]. In cultured astrocytes the swelling induced by conditions simulating ischemia, such as hypoxia/hypoglycemia, or high $[K^+]_o$ is reduced by bumetanide [11, 12] and in the same line, swelling is notably lower in astrocytes from a NKCC1 null-mice [13]. The ion gradient collapse and the cell volume increase characteristic of ischemia have another detrimental consequence: the rise in extracellular glutamate levels. Besides its effect as neurotoxin, glutamate further contributes to astrocyte swelling by increasing the NKCC1 activity, among other mechanisms [14]. The link between NKCC1 and glutamate is suggested by the effect of bumetanide or the genetic deletion of NKCC1 decreasing the ischemia-induced excitotoxicity [6].

Cerebral edema is a main complication of head trauma and it is of decisive importance in the evolution of the pathology. Brain edema related to head trauma has been thought for long time to be vasogenic in origin, but there is now evidence in support of the cytotoxic astrocyte swelling as the early and most prominent component of the edematous condition [15]. Directly related to swelling is the post-traumatic widespread depolarization, which increases $[Na^+]_i$ and $[Cl^-]_i$, elevates $[K^+]_o$ and drives cell water influx. Transient sheer forces derived from trauma, mechanically deform membranes and alter ion permeability also leading to intracellular water increase. If hemorrhagic episodes concur with trauma, as is often the case, the cascade of reactions characteristic of brain ischemia is also triggered. An additional indirect mechanism of swelling after brain trauma is the hyponatremia evolving as consequence of a disturbed secretion of the antidiuretic hormone.

NKCC1 also contributes to astrocyte swelling in brain and spinal cord trauma. NKCC1 activates in experimental in vivo models of head trauma and its functional inhibition or genetic blockade reduce astrocyte swelling and brain edema [15]. These results reproduce in cultured astrocytes exposed to experimental models of trauma [8, 16].

A note of caution on the role of NKCC in cell swelling comes from a study in Xenopus oocytes showing that bumetanide has a direct effect reducing water fluxes through AQP1 and AQP4. Synthetic bumetanide derivatives showed that this occurs directly by occlusion of the water pore of AQPs at the cytoplasmic site [17]. If these results are confirmed by further evidence, the involvement

of NKCC1 on cytotoxic swelling evaluated by the effect of bumetanide should be reappraised.

The two-pore K^+ channels may also participate in the integral brain response to ischemia. The TWIK-1 channel appears to contribute to K^+ influx during oxygen-glucose deprivation, particularly at a specific subpopulation of mice cortical astrocytes, more susceptible to swelling in the ischemic condition [18].

The SUR1/$NC_{ca\text{-}ATP}$ Channel

Since NKCC activity depends on the energy provided by transmembrane ion gradients, its role in astrocyte swelling is relevant at the penumbra, area in which ion gradients are sustained by partial oxidative synthesis of ATP, or/and by the activated astrocyte glycolytic anaerobic pathway. Astrocyte swelling in this area may be transient or prolonged, according to the magnitude of $[K^+]_o$ elevation, the efficiency of the spatial buffering and the remnant function of the Na^+/K^+ ATPase. Once the ATP synthesis machinery is definitely arrested, passive mechanisms of ion fluxes activate, aggravating cytotoxic astrocyte swelling, and ultimately leading to ionic edema, which if concurrent with disruption of the tight junctions results in vasogenic edema. The non-selective cation Ca-ATP channel ($NC_{Ca\text{-}ATP}$) seems to be a key player in this process (Fig. 1). The $NC_{Ca\text{-}ATP}$ channel was first characterized in reactive, freshly obtained reactive astrocytes exposed to low oxygen and ATP depletion [19]. The channel permeates monovalent cations, with similar permeability for Na^+ or K^+, is impermeant to anions and divalent cations, requires nanomolar concentrations of Ca^{++} and is activated by intracellular ATP depletion [20]. The $NC_{Ca\text{-}ATP}$ channel is regulated by the sulfonylurea receptor 1 (SUR1) possibly associated with a TRPM4-like channel. This channel is not constitutively expressed in brain but is transcriptionally upregulated by hypoxic conditions and opened by depletion of intracellular ATP, i.e. the salient features of ischemia [21]. The $NC_{Ca\text{-}ATP}$ channel is expressed in neurons and capillary endothelial cells at the ischemic core and in astrocytes as well at the peri-infarct zone [21].

The $NC_{Ca\text{-}ATP}$ channel contributes critically to generate ionic brain edema, defined as the transcapillary ion and water fluxes into the brain parenchyma. The ionic shifts in glial cytotoxic swelling cause a decrease of Na^+ and water concentration at the extracellular space, generating new gradients between the intravascular space and the brain extracellular space, through capillary endothelial cells. This creates the driving force for passive transcapillary movement of ions and water. The $NC_{Ca\text{-}ATP}$ channel is proposed as the pathway for these transendothelial passive fluxes [7, 21] (Fig. 1).

The contribution of the $NC_{Ca\text{-}ATP}$ channel to ischemic brain edema is supported by the effect of glibenclamide, a highly selective SUR1 blocker, reducing the increase in water content in the ischemic area, particularly at the peri-infarct region [22]. Further studies have shown a beneficial effect of glibenclamide in three ischemia models, reducing cell mortality, linked to its effect preventing brain edema [21, 22]. A role for the $NC_{Ca\text{-}ATP}$ channel in the generation

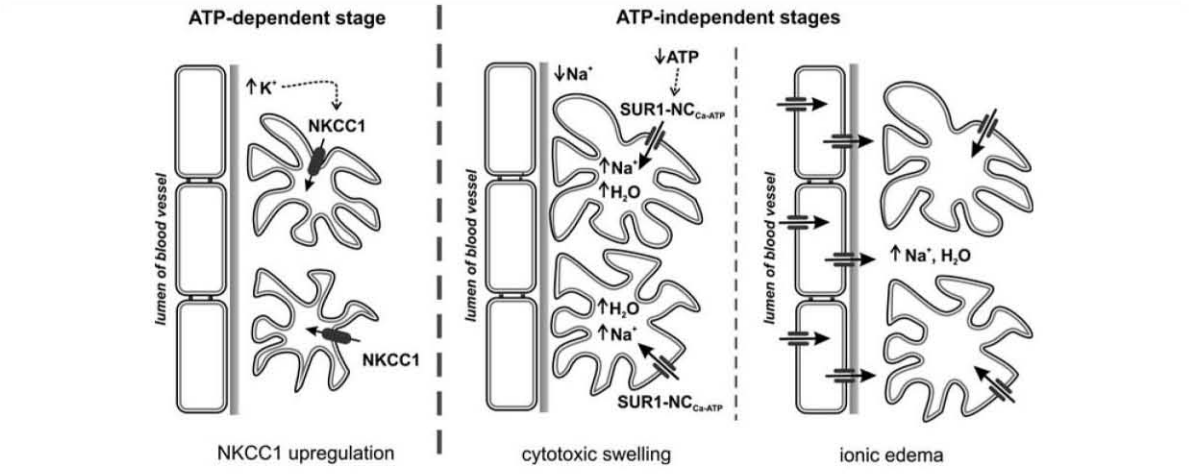

Fig. 1 The NKCC1/$NC_{Ca\text{-}ATP}$ -mediated chain of events leading to cytotoxic astrocyte swelling and ionic edema in ischemia. During early phases of ischemia when ion gradients are not totally collapsed, astrocyte swelling is driven by the elevated of $[K]_o$ extracellular potassium upregulating NKCC1. In later stages of ischemia, ATP depletion triggers the opening of the (SUR1)-regulated NC_{Ca}-ATP channel, increasing intracellular Na^+ and osmotic Cl^+ and water fluxes. Ionic edema occurs when the reduction in $[Na^+]_o$ and water levels, creates new ionic and osmotic gradients between plasma and interstitial fluid, driving transcapillary fluxes of Na^+ and osmotically obligated water. From results reviewed in [6, 7, 21, 24]

of edema has been also proposed for brain and spinal cord trauma, based on the beneficial effect of glibenclamide reducing the functional injury caused by trauma or hemorrhage [23, 24].

Transporters and Channels in Hepatic Encephalopathy

Hepatic encephalopathy is a complex neurological syndrome derived from acute liver failure. Brain edema is a most detrimental consequence and the frequent cause of death in this pathology. The blood–brain barrier appears intact and the edema results primarily from astrocyte cytotoxic swelling, which may be followed by transendothelial ion and water fluxes [25, 26]. Studies on the mechanisms of astrocyte swelling during hepatic encephalopathy underline the importance of ammonium as a trigger factor of the astrocyte volume increase. The liver failure leads to a dramatic rise in plasma ammonia levels, and enhances markedly the ammonia blood/brain ratio [25, 26]. Ammonia is accumulated by astrocytes as part of their specific function in brain ammonia detoxification, via the synthesis of glutamine. The abnormally high ammonia levels in astrocytes initiate a chain of deleterious reactions, which end up in oxidative/nitrosative stress, induction of the mitochondrial transition pore, ion overload and water accumulation. A synergistic action of ammonia and pro-inflammatory cytokines in the pathogenia of astrocyte swelling and brain edema has been recently suggested [27].

One of the first consequences of ammonia accumulation in astrocytes is the increase of intracellular Na^+ up to 20-30 mM. This effect is reduced by bumetanide, a result which point to the involvement of the cotransporter NKCC1 in the $[Na^+]_i$ increase. A link between ammonia and NKCC1 is suggested by the following evidence: (1) activation of NKCC1 by ammonia in cultured astrocytes and in astrocytes from hippocampal slices [28], (2) reduction by bumetanide of the ammonia-induced astrocyte swelling [28], (3) increased NKCC1 activation and total protein upregulation in a model of acute liver failure (thioacetamide) in vivo, and iv) decrease by bumetanide of brain edema in thioacetamide-treated rats [8]. The mechanisms of the ammonia-induced NKCC1 activation are not fully clarified. A chain of reactions is proposed including glutamine catabolism in the mitochondria by the phosphate-activated glutaminase, increase of mitochondrial ammonia and oxidative stress generation, ultimately leading to activation of MAP kinases and NKCC1 phosphorylation. Furthermore, the MAP kinase-dependent activation of the transcription factor NF-kB, increases NKCC1 protein expression [28, 29] (Fig. 2).

The role of the Kir4.1 channel on brain edema in hepatic encephalopathy has been examined. A significant down-regulation of the astrocytic *Kir4.1* gen has been reported in the hyperammonemic OTC^{spf} mice [30], and a possible link between this effect and astrocyte swelling and brain edema in hepatic encephalopathy has been proposed [30]. Hyponatremia is a common complication of hepatic failure and may additionally contribute to astrocyte swelling and brain edema in hepatic encephalopathy. The inwardly rectifying K^+ channel Kir4.1 may contribute to relieve the hyponatremic astrocytic swelling. Glutamine, at levels present in hepatic encephalopathy, induces downregulation of Kir4.1, and then impairs the ability of astrocyte for volume recovery, further contributing to brain edema in the pathological condition [31]. Consistent with these results, overexpression the Kir4.1 channel in HEK cells influence K^+ fluxes in a direction which attenuates astrocytic swelling in ammonia/hiponatremia conditions [32].

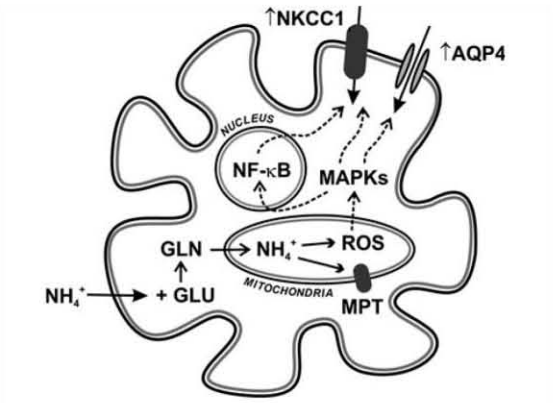

Fig. 2 Proposed cascade of events leading to NKCC1 activation and astrocyte swelling during hepatic encephalopathy. Hyperammonemia induced by acute liver failure increases the astrocyte ammonia levels, which conjugates with glutamate to form glutamine via the glutamine synthetase. Glutamine enters the mitochondria and generates ammonia by the phosphate-activated glutaminase. Mitochondrial ammonia induces mitochondrial permeability transition and ROS generation. The oxidative stress activates NKCC1 phosphorylation and NFκB-mediated expression via the activity of MAPKs. All this results in a marked increase of Na^+, K^+ and Cl^- influx, along with osmotically obligated water. Activated MAPKs are presumable also involved in regulation of the astrocyte water channel AQP4. *GLU* glutamate, *GLN* glutamine, NH_4^+ ammonia, *ROS* reactive oxygen species, *MPT* membrane permeability transition. From results in [8, 28, 29, 60]

There is no information so far about a role for the $NC_{Ca\text{-}ATP}$ channel in astrocyte swelling and brain edema in hepatic encephalopathy. Since there are no episodes of anoxia in this pathology, the conditions for activation of these channels may not be present. Though, there is evidence of ionic edema, as indicated by swelling of capillary endothelial cells, but this may involve other mechanisms related with generation of oxidative/nitrosative stress. The high circulating levels of ammonia, as well as inflammatory factors such as interleukins and the tumor necrosis-α factor, may generate nitrossative stress in the endothelial

cells, disruption of ion homeostasis and cell swelling. This proposed mechanism is supported by the positive correlation found between circulating levels of TNFa and the development of intracranial pressure during hepatic encephalopathy [33]. The intercellular tight junctions do not appear severely affected in hepatic encephalopathy and the blood brain barrier looks well preserved. Although some subtle changes in permeability through the junctions may have been induced by circulating ammonia [34] no predominant contribution of vasogenic edema to hepatic encephalopathy has been established.

Chloride Channels

Passive Cl^- fluxes are key elements in astrocyte swelling as well as in the transcapillary ion fluxes producing ionic brain edema. Though, there is no much information about the molecular entity in charge of Cl^- permeation. When edema is due to the increased function of electroneutral carriers, Cl^- and cations are cotransported with a stoichiometry preserving neutrality, but when swelling occurs by a channel-mediated accumulation of cations, a parallel Cl^- influx is required to preserve electrical and osmotic equilibrium. The route for these passive Cl^- fluxes has not been explored in detail. The volume-sensitive Cl^- channel [35] is a natural candidate for this role, but its participation in brain edema has not been conclusively established. A recent study reports decreased swelling upon blockade of the volume-sensitive Cl^- channel on a subclass of cortical astrocytes more sensitive to ischemic swelling [18]. The maxi-anion channel is another likely candidate to permeate Cl^- fluxes during swelling induced by hypoxia or ischemia. This is a volume-sensitive channel, functionally expressed in a large variety of cells, including astrocytes [36, 37]. The maxi-anion channel has a large unitary conductance (approximately 400 pS) and inactivation kinetics at potentials more positive than +20 mV or more negative than −20 mV. It has a wide pore allowing passage of large organic anions as glutamate and ATP [36, 37]. Of interest for the ischemia-induced brain edema, ATP depletion during chemical ischemia activates the maxi-anion channel in astrocytes. This particular feature makes it a candidate pathway to equilibrate the cation fluxes through the $NC_{Ca\text{-}ATP}$ channel and other K^+ or Na^+ channels involved in cytotoxic astrocyte swelling.

The Water Channels

There is substantial evidence on the role of the water channels aquaporins in the water homeostatic balance between blood and cerebrospinal fluid, and the brain interstitial and intracellular compartments. Aquaporins are also prominently involved in the pathogenesis of brain edema. Water fluxes across the cells occur either by directly crossing the membrane lipid bilayer or by via aquaporins, through which water permeates following its concentration gradient. The aquaporin family consists of thirteen members, with three of them AQP4, AQP1 and AQP9 present in brain. AQP4 is the predominant isoform in brain [38]. It is expressed in astrocytic processes surrounding neurons but more robustly at the endfeet of astrocytes forming the glia limitans of the blood/brain barrier and at the interface between the brain parenchyma and the cerebrospinal fluid. The spatial distribution of AQP4 support the key role played by this channel on the water exchange between the brain and spinal parenchyma and the major fluid compartments, blood and cerebrospinal fluid. AQP4 is expressed in two spliced isoforms, M1 and M23. The M23 isoform is predominant in brain [39]. The role of AQP4 in the development and resolution of brain edema in various pathologies is now well recognized. As below discussed, AQP4 is implicated in brain edema during acute hyponatremia, ischemia, trauma and hepatic encephalopathy.

Hyponatremia

Hyponatremia is often the consequence of clinical conditions such as congestive heart failure, chronic liver or renal failure, trauma, the syndrome of inappropriate antidiuretic hormone secretion, adrenal insufficiency and hypothyroidism, or occurs primarily by excessive water intake as in psychotic polydipsia or strenuous exercise. Acute and severe hyponatremia leads to neurological symptoms and frequent morbidity and mortality, mostly related to brain edema. Astrocytes are the cells which predominantly swell in acute hyponatremia, while neurons appear less affected. This may be due to differences in water permeability or/and to osmolyte redistribution as part of a protective action of astrocytes sparing neurons from swelling [40]. When hyponatremia develops chronically, as during pregnancy or elderly it is well tolerated due to efficient mechanisms of astrocyte volume regulation. Evidence on the involvement of AQP4 in hyponatremic brain edema includes (1) increase of AQP4 immunoreactivity in brain of mice during systemic hyponatremia [41], (2) decreased hyponatremic brain edema in AQP4-null mice, as compared to wild-type animals [42], (3) resistance to neurological injury and less mortality in animals with decreased AQP4 functional activity facing water intoxication [42], and (4) a delay in the onset of brain edema and reduced mortality in acute hyponatremia when AQP4 is mislocalized in dystrophin- or α-syntrophin-null mice [41, 43]. Consistent with these results, overexpression of AQP4 enhances brain swelling, intracranial pressure and mortality. AQP4 is functionally expressed in astrocytes in culture, although it is found

throughout the astrocyte cell membrane, without the polarization observed in vivo [44]. Hypotonic exposure of AQP4-defective cultured astrocytes derived from $AQP^{-/-}$ mice or by siRNA decreased AQP4 expression, show a remarkable decrease in water permeability and less swelling as compared to AQP4-expressing cells [45].

Ischemia

The role of AQP, particularly AQP4 in ischemic brain edema is well documented. Early events of ischemia in mice, rats and human brain enhance AQP4 expression in astrocytes endfeet [46] Conversely, less brain edema, infarct size and reduced lesion volume is observed by AQP4 suppression as in AQP-null mice as compared to the wild-type mice. Less mortality and better neurological performance is also observed in these $AQP4^{-/-}$ mice [42]. Prevention or reduction of these deleterious consequences of brain ischemia are observed in mice with mislocalized AQP4 by suppression of α-syntrophin or dystrophin [47]. In cultured astrocytes, AQP4 exposed to conditions simulating ischemia, AQP4 expression increases at the plasma membrane. This increase occurs without changes in mRNA suggesting a redistribution or translational modifications as the mechanisms of AQP overexpression [48]. Lactacidosis, as occurs at the penumbra when the anaerobic glycolitic pathway is the main source of ATP, generates cytotoxic astrocyte swelling. In cultured astrocytes exposed to high levels of lactic acid there is a significant increase in AQP4 mRNA expression that may contribute to cytotoxic swelling [48]. Besides AQP4, AQP-3-5 and -8 are found expressed in rat brain during permanent focal ischemia, and may also participate in the generation of edema [49]. In contrast to the beneficial role of reducing water permeability through the AQP4 in cytotoxic swelling, AQP4 suppression aggravates vasogenic edema as found in brain tumours or infectious diseases [38].

Trauma

Experimental models of brain and spinal cord injury are largely heterogeneous, but despite their differences, a consistent result is the increase in AQP4 expression in response to traumatic injury. Most studies showing AQP4 upregulation are in rodent brain [50] in which AQP increased expression correlates with the magnitude of the edema. The same increased AQP4 expression is observed in samples of ischemic tissue obtained from human brain [51]. Agents reducing AQP4 activity or expression such as acetazolamide, phorbol dibutyrate, Mg^{++} or the V1a receptor antagonist [52, 53], all decrease brain water content; in the same line, AQP4 deletion or silencing by AQP4 siRNA decrease brain water mobility [53]. Trauma-induced AQP4 upregulation is also observed in cultured astrocytes. Fluid percussion injury generates astrocyte swelling and increased AQP4 expression at the plasma membrane. These two facts appear causally correlated since silencing the AQP4 gene by siRNA reduces astrocyte swelling [54]. These results, altogether, point to AQP4 as a causal factor of brain edema in trauma. The mechanisms and regulation of the AQP4 increased expression by brain traumatic injury are not well known. In contrast to results in cultured astrocytes exposed to ischemic conditions, AQP4 upregulation in a model of trauma in cultured astrocytes seems to come from the novo synthesis of the molecule [54].

AQP4 seems also involved in water movements in spinal cord injury. Spinal trauma evokes acute and chronic changes in the channel expression [55]. Consistent with this suggested role for AQP4, AQP-null mice exposed to spinal cord compression show reduced swelling and pressure, less neuronal death and better neurological outcome than the corresponding wild animals [56]. These results suggest AQP4 as the molecule propitiating water influx and astrocyte swelling. Besides AQP4, AQP1 may be involved in the brain response to trauma.

Hepatic Encephalopathy

Early evidence relating AQP4 to brain edema in hepatic encephalopathy was the channel upregulation found in brain of rats in experimental models of acute liver failure [57]. More detailed studies in experimental models of acute liver failure, conclusively demonstrated an increase of AQP4 levels in the membrane enriched cortical brain tissue plasma membrane fraction, a change occurring without an increase in *Aqp4* mRNA [58, 59]. In cultured astrocytes, the early study by Rama Rao et al. [60] showed AQP4 upregulation in ammonia-treated astrocytes. A recent study identified a synergistic effect of ammonia and proinflammatory factors, as inductors of astrocyte swelling in experimental models of hepatic encephalopathy, via oxidative-stress exacerbation [61].

AQP4 Regulation

All these observations underline the prominent role of AQP4 in astrocyte swelling and brain edema, and makes the channel a potential target for the control of brain edema. Consequently, knowledge about AQP4 regulation is of particular interest. A recent review on this topic offers a comprehensive overview of the short term and long term regulation of AQP4 including that expressed in astrocytes [62]. Only a summary of this information is exposed here, and readers are referred to the cited review for details. AQP4 can be regulated by reversible phosphorylation. The molecule has several potential phosphorylation sites, and a

variety of Ser and Thr kinases may act as regulatory molecules. Ser 111 and Ser 180 have been identified as phosphorylation sites important for AQP4 function in the kidney, linked to the NO- NOS-PKG signaling chain, but this has not been so far established in astrocytes.

AQP4 regulation may also occur via its interactions with other molecules. AQP4 in the brain is found associated by a chemical cross-linking interaction with dystrophin, syntrophin and β-dystroglycan. This dystrophin-glycoprotein complex has a prominent role in AQP4 localization and distribution, as well as in its interaction with the extracellular matrix. As mentioned before, in acute hyponatremia, ischemia and epilepsy, disruption of this complex by dystrophin or syntrophin deletion leads to AQP4 mislocalization, in turn affecting brain edema and its neurological consequences. The dystrophyn/glycoprotein complex also connects AQP4 with the K^+ channel Kir4.1. Though, no functional link has been found between the two channels in astrocytes. A recent report provides evidence on the effect of endotelin decreasing the expression of AQP4 and AQP1 and water permeability in cultured astrocytes, via an interaction with the endothelin B receptor [63]. An interesting interaction has been found in cerebellum between AQP4 and the metabotropic glutamate receptors. Activation of GluR1 increases AQP4 water permeability via CaMKII, NOS and PKG [64]. This association suggests an influence of glutamate on AQP4 regulation, increasing water permeability. This functional link may provide a mechanism for the exit of water accumulated during astrocyte glutamate clearance. Vasopressin is known to exacerbate brain edema after focal ischemia or trauma, while a decrease is induced by vasopressin deficiency or antagonists of the vasopressin receptor. The correlation of these effects with AQP4 in brain ischemia has not been conclusively established, and results suggest a possible bimodal effect of the hormone with different actions at early and late times after the ischemic episode [rev. in 62]. Many other agents and conditions have been proposed as regulators of AQP4 expression, whose effects are revised in detail in Zelenina [62]. This wide range of possibilities offer an interesting, though complex panorama of therapeutic options to control brain edema having AQP4 as target.

Concluding Remarks

Cytotoxic astrocyte swelling is a causal effect common to the pathogenesis of brain edema in acute hyponatremia, ischemia, trauma and hepatic encephalopathy. Astrocyte swelling is a physiopathological response to a plethora of factors originated by these clinical conditions, some of them resulting from the astrocyte function regulating homeostasis of ions and water between the intracellular and extracellular compartments in brain and the vascular and cerebrospinal fluid compartments. Prevention of astrocyte swelling is a complex situation, which has to consider preserving the protective role of astrocytes on neuron cytoarchitecture and excitability, via the precise balance of ion distribution and direction of water fluxes. Better understanding of the various mechanisms implicated in astrocyte swelling, the dynamics of their regulation and the interplay among them is necessary for an effective and safe treatment of brain edema.

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