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Evidence for two mechanisms of amino acid osmolyte release from hippocampal slices

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Abstract
A 30% decrease in osmolarity stimulated ^{3}H-taurine, ^{3}H-GABA and glutamate (followed as ^{3}H-D-aspartate) efflux from rat hippocampal slices. ^{3}H-taurine efflux was activated rapidly but inactivated slowly. It was decreased markedly by 100 μM 5-nitro-(3-phenylpropylamino) benzoic acid (NPPB) and 600 μM niflumic acid and inhibited strongly by tyrphostins AG18, AG879 and AG112 (25–100 μM), suggesting a tyrosine kinase-mediated mechanism. Hyposmolarity activated the mitogen-activated protein kinases (MAPK) extracellular-signal-related kinase-1/2 (ERK1/ERK2) and p38, but blockade of this reaction did not affect ^{3}H-taurine efflux. Hyposmosis also activated phosphatidyl-inositide 3-kinase (PI3K) and its prevention by wortmannin (100 nM) essentially abolished ^{3}H-taurine efflux. ^{3}H-taurine efflux was insensitive to the protein kinase C (PKC) blocker chelerythrine (2.5 μM) or to cytochalasin E (3 μM). The release of ^{3}H-GABA and ^{3}H-D-aspartate occurred by a different mechanism, characterized by rapid activation and inactivation, insensitivity to NPPB, niflumic acid, tyrphostins, or wortmannin. ^{3}H-GABA and ^{3}H-D-aspartate efflux was not due to external [NaCl] decrease, cytosolic Ca^{2+} increase or depolarization, or to reverse operation of the carrier. This novel mechanism of amino acid release may be mediated by Ca^{2+}-independent exocytosis and modulated by PKC and actin cytoskeleton disruption, as suggested by its inhibition by chelerythrine and potentiation by 100 nM phorbol-12-myristate-13 acetate (PMA) and cytochalasin E. GABA and glutamate osmosensitive efflux may explain the hyposmolarity-elicited increase in amplitude of inhibitory and excitatory postsynaptic potentials in hippocampal slices as well as the hyperexcitability associated with hyponatraemia.

Keywords: taurine, hyposmolarity, swelling, regulatory volume decrease

Introduction

Amino acids are part of the organic osmolyte pool contributing to regulatory volume decrease (RVD) in most cells [34]. Among them, taurine has been studied in detail mainly because of its metabolic inertness and is often considered to represent osmolyte amino acids. In cultured cells, taurine is released upon hyposmosis through a leak pathway, with essentially no contribution of energy-dependent carriers [15, 35]. Remarkably, osmosensitive taurine release is sensitive to Cl^- channel blockers, suggesting a common pathway for efflux of Cl^- and amino acids, and possibly other organic osmolytes as well [22, 36, 39]. This pathway may be an anion channel–like molecule of broad specificity. Even though the electrophysiological properties and pharmacological profile of this pathway have been characterized in detail, its molecular identity is still unknown [30, 31]. The activation signal and the transduction intermediates are not well defined. Ca^{2+} appears to be a good candidate as messenger in transduction because, in most cells, swelling elicits an increase in cytosolic Ca^{2+} [32]. Moreover, recent evidence for volume-associated phosphorylation of tyrosine kinases and the sensitivity of osmolyte translocation pathways to tyrosine kinase blockers [43] has indicated the involvement of such kinases in the modulation of the volume-activated anion channel. Most reports have focused on the volume-sensitive Cl^- channel with less information available for osmosensitive taurine fluxes. Essentially no studies have addressed the features of the release of other organic osmolytes, including amino acids, which, similar to taurine, are released from brain cells in response to hyposmolarity [21, 33]. In the present study in hippocampal slices, we compared some of the properties of osmosensitive efflux of ^{3}H-taurine, ^{3}H-GABA, and glutamate (followed as ^{3}H-D-aspartate), including the time course of release activation and inactivation, the effect of Cl^- channel blockers, the involvement of tyrosine kinases, in particular the mitogen-activated protein kinases (MAPK) extracellular-signal-related kinase-1/2 (ERK1/2) and MAPK-p38, and of phosphatidylinositide 3-kinase (PI3K) and the effect of phospholipase blockers. The efflux of taurine had properties remarkably different from those of GABA and glutamate. These may represent either a different mechanism of osmosensitive release or a response not related directly to the volume regulatory process but rather to epiphenomena associated with the complex mechanism of swelling and volume regulation.

Materials and methods

Materials

Anti-MAPK-p38 and anti-ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, California, USA), p-Akt antibody was from Cell Signaling Technology (Beverly, Massachusetts, USA), ATF2 (activating transcription factor 2, recombinant protein fusion of residues 19–96, and glutathione S-transferase) was from New England Biotechnology (Beverly, Massachusetts, USA) and okadaic acid was from GIBCO (Gaithersburg, Maryland, USA). 5-Nitro-(3-phenylpropylamino) benzoic acid (NPPB), BAPTA-AM and wortmannin were

from RBI (Natick, Massachusetts, USA); tyrphostins (AG18, AG112, and AG879), lavendustin A, herbimycin A, dephostatin, phorbol-12-myristate-13 acetate (PMA), LY294002, PD98059, SB202190, arachydonyltrifluoromethyl ketone (AACOCF3), methyl arachidonyl fluorophosphonate (MAFP), 7,7-dimethyl-5,8-eicosadienoic acid (DEDA), chelerythrine, cytochalasin E and EGTA-AM were from Calbiochem-Novabiochem (San Diego, California, USA). [^{3}H]-taurine, [^{3}H]-GABA, and [^{3}H]-D-aspartate were from New England Nuclear (Boston, Massachusetts, USA). [γ^{32}-P]ATP was from Amersham Pharmacia Biotech. Dihydrokainic acid was from Tocris Cookson (Ballwin, Missouri, USA). Niflumic acid, 4,4′-diisothiocyanato-stilbene-2,2′-disulphonic acid (DIDS), 4-bromophenacylbromide (4BpB), 1-(2-{[(diphenylmetilene)-imino]oxy}etil)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO-711), *o*-vanadate, and all other reagents were of analytical grade and from Sigma (St. Louis, Missouri, USA).

Hippocampal slices

Male adults Wistar rats (weighing 200–250 g) were used throughout the study. Animals were killed by decapitation and brains rapidly removed and submerged in cold Krebs-HEPES medium containing (in mM): 135 NaCl, 1.0 $CaCl_2$, 1.17 $MgCl_2$, 1.7 KH_2PO_4, 5 KCl, 5 dextrose, and 10 HEPES (300 mOsm/l, isosmotic, pH 7.4). Hippocampal slices, 400 μm thick, obtained as earlier described [11] were submerged immediately in isosmotic medium continuously bubbled with O_2 and kept at room temperature at least for 30 min before the assays. The hyposmotic solution medium (osmolarity reduced by 30% to 210 mOsm/l), was prepared by reducing the [NaCl]. Final osmolarities were verified with a freezing-point osmometer (Osmette A, Precision Systems, Natick, Massachusetts, USA).

Labeled amino acid efflux

Hippocampal slices were incubated in isosmotic medium with the labeled amino acids ^{3}H-taurine (60 min, 55.5 MBq/ml), ^{3}H-GABA, or ^{3}H-D-aspartate (as a metabolically inert analogue of glutamate, 30 min, 37 MBq/ml). Then, slices were transferred into perfusion chambers (0.4 ml volume) and washed by perfusion with warmed (37°C), isosmotic medium at 1 ml/min for 15 min. Thereafter, samples were collected every minute. After 5–10 min of basal release, the osmolarity of the perfusion medium was changed suddenly to 30% hyposmotic medium and samples were collected for 25 min. Preincubation times with drugs varied depending upon the experiment and are indicated in the corresponding figure legend. Controls contained the corresponding vehicle. Amino acid release data are expressed as radioactivity released per minute as a percentage of the total incorporated. When amino acid release was calculated as efflux rate constants, i.e., the radioactivity released at any given time as percentage of total radioactivity present in the tissue at that time, the same kinetic profile was obtained [35].

MAPK-p38 Kinase Assay

MAPK-p38 kinase activity was measured by an immune complex kinase assay using ATF2 as substrate. Briefly, hippocampal slices were incubated for 5 min in isosmotic or hyposmotic medium and, where indicated, 50 μM SB202190 added. The reactions were terminated by addition of lysis buffer containing 20 mM TRIS pH 7.5, 1% Triton X-100, 150

mM NaCl, 20 mM NaF, 0.2 mM sodium o-vanadate, 1 mM EDTA, 1 mM EGTA, and 5 mM phenylmethylsulphonyl fluoride. Samples were homogenized and sonicated. Following centrifugation at 15,000 *g* for 15 min at 4°C, cleared lysates (approximately 1.5 mg protein) were incubated with 4 μl/sample anti-p38 antibody plus protein A-sepharose beads (3:1 v/v) and incubated for 90 min at 4°C to precipitate the immune complexes. Samples were centrifuged at 15,000 *g* for 10 min and the beads then washed twice in kinase buffer containing 25 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol and 0.1 mM sodium *o*-vanadate. The kinase assay was initiated by the addition of 20 μl kinase buffer containing 10 μCi [γ^{32}P]ATP and 50 ng ATF2 to the washed beads. Reactions were incubated for 20 min at 30°C and then terminated by the addition of 8 μl 5×Laemmli SDS sample buffer. Samples were boiled and centrifuged briefly and the products resolved by 15% SDS-PAGE. The incorporation of ^{32}P was visualized by autoradiography and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA). Western blots for immunoprecipitated MAPK-p38 were made and developed with enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

ERK1/2 assay

ERK1/2 activity was followed by myelin basic protein (MBP) phosphorylation as described elsewhere [29].

PI3K activity

PI3K activity was determined indirectly by phosphorylation of Akt protein. Samples were prepared as described above, 2 mg of protein dissolved in lysis buffer was immunoprecipitated with a phospho-specific antibody that recognizes Akt phosphorylated at Ser-473. The phosphorylated form of Akt was detected by immunoblotting using the same antibody. These commercially available reagents were used according to the manufacturers' recommendations.

Results

Release pattern and effect of ion replacement

Figure 1 shows the time course of ^{3}H-taurine, ^{3}H-GABA, and ^{3}H-D-aspartate release from hippocampal slices in response to 30% hyposmotic solutions. Marked differences were observed in the efflux pattern among the three amino acids. While the release of ^{3}H-D-aspartate and ^{3}H-GABA was rapidly activated and inactivated, release of ^{3}H-taurine was delayed, reaching a peak only 6–7 min after the stimulus, and essentially no inactivation was observed up to 20 min. At the end of the experiment, 20 min after the stimulus, the total amount of ^{3}H-taurine released corresponded to 50% of the label accumulated in the slice, while for ^{3}H-GABA and ^{3}H-D-aspartate total release corresponded to 25% and 35%, respectively (fig. 1). Hyposmotic solutions were made by decreasing the [NaCl] in the medium. To investigate whether this decrease, rather than the hyposmolarity itself, was the trigger for amino acid release, slices were exposed to media containing a decreased amount of NaCl as in the hyposmotic solution, but made isosmotic again with mannitol. Under these

conditions no increase in amino acid efflux over basal levels was detected, indicating a specific response to hyposmolarity. We next examined the effect of ion replacement in the hyposmotic solution. Replacing all the Na^+ in the medium by choline did not affect the release of ^{3}H-taurine but delayed the inactivation phase of ^{3}H-D-aspartate and ^{3}H-GABA efflux, resulting in increased percentage release (table 1). Replacing Cl^- by gluconates did not affect the efflux of any of the amino acids (table 1). Omission of Ca^{2+} and addition of 0.5 mM EGTA had no effect on the fluxes of any of the amino acids. Reducing cytosolic Ca^{2+} by treatment with BAPTA-AM did not affect the efflux of ^{3}H-taurine or ^{3}H-GABA but increased that of ^{3}H-D-aspartate (table 1).

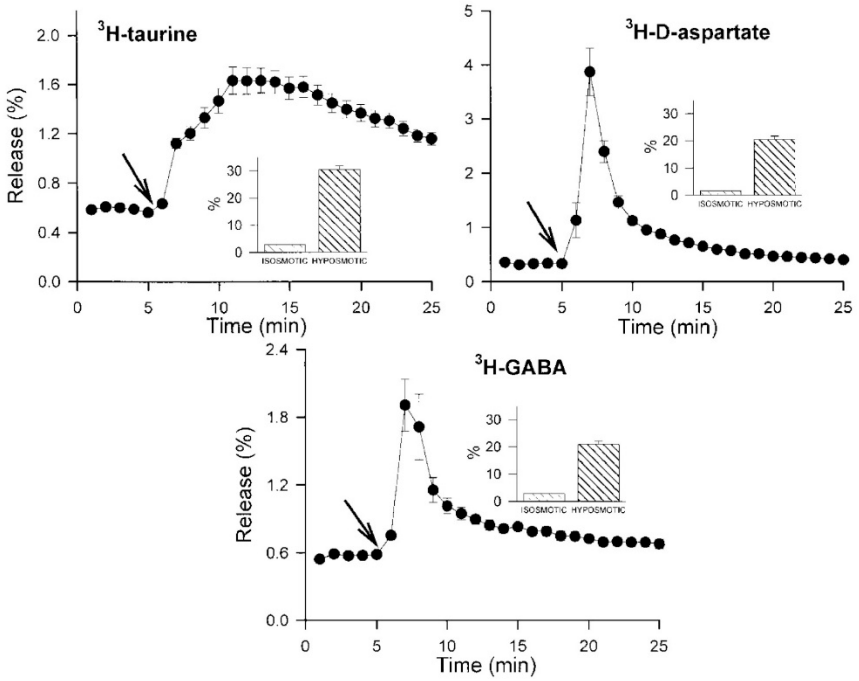

Figure 1. Amino acid release from rat hippocampal slices exposed to 30% hyposmotic medium. Slices preloaded with ^{3}H-taurine, ^{3}H-D-aspartate, or ^{3}H-GABA were superfused for 5 min with isosmotic medium. Thereafter (*arrow*), the medium was replaced by 30% hyposmotic solution. One-min fractions were collected for 20 min. Data represent the radioactivity released per minute expressed as percentage of the total incorporated and are means ± SE. The *insets* show the percentage (mean ± SE) of total release of the corresponding labeled amino acid during the period of exposure to the hyposmotic solution (min 6–25) and during the basal collection period (min 1–5).

Table 1. Effect of ionic replacement and Ca^{2+} dependency of amino acid osmosensitive release from hippocampal slices. Slices were loaded with labeled amino acids and superfused as described in Materials and Methods. Data indicate the release (expressed as the percentage of the total label incorporated) during a 20-min exposure to medium made 30% hyposmotic by decreasing [NaCl]. In Cl^- and Na+-free media, Cl^- was replaced by gluconates and Na+ by choline, respectively. In Ca^{2+}-free medium, Ca^{2+} was omitted and 0.5 mM EGTA was added to the solution. To chelate intracellular Ca^{2+}, slices were treated with 30 μM EGTA-AM or BAPTA-AM for 20 min prior to superfusion. Means ± SE, n = 4–16 experiments

Condition	^{3}H-taurine release (%)	^{3}H-D-aspartate release (%)	^{3}H-GABA release (%)
Control (NaCl)	30.3 ± 1.41	20.9 ± 1.20	0.9 ± 1.61
Cl^--free	31.0 ± 1.26	19.5 ± 1.40	25.9 ± 0.76
Na+-free	30.9 ± 1.03	30.4 ± 2.90	25.9 ± 0.76
Ca^{2+}-free	29.5 ± 1.06	22.9 ± 1.23	20.7 ± 1.10
EGTA-AM	30.9 ± 1.32	24.7 ± 2.93	21.7 ± 1.56
BAPTA-AM	32.5 ± 1.72	24.5 ± 1.50	23.5 ± 1.56

The effect of Cl^- channel blockers

The osmosensitive release of ^{3}H-taurine is sensitive to Cl^- channel blockers in a variety of cell types, including cultured neurons and astrocytes, in the supraoptic nucleus, and in vivo in a cortical cup preparation [4, 8, 18, 36, 37]. Consistent with these results, niflumic acid and NPPB (100 μM) essentially abolished ^{3}H-taurine efflux in hippocampal slices (fig. 2). DIDS reduced taurine release only at a high concentration (600 μM). Interestingly, the efflux of ^{3}H-GABA and ^{3}H-D-aspartate was notably less sensitive to these agents. In fact, only DIDS exerted a significant inhibitory effect, but niflumic acid and NPPB, in clear contrast to ^{3}H-taurine efflux, were without effect (fig. 2). Noteworthy in these experiments was an early ^{3}H-taurine release fraction that was resistant to the Cl^- channel blockers and showed an efflux pattern similar to those of ^{3}H-GABA and ^{3}H-D-aspartate, i.e., rapid activation and inactivation phases (fig. 2).

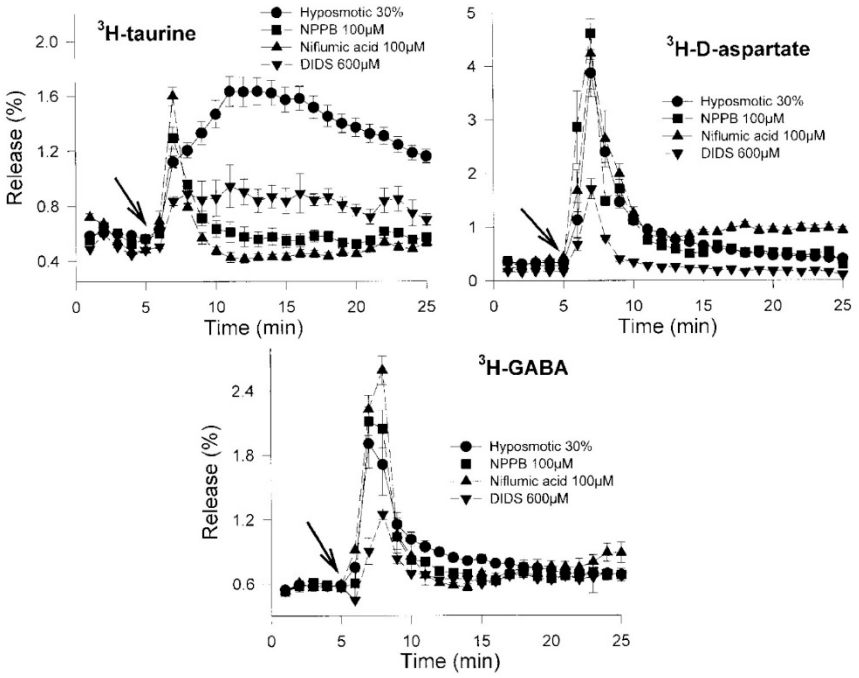

Figure 2. Effect of anion channel blockers on amino acid release from rat hippocampal slices. Slices preloaded with amino acid tracers were preincubated for 15 min in isosmotic medium with 100 μM NPPB (■), 100 μM niflumic acid (▲), or 600 μM DIDS (▼) (vehicle controls ●). Thereafter, slices were superfused and data presented as in figure 1. Blockers were present in all solutions used throughout the experiment. Means ± SE, $n = 4–10$. *$P <$ 0.001 in fractions 10–25 for all blockers in taurine efflux curves; **$P < 0.05$ in fractions 7–25 for the effect of DIDS on D-aspartate efflux curve and for fractions 7–16 for GABA efflux curves.

The involvement of tyrosine kinases

The osmosensitive efflux of taurine is influenced notably by manipulation of tyrosine kinase activity in cultured brain cells [28, 29], in the supraoptic nucleus [7], and in skate red cells [17]. In these preparations, taurine efflux is inhibited markedly by tyrphostins, which block tyrosine kinases, and potentiated by *o*-vanadate, which maintains tyrosine phosphorylation. In hippocampal slices, tyrphostins AG18 (50 μM), AG879 (25 μM), and AG112 (100 μM) all inhibited the release of ^{3}H-taurine potently (fig. 3). Again, there was an early component of the efflux curve that, as with the Cl–channel blockers, was unaffected by tyrphostins (fig. 3). The nonfunctional analogue of tyrphostin AG18, AG9 was without effect. The other tyrosine kinase blockers tested, genistein (up to 100 μM), herbimycin A (1 μM), and lavendustin A (10 μM), failed to modify ^{3}H-taurine release (table 2). In contrast to observations in cell lines and in primary cell cultures, no effect on ^{3}H-taurine release was observed with the tyrosine kinase blockers *o*-vanadate (up to 1 mM) and dephostatin (20 μM) (table 2). The effect of AG18 was tested on the release of ^{3}H-GABA and ^{3}H-D-aspartate.

In contrast to its potent inhibition of ^{3}H-taurine efflux, this blocker did not affect the release of ^{3}H-GABA and ^{3}H-D-aspartate.

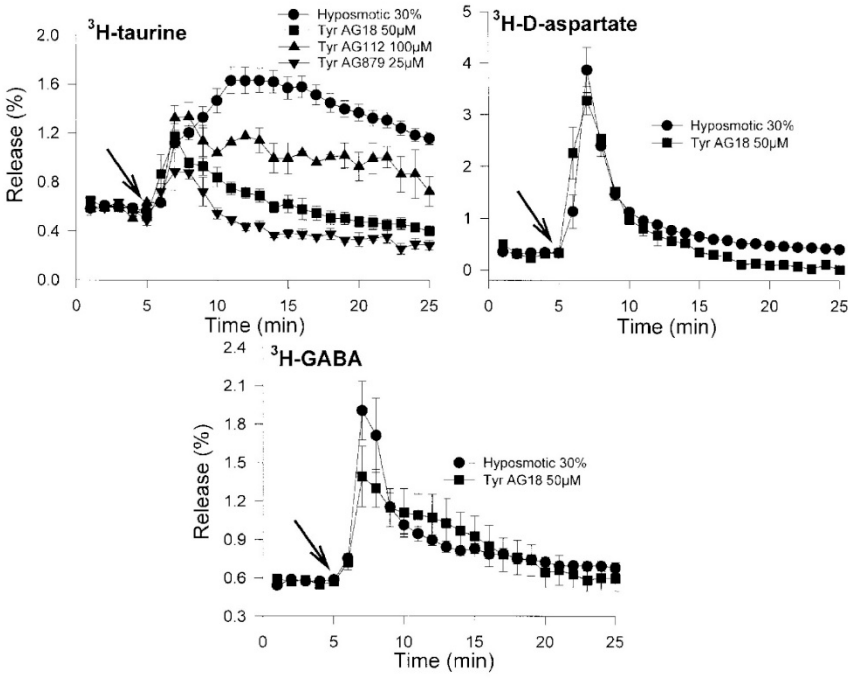

Figure 3. Effect of tyrphostins on hyposmolarity-induced amino acid release from rat hippocampal slices. Slices preloaded with amino acid tracers were preincubated for 1 h in isosmotic medium without (●) or with 50 μM tyrphostin AG18 (■), 100 μM AG112 (▲) or 25 μM AG879 (▼). Thereafter, slices were superfused and data presented as in figure 1. Tyrphostins were present in all solutions throughout the experiment. Means ± SE, n = 4–10.

Table 2. Effect of tyrosine kinase and tyrosine phosphatase blockers on the osmosensitive efflux of taurine. Hippocampal slices were loaded with ^{3}H-taurine and preincubated with the drugs for 60 min. The slices were the washed and superfused with isosmotic medium and with 30% hyposmotic medium, containing the same concentration of the drugs tested. Data represent the release (percentage of total incorporated label) during the 20-min exposure to hyposmotic medium minus the isosmotic release in the same period. Means ± SE, n = 4–16 experiments

Drug	Concentration (μM)	^{3}H-taurine release (%)
Control		23.31 ± 1.41
Tyrosine kinase blockers		
Tyr AG18	50	8.93 ± 1.30*
Tyr AG112	100	16.52 ± 1.82*
Tyr AG879	25	5.14 ± 1.10*
Genistein	100	26.34 ± 2.11
Herbimycin A	1	28.68 ± 4.95
Lavendustin A	10	23.86 ± 1.34
Tyrosine phosphatase blockers		
o-Vanadate	1000	24.52 ± 1.31
Dephostatin	20	26.69 ± 1.14

*P < 0.001, **P < 0.005 vs. control

The effect of tyrphostins on ^{3}H-taurine osmosensitive efflux is suggestive of one or several steps mediated by tyrosine kinases, as part of the signaling cascade for activation or operation of the taurine translocation pathway. To contribute to the identification of the kinases involved, we examined the effect of hyposmolarity on the activity of the MAPK ERK1/2 and p38 and on the tyrosine-kinase-activated PI3K, as well as the consequences of inhibiting their activity on the osmosensitive ^{3}H-taurine efflux. These kinases are activated in response to hyposmolarity in a variety of cell types (review in [13]). Figure 4 shows the effect of swelling on ERK1/2 phosphorylation in hippocampal slices. In contrast to cells in culture, the hyposmotic stimulus activated ERK1/2 only marginally in our preparation. PD98059 is used widely to prevent ERK1/2 activation and its effect was tested on taurine efflux in the hippocampal slices. Despite a slight effect of this blocker reducing ERK1/2 activity in hyposmotic conditions to levels slightly below the isosmotic activity, PD98059 (50 μM), had no influence on ^{3}H-taurine efflux (fig. 4). Another tyrosine kinase seemingly involved in cell response to hyposmosis is MAPK-p38. Figure 5 shows a clear activation of p38 following the hyposmotic stimulus. This effect was efficiently prevented by the blocker SB202190 (50 μM), which, however, did not decrease ^{3}H-taurine efflux.

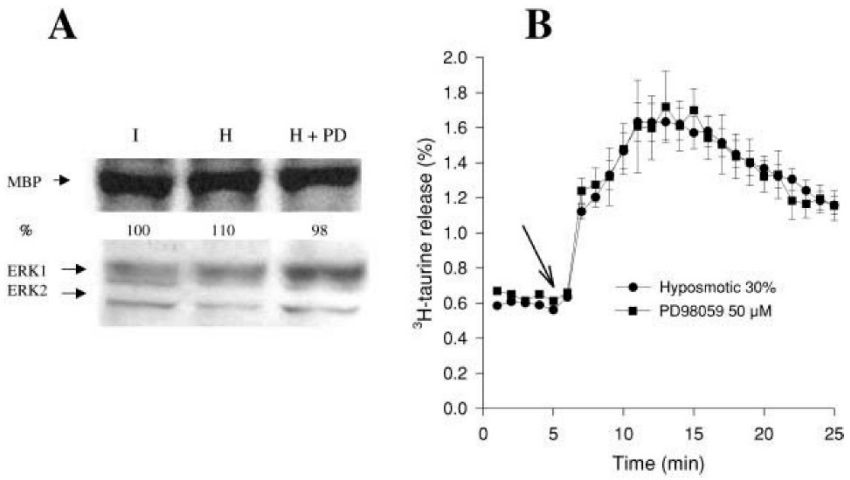

Figure 4. A Extracellular signal-related kinase 1/2 (ERK1/2) activity in response to hyposmolarity in hippocampal slices. Slices were exposed to media: isosmotic (*I*), 30% hyposmotic (*H*), or 30% hyposmotic+50 µM PD98059 (*H+PD*) for 5 min. Representative results of three independent experiments are shown. Myelin basic protein (*MBP*) phosphorylation (*upper panel*) was assayed using the immune-complex against antibody ERK1/2. The effect of hyposmolarity with or without PD98059 is shown as a percentage of isosmotic activity. A Western blot of ERK1/2 for the corresponding assay is shown in *lower panel*. **B** Slices preloaded with ^{3}H-taurine were preincubated for 1 h in isosmotic medium without (●) or with 50 µM PD98059 (■). Thereafter, slices were superfused and data presented as in figure 1. PD98059 was present in all solutions throughout the experiment. Means ± SE, $n = 6$.

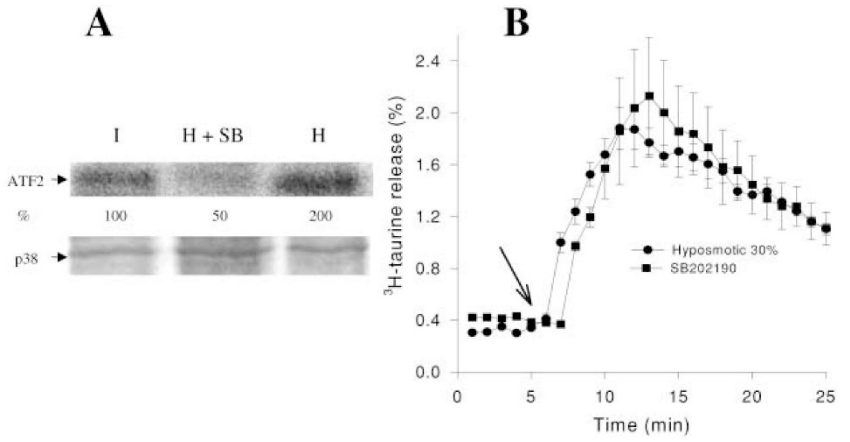

Figure 5. A Mitogen-activated protein kinase-p38 (MAPK-p38) activity in response to hyposmolarity in hippocampal slices. Slices were exposed to media: isosmotic (*I*), 30% hyposmotic+50 µM SB202190 (*H+SB*), or 30% hyposmotic (*H*) for 5 min. Representative results of three independent experiments are shown. Activating transcription factor 2 (*ATF2*) phosphorylation (*upper panel*) was assayed using the immune-complex against

MAPK-p38 antibody. The effect of hyposmolarity with or without SB202190 is shown as percentage of isosmotic activity. A Western blot of p38 for the corresponding assay is shown in the *lower panel*. **B** Slices preloaded with ^{3}H-taurine were preincubated for 1 h in isosmotic medium without (●) or with 50 μM SB202190 (■). Thereafter, slices were superfused and data presented as in figure 1. SB202190 was present in all solutions throughout the experiment. Means ± SE, $n = 6$.

Activation of PI3K by hyposmosis has been reported in intestinal 407 and hepatoma cells and in cholangiocytes [10, 24, 40]. In the present study PI3K activity was assessed by measuring the phosphorylation of Akt. Figure 6 shows the clear enhancement of PI3K activity upon hyposmotic stimulus. This reaction was blocked by wortmannin (100 nM) and, to a lesser extent, by LY294002 (25 μM). Wortmannin was a very potent blocker of ^{3}H-taurine efflux. As shown in figure 6, the magnitude of the inhibition by this agent was almost the same as that of tyrphostin. Similar to the effect of the Cl^- channel blockers and of tyrphostin, an early peak of taurine release was insensitive to wortmannin (fig. 6). LY294002 was clearly less effective than wortmannin in decreasing ^{3}H-taurine efflux. Similar to tyrphostins, the fluxes of ^{3}H-D-aspartate and ^{3}H-GABA were essentially unaffected by wortmannin (fig. 6).

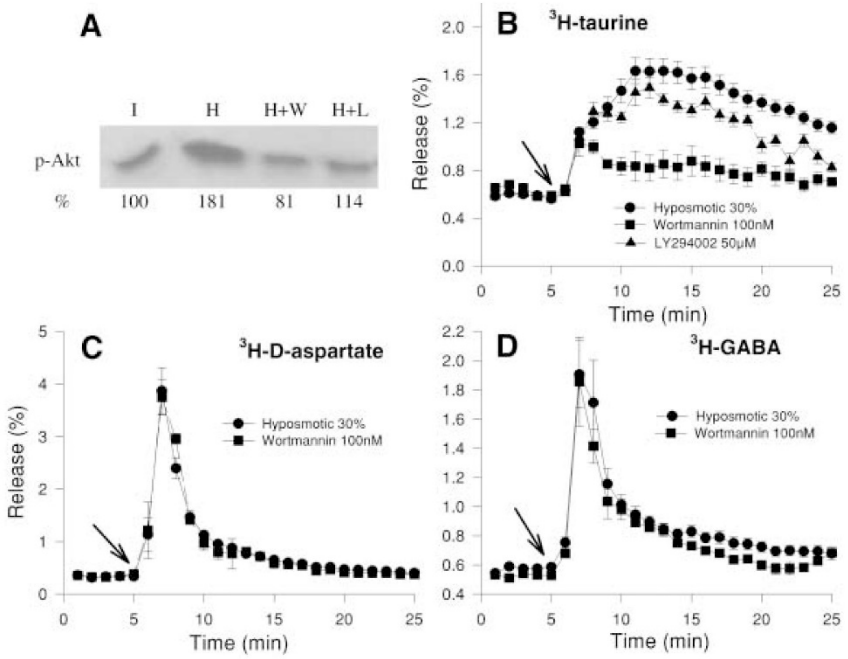

Figure 6. A–D Phosphatidylinositide 3-kinase (PI3K) activity in response to hyposmotic solution in hippocampal slices. **A** Slices were exposed to media: isosmotic (*I*), 30% hyposmotic (*H*), 30% hyposmotic+100 nM wortmannin (*H+W*), or 30% hyposmotic +25 μM LY294002 (*H+LY*) for 5 min. Representative results of three independent experiments are shown. PI3K activity was quantified by detection of the phosphorylated form of Akt by immunoblotting with a phospho-specific antibody that recognizes Akt phosphorylated at

Ser-473. **B** Slices preloaded with ^{3}H-taurine were preincubated for 1 h in isosmotic medium without (●) or with 100 nM wortmannin (■) or 25 μM LY294002 (▲). **C**, **D** Slices loaded with ^{3}H-D-aspartate and ^{3}H-GABA treated with wortmannin as in **B.** Thereafter, slices were superfused and data presented as in figure 1. Blockers were present in all solutions throughout the experiment. Means ± SE, $n = 6$. *P<0.001 from fractions 9–25; **P < 0.05 in fractions 13–19; ***P < 0.01 in fractions 20–25.

Phospholipase blockers

Involvement of phospholipases in the osmosensitive ^{3}H-taurine flux from hippocampal slices was examined using the general blockers 4BpB and DEDA, as well as AACOCF3 and MAFP, agents more specific for the cytosolic form of phospholipase A2, for which a role in ^{3}H-taurine efflux in CHP-100 neuroblastoma cells has been demonstrated clearly [3]. Only 4BpB (20 μM) significantly reduced ^{3}H-taurine fluxes (control 30.31 ± 1.41; 4BpB 18.103 ± 0.78; $n = 4$, $P < 0.001$). None of the other blockers influenced this release: MAPF (20 μM) 31.46 ± 1.99, $n = 4$; AACOCF3 (50 μM) 30.57 ± 2.9, $n = 4$. The latter was also tested at up to 150 μM without any effect.

Possible mechanisms for GABA and D-aspartate release

The above results clearly show different mechanisms for the release of ^{3}H-GABA and ^{3}H-D-aspartate as compared with taurine. We explored two alternatives. One was that ^{3}H-GABA and ^{3}H-D-aspartate fluxes occur by the reverse operation of Na+-dependent transporters. This was assessed by the effect of the carrier blockers NO-711 (GABA) and dihydrokainic acid (glutamate), agents known to block amino acid transport in hippocampal slices effectively [25]. The release of ^{3}H-D-aspartate was unaffected by 1 mM dihydrokainic acid (control 20.5 ± 1.20; dihydrokainic acid 19.51 ± 2.29 $n = 4$). Treatment with NO-711 (20μM) delayed the inactivation phase of ^{3}H-GABA efflux, thus increasing the total amount released (control 20.98 ± 1.61; NO-711 26.3 ± 2.15, $n = 4$), but the peak release and activation time were unaffected.

Another possibility for the mechanism of amino acid release addressed here was that of exocytosis, mediated by a change in the actin cytoskeleton modulated by PKC. For this purpose, the effect of PKC modulation and cytoskeletal disruption was examined for ^{3}H-taurine and ^{3}H-D-aspartate release. Figure 7 shows a decrease of ^{3}H-D-aspartate release by the PKC blocker chelerythrine and potentiation by up-regulation with PMA. None of these treatments affected ^{3}H-taurine efflux (fig. 7). Disruption of the actin cytoskeleton with cytochalasin E also failed to affect ^{3}H-taurine efflux but potentiated the release of ^{3}H-D-aspartate (fig. 7).

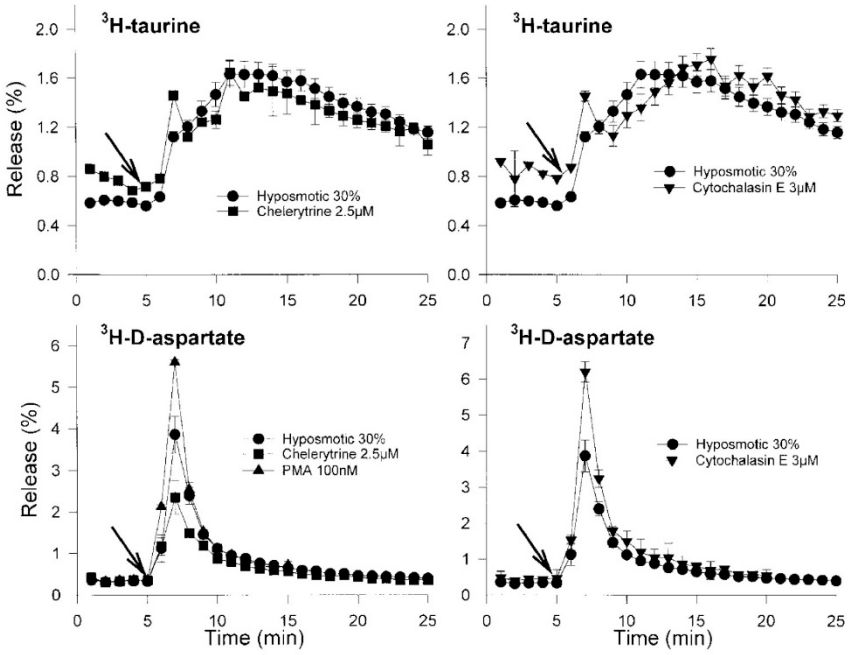

Figure 7. Effect of modulation of protein kinase C (PKC) activity and of cytochalasin E on ^{3}H-taurine and ^{3}H-D-aspartate release from rat hippocampal slices exposed to 30% hyposmotic medium. Slices were loaded with the labeled amino acids and then treated for 30 min with 2.5 μM chelerythrine (■) or 100 nM PMA (▲) or for 2 h with 3 μM cytochalasin E (▼) (vehicle controls ●). Thereafter, slices were superfused and data presented as in figure 1. Drugs were present in all solutions throughout the experiment. Means ± SE, n = 4–6; *$P < 0.001$.

Discussion

The present results showed the prompt release of ^{3}H-taurine, ^{3}H-GABA, and glutamate (followed as ^{3}H-D-aspartate) from hippocampal slices in response to hyposmolarity. These amino acids are those released preferentially under hyposmotic conditions from hippocampus, both in vitro and in vivo preparations [12, 27, 38]. The characterization of the amino acid osmosensitive release carried out in the present work, showed that while taurine efflux exhibited, in general, the features typically described for most cells, the mechanism of GABA and glutamate release was notably different. This interesting observation raises a number of questions about the source and the function of this release.

Taurine efflux

The hyposmolarity-induced taurine efflux from hippocampal slices showed the general pattern and pharmacological profile previously described for brain cells [36, 37]. Taurine release was inhibited by niflumic acid, NPPB, and DIDS and was sensitive to blockers of protein tyrosine kinases. Involvement of tyrosine kinases in the mechanism of taurine release in response to hyposmotic stress has been suggested in various cell types, including

brain cells, on the basis of the effects of tyrosine kinase and phosphatase blockers [7, 17, 19, 26, 28, 29]. Not all blockers exert the same effect, particularly in brain cells where tyrphostins are more potent than genistein. Astrocytes and cerebellar granule neurons [28, 29], and the hippocampal slices here reported, are particularly sensitive to tyrphostins, clearly more so than skate erythrocytes [17]. Herbimycin and lavendustin have no effect on taurine efflux in hippocampal slices, as is the case in Jurkat T-lymphocytes [26]. The meaning of these differences is still obscure but may be related to the involvement of tyrosine kinases with different sensitivity to the blockers in the different cell types. Inhibition of tyrosine phosphatases by *o*-vanadate potentiates osmosensitive taurine efflux in cultured cerebellar granule neurons and in rat supraoptic nucleus [7, 29]. This effect was not observed here in hippocampal slices or in trout red blood cells [19], despite the inhibitory effect of tyrosine kinase blockers in this preparation. It is unclear so far whether this is due to permeability restriction in hippocampal slices compared with cell monolayers or to a different sensitivity of the phosphatases involved.

While a role for tyrosine kinases in the mechanisms of hyposmolarity-induced taurine efflux seems well supported, the specific enzymes involved are not yet fully identified. Some possibilities were examined in the present study. The MAPK ERK1/2 were among the first kinases found to be activated by hyposmolarity in a variety of cell types, including epithelial cells, brain cells, and myocytes [13]. However, this reaction seems to be unrelated to the mechanisms of osmolyte extrusion, since its prevention by specific MAPK/ERK-kinase (MEK) blockers such as PD98059 consistently fails to modify Cl^- and taurine osmosensitive pathways [29, 42] (an exception is the volume-activated Cl^- currents in cortical astrocytes that are decreased by PD98059 [6]. Much the same can be concluded for MAPK-p38 that, despite being activated by hyposmolarity, appears to be unrelated to the volume-activated Cl^- pathway [41]. This lack of connection was also observed in the present study in hippocampal slices in which ERK1/2 and particularly p38 were activated by swelling but had no effect on taurine fluxes. These observations suggest that the activation of these MAPK may be epiphenomena, probably associated with some of the numerous events concurrent with cell volume changes, such as stress, adhesion, and cytoskeletal rearrangements, but clearly unrelated to the activation or operation of osmolyte extrusion pathways. In contrast, the tyrosine-activated kinase PI3K appears to play a key role in the signaling cascades leading to osmotransduction. Activation by hyposmolarity of PI3K and its prevention by wortmannin have been reported in Intestine 407 and hepatoma cells and in cholangiocytes [10, 24, 40]. In these cells wortmannin also blocks the volume-activated Cl^- currents [10, 40]. In hippocampal slices a robust activation of PI3K by hyposmolarity was observed, which was essentially abolished by wortmannin, and this blockade led to a dramatic inhibition of taurine efflux. Previously, we have observed potent inhibition by wortmannin in cultured cerebellar granule neurons [29]. LY294002 also decreased PI3K stimulation by hyposmosis, with lower potency, and, accordingly, had a weaker effect in decreasing taurine efflux. These results underline the essential role of this enzyme in the osmosensitive taurine pathway. The step within the taurine efflux transduction cascade where PI3K functions and its place in the hierarchy of signaling remain to be defined.

The involvement of phospholipases on taurine efflux activated by swelling was also examined in the present study. The most compelling evidence connecting phospholipases

to taurine efflux comes from a study in CHP-100 neuroblastoma cells, showing a concomitant hyposmolarity-induced release of arachidonic acid and taurine, both inhibited by AACOCF3, a specific blocker of the 85-kDa PLA2 [3]. This agent was ineffective in hippocampal slices and the more potent, irreversible blocker of this phospholipase, MAPF, was similarly ineffective. The two general blockers of phospholipases also tested, DEDA and 4BpB, had different effects. DEDA was ineffective whilst 4BpB was very potent. Considering the insensitivity of taurine fluxes to all other phospholipase blockers, the effect of 4BpB suggests an action different from phospholipase inhibition and allows the conclusion that, in our preparation, these enzymes do not participate in the taurine efflux activation. Similarly, in the brain in vivo, taurine efflux is insensitive to any of the phospholipase blockers, with exception of marginal inhibition by AACOCF3 [9].

The modulation by PKC, in connection with membrane actin cytoskeleton as a possible mechanism for amino acid release, was also examined in the present work. In hippocampal slices, taurine efflux was not influenced by PKC blockers or by actin cytoskeleton disruption, consistent with results in various cell types. These results further emphasize the marked differences here reported between the osmosensitive fluxes of taurine in comparison to those of GABA and glutamate.

An interesting finding of the present study was the detection of an early component of taurine efflux, uncovered by its resistance to both Cl^- channel blockers and tyrosine kinase inhibitors. This fraction, accounting for about 17% of the total amount of taurine released by hyposmolarity, activated and inactivated within the first 1–3 min after the stimulus and seemed, in general, similar to the efflux of GABA and D-aspartate, the features of which are discussed below.

GABA and glutamate (D-aspartate) efflux

Hyposmolarity elicited a rapid efflux of GABA and D-aspartate from hippocampal slices, with very rapid activation and inactivation. This time course contrasts with the delayed and sustained efflux of ^{3}H-taurine. The insensitivity of D-aspartate and GABA release to Cl^- channel blockers, which consistently inhibit the osmosensitive leak pathway for organic osmolytes, including taurine, suggests a different pathway for translocation of GABA and glutamate. Furthermore, this mechanism is not modulated by tyrosine kinases, which are clearly involved in the volume-activated osmolyte pathway in most cell types so far examined. A report on cultured astrocytes has also documented the insensitivity of the osmolarity-sensitive efflux of glutamate to tyrphostins [28]. All together, these results clearly indicate different translocation mechanisms for the osmolarity-evoked release of GABA and glutamate and possibly also different activation signals. A possibility explored in the present study was the reverse operation of the carrier. However, neither the ion dependence nor the sensitivity to specific carrier blockers supported this hypothesis. Regarding the activation signals, GABA and glutamate release may respond to other stimuli associated with events concurrent with swelling or/and volume regulation. The efflux may occur subsequent to an increase in cytosolic Ca^{2+} or to swelling-associated depolarization, known to occur in various cell types, including astrocytes [20, 32]. However, this response is likely to be due to rapid Cl^- extrusion through the volume-activated pathway and, consequently, should be prevented by niflumic acid or NPPB. Thus, the insensitivity of

GABA/glutamate release to these blockers is evidence against the notion of depolarization as the trigger for this release. In addition, there is no evidence of depolarization linked to swelling in hippocampal slices [5].

A further possibility is an exocytosis-mediated mechanism of release for GABA and glutamate, since hyposmotic stimulation leads to a robust increase in the rate of both endo- and exocytosis [43]. In support of this option are the present results suggesting that PKC and cytoskeleton could be effectors of the osmosensitive efflux of these amino acids (but not for taurine release). Much evidence indicates that the membrane actin network may act as a barrier to exocytotic phenomena, and that its disassembly dynamics are modulated by PKC. The Ca^{2+} independence of amino acid release here observed would argue against the possibility of vesicular exocytosis (typically Ca^{2+} dependent), as the mechanism of osmosensitive release. However, there is increasing evidence showing stimulation of exocytosis by phosphorylation reactions (PKC) without a Ca^{2+} signal, either directly releasing the brake for exocytosis, or increasing the Ca^{2+} sensitivity for exocytotic release [14, 23]. In this way, residual cytosolic Ca^{2+} may be a sufficient trigger for release in connection with phosphorylation reactions. Ca^{2+}-independent, PKC-mediated exocytosis, occurring via small GTPases has also been suggested as an alternate mechanism. These are interesting avenues for future research aimed to clarify the mechanisms of the osmosensitive release of some amino acids. In the meantime, the present results may contribute to explaining the effects of hyposmolarity on synaptic currents in hippocampal slices. Lowering osmolarity causes reversible, osmolarity-dependent enhancement of up to 400% of excitatory postsynaptic currents and of about 180% of the inhibitory postsynaptic currents [16]. These effects may be due in part to the enhanced efflux of glutamate and GABA reported in the present study. These results may also contribute to explain the increased seizure susceptibility observed during hyponatraemia [1, 2].

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