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Osmosensitive Release of Neurotransmitter Amino Acids: Relevance and Mechanisms

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Abstract
Hyposmolarity activates amino acid efflux as part of the corrective volume process in a variety of cells. This review discusses the mechanism of amino acid release in brain cells preparations. Results present evidence of substantial differences between the efflux of taurine and that of GABA and glutamate, which besides a possible role as osmolytes, have a main function as synaptic transmitters. The differences found concern the efflux time course, the sensitivity to Cl^- channel blockers, the modulation by tyrosine kinases, the influence of PKC and the effect of cytoskeleton disruptive agents. While taurine efflux features fit well with the mechanisms so far described in most cell types, the efflux of GABA and glutamate does not. Alternate mechanisms for the release of these two amino acids are discussed, including a PKC-modulated, actin-dependent exocytosis.

Keywords: hyposmolarity, tyrosine kinases, hyperexcitability, PKC

Introduction

Most cells respond to decreases in external osmolarity by rapid swelling, followed by a corrective process leading to cell volume recovery, usually referred as regulatory volume decrease (RVD) (1). This is an active process accomplished by the extrusion of intracellular osmolytes, occurring essentially through leak pathways. The main intracellular ions K^+ and Cl^- are important osmolytes, but also a number of organic molecules, polyols, organic amines and particularly amino acids, are significantly involved in RVD (fig. 1). In fact, the contribution of organic osmolytes in brain during chronic hyponatremia, is crucial for maintaining the brain water content within limits compatible with survival (2) (fig. 1).

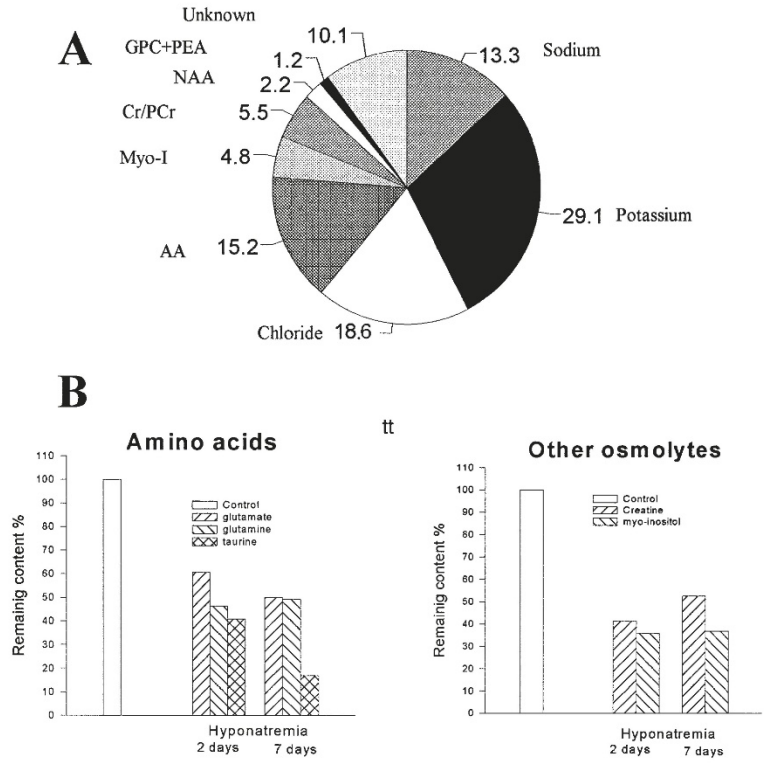

Figure 1. Inorganic and organic osmolytes involved in the control of brain water content during chronic hyponatremia in mice. **A:** Relative contribution of the various osmolytes to cell volume correction. **B:** Decrease in the brain content of amino acids, creatine, and myo-inositol after 2–7 days of chronic hyponatremia in mice. Empty bar: normal brain content of osmolytes. Recalculated from (2). GPC: glycerophosphorylcholine, PEA: phosphatidyl ethanolamine, NAA: N-acetyl aspartate, Cr/PCr: creatine/phosphocreatine, Myo-I: myo-inositol, AA: amino acids.

A simple approach to consider a molecule as potential osmolyte is to examine its response to hyposmotic swelling. A large number of reports in the last years document the osmosensitive efflux of amino acids in many cell types, including brain cells and glial cells

as well as neurons (3–5). Amino acid release has been also observed in more integrated preparations such as brain slices (6–8), supraoptic nucleus (9), and in the brain in vivo using paradigms of microdialysis or superfusion (10,11). Amino acids released upon hyposmosis are preferentially taurine, GABA, glycine, and glutamate. N-acetyl aspartate also seems to be released by hyposmolarity, particularly from neurons (12). The increased efflux of amino acids consistently observed in all these preparations has its counterpart in the decrease in tissue levels (10). In chronic hyponatremic mice, taurine concentrations are reduced to only 15% of its initial value, while glutamate levels are reduced to 60%. Studies about the mechanism of this release have been carried out preferentially on taurine because of its particular features such as metabolic inercy, high concentrations, and prompt release upon the hyposmotic stimulus (13). For these reasons, taurine has been considered as representative of amino acid osmolytes, and it has been currently assumed that results obtained for taurine are valid for other amino acids as well. In the present work, we present evidence about substantial differences between the mechanisms of hyposmolarity-activated release of taurine and those of GABA and glutamate, which not only suggest differences in the pathways but likely also in the functional meaning of this release.

The Efflux Pathway

Studies in cultured astrocytes and neurons document the efflux of taurine, GABA, glutamate, and glycine in response to hyposmotic stimulus (3). Taurine efflux occurs through a leak pathway, with essentially no contribution of the energy-dependent carrier (4,5). The diffusive nature of the taurine permeation pathway is suggestive of a channel-like molecule as the mechanism for efflux. Since osmosensitive taurine release in most cell types, including cultured astrocytes and neurons, is sensitive to Cl^- channel blockers (14), an anion channel-like molecule has been proposed as the common pathway for the corrective fluxes for Cl^- and amino acid during RVD. In fact, it is known that the volume-activated Cl^- channel exhibits a broad range of permeability, which includes all monovalent anions and even large molecules such as benzoate (15). Taurine, glutamate, and aspartate, all permeate through this channel when they are present in anionic form (15), suggesting that the size of the pore is large enough to allow the passage of these amino acids. Still, the question remains about how amino acids, which are found in the cell mostly as zwitterions, may translocate through an anion channel.

It has been often assumed that other amino acids released by hyposmolarity permeate by a similar pathway as taurine, but this has not been studied in detail. In a study in hippocampal slices directed to address to this question, we found remarkable differences between the efflux of taurine and the release of GABA and glutamate (16). The first main difference is the time course, as illustrated in figure 2A. While the efflux of taurine slowly activates and essentially does not inactivate, those of glutamate and GABA are characterized by rapid activation and inactivation. The sensitivity to Cl^- channel blockers further reveals these differences. In most cell types, the osmosensitive efflux of taurine is markedly decreased by agents such as NPPB (5-Nitro-[3-phenylpropylamino]benzoic acid), DDF (dideoxyforskolin), niflumic acid, and DIDS (4,4′-diisothiocyanato-stilbene-2-2′ disulfonic

acid), which also effectively block the volume-activated Cl^- channel (1,17). A report in cultured astrocytes shows inhibition by DIDS of glutamate efflux but none of the other blockers was examined (18). The referred study in hippocampal slices revealed the insensitivity of the release of GABA and glutamate to NPPB and niflumic acid, which are potent blockers of taurine efflux also in this preparation (16) (fig. 2B). Only DIDS, at high concentrations, was an efficient blocker of the efflux of GABA and glutamate.

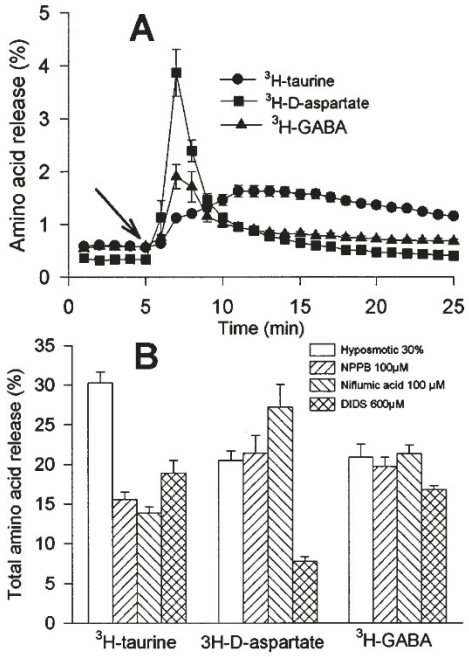

Figure 2. Time course and pharmacological profile of hyposmosis-induced amino acid efflux from rat hippocampal slices. **A:** Efflux time course. Slices were loaded with [^{3}H]taurine, [^{3}H]GABA, and [^{3}H]D-aspartate (as tracer for glutamate), washed and superfused with warmed isosmotic medium (1 ml/min) and after reaching stable basal efflux (arrow), superfusion continued with 30% hyposmotic medium, made by reducing NaCl. **B:** Effect of Cl^- channel blockers. Slices were preincubated 15 min in isosmotic medium with 100 mM NPPB, 100 µm niflumic acid or 600 µM DIDS. Blockers were present in all superfusion solutions. Bars represent the amino acid released during 20 min of exposure to hyposmotic medium minus the release in isosmotic medium during the same time period. Data are arranged from Franco et al. (16).

Release of taurine, glutamate, and GABA in response to hyposmolarity has also been observed in vivo using microdialysis or superfusion of the cortex surface (10,11). Here again, the release of aspartate and glutamate was insensitive to Cl^- channel blockers (11). All together, these findings showing major differences in the mechanism of amino acid release are suggestive of different pathways, which in the case of GABA and glutamate would also considerably differ from the volume-sensitive Cl^- channel.

Transduction Signaling

Volume regulation is a complex process which initiates by detection of the change in cell volume, followed by the trigger of signaling cascades, ultimately leading to activation of effectors, i.e., the various translocation pathways in charge of the osmolyte extrusion necessary for volume correction. At a certain point, the cell should "remember" its initial value and inactivate the efflux pathways.

Most work about the mechanisms of RVD has focused on the characterization of the osmolyte efflux pathways, and it is only recently that other aspects of the volume adjustment process have been examined. As the above mentioned, the first signal in the chain is necessarily the detection of the change in cell volume, and nevertheless, this volume sensor mechanism is essentially unknown in all cell types. As for the transduction signaling, some recent evidence relates second messengers such as Ca^{2+}, such protein phosphorylation and phospholipases as elements of the transduction cascade (19–22). Here again, marked differences have been found between a typical organic osmolyte such as taurine, and amino acids such as GABA and glutamate.

Calcium

A change in cytosolic calcium ($Ca^{2+}i$) could be a key transduction messenger, since with few exceptions, hyposmotic swelling always results in an increase in $Ca^{2+}i$ levels (20). In spite of this general response, RVD and osmolyte fluxes are either Ca^{2+}-dependent or Ca^{2+}-independent in the different cell types. Activation of the volume-sensitive Cl^- channel is essentially Ca^{2+}-independent in most cells. (rev. in 20). Similarly, taurine efflux is insensitive to changes in $Ca^{2+}i$ in most cell types, including cultured neurons and astrocytes (23,24). Taurine release from brain slices is also essentially Ca^{2+}-independent (16). The influence of Ca^{2+} on the efflux of glutamate and GABA has not been examined in detail. In hippocampal slices, it seems independent of external and internal Ca^{2+}, although cytosolic Ca^{2+} cannot be fully depleted (16). In contrast to taurine and Cl^- fluxes, the volume-activated K^+ fluxes are Ca^{2+}-dependent in numerous cell types but not in many others (20). It is worthy to note that most cells showing these Ca^{2+} requirement for swelling-activated K^+ channels, are epithelial cells and that the Ca^{2+}-activated BK-type channels appear those involved in K+ permeation during swelling. Obviously, in these cells, RVD is also Ca^{2+}-dependent, while it is not in cells where all the permeation pathways activated by swelling do not require Ca^{2+} (19).

Protein Phosphorylation

Recent evidence points to a key role of protein kinases as part of the signaling cascades to activate osmolyte fluxes (22,25). Tyrosine kinases appear involved in the mechanisms of osmosensitive release of Cl^- and taurine, as shown by the inhibitory effect of tyrosine kinase blockers such as tyrphostins, genistein, herbymicin, and lavendustin (19,26,27). Accordingly, blockers of tyrosine phosphatases, such as *ortho*-vanadate, which prolong the tryosine phosphorylation reactions, lead to marked potentiation of taurine and Cl^- fluxes in various cell types, including cultured neurons and astrocytes (19,28). These results further emphasize the close similarities between Cl^- and taurine translocation pathways. The

specific tyrosine kinases involved in osmolyte fluxes are not well identified. This is further complicated by the known activation of a number of tyrosine kinases by hyposmolarity and swelling, which however, appear unrelated to the operation of the osmolyte efflux pathways. This is the case of the MAP kinases ERK1/ERK2 and p38, which are indeed activated during swelling and RVD but have no influence on taurine and Cl^- release in various cell types, including neurons (28,29), and in hippocampal slices (fig. 3) (16). (An exception are cortical astrocytes in which ERK1/ERK2 appear connected with the volume-activated Cl^- channel) (30). This is not unexpected, since the hyposmotic shock and the subsequent swelling are complex phenomena, involving numerous processes such as cell adhesion and retraction, as well as dramatic changes in the cytoskeleton organization. All these adaptations represent also stressful situations for the cell and some of these kinases are stress-activated enzymes. Therefore, it is necessary to discriminate among the plethora of signals activated by hyposmosis and swelling, those being strictly connected with the activation of corrective osmolyte pathways leading to RVD.

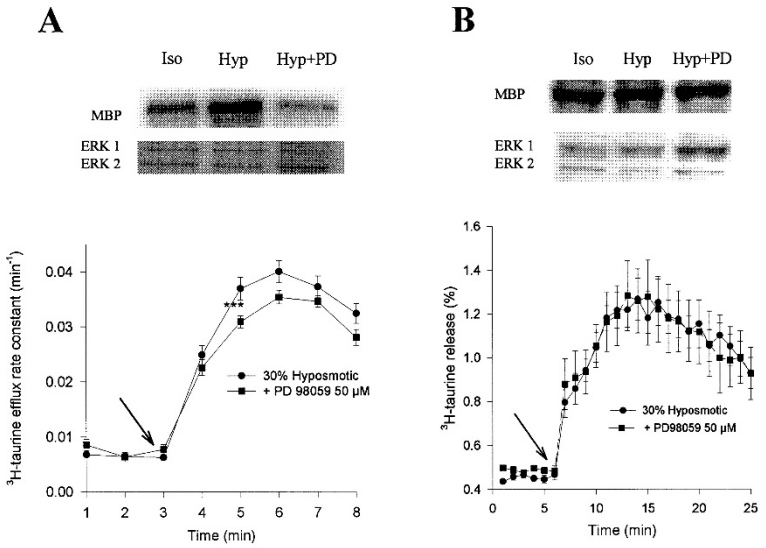

Figure 3. Activation of ERK1/2 by hyposmosis in cerebellar granule neurons and in hippocampal slices, and lack of connection of this activity with the osmosensitive taurine efflux. Cultured cerebellar granule neurons (**A**) or hippocampal slices (**B**) were exposed to media: isosmotic (Iso), 30% hyposmotic (Hyp), or 30% hyposmotic + 50 µM PD98058 (Hyp + PD). Upper panel: ERK1/ERK2 activity in the different conditions. ERK1/ERK2 assay was performed as described in Morales-Mulia et al. (26). Briefly, aliquots of the cell lysate were immunoprecipitated with a polyclonal antibody to ERK1/ERK2. Immune complexes were collected by protein A sepharose and incubated with myelin basic protein (MBP) as substrate and [^{32}P]ATP. The activity of ERK1/ERK2 was monitored via autoradiography after SDS/PAGE electrophoresis. Lower panel: Lack of effect of the ERK1/2 activation blocker PD98058 on the hyposmolarity-induced release of taurine, despite the reduction in ERK activity. A. reproduced from Morales-Mulia et al. (26) and B. from Franco et al. (16).

Studies so far have identified the tyrosine-kinase activated kinase P13K, as one directly related to taurine efflux during RVD. P13K is activated by hyposmolarity, and this reaction is blocked by wortmannin and LY294002 (16,28,31). These agents, particularly wortmannin, markedly inhibit taurine (and Cl^-) fluxes (16,28,31). The magnitude and the time course of taurine efflux reduction by P13K blockers are rather similar to those due to general blockers of tyrosine kinases, suggesting this enzyme as a main target of tyrosine kinase activity. A noteworthy effect of the Cl^- channel and tyrosine kinase blockers observed in hippocampal slices is the persistence of a small peak of taurine, evident only when the blockers have blunted most of the efflux. This component, which accounts for about 17%, shows rapid activation and inactivation and seems similar in all respects, to the D-aspartate efflux (fig. 4).

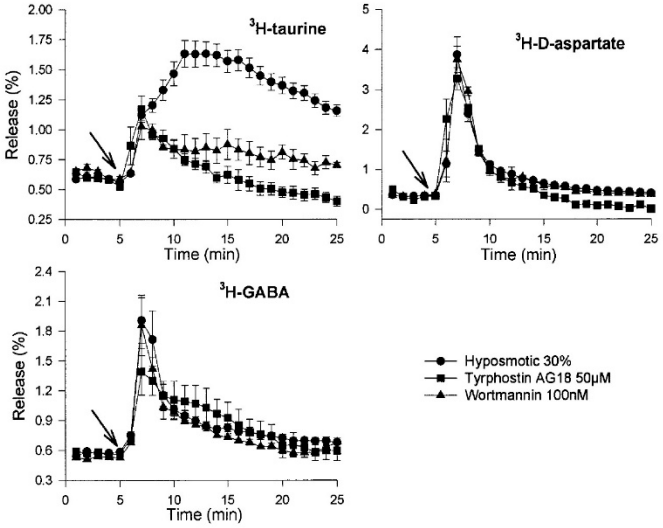

Figure 4. Effects of tyrosine kinase blockers on hyposmolarity-elicited release of taurine, GABA, and D-aspartate. Loading and superfusion of slices as in figure 2. Tyrphostine AG18 (50 μM), a blocker of tyrosine kinases, markedly reduced [^{3}H]taurine release without significantly affecting the efflux of [^{3}H]GABA and [^{3}H]D-aspartate. The same different effects are observed in the presence of wortmannin (100 nM), a blocker of the tyrosine kinase-activated kinase P13K. Both blockers revealed a resistant component of taurine efflux which, similar to those of GABA and glutamate, appears not modulated by tyrosine kinases. Data arranged from Franco et al. (16).

In contrast to these important influences of tyrosine and tyrosine-activated kinases on taurine fluxes, D-aspartate efflux is resistant to these agents, as reported in cultured astrocytes and hippocampal slices (16,32) (fig. 4). This remarkable difference is a clear indication of different signaling pathways for trigger of taurine and glutamate release. Being glutamate a neurotransmitter, its release may respond to other stimuli associated with events concurrent with swelling or/and volume regulation, such as swelling-associated depolarization, known to occur in various cell types, including astrocytes. However, this response

is likely due to rapid Cl^- extrusion through the volume-activated pathway, and should be consequently prevented by niflumic acid or NPPB. Thus, the insensitivity of GABA/glutamate release to these blockers is against the notion of depolarization as the trigger for glutamate release. The Na^+ and Cl^- independence of GABA and glutamate release as well as the insensitivity of this efflux to carrier blockers observed in hippocampal slices (16) seem to exclude the reverse operation of the energy-dependent transporter. The efflux is also independent of external Ca^{2+}, and insensitive to internal Ca^{2+} stores depletion, thus making unlikely the involvement of the typically Ca^{2+}-dependent vesicular release. However, this possibility cannot be definitely ruled out in the light of some recent evidence next discussed.

Calcium-Independent Exocytosis?

The hyposmotic stimulus trigger active phenomena of exocytosis in various cell types, which are either Ca^{2+}-dependent or -independent (33). This raises the possibility of exocytosis as the mechanism of GABA and glutamate release associated with swelling, although the vesicular release of these amino acids is typically Ca^{2+}-dependent. However, recent evidence points to occurrence of exocytotic release without a previous Ca^{2+} signal (34,35). The focus of this argument is that the submembrane actin filaments are organized as a mesh preventing exocytosis of the pool of synaptic vesicles trapped in this network. The disassembly dynamics occurs via Ca^{2+} and associated proteins and seems modulated by PKC, likely via an effect increasing vesicular release sensitivity to Ca^{2+}. Thus it can be speculated whether hyposmolarity in association with PKC could activate exocytosis at basal Ca^{2+} levels, or even at residual Ca^{2+} levels in Ca^{2+}-depleted preparations. In support of this hypothesis are results in hippocampal slices (16) showing that the osmosensitive release of glutamate is modulated by PKC (reduced by the blocker chelerythrine and enhanced by phorbol-12-myristate-13 acetate) and potentiated by the cytoskeleton disruption elicited by cytochalasin E. Noteworthy, these maneuvers do not affect taurine release, further emphasizing the differences in the mechanisms for release between these two amino acids.

These findings, although intriguing, have to be considered as preliminary, since they have so far been observed in only a few preparations. Such kind of studies should be extended to other brain preparations, including neurons of various types and different brain regions, and more importantly, to nonnervous cell types. It is not unlikely that in cells where these amino acids do not play a role as synaptic transmitters, their response to hyposmotic swelling is restricted to an osmolyte type and the mechanisms of release is in all alike to that of taurine.

In any event, the observations here presented explain a number of effects of osmolarity described in hippocampal slices, such as the reversible hyposmolarity-dependent enhancement of excitatory postsynaptic potentials, and to a lesser extent, also the inhibitory postsynaptic currents (36). This response of brain tissue to hyposmolarity, likely mediated by the mechanisms here described, may also explain the increased seizure susceptibility observed during hyponatremia (37).

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