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Depolarization, exocytosis and amino acid release evoked by hyposmolarity from cortical synaptosomes

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Abstract

External osmolarity reduction (20%) led to labelled glutamate, GABA and taurine release from rat brain cortical synaptosomes. A Cl^--independent, Na^+-dependent, La^{3+}-sensitive and tetrodotoxin (TTX) reduced depolarization of synaptosomes occurred upon hyposmolarity, suggestive of Na^+ entry through nonselective cation channels. This depolarization, together with cytosolic Ca^{2+} ($[Ca^{2+}]_i$) increase, resulted in exocytosis, monitored by FM1-43. The release fraction resulting from these phenomena was estimated, by its decrease, by La^{3+}, EGTA-AM and tetanus toxin (TeTX), as 34–44% for glutamate, 21–29% for GABA and 18–22% for taurine. Protein kinase C (PKC) activation by phorbol-12-myristate-13-acetate (PMA) increased the hyposmolarity-elicited exocytosis and this activation increased glutamate (80%), GABA (51%) and taurine (42%) hyposmotic efflux. Inhibition by chelerythrine reduced glutamate, GABA and taurine efflux by 64%, 50% and 24%, respectively. The Na^+-dependence of amino acid release (glutamate 63%, GABA 46% and taurine 29%) may result from both, prevention of the depolarization-exocytosis efflux, and blockade of the carrier reversal operation. Carrier blockade by DL-threo-β-benzyloxy aspartate (TBOA) and NO-711 resulted in 37% and 28% reduction of glutamate and GABA release, respectively. Contribution of the osmolyte leak pathway to amino acid release, estimated by the influence of Cl^- (NPPB) and tyrosine kinase (AG18) blocker, was up to 55% for taurine, but only 10–18% for GABA, with apparently no contribution for glutamate. The predominant osmolyte-type mechanism of taurine release suggest its function in volume control in nerve endings, while glutamate and GABA respond to events concurrent with hyposmolarity by a neurotransmitter-like release mechanism. The hyposmolarity-induced amino acid efflux from nerve endings may have consequences for neuronal excitability during hyponatremia.

Introduction

The ability to regulate volume after hyposmotic swelling has been preserved in most cells, even when surrounded by an external environment of controlled osmolarity (Lang *et al*., 1998). This persistent ability may be necessary for cells to correct local and transient volume changes associated with normal functions and metabolic reactions as well as in pathological situations. Such a requirement to keep cell volume constant has been proven critical in brain, where oedema is a most severe sequel event in numerous pathologies (Andrew, 1991; Kimelberg, 1999). In brain cells, as in many other cells, hyposmolarity leads to rapid swelling followed by a volume regulatory process, via the extrusion of K^+, Cl^- and some organic molecules, primarily amino acids (Kimelberg *et al*., 1990; Pasantes-Morales *et al*., 1993; Pasantes-Morales *et al*., 1994a,b). Less is known about the occurrence of such mechanisms in intracellular compartments or in specialized regions of neurons, such as dendrites or nerve endings, despite the fact that dendrites are particularly sensitive to swelling and nerve endings are continuously exposed to osmotic gradients due to the constant influx and efflux of ions and neurotransmitters. Work by Babila *et al*. (1990) showed that isolated nerve endings exposed to hyposmotic solutions first swell and then rapidly recover their original volume. A more recent report (Mongin *et al*., 1997) shows an increase in $[Ca^{2+}]_i$ as a consequence of swelling in synaptosomes but the influence of Ca^{2+} on the osmolyte corrective fluxes has not been addressed.

In the present study we investigated whether hyposmolarity, and the resulting swelling, activate amino acid extrusion from synaptosomes. Taurine acts as an osmolyte in many cell types, including brain cells, and may also play this role in synaptic endings, where it is present in high concentrations, in spite of its marginal function as neurotransmitter (Kontro *et al*., 1980). Other amino acids, such as GABA and glutamate – with a prominent role as neurotransmitters – are also involved in volume regulation mechanisms in brain cells and slices. The large concentrations (mM) of these amino acids in nerve endings (Lahdesmaki *et al*., 1977), largely exceeding those required for a synaptic transmitter function, is suggestive of an osmolyte role in nerve endings too. A hyposmolarity-evoked release of amino acids from synaptosomes may occur either by the leak pathway, well characterized in cultured cells, or by events concurrent with hyposmolarity, particularly depolarization, that in turn trigger other mechanisms of release. The first possibility was tested by the extent of inhibition by Cl^- channel blockers and by the dependence on tyrosine kinase reactions, as markers for the osmolyte-type release. As for the second possibility, as we found that hyposmolarity indeed results in synaptosome depolarization, we examined two possible mechanisms for release: (i) a Ca^{2+}-dependent exocytosis and (ii) the reversal of the carrier. All of these mechanisms may operate and contribute to the total release observed.

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Materials and methods

Materials

Salts (NaCl, KCl, KH_2PO_4, $CaCl_2$, $MgSO_4$ and $MgCl_2$), *N*-2-hydroxyethyl piperazine-*N*′-(2-ethanesulphonic acid) (HEPES) and glucose were from T. J. Baker (Xalostoc, Mexico); wortmannin, tyrphostin AG18, phorbol-12-myristate-13-acetate (PMA), chelerythrine chloride, cytochalasin E, 5-nitro-3-(3-phenylpromylamino)benzoic acid (NPPB), ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester (EGTA-AM) and tetanus toxin (TeTX) were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA); fura-2 acetoxymethyl ester (fura-2/AM), FM1-43 and bis-(1–3diethylthiobarbituric acid) trimethine oxonol [DiSBAC2(3)] (bisoxonol) were from Molecular Probes (Eugene, OR, USA). Tetrodotoxin (TTX) was from Alomone Laboratories (Jerusalem, Israel). [^{3}H]Taurine, [^{3}H]L-glutamate and [^{3}H]GABA were from New England Nuclear (Boston, MA, USA). NO-711 hydrochloride, DL-threo-β-benzyloxy aspartate (TBOA), 4,4′-diisothiocyanato-stilbene-2,2′ disulphonic acid (DIDS), bovine serum albumin (BSA), triton X-100, choline chloride and gluconate salts were from Sigma Chemical Co. (St. Louis, MO, USA).

Synaptosomal preparation and superfusion

Cerebral cortex was excised from male Wistar adult rats of about 180, after decapitation. A crude synaptosomal fraction was prepared by differential centrifugation according to Whittaker & Barker (1972). Purified synaptosomes were obtained as described elsewhere (Hajos, 1975) in a sucrose discontinuous gradient. Synaptosome suspensions were incubated under shaking with [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA (5 μCi/mL) during 30 min in isosmotic medium containing (in mM): NaCl, 135; KCl, 1; $CaCl_2$, 1; $MgSO_4$, 1.17; KH_2PO_4, 1.7; HEPES, 10 and glucose, 10. Hyposmotic media (10%, 270 mOsm; 20%, 240 mOsm) were prepared by reducing the necessary amount of NaCl. Osmolarity of all solutions was measured in a freezing point osmometer (Osmette A, Precision Systems Inc., Natick, MA, USA). After incubation, synaptosomes were separated from the loading solution by rapid filtration through a Millipore filter (pore size 0.45 μm) and rinsed with isosmotic medium. The filter membrane containing synaptosomes was transferred to a superfusion chamber (0.7 mL) and superfused with isosmotic medium at a flow rate of 1 mL/min. After a wash period of 15 min, fractions of the perfusate medium were collected at 1 min intervals into scintillation vials. The baseline was attained at about 3 min, after which the isosmotic medium was replaced by 20% or 10% hyposmotic medium and superfusion continued for further 12 min. At the end of the superfusion, synaptosomes were solubilized and the radioactivity of collected fractions and that remaining in synaptosomes was measured by scintillation spectrometry. Preincubation times with drugs varied depending on the experiment and are indicated in the corresponding figure legends. When required, controls always contained the vehicle. Results are expressed in percentage release in each fraction of the total radioactivity (i.e., radioactivity in samples plus radioactivity remaining in synaptosomes at the end of superfusion). The radioactivity of the hyposmotic superfusion fractions representing authentic GABA and glutamate was found to be 82.7% and 74.3%, respectively, as assessed by paper chromatography.

Depolarization measurement

Depolarization was measured by spectrofluorometry using an Aminco-Bowman luminescence spectrometer (series 2, SLM AMINCO; Rochester, NY, USA), equipped with a magnetic ministirrer and the fluorophore bisoxonol DiSBAC2(3) (150 nM). Bisoxonol was added to 2 mL of isosmotic medium in a quartz cuvette and the reaction started by addition of synaptosomes (40–50 μg protein, determined by the Bradford method; Bradford, 1976). After 30 s, the medium was diluted to reach 20% hyposmolarity and fluorescence changes were followed during further 60 s. Fluorescence intensity of the dye was recorded at excitation and emission of 540 and 580 nm, respectively (5 nm slits for both excitation and emission wavelengths). Bisoxonol fluorescence intensity variations were not converted into absolute membrane potential values as the valinomycin nullpoint method (Waggoner, 1976) could not be applied due to the formation of complexes between the lipophilic anion bisoxonol and the positively charged molecule of valinomycin.

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

Synaptosomes were incubated under shaking with the fluorescent Ca^{2+} indicator, fura-2/AM (2 μM) during 60 min. After this period, synaptosomes were spun at 25 000 ***g*** for 5 min to remove the extracellular dye. The synaptosomal pellet was resuspended in isosmotic medium and maintained with shaking during 30 min. Then, the preparation was centrifuged (25 000 ***g*** for 5 min) four times and the pellet resuspended in isosmotic medium. Changes in $[Ca^{2+}]_i$ were determined in an Aminco-Bowman luminescence spectrometer equipped with a magnetic ministirrer. Excitation wavelength was alternated between 340 and 380 nm and fluorescence intensity was monitored at 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the synaptosomes with 10% triton X-100, and the minimum fluorescence obtained after buffering the Ca^{2+} in the solution with 250 mM EGTA. The values obtained through this procedure were used to calculate the intracellular Ca^{2+} concentration according to previously published equations (Grynkiewicz *et al*., 1985).

Exocytosis measurements

Exocytosis was assayed by spectrofluorimetry as described by Guatimosim *et al*. (1997) using the probe FM1-43. Synaptosomes were incubated with 4 μM FM1-43 for 10 min in isosmotic medium and then during 1 min in medium containing 40 mM KCl. Loaded synaptosomes were washed three times with isosmotic medium plus BSA (1 mg/mL) by centrifugation and resuspension cycles, and transferred to a quartz cuvette for the fluorimetric assay. The experiment was performed on a luminescence spectrometer with continuous stirring. Samples were excited at 488 nm and the fluorescence emission was recorded at 560 nm.

Statistical analysis

Significance of differences in mean values was calculated using the two-tailed Student's *t*-test.

Results

Efflux of amino acids in response to hyposmolarity

Synaptosomes loaded with [^{3}H]taurine, [^{3}H]GABA, or [^{3}H]glutamate, and superfused with isosmotic medium, exhibited a basal efflux corresponding to 1–1.3% of the total radioactivity accumulated during loading (Fig. 1). Stimulation with 20% hyposmotic medium (−60 mOsm), led to an immediate increase in amino acid efflux, which reached a peak 2–3 min after the stimulus. At this time, the percentage efflux increased to a maximal of 5.8%, 5.5% and 3.7% for [^{3}H]glutamate, [^{3}H]taurine, and [^{3}H]GABA, respectively. After this maximal, the amino acid efflux decreased towards the initial values of release, despite the persistence of the hyposmotic condition. The time course of the osmosensitive release was rather similar for the three amino acids but with a more rapid inactivation for glutamate efflux

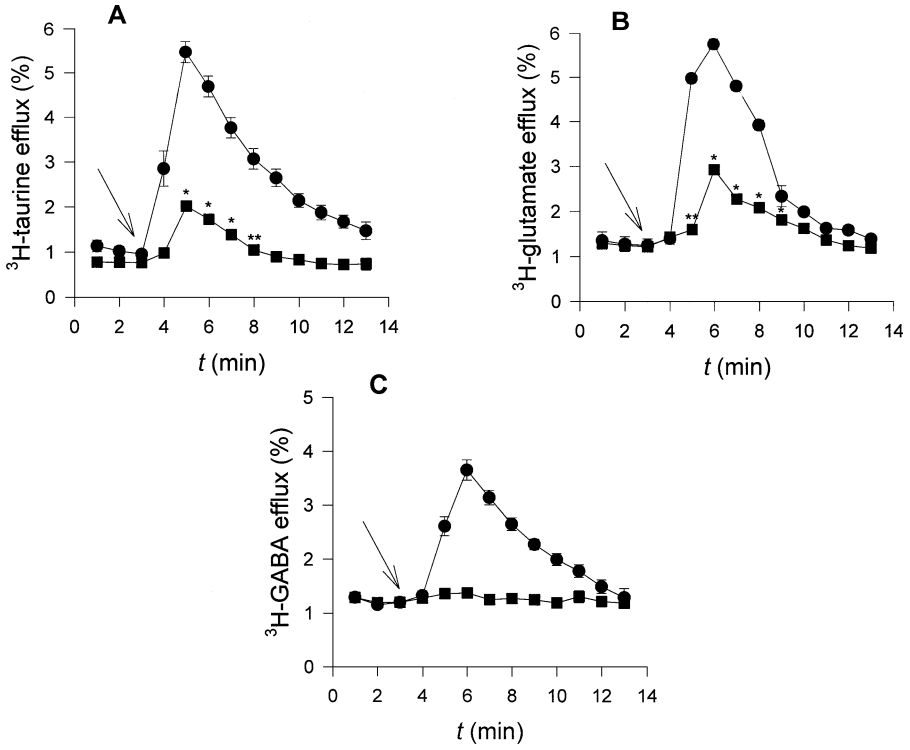

FIG. 1. Amino acid release from rat cerebral cortex synaptosomes exposed to 20% or 10% hyposmotic medium. Synaptosomes were preloaded with [^{3}H]taurine (A), [^{3}H]glutamate (B) or [^{3}H]GABA (C), washed and prepared for superfusion as described in Methods. Then, synaptosomes were superfused (1 mL/min) during 3 min with isosmotic medium to obtain a constant basal efflux. At the arrow, the medium was replaced by 20% (●), or 10% (■) hyposmotic solutions, and superfusion continued for 10 min. Fractions were collected every minute. Results are expressed as radioactivity released per min as a percentage of the total radioactivity incorporated. Data are means of 6–15 experiments. SE is represented as vertical bars when they exceed the size of symbols. $^*P < 0.001$, $^{**}P < 0.05$ with respect to the last point in isosmotic.

(Fig. 1A–C). The total amount released during the 10 min of stimulus was 28%, 27% and 19%, for [^{3}H]glutamate, [^{3}H]taurine and [^{3}H]GABA, respectively. A significant increase in glutamate and taurine efflux, but not in GABA efflux, was elicited by 10% hyposmotic medium (−30 mOsm relative to normal levels) (Fig. 1A–C). Amino acid release is a consequence of the osmolarity reduction and not of the decrease in NaCl, as no amino acid efflux was observed in solutions with low NaCl but made isosmotic with sucrose or mannitol. It is neither due to synaptosomal damage as no leakage of lactate dehydrogenase (LDH) was observed (results not shown).

Hyposmolarity-induced depolarization

Exposure to 20% hyposmotic medium resulted in a marked depolarization of nerve endings (Fig. 2). Depolarization may result from Cl^- efflux as part of the volume corrective process. However, depolarization was not affected by a prolonged exposure to Cl^--free medium (replaced by gluconate) nor by blockade of Cl^- fluxes by NPPB (Fig. 2A). In contrast, external Na^+ depletion essentially abolished it, suggesting that Na^+ influx caused depolarization. TTX (5 μM) decreased the hyposmolarity-induced depolarization by about 43% (Fig. 2B). Depolarization was unaffected in Ca^{2+}-free medium conditions (results not shown). Addition of 100 μM La^{3+} suppressed depolarization (Fig. 2B), implicating nonselective cation channels as a possible mechanism.

To evaluate the contribution of the hyposmolarity-induced depolarization to hyposmotic amino acid efflux, the effects of Na^+-free medium (NaCl replaced by choline Cl) or of La^{3+}, were then examined. In the presence of 100 μM La^{3+}, the efflux of glutamate, GABA and taurine decreased 44, 29 and 22%, respectively (Fig. 3A). In Na^+-free medium, glutamate, GABA and taurine release was reduced by 63%, 46%, 29%, respectively (Fig. 3B).

Effect of carrier blockers

The Na^+-dependence of amino acid efflux may represent either the release fraction responding to depolarization, or that occurring by reversal of the carrier operation. This later possibility was examined in synaptosomes treated with competitive, nontransportable blockers of the GABA and glutamate carriers. Figure 3C shows the effect of NO-711 and of TBOA, nontransportable blockers of GABA and glutamate transporters, respectively, on the hyposmotic release of amino acids. NO-711 reduced GABA release by 28% and TBOA decreased glutamate release by almost 37% (Fig. 3C). Blockade of taurine transport was not performed due to lack of nontransportable carrier inhibitors.

Ca^{2+} and PKC

Hyposmotic swelling significantly increased $[Ca^{2+}]_i$ in nerve endings as reported previously by Mongin *et al*. (1997). Upon 20% reduction in osmolarity, the initial $[Ca^{2+}]_i$ of 365 nM increased to 580 nM. This $[Ca^{2+}]_i$ elevation is due predominantly to an influx of external Ca^{2+}, as in Ca^{2+}-free medium containing 0.1 mM EGTA plus 10 mM $MgCl_2$, hyposmolarity elicited only a marginal increase in $[Ca^{2+}]_i$. Under this condition, the basal $[Ca^{2+}]_i$ levels showed a marked decrease to 230 nM (Fig. 4A). In synaptosomes treated with EGTA-AM, $[Ca^{2+}]_i$ basal levels further decreased to 105 nM and no increase was induced by the hyposmotic condition (Fig. 4A). The effect of $[Ca^{2+}]_i$ reduction was examined in the hyposmotic release of amino acids in synaptosomes treated with EGTA-AM. A decrease in the efflux of all amino acids was observed, with glutamate the most sensitive to this treatment

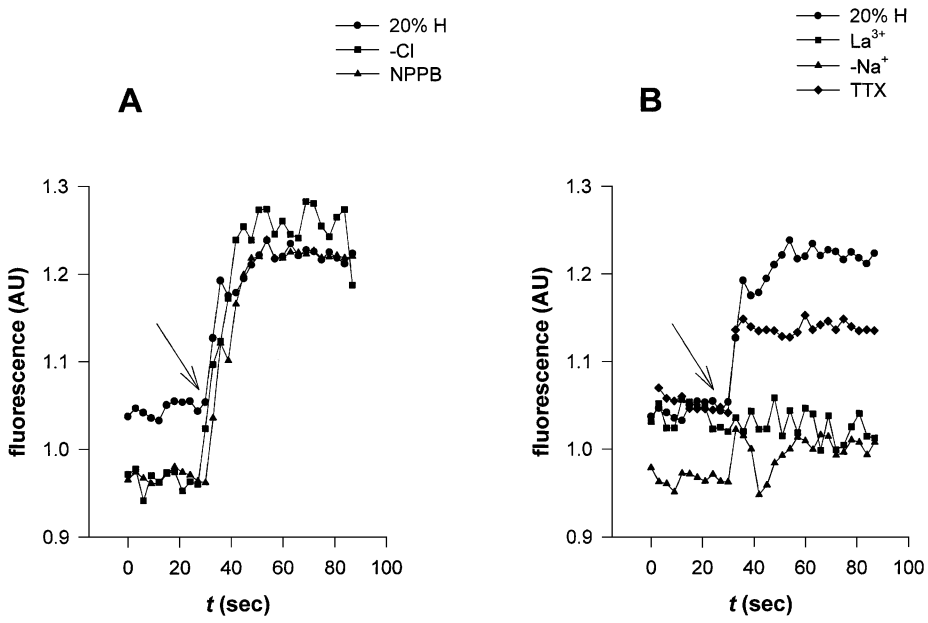

FIG. 2. Depolarization elicited by hyposmotic medium. Membrane potential was monitored by changes in fluorescence in synaptosomes loaded with bisoxonol, as described in Methods. Fluorescence was measured in isosmotic medium (30 s) and at the arrow, the medium was diluted up to reach 20% hyposmolarity, and fluorescence changes were followed for a further 60 s. (A) Control, 20% hyposmotic (●). Cl^- free medium (Cl^- replaced by gluconates) (-Cl^-; ■); 100 μM NPPB (NPPB; ▲). (B) Control, 20% hyposmotic (●). Na^+-free medium (Na^+ replaced by choline chloride) (–Na^+ ▲); 100 μM La^{3+} (La^{3+} ■); 5 μM TTX (TTX; ◆). Synaptosomes were exposed to the Cl^- or Na^+-free solutions during 15 min before and through all the experiment and during 10 min to TTX. NPPB and La^{3+} were added together with water at the hyposmotic stimulus. Values represent fluorescence arbitrary units (AU), after subtracting the dilution factor. Representative results of 3–4 experiments.

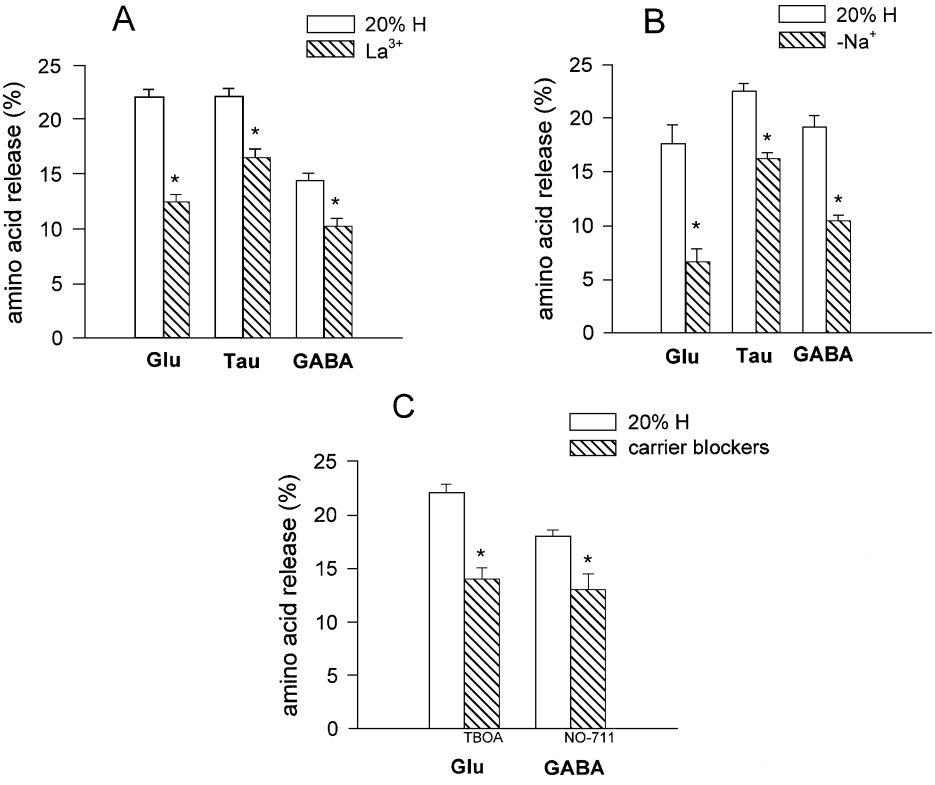

FIG. 3. Effects of Na^+-free medium, La^{3+} and carrier blockers on the hyposmotic release of amino acid from synaptosomes. Synaptosomes were preloaded with [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA and superfused as in Fig. 1. Bars represent the radioactivity released (%) at the peak release fractions (4–8 for taurine and 5–9 for glutamate and GABA). Empty and dashed bars correspond to control and experimental conditions, respectively. (A) Effect of 100 μM La^{3+} (La^{3+}). La^{3+} was present only in the hyposmotic medium. (B) Effect of Na^+-free medium (–Na^+). Synaptosomes were exposed to the Na^+-free medium (NaCl replaced by choline chloride, starting at washing and through all the experiment). (C) Effect of TBOA (100 μM) or NO-711 (20 μM). Synaptosomes were treated with the carrier blockers during 15–20 min prior to superfusion and were present throughout the experiment. Data are means ± SE of 6–17 experiments. $^*P < 0.001$ with respect to controls.

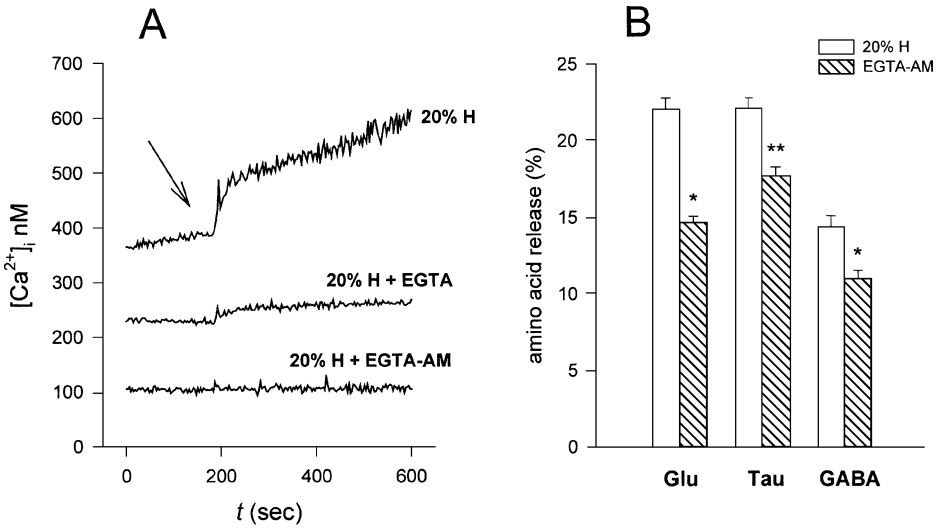

FIG. 4. Change in synaptosomal $[Ca^{2+}]_i$, elicited by hyposmolarity and its effect on amino acid efflux. (A) Synaptosomes were loaded with fura-2/AM and $[Ca^{2+}]_i$ determined as described in Methods. $[Ca^{2+}]_i$ was measured in isosmotic medium in synaptosomes under continuous stirring. At the arrow, the medium was diluted up to reach 20% hyposmolarity (20% H). In 20% H + EGTA, the experiment was carried out in Ca^{2+}-free medium containing 10 mM $MgCl_2$ plus 0.1 mM EGTA. In 20% H + EGTA-AM, synaptosomes were preincubated (15 min) with 50 μM EGTA-AM in a Ca^{2+}-free medium. Fluorescence units were transformed into Ca^{2+} concentration as described in Methods. Curves representative of 3–5 separate experiments. (B) Effect of EGTA-AM (dashed bars) on the hyposmotic (20% H, empty bars) release of amino acids. Data are means ± SE of 4–17 experiments. $^{*}P < 0.001$, $^{**}P < 0.01$.

(34% decrease). GABA and taurine efflux decreased 24% and 20%, respectively (Fig. 4B).

The effect of modulating PKC activity, i.e. inhibition by chelerythrine and activation by PMA, was examined on the hyposmotic amino acid efflux. Chelerythrine (2.5 μM) reduced (> 64%) the efflux of [^{3}H]glutamate and PKC activation by PMA (100 nM) increased it almost 80% (Fig. 5A). Chelerythrine reduced (50%) the efflux of GABA, and PMA increased it by 51%. The efflux of taurine was decreased by chelerythrine (24%) and enhanced by PMA (42%; Fig. 5A). Potentiation by PKC of glutamate release was essentially abolished in synaptosomes treated with EGTA-AM (Fig. 5B). Replacement of Na^+ by choline Cl or *N*-methyl-D-glucamine, also suppressed the stimulatory effect of PKC (Fig. 5B) (taurine and GABA were not examined).

Hyposmolarity-induced exocytosis

Exocytosis associated with hyposmolarity was monitored by the decrease in fluorescence release of FM1-43 dye, previously incorporated into synaptic vesicles by synaptosomal exposure to 40 mM KCl. The accumulated dye is released into the extracellular space, where it looses its fluorescence when the vesicle lumen is exposed to the extracellular medium. Release of FM1-43 is then detected as a fluorescence decrease, reflecting the exocytosis extent. As shown in Fig. 6, hyposmolarity induced a rapid decrease in FM1-43 fluorescence intensity, which exceeded the change in isosmotic conditions, due to medium dilution. Hyposmotic-induced drop in FM1-43 fluorescence was prevented by preloading synaptosomes with EGTA-AM, and in free-Na^+ conditions, reflecting both Ca^{2+} and Na^+-dependence of

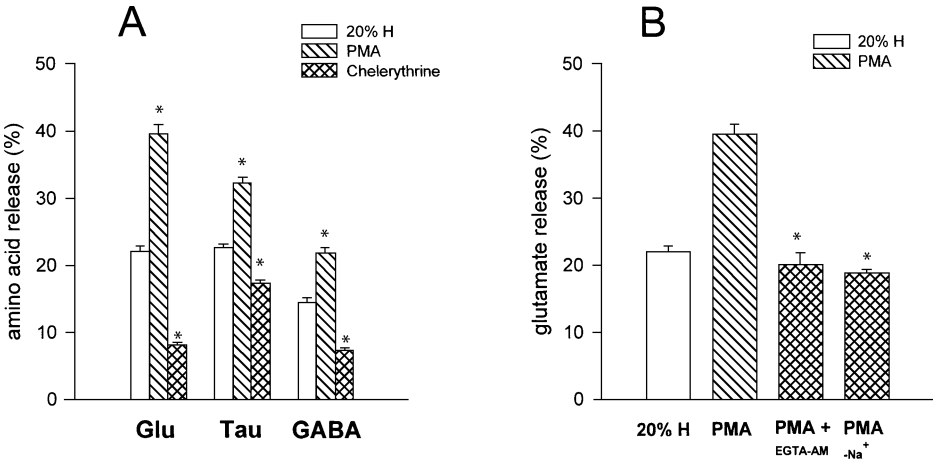

FIG. 5. Effect of PKC activity modulation on hyposmolarity induced amino acid release from synaptosomes. (A) Effects of 100 nM PMA and 2.5 μM chelerythrine (15 min preincubation and throughout the experiment) on [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA release. Data expressed as in Fig. 3, are means ± SE of 4–8 experiments. Significantly different from 20% H without additions, by $^{*}P < 0.001$. (B) Effect of Ca^{2+}- and Na^+-free media on the PMA-potentiated 20% hyposmotic release of glutamate. Sodium substitution, PMA, -Na^+ and EGTA-AM conditions were as described in Methods. In all cases, bars represent the radioactivity released by hyposmolarity as in Fig. 3. Data are means ± SE of 4–8 experiments. $^{*}P < 0.001$ with respect to PMA-treated condition.

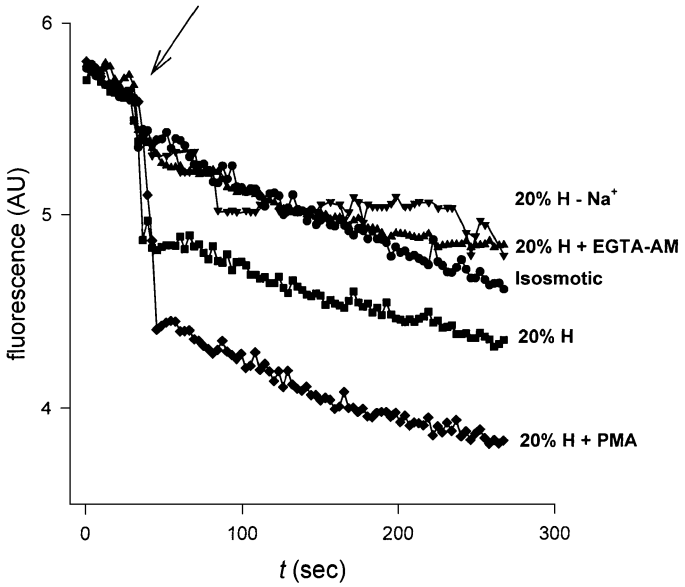

FIG. 6. Hyposmolarity-elicited exocytosis in synaptosomes. Exocytosis was monitored by FM1-43 fluorescence decrease as described in Methods. After 30 s in isosmotic condition (arrow), the medium was diluted to reach 20% hyposmolarity (■). Control condition (isosmotic) (●) was made using the same volume addition of isosmotic medium. The Na^+-free (▼) EGTA-AM (▲) and PMA (◆) pretreatment was as described in Methods. Data are expressed as fluorescence arbitrary units (AU). Each trace is representative of three separate experiments.

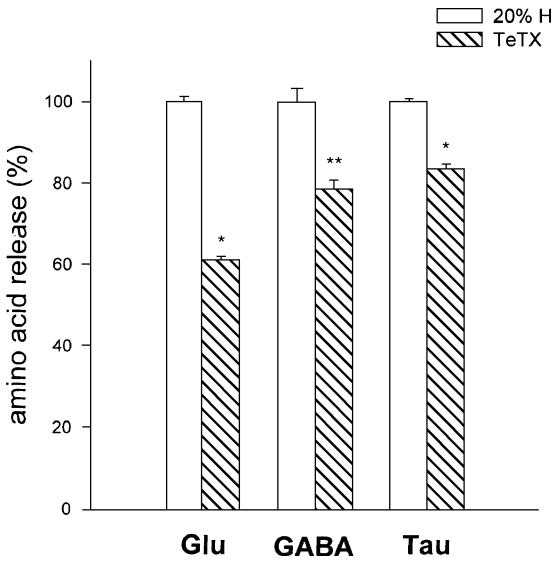

FIG. 7. Effect of TeTX on hyposmotic amino acid release from synaptosomes. Synaptosomes preloaded with [^{3}H]glutamate, [^{3}H]GABA and [^{3}H]taurine were preincubated during 90 min in isosmotic medium in the presence of 50 nM TeTX with 0.1% BSA. Thereafter, synaptosomes were superfused as in Fig. 1. Empty bars represent net release (hyposmotic minus isosmotic release) as 100%. Dashed bars correspond to percentage decrease by the toxin. Data are means ± SE of three to six experiments. $^*P < 0.001$, $^{**}P < 0.01$.

hyposmolarity-induced exocytosis. PMA pretreatment markedly potentiated hyposmolarity-evoked exocytosis (Fig. 6).

The effect of tetanus toxin (TeTX) on amino acid release

The above results raise the possibility of vesicular release as the mechanism for a fraction of the hyposmotic amino acid efflux. To test this hypothesis, the efflux of GABA, glutamate and taurine was measured in synaptosomes treated with tetanus toxin (50 nM). The treatment resulted in a significant reduction of 39%, 21% and 18% for glutamate, GABA and taurine, respectively (Fig. 7). The effect of this toxin could not be tested directly on exocytosis due to interference of the toxin with the fluorophore used.

Effect of Cl^- channel blockers and of inhibition of protein tyrosine kinase

In a large variety of cells, including neurons in culture, the efflux of taurine activated by hyposmolarity occurs through a leak pathway markedly sensitive to anion channel blockers (Pasantes-Morales & Schousboe, 1997). Two of those agents, NPPB and DIDS, were tested on the osmosensitive release of amino acids from synaptosomes, at concentrations that effectively reduce hyposmotic taurine efflux in cultured neurons. The release of taurine was reduced (53–55%) by 100 μM NPPB and 600 μM DIDS. These agents decreased GABA efflux by 18% and 15% but only DIDS decreased glutamate efflux by 12% (Table 1).

Osmosensitive fluxes of taurine in neurons and in other cell types are markedly dependent on the activity of tyrosine kinases, being reduced particularly by tyrphostins (Pasantes-Morales *et al*., 2000). The effect of the tyrosine kinase general blocker, tyrphostin AG18 (50 μM) was tested on amino acid fluxes from synaptosomes and results are shown in Table 1. Marked differences were observed in the effect of this agent between the three amino acids. Whereas taurine efflux was decreased (41%), that of [^{3}H]GABA was reduced only by about 10%, and that of [^{3}H]glutamate was unaffected (Table 1).

Discussion

Mild decreases in osmolarity led to taurine, GABA and glutamate efflux from synaptosomes. This release may occur by the well characterized leak pathway, through which organic osmolytes are extruded in response to hyposmolarity, as part of the volume corrective process (Kimelberg *et al*., 1990; Pasantes-Morales *et al*., 1993; Junankar & Kirk, 2000; van der Wijk *et al*., 2000). Alternately, or in addition, the efflux of amino acids, particularly that of GABA and glutamate – that play a prominent role as neurotransmitters – may result in part, from activation of synaptic mechanisms triggered by the hyposmolarity-induced depolarization found in our study. The present results showed that these two mechanisms are indeed operating during amino acid release, with differences in contribution for each amino

TABLE 1 Effect of the Cl^- channel blockers NPPB and DIDS, and the tyrosine kinase blocker tyrphostin AG18 on hyposmolarity-evoked amino acid release

	Amino acid release (%)		
Blockers (μM)	Glutamate	GABA	Taurine
Control hyposmotic	22.0 ± 0.71	14.3 ± 0.71	21.3 ± 0.72
NPPB (100)	21.9 ± 1.16	11.7 ± 0.75**	9.5 ± 0.71*
DIDS (600)	19.3 ± 1.12	12.1 ± 0.90**	10.0 ± 0.93*
AG18 (50)	23.6 ± 0.35	12.0 ± 0.23**	12.7 ± 0.26*

Synaptosomes preloaded with [^{3}H]taurine, [^{3}H]glutamate or [^{3}H]GABA were preincubated during 30 min in isosmotic medium in the presence of the blockers, and superfused as in Fig. 1. The blockers were present in all solutions throughout the experiment. The numbers correspond to radioactivity released at the peak release fractions as in Fig. 3 and are means ± SE of 4–11 experiments. $^*P < 001$, $^{**}P < 0.05$ with respect to controls.

acid. The reversal operation of the carrier also contributes to GABA and glutamate release.

Osmosensitive amino acid release mediated by depolarization and exocytosis

Hyposmolarity caused a large depolarization in synaptosomes. Depolarization evoked by hyposmolarity occurs in a variety of cell types and is mediated by activation of Cl^- exit through conductive channels (Diener & Scharrer, 1995; Hallows & Knauf, 1994; Kinard *et al*., 2001), or by cation influx, mainly Na^+ (Kim & Fu, 1993; Welsh *et al*., 2000). Depolarization in synaptosomes was unrelated to Cl^- efflux but resulted from Na^+ influx, possibly through nonspecific cation channels, as suggested by its prevention in Na^+-free medium or by La^{3+}. Activation of nonselective cation channels by hyposmolarity has been described in smooth muscle cells (Welsh *et al*., 2000), in atrial cells (Kim & Fu, 1993; Crumb *et al*., 1995) and in the A6 epithelial cell line (Li *et al*., 1998). Opening of these channels leads to membrane depolarization, carried by Na^+. This type of channel may be responsible for the swelling-induced depolarization in synaptosomes. This initial depolarization probably activates voltage-dependent Na^+ channels, as shown by partial TTX inhibition, which will further contribute to the observed depolarization. An interesting finding in the present study was the occurrence of hyposmolarity-induced exocytosis, which was depolarization- and $[Ca^{2+}]_i$-dependent. A fraction of amino acid extrusion in hyposmotic conditions occurs via this depolarization-exocytosis mechanism, as indicated by: (i) its reduction by preventing depolarization, namely in the absence of Na^+ and in the presence of La^{3+} (ii) its decrease in conditions of low $[Ca^{2+}]_i$, which abolished the hyposmolarity-induced exocytosis, and (iii) its reduction by TeTX. The contribution of this mechanism to amino acid release can be estimated by the magnitude of the Ca^{2+}-dependent fraction and the extent of reduction by La^{3+} and TeTX. The decrease in Na^+-free medium is not conclusive for this estimation, as in this condition, the carrier-mediated release is also affected. According to these estimations, the fraction responding to depolarization-exocytosis ranges form 34 to 40% for glutamate, 21 to 29% for GABA and 18 to 22% for taurine (Table 2). PKC activation by PMA increased the hyposmolarity-evoked exocytosis, a finding in line with the facilitatory action of PKC on vesicular release described in several preparations, including nerve endings (Majewski & Iannazzo, 1998; Vaughan *et al*., 1998). Thus, the influence of PKC is also indicative of the importance of exocytosis as a mechanism for amino acid release. In agreement with the different contribution of exocytosis as a mechanism for amino acid release, i.e. higher for glutamate and lower for taurine, glutamate efflux was the most sensitive and taurine the least sensitive to manipulations of PKC activity. It was found that PKC activation increased the hyposmotic efflux of glutamate, GABA and taurine by 80%, 51% and 42%, respectively, while PKC blockade by chelerythrine decreased the amino acid hyposmotic efflux as follows: 64% for glutamate, 50% for GABA and 24% for taurine. PKC modulates hyposmotic glutamate (but not of taurine) efflux in hippocampal slices (Franco *et al*., 2001), and some influence of PKC on hyposmotic amino acid release has been also reported in brain *in vivo* (Estévez *et al*., 1999b).

Altogether, these results suggest a chain of events for the hyposmolarity-induced release of amino acids with the following sequential steps: (i) hyposmolarity activation of nonspecific cation channels; (ii) Na^+ entry and subsequent nerve ending depolarization; (iii) Na^+- and Ca^{2+}-dependent exocytosis and (iv) PKC-modulated exocytotic release of amino acids. The initial step in this cascade of phenomena elicited by hyposmolarity seems to be the activation of nonselective cation channels, but the mechanism of this activation has not been explored in detail in the present study. Swelling-activated nonselective cation channels have been reported in atrial cells (Kim & Fu, 1993) and in renal epithelial A6 cells (Marunaka *et al*., 1997). In cerebrovascular smooth muscle, cation channels mediating depolarization have been found activated by swelling or intravascular pressure (Welsh *et al*., 2000).

The hyposmotic amino acid release occurs in part by the carrier reversal operation

The Na^+ entry which seems to occur upon hyposmolarity may activate the reversal operation of the carrier, a mechanism responsible for amino acid release in a variety of conditions, including ischemia and energetic failure (Attwell *et al*., 1993; Katsumori *et al*., 1999; Rossi *et al*., 2000). A fraction of 28% of the release of GABA and 37% for glutamate, implicates this mechanism, as shown by the efflux reduction in the presence of the competitive, non transportable carrier blockers NO-711 and TBOA. The contribution of this mechanism to taurine efflux could not be investigated due to the lack of reliable blockers of taurine transport.

Contribution of the osmolyte leak pathway

The depolarization-/exocytosis-independent fraction of glutamate, GABA and taurine osmosensitive release may occur through the well known leak pathway, typical for organic osmolyte extrusion. This pathway is characteristically sensitive to Cl^- channel blockers and is modulated by tyrosine kinase activity (Junankan & Kirk, 2000; van der Wijk *et al*., 2000; Pasantes-Morales & Franco, 2002). Considering these two properties as markers for the leak pathway, we present here results suggesting that a substantial amount of taurine efflux (up to 55%) occurs through this pathway, while this mechanism appears to contribute only about 10–18% to the release of GABA (Table 2). The insensitivity of glutamate release to tyrphostins (Mongin *et al*., 1999) and to NPPB, suggests that the leak pathway is not implicated. The decrease in glutamate release by DIDS may be due to an effect of this agent on the nonselective cation channel activity (Welsh *et al*., 2000).

These results suggest that the release of taurine from synaptosomes mainly reflects its osmolyte role, which is widely documented in numerous brain preparations including neurons and astrocytes in culture (Pasantes-Morales & Schousboe, 1997), brain slices (Oja & Saransaari, 1992; Deleuze *et al*., 1998; Franco *et al*., 2001) as well as in brain *in vivo* (Estévez *et al*., 1999a). An osmolyte role for taurine in nerve endings is further suggested by its high concentrations in

TABLE 2. Contribution of depolarization-exocytosis, leak pathway and carrier reversal to the hyposmolarity evoked release of glutamate, GABA and taurine

	Fraction release (%)		
	Glutamate	GABA	Taurine
Depolarization-exocytosis†	34–44	21–29	18–22
Leak pathway‡	–	10–18	41–55
Carrier reversal§	37	28	ND

†The contribution of depolarization-exocytosis was estimated by the effect reducing amino acid release of La^{3+}, EGTA-AM and TeTX. ‡Estimated by the inhibitory effect of Cl^- channel- and tyrosine kinase-blockers. §Estimated by the reduction observed in the presence of the competitive, nontransportable blockers NO-711 for GABA and TBOA for glutamate. ND, not determined. As described in Methods, the efflux of labelled GABA and glutamate after the time required to complete the experiments, corresponds to about 80% of the nonlabelled amino acids. Thus, the fraction of release through the different routes, illustrated in this Table may have been overestimated, but as the labelled/unlabelled ratio was found essentially constant in all conditions, the relative contribution of each pathway likely remains the same.

synaptosomes and in synaptic vesicles, despite its negligible function as neurotransmitter (Lahdesmaki *et al*., 1977; Kontro *et al*., 1980). Also along this line is the presence of taurine in the purely cholinergic nerve endings of the *Torpedo* electric organ (Vyas & Bradford, 1987) and the corelease of taurine and glutamate from cerebellar granule neurons (Holopainen *et al*., 1989). Thus, the synaptic taurine pool may be released in connection with swelling events concurrent with synaptic function. Even the taurine release fraction associated with exocytosis may result from a vesicular pool involved in the osmotic control of the vesicular compartment. The significance of the glutamate and GABA osmosensitive release is less clear. The mechanism of this release shows the characteristic features of vesicular release, but the amino acid concentrations in nerve endings largely exceed those required for a neurotransmitter function (Kontro *et al*., 1980), and some implication in an osmolyte role cannot be excluded.

Consequences of the osmosensitive release of amino acids from nerve endings

Hyposmotic swelling in brain occurs in numerous pathologies associated with hyponatremia. This condition occurs either from water excess or from a Na^+ deficit. Water excess may come from excessive oral intake, as in psychotic polydipsia, or more commonly from impaired renal water elimination as a consequence of inappropriate secretion of antidiuretic hormone, glucocorticoid deficiency, hypothyroidism, and renal or hepatic failure. In addition, a variety of diseases or conditions such as head trauma, brain tumour and cerebrovascular accidents result in hyponatremia associated with the syndrome of inappropriate secretion of antidiuretic hormone or the cerebral salt wasting syndrome. Hyponatremia also results from Na^+ loss due to mineralocorticoid deficiency, nephrotic syndrome, osmotic diuresis, vomiting or diarrhea, or during rapid correction of uremia by excessive haemodialysis and by infusion of hypotonic solutions in the perioperative period. Hyponatremia is a common state in the elderly and during pregnancy (Law, 1989; Verbalis, 1998; Fall, 2000). Fatal hyponatremia-induced cerebral oedema has been recently associated with 'ecstasy' use (Holmes *et al*., 1999).

The extent of swelling in different brain cell types or in discrete neuronal regions has not been examined in detail, although swelling in dendrites has been observed in hyposmotic conditions (Andrew *et al*., 1997). Nerve ending swelling has been reported in head trauma (Castejon *et al*., 1995), kainate-induced seizures (Sperk *et al*., 1983) and kindling (Langmeier *et al*., 1982). It may occur also in hyponatremia, as suggested by studies in hippocampal slices, where osmolarity reduction causes an increase in amplitude of evoked field potentials and of excitatory postsynaptic potentials which is inversely related to osmolarity (Chebabo *et al*., 1995; Schwartzkroin *et al*., 1998). Hyposmolarity does not affect cell properties such as resting membrane potential, cell input resistance and action potential threshold and duration, suggesting a hyperfunction of excitatory synapses (Chebabo *et al*., 1995). Hyposmolarity similarly affects the inhibitory postsynaptic potentials, with notably less effect, though, than on the excitatory potentials (Andrew, 1991; Chebabo *et al*., 1995; Huang *et al*., 1997). The release of glutamate and GABA from nerve terminals documented here may be responsible for these effects. This finding may also contribute to explain the generation of epileptiform activity and increased susceptibility to seizures in chronic and acute hyponatremia (Andrew, 1991).

The swelling-evoked taurine release from nerve endings, and probably also from synaptic vesicles, is an interesting finding as taurine has a marginal (if any) role as a neurotransmitter. The presence of this swelling-responsive synaptic taurine pool may reflect a need for volume correction mechanisms in the nerve terminal and/or in synaptic vesicles under physiological conditions, resulting from ion redistribution related to synaptic activity. In pathological conditions, the release of taurine may have a dual benefit, relieving swelling first, and once translocated into the extracellular space, acting as neuroprotectant, a well documented action of this amino acid (Saransaari & Oja, 2000).

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