European Journal of Neuroscience, Vol. 22, pp. 1636–1642, 2005

Hyposmolarity evokes norepinephrine efflux from synaptosomes by a depolarization- and Ca^{2+}-dependent exocytotic mechanism

K. Tuz and H. Pasantes-Morales
Department of Biophysics, Institute of Cell Physiology, National University of Mexico, UNAM, Apartado Postal 70–253, 04510 México D.F., Mexico City, Mexico

Keywords: exocytosis, hyposmolarity, nerve endings, norepinephrine

Abstract

Osmolarity reduction (20%) elicited ^{3}H-norepinephrine (NE) efflux from rat cortical synaptosomes. The hyposmotic NE release resulted from the following events: (i) a Na^+-dependent and La^{3+}-, Gd^{3+}- and ruthenium red-sensitive depolarization; (ii) a cytosolic Ca^{2+} ($[Ca^{2+}]_i$) rise with contributions from external Ca^{2+} influx and internal Ca^{2+} release, probably through the mitochondrial Na^+–Ca^{2+} exchanger; and (iii) activation of a $[Ca^{2+}]_i$-evoked, tetanus toxin (TeTX)-sensitive, PKC-modulated NE efflux mechanism. This sequence was established from results showing a drop in the hyposmotic $[Ca^{2+}]_i$ rise by preventing depolarization with La^{3+}, and by the inhibitory effects of Ca^{2+}-free medium (EGTA; 50%), CGP37157 (the mitochondrial Na^+–Ca^{2+} exchanger blocker; 48%), EGTA + CGP37157 or by EGTA-AM (> 95% in both cases). In close correspondence with these effects, NE efflux was 92% decreased by Na^+ omission, 75% by La^{3+}, 47% by EGTA, 50% by CGP37157, 90% by EGTA + CGP37157 and 88% by EGTA-AM. PKC influenced the intracellular Ca^{2+} release and, mainly through this action, modulated NE efflux. TeTX suppressed NE efflux. The K^+-stimulated NE release, studied in parallel, was unaffected by Na^+ omission, or by La^{3+}, Gd^{3+} or ruthenium red. It was fully dependent on external Ca^{2+}, insensitive to CGP37157 and abolished by TeTX. These results suggest that the hyposmotic events, although different from the K^+-evoked depolarization and $[Ca^{2+}]_i$ rise mechanisms, are able to trigger a depolarization-dependent, Ca^{2+}-dependent and TeTX-sensitive mechanism for neurotransmitter release.

Introduction

The osmolarity of the fluids surrounding animal cells is tightly controlled in physiological conditions but it may be altered in pathological states, particularly in those with associated hyponatremia. Severe chronic hyponatremia leads to cellular brain oedema which, in turn, evokes a complex syndrome including coma, a dazing state and higher susceptibility to seizures. This suggests an effect of the hyposmotic condition on brain excitability, a notion supported by studies *in vitro* in hippocampal slices showing an increase in amplitude and duration of excitatory postsynaptic potentials (Chebabo *et al*., 1995). This effect suggests an increase in glutamate efflux during the hyposmolar condition. This was examined in our previous study in rat cortical synaptosomes, in which we found a hyposmolarity-induced release of glutamate, GABA and taurine (Tuz *et al*., 2004). These three amino acids participate as osmolytes in brain *in vivo* as well as in neurons and astrocytes in culture (Verbalis & Gullans, 1991; Pasantes-Morales *et al*., 1993). Glutamate and GABA, on the other hand, have a role as main neurotransmitters. In our study we found that hyposmolarity induces a Na^+-dependent depolarization, $[Ca^{2+}]_i$ rise and increased exocytosis. A fraction of the hyposmotically induced release of amino acids, particularly large for glutamate, was found to be depolarization- and Ca^{2+}-dependent and blocked by tetanus toxin (TeTX), suggesting that this release occurs by exocytosis (Tuz *et al*., 2004). All these results indicate that hyposmolarity elicits in nerve endings, a number of processes which mimic those of depolarization–secretion exocytosis, and raise the question of whether other neurotransmitters, not involved as osmolytes in the response to swelling, may also be responsive to hyposmolarity. To test this possibility we examined the effect of hyposmolarity on the release of norepinephrine (NE) from rat cortical synaptosomes, the same preparation used in our previous study. Norepinephrine is an important synaptic transmitter in rat cerebral cortex, and its release fits well with the typical depolarization–secretion vesicular process. The effect of hyposmolarity and the features of the NE efflux in this condition were studied in parallel with the release evoked by depolarizing concentrations of KCl (20 mM), a well studied process which follows the classical vesicular release pattern.

Correspondence: Dr Herminia Pasantes-Morales, as above.
E-mail: hpasante@ifc.unam.mx

Received 11 May 2005, revised 25 July 2005, accepted 26 July 2005

Materials and methods

Materials

Salts ($CaCl_2$, KCl, KH_2PO_4, $MgCl_2$, $MgSO_4$ and NaCl), 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES) and glucose were from T. J. Baker (Xalostoc, Mexico); ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester (EGTA-AM), TeTX and Gö6976 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 (bisoxonol) were

doi:10.1111/j.1460-9568.2005.04344.x

from Molecular Probes (Eugene, OR, USA). L-[7,8–^{3}H]-norepinephrine (NE) was from Amersham (UK). Bovine serum albumin (BSA), triton X-100, pargyline, ascorbic acid, ruthenium red, $LaCl_3$, $GdCl_3$, choline chloride, gluconate salts, phorbol-12-myristate-13-acetate (PMA), ω-conotoxin MVIIC, thapsigargin and dantrolene were from Sigma Chemical Co. (St Louis, MO, USA). Nimodipine and nitrendipine were from RBI (Natick, MA, USA) and CGP 37157 was from Tocris (Ellisville, MO, USA).

Synaptosomal preparation and superfusion

Cerebral cortex was excised from decapitated male Wistar adult rats of ~180 g. 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-norephinephrine (2.5 μCi/mL) in the presence of 10 μM pargyline, 0.1% BSA and 0.0002 g/mL ascorbic acid, for 20 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 (−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 18 min, fractions of the perfusate medium were collected at 1-min intervals into scintillation vials. The baseline was attained at ≈3 min, after which the isosmotic medium was replaced by 20% hyposmotic or isosmotic medium plus 20 mM KCl and superfusion continued for a further 7 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 time with drugs varied depending on the experiment and are indicated at the corresponding figure legends. When required, controls contained the vehicle. Results are expressed as percentage release in each fraction of the total radioactivity (i.e. radioactivity in samples plus radioactivity remaining in synaptosomes at the end of superfusion).

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 (150 nM). The fluorophore 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). After 30 s, the medium was diluted up to reach −20% hyposmolarity and fluorescence changes were followed for a 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 because 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. Therefore, results of depolarization are expressed in arbitrary units (AU).

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

Synaptosomes were incubated under shaking with the fluorescent Ca^{2+} indicator fura-2 AM (2 μM) for 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 for 30 min. The preparation was then 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 for 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 NE in response to hyposmolarity

Synaptosomes loaded with ^{3}H-NE were washed and superfused with isosmotic medium and, after a short period, when the basal efflux was attained, superfusion continued with a 20% hyposmotic medium (−60 mOsm) or with isosmotic medium containing 20 mM KCl. Hyposmolarity elicited a rapid increase in NE efflux, with a peak attained 2 min after the stimulus. After this maximum, efflux slowly inactivated despite the persistence of hyposmolarity, decreasing efflux towards basal levels (Fig. 1A). The net NE release, i.e. hyposmotic minus isosmotic release over the 5 min immediately following the stimulus is shown in the inset bars (Fig. 1A). Upon stimulation with a depolarizing concentration of KCl, NE release showed an increase similar in magnitude to that evoked by hyposmolarity, with a maximal efflux after 3 min and a faster inactivation phase (Fig. 1B).

Hyposmolarity-induced depolarization and NE release

Hyposmolarity induces depolarization in synaptosomes as shown in our previous study on hyposmolarity-evoked amino acid efflux (Tuz *et al*., 2004). This depolarization was independent of Cl^- but strictly dependent on the presence of Na^+. Figure 2A shows the effect of La^{3+} or Gd^{3+} (100 μM) in preventing depolarization. These results suggest a

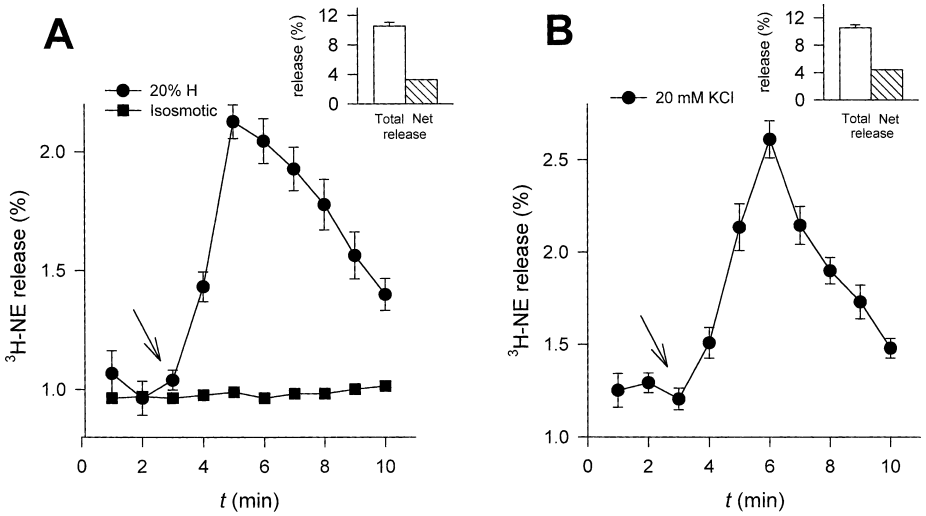

FIG. 1. Norepinephrine release from synaptosomes of rat cerebral cortex exposed to 20% hyposmotic or 20 mM KCl medium. Synaptosomes were incubated with ^{3}H-NE, washed and prepared for superfusion as described in Materials and Methods. Then, synaptosomes were superfused at a flow rate of 1 mL/min with isosmotic medium (■) to obtain a constant basal efflux and, at the arrow, the medium was replaced by (A) 20% hyposmotic (●) or (B) 20 mM KCl. Results are expressed as radioactivity released per min as a percentage of the total radioactivity incorporated. Insets show total release (the sum of release in the higher five points after the stimulus) (open bars) and net release (total release minus basal release; hatched bars). Data are means of 3–9 experiments.

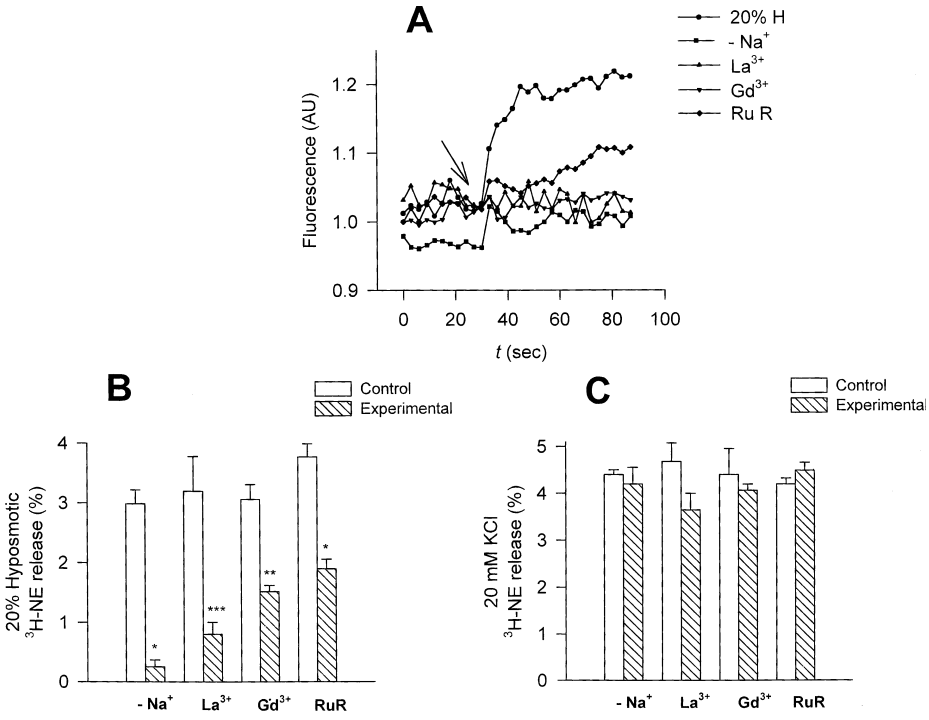

FIG. 2. Hyposmolarity-induced depolarization and its effect on NE release. (A) Depolarization was measured by bisoxonol fluorescence as described in Materials and Methods. Fluorescence was measured in isosmotic medium and, at the arrow, medium was diluted up to reach −20% hyposmolarity. Fluorescence changes were followed for a further 60 s. Control, 20% hyposmotic (●); Na^+ free medium (Na^+ replaced by choline chloride; $-Na^+$ ■), 100 μM La^{3+} (La^{3+} ▲), 100 μM Gd^{3+} (Gd^{3+} ▼) and 1 μM ruthenium red (RuR; ◆). Synaptosomes were exposed to the different conditions for 15 min before and throughout the experiment. Values represent fluorescence arbitrary units (AU) after subtracting the dilution factor. Representative results of three experiments. (B and C) Effects of Na^+-free medium, La^{3+}, Gd^{3+} and RuR on the NE release elicited by (B) 20% hyposmotic or (C) 20 mM KCl. Synaptosomes were preloaded with ^{3}H-NE and superfused as in Fig. 1. Bars represent the radioactivity released (%) in five fractions after the stimulus minus the basal release in the same time. Empty and hatched bars correspond to control and experimental conditions, respectively. Data are means ± SEM of 3–6 experiments. *$P < 0.001$, **$P < 0.01$ and ***$P < 0.02$ with respect to controls.

nonselective cation channel as a possible mechanism for this hyposmolarity-evoked, Na^+-dependent depolarization. Nonspecific cation channels of the transient receptor potential (TRP) channel family may be candidates for this role because one subtype, the TRVP4, is present in brain, is activated by stretch and by hyposmolarity, and is sensitive to La^{3+} and Gd^{3+} (Gunthorpe *et al.*, 2002). The activity of this TRP channel subtype is also reduced by ruthenium red. Figure 2A shows that this agent markedly decreased the hyposmolarity-induced

depolarization, in further support of the possible involvement of nonspecific cation channels in this phenomenon. Next, the effect of these conditions or agents in reducing the hyposmotic depolarization was tested on NE efflux. Figure 2B shows that hyposmotic NE efflux was essentially prevented in a Na^+-free medium and was also notably decreased by La^{3+} (75%) and reduced (51%) by Gd^{3+}. Ruthenium red decreased it by 50%. In contrast, NE release evoked by 20 mM KCl was unaffected by the Na^+-free condition, was slightly reduced by La^{3+} and was insensitive to Gd^{3+} and ruthenium red (Fig. 2C).

The influence of Ca^{2+} and protein kinase C (PKC)

The hyposmotic condition is known to increase $[Ca^{2+}]_i$ in most cell types (Pasantes-Morales & Morales-Mulia, 2000). This also occurs in synaptosomes as previously reported by Mongin *et al.* (1997) and confirmed in our previous study (Tuz *et al.*, 2004). Upon 20% reduction in osmolarity, the initial $[Ca^{2+}]_i$ of 345 nM increased to 580 nM. In conditions which were nominally externally Ca^{2+}-free (no Ca^{2+} plus EGTA), the increase in $[Ca^{2+}]_i$ was markedly reduced but not abolished (Fig. 3A). In synaptosomes treated with EGTA-AM, $[Ca^{2+}]_i$ basal levels markedly dropped to 125 nM and no increase was induced by the hyposmotic condition (Fig. 3A). These results suggest that the $[Ca^{2+}]_i$ increase results from influx through membrane channels as well as from release from internal stores. The hyposmotic Ca^{2+} influx was insensitive to L-type voltage-gated Ca^{2+} channel blockers nimodipine and nitrendipine, but was reduced by $\approx$30% by ω-conotoxin MVIIC, a blocker of the P/Q type channels (Fig. 3B).

The internal Ca^{2+} released by hyposmolarity seems to come essentially from the mitochondrial store, involving the Na^+–Ca^{2+} exchanger as a mechanism for release. This is suggested by the effect of the mitochondrial Na^+–Ca^{2+} exchange blocker CGP37157, decreasing by 48% the $[Ca^{2+}]_i$ rise (Fig. 3A). In the absence of external Ca^{2+}, CGP37157 fully suppressed the $[Ca^{2+}]_i$ elevation (Fig. 3A). La^{3+}, at the same concentration that prevented depolarization (100 μM), markedly reduced the $[Ca^{2+}]_i$ increase (Fig. 3B).

The effect of these treatments and agents on NE release was next examined. The osmosensitive NE efflux was ~47% reduced by external Ca^{2+} omission, was insensitive to the dihydropyridines but was markedly reduced by EGTA-AM, suggesting an important contribution of Ca^{2+} from internal sources (Fig. 3C). Treatment with thapsigargin or dantrolene did not reduce NE efflux (control, 3.65 ± 0.02; dantrolene, 3.69 ± 0.09; thapsigargin, 3.52 ± 0.08, $n = 3$). The mitochondrial Ca^{2+} which, as shown above, substantially contributed to the hyposmolarity-induced $[Ca^{2+}]_i$ elevation, significantly supported NE efflux as shown by its 50% reduction when the mitochondrial Na^+–Ca^{2+} exchanger was blocked with CGP37157. The effect of external Ca^{2+} omission plus that of CGP37157 were additive and, when the two conditions were tested together, NE efflux was abolished as shown in Fig. 3C. The K^+-stimulated NE release exhibited features different from those of the hyposmolarity-sensitive release regarding the Ca^{2+} influence. The K^+-induced efflux was almost 90% inhibited in the absence of external Ca^{2+} or by treatment with EGTA-AM. It was 43–55% reduced by the dihydropyridines, while it was unaffected by CGP37157 (Fig. 3D).

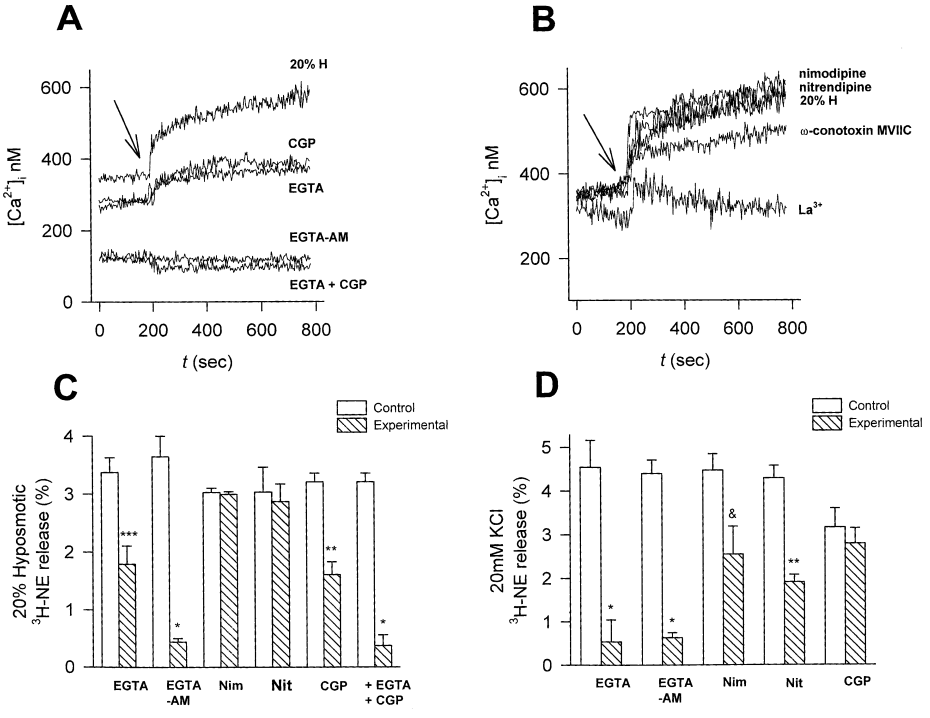

FIG. 3. Changes in $[Ca^{2+}]_i$ elicited by hyposmolarity and its effect on NE efflux. (A) Synaptosomes were loaded with fura-2 AM and $[Ca^{2+}]_i$ was estimated as described in Materials and Methods. $[Ca^{2+}]_i$ was measured in isosmotic medium and, at the arrow, the medium was diluted up to reach −20% hyposmolarity (20% H). EGTA, synaptosomes treated with a Ca^{2+}-free medium containing 0.1 mM EGTA plus 10 mM $MgCl_2$; EGTA-AM, synaptosomes 15 min preincubated with 50 μM EGTA-AM in Ca^{2+}-free medium; CGP, incubation with 10 μM CGP37157; CGP + EGTA, treatment with CGP37157 in a Ca^{2+}-free medium. Fluorescence units were transformed into Ca^{2+} concentration as described in Materials and Methods. (B) Effect of nimodipine, nitrendipine, ω-conotoxin MVIIC and La^{3+} on $[Ca^{2+}]_i$ rise evoked by 20% hyposmolarity. Nim, nimodipine, 10 μM; Nit, nitrendipine, 10 μM; ω-conotoxin MVIIC, 100 nM; La^{3+}, 100 μM. The blockers were present for 15 min before and throughout the experiment. Curves representative of three or four separate experiments. (C and D) Effects of treatments and agents described in A and B (except ω-conotoxin MVIIC and La^{3+}) on (C) the hyposmolarity- or (D) the KCl-stimulated NE release. Data are expressed as in Fig. 2 and are means ± SEM of 3–6 experiments. $^*P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.02$ and $^{\&}P < 0.05$ with respect to controls.

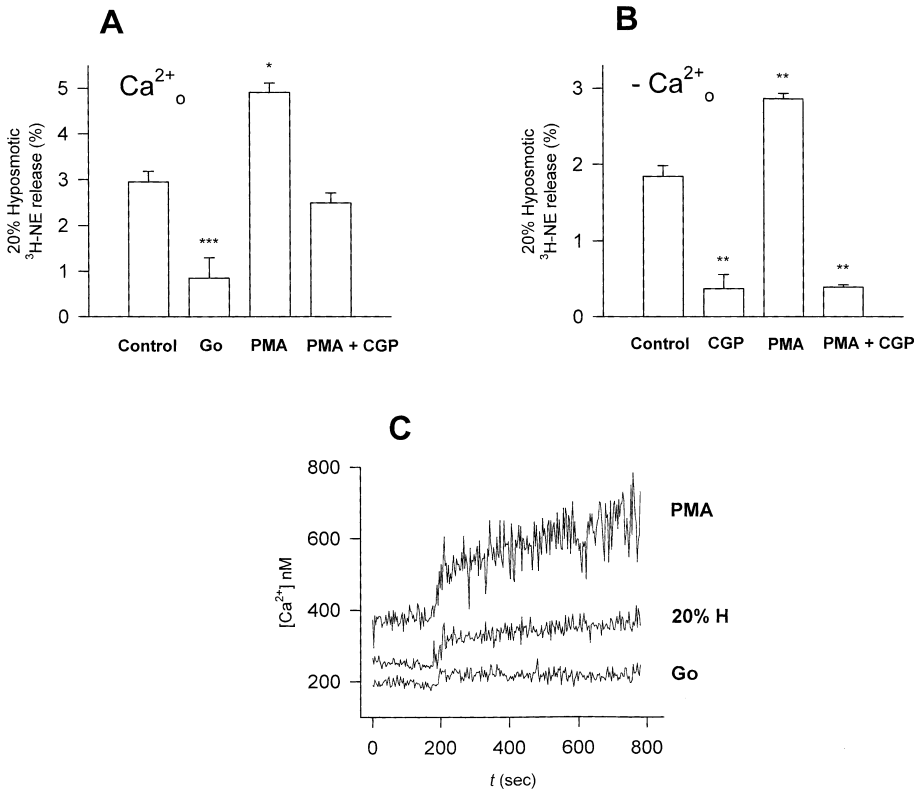

FIG. 4. Effect of modifying PKC activity on the $[Ca^{2+}]_i$ changes and NE release elicited by hyposmolarity. (A) Effect of Gö6976, PMA or CGP37157 on NE release evoked by 20% hyposmolarity in the presence of external Ca^{2+}. PMA + CGP, 75 nM PMA plus 10 μM CGP37157. (B) Effect of PMA and/or CGP37157 on NE release elicited by hyposmolarity in a Ca^{2+}-free medium. Synaptosomes were preloaded with ^{3}H-NE and superfused as in Fig. 1. The agents were present for 15 min before (except PMA, 3.5 min) and throughout the experiment. Data are expressed as in Fig. 2 and are means ± SEM of 3–8 experiments. $*P < 0.001$, $**P < 0.01$ and $***P < 0.02$ with respect to controls. (C) Effect of Gö6976 and PMA on hyposmotic $[Ca^{2+}]_i$ changes in a Ca^{2+}-free medium (prepared as in Fig. 3). Synaptosomes were loaded with fura-2 AM and $[Ca^{2+}]_i$ was measured as described in Fig. 3. Go, 1 μM Gö6976 (15 min preincubated); PMA, 75 nM PMA (3.5 min preincubated).

The influence of PKC on the hyposmotic NE release is shown by the effect of the PKC blocker Gö6976, reducing the release by 72%, and the stimulatory effect of PMA, increasing NE efflux by ~66% (Fig. 4A). PKC increased the hyposmotic NE efflux in both the presence and absence of Ca^{2+} (Fig. 4A and B). In the two conditions, CGP37157 markedly inhibited the PKC-induced NE efflux increase (Figs 3C and 4B). PKC seems to influence NE efflux through an effect on the mechanism of internal Ca^{2+} release because, in the absence of external Ca^{2+}, the hyposmotic $[Ca^{2+}]_i$ elevation was markedly reduced by Gö6976 and potentiated by PMA (Fig. 4C).

Increased exocytosis by hyposmolarity and the effect of TeTX on NE efflux

In our previous study (Tuz *et al*., 2004) we found an effect of hyposmolarity in enhancing exocytosis in synaptosomes, as monitored by the decrease in the fluorescent dye FM1-43 from previously loaded synaptosomes (Guatimosim *et al*., 1997; Fig. 5A). The hyposmotic-induced exocytosis was prevented in Na^+-free medium (Na^+ replaced by choline chloride) and in synaptosomes treated with EGTA-AM (Fig. 5B), and it was increased by PMA (Fig. 5B). All these conditions, as shown above, modified the hyposmotic NE efflux, suggesting an exocytotic mechanism for its release. To further support this possibility, synaptosomes were treated with TeTX, an agent known to disrupt the vesicular release. Figure 5C shows that this toxin decrease the hyposmotic NE efflux by >90%. NE release evoked by high K^+ concentration was even more reduced by treatment with the toxin (Fig. 5D).

Discussion

The present results show a hyposmolarity-evoked NE efflux from synaptosomes, resulting from a series of events which reproduce those of the excitation–secretion process, i.e. depolarization, $[Ca^{2+}]_i$ rise and vesicular exocytosis. The trigger for all these reactions eventually resulting in NE release seems to be the La^{3+}-, Gd^{3+}- and ruthenium red-sensitive depolarization, probably due to Na^+ influx through nonspecific cation channels, possibly of the TRP class. A subtype of this family of channels, the TRPV4, is almost exclusively present in the nervous tissue, is osmotically and mechanically sensitive and is blocked by Gd^{3+}, La^{3+} and ruthenium red (Gunthorpe *et al*., 2002).

The event subsequent to the hyposmotic depolarization is a $[Ca^{2+}]_i$ increase. The fact that preventing depolarization with La^{3+} abolished this Ca^{2+} response established the link between these two events. The hyposmotic $[Ca^{2+}]_i$ increase was characterized in the present study and found to be different in some respects from that evoked by depolarizing K^+ concentration. The mechanisms and sources contributing to the increase in $[Ca^{2+}]_i$ are different in the two paradigms. While in the K^+ model $[Ca^{2+}]_i$ elevation was, as expected, essentially dependent on external Ca^{2+}, elevation elicited by hyposmolarity resulted from some external Ca^{2+} entry as well as from a significant contribution of Ca^{2+} released from internal sources. The mechanism of

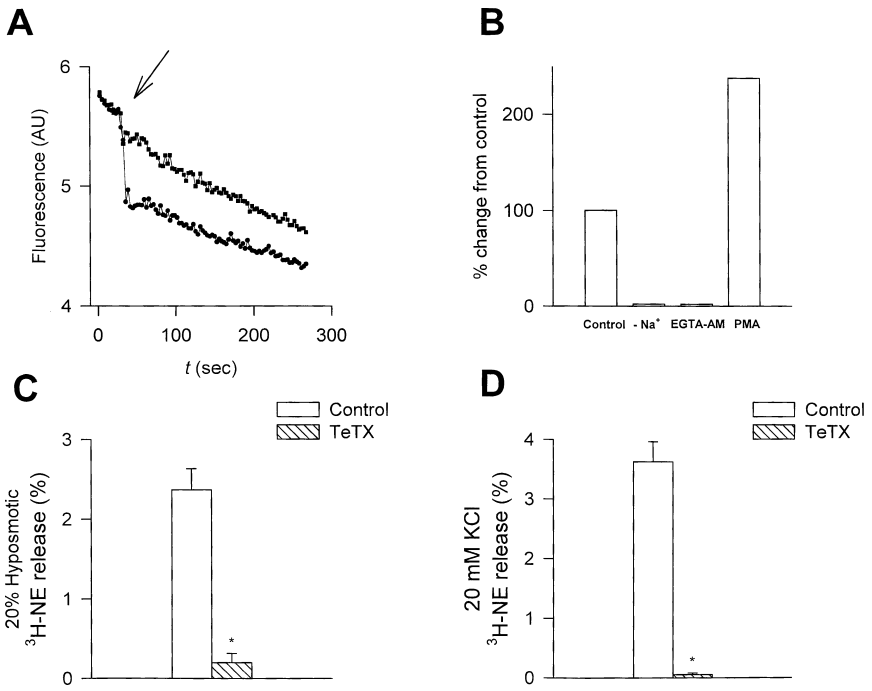

FIG. 5. Hyposmolarity-induced exocytosis and effect of TeTX on NE release from synaptosomes. (A) Exocytosis was monitored by changes in fluorescence in synaptosomes loaded with 4 μM FM1-43 for 10 min in isosmotic medium and 1 min in 40 mM KCl to internalize the fluorophore, as described in Materials and Methods. After 30 s in isosmotic conditions, the medium was diluted up to reach −20% hyposmolarity (arrow) and the FM1-43 release in this condition (●) was compared with that under a continuous isosmotic medium (■). Data are expressed as fluorescence arbitrary units (AU). The figure is a representative of three experiments. (B) Effect of Na^+-free medium (–Na^+), EGTA-AM and PMA on the hyposmolarity-induced exocytosis. (C and D) Effect of TeTX on NE efflux evoked by (C) hyposmolarity or (D) depolarizing K^+ concentrations. Synaptosomes preloaded with ^{3}H-NE were incubated for 90 min in isosmotic medium in the presence of 50 nM TeTX with 0.1% BSA. Thereafter, synaptosomes were superfused as in Fig. 1. Open bars, controls; hatched bars, TeTX. Data are expressed as in Fig. 2 and are means ± SEM of three experiments. *$P < 0.01$ with respect to controls.

external Ca^{2+} influx also showed marked differences between the two stimulatory conditions, with null participation of the voltage-gated L-type Ca^{2+} channels in the hyposmotic $[Ca^{2+}]_i$ increase, in contrast to their significant role in the high-K^+ condition. A $[Ca^{2+}]_i$ elevation evoked by hyposmolarity has been reported in hippocampal pyramidal neurons (Borgdorff *et al*., 2000), with contributions of extracellular Ca^{2+} as well as of Ca^{2+} released from internal sources. In that study, the external Ca^{2+} influx is attributed to the NaCl decrease in the hyposmotic medium, whereas it is proposed that the internal Ca^{2+} is elicited by stretch or swelling. In the present study in synaptosomes we further explored the mechanisms of the two sources of hyposmotic $[Ca^{2+}]_i$ elevation. Similar to the study in neurons, we found a contribution of external Ca^{2+} influx occurring not via the voltage-gated L-type Ca^{2+} channels but partly (30%) through P/Q type channels. The mechanism of Ca^{2+} influx insensitive to the voltage-gated channel blockers was not identified in the present study. As for the internal source of Ca^{2+} released by hyposmolarity, our results suggest the mitochondrial pool, with the release involving the mitochondrial Na^+–Ca^{2+} exchanger, which is activated by an increase in cytosolic Na^+ (Adam-Vizi, 1992; Hayasaki-Kajiwara *et al*., 1999; Raiteri *et al*., 2002). This is the notion suggested by the effect of the mitochondrial Na^+–Ca^{2+} exchanger blocker CGP37157 reducing by 48% the hyposmotic $[Ca^{2+}]_i$ rise and fully preventing it when tested in the absence of external Ca^{2+}.

The hyposmotic NE release responded in full correspondence to each one of the precedent events, i.e. depolarization and $[Ca^{2+}]_i$ elevation. Preventing or reducing depolarization by Na^+ omission, or by La^{3+}, Gd^{3+} or ruthenium red, consequently reduced NE efflux. Also, NE release closely paralleled the changes in $[Ca^{2+}]_i$ in the different conditions tested. Thus, ∼53% of the hyposmotic efflux persisted in the absence of external Ca^{2+}, 50% remained after blockade of the internal Ca^{2+} release (CGP37157) and >90% was inhibited when the two conditions were tested together.

PKC was found to modulate NE efflux, which was inhibited by PKC blockade (Gö6976) and potentiated by PKC activation (PMA). The kinase activation may result from the hyposmotic simulation of phospholipases (Thoroed *et al*., 1997; Tomassen *et al*., 2004) or from an increase in cytosolic Na^+ (Hayasaki-Kajiwara, 1999). Once activated, PKC may act directly on the exocytotic process (Vaughan *et al*., 1998), by recruiting vesicles or/and by increasing Ca^{2+} affinity for the vesicular release (Keenan & Kelleher, 1998; Hille *et al*., 1999). PKC may also act by enhancing the Ca^{2+} mobilization from the mitochondrial reservoir through an action on the Na^+–Ca^{2+} exchanger, as has been recently described in motoneurons (Yang *et al*., 2003). Even though the two possibilities are plausible, our results favour a major effect on the mitochondrial Ca^{2+} release because: (i) PKC activation increased and PKC inhibition decreased the hyposmolarity-induced $[Ca^{2+}]_i$ rise in the absence of external Ca^{2+} and (ii) blockade of the mitochondrial Ca^{2+} release by CGP37157 in either the presence or absence of Ca^{2+} prevented the PKC potentiation of NE efflux.

The events inducing the hyposmotic NE efflux, i.e. depolarization and $[Ca^{2+}]_i$ rise, are typical of the vesicular mechanism by which neurotransmitters are released by exocytosis. The contribution of this mechanism to NE release is suggested by the effect of TeTX in preventing hyposmotic NE efflux. The fact that this toxin similarly affected the K^+-stimulated NE efflux, known to occur by vesicular exocytosis, further supports this notion.

In summary, the present results suggest the following serial events evoked by hyposmolarity, ultimately leading to NE efflux: (i) the Na^+-dependent depolarization of the terminal, probably mediated by

osmotically or stretch sensitive nonspecific cation channels; (ii) the activation of voltage-dependent Na^+ and Ca^{2+} channels; (iii) a consequent $[Ca^{2+}]_i$ elevation resulting from both Ca^{2+} entry and a PKC-modulated, Na^+–Ca^{2+} exchange-mediated Ca^{2+} release from mitochondrial stores; (iv) the $Ca^{2+}{}_i$-evoked TeTx-sensitive release of NE. As above discussed, the pathways of hyposmolarity-triggered depolarization and $[Ca^{2+}]_i$ elevation differ in some respects from those of the classical depolarization–secretion here exemplified by the K^+-stimulated NE release, but the resulting $[Ca^{2+}]_i$ increase seems to trigger in both cases a TeTX-sensitive mechanism which may not differ from the vesicular exocytosis characteristic of neurotransmitter release.

In a previous study (Tuz *et al*., 2004) we found a hyposmotic release of glutamate, the major excitatory neurotransmitter. Glutamate release was partly Ca^{2+}-dependent and TeTX-sensitive, suggesting the contribution of exocytosis, but the reversal operation of the carrier was contributing as well. The glutamate release may explain the effect of hyposmolarity increasing the amplitude and duration of excitatory postsynaptic potentials (Chebabo *et al*., 1995), suggesting that the hyposmotic stimulus might potentiate the impulse-evoked release, acting probably via an additive mechanism. This could be also the case for NE, because the differences observed between the K^+- and the hyposmolarity-evoked release may result in additive effects of the two stimulatory conditions. Although this was not explored in the present study in which the hyposmolarity effects were examined only on the basal NE efflux, the potentiation of synaptic transmission by hyposmolarity and/or swelling opens an interesting avenue for future studies on the intriguing possibility of a modulation of synaptic function by conditions leading to volume increase in nerve terminals, in both pathological and physiological conditions.

Acknowledgements

This work was supported in part by grant No. IN204900 from DGAPA, UNAM and 4646 5-4 from CONACyT.

Abbreviations

$[Ca^{2+}]_i$, cytosolic Ca^{2+}; bisoxonol, bis-(1,3diethylthiobarbituric acid) trimethine oxonol; BSA, bovine serum albumin; fura-2 AM, fura-2 acetoxymethyl ester; NE, norepinephrine; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; TeTX, tetanus toxin; TRP, transient receptor potential.

References

Adam-Vizi, V. (1992) External Ca^{2+}-independent release of neurotransmitters. *J. Neurochem.*, **58**, 395–405.

Borgdorff, A.J., Somjen, G.G. & Wadman, W.J. (2000) Two mechanisms that increase free intracellular calcium in rat hippocampal neurons during hyposmotic and low NaCl treatment. *J. Neurophysiol.*, **83**, 81–89.

Chebabo, S.R., Hester, M.A., Aitken, P.G. & Somjen, G.G. (1995) Hypotonic exposure enhances synaptic transmission and triggers spreading depression in rat hippocampal tissue slices. *Brain Res.*, **695**, 203–216.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

Guatimosim, C., Romano-Silva, M.A., Cruz, J.S., Beirao, P.S., Kalapothakis, E., Morales-Santos, T., Cordeiro, M.N., Diniz, C.R., Gomes, M.V. & Prado, M.A. (1997) A toxin from the spider *Phoneutria nigriventer* that blocks calcium channles coupled to exocytosis. *Br. J. Pharmacol.*, **122**, 591–597.

Gunthorpe, M.J., Benham, C.D., Randall, A. & Davis, J.B. (2002) The diversity in the vanilloid (TRPV) receptor family of ion channels. *Trends Pharmacol. Sci.*, **23**, 183–191.

Hajos, F. (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.*, **93**, 485–489.

Hayasaki-Kajiwara, Y., Kitano, Y., Iwasaki, T., Shimamura, T., Naya, N., Iwaki, K. & Nakajima, M. (1999) Na^+ influx via Na^+/H^+ exchange activates protein kinase C isozymes delta and epsilon in cultured neonatal rat cardiac myocytes. *J. Mol. Cell Cardiol.*, **31**, 1559–1572.

Hille, B., Billard, J., Babcock, D.F., Nguyen, T. & Koh, D.S. (1999) Stimulation of exocytosis without a calcium signal. *J. Physiol. (Lond.)*, **520**, 23–31.

Keenan, C. & Kelleher, D. (1998) Protein kinase C and the cytoskeleton. *Cell Signal*, **10**, 225–232.

Mongin, A.A., Aksentsev, S.L., Orlov, S.N. & Konev, S.V. (1997) Hypoosmotic shock activates Ca^{2+} channels in isolated nerve terminals. *Neurochem. Int.*, **31**, 835–843.

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

Pasantes-Morales, H. & Morales-Mulia, S. (2000) Influence of calcium on regulatory volume increase: role of potassium channels. *Nephron*, **86**, 414–427.

Raiteri, L., Stigliani, S., Zedda, L., Raiteri, M. & Bonanno, G. (2002) Multiple mechanisms of transmitter release evoked by 'pathologically' elevated extracellular $[K^+]$: involvement of transporter reversal and mitochondrial calcium. *J. Neurochem.*, **80**, 706–714.

Thoroed, S.M., Lauritzen, L., Lambert, I.H., Hansen, H.M. & Hoffmann, E.K. (1997) Cell swelling activates phospholipase A2 in Ehrlich ascites tumor cells. *J. Membr. Biol.*, **160**, 47–58.

Tomassen, S.F.B., van der Wijk, T., de Jonge, H.R. & Tilly, B.C. (2004) Activation of phospholipase D by osmotic cell swelling. *FEBS Lett.*, **566**, 287–290.

Tuz, K., Peña-Segura, C., Franco, R. & Pasantes-Morales, H. (2004) Depolarization, exocytosis and amino acid release evoked by hyposmolarity from cortical synaotosomes. *Eur. J. Neurosci.*, **19**, 916–924.

Vaughan, P.F.T., Walker, J.H. & Peers, C. (1998) The regulation of neurotransmitter secretion by protein kinase C. *Mol. Neurobiol.*, **18**, 125–155.

Verbalis, J.G. & Gullans, S.R. (1991) Hyponatremia causes large sustained reductions in brain content of multiple organic osmolytes in rats. *Brain Res.*, **567**, 274–282.

Waggoner, A. (1976) Optical probes of membrane potential. *J. Membr. Biol.*, **27**, 317–334.

Whittaker, V.P. & Barker, L.A. (1972) The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In Fried, R. (ed.), *Methods of Neurochemistry*. Marcel Dekker, New York.

Yang, F., Xiang-Ping, H., Russell, J. & Lu, B. (2003) Ca^{2+} influx-independent synaptic potentiation mediated by mitochondrial Na^+–Ca^{2+}exchanger and protein kinase C. *J. Cell Biol.*, **163**, 511–523.