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European Journal of Pharmacology 566 (2007) 103–112
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Ethanolamine and related amino alcohols increase basal and evoked release of [^{3}H]-D-aspartic acid from synaptosomes by enhancing the filling of synaptic vesicles

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Received 19 January 2007; received in revised form 7 March 2007; accepted 8 March 2007
Available online 24 March 2007

Abstract

This research examines the effects of ethanolamine and other amino alcohols on the dynamics of acridine orange (AO), oxonol V, and [^{3}H]-D-aspartic acid in synaptic preparations isolated from mammalian brain. Ethanolamine concentration-dependently enhanced AO release from synaptosomes. Similar effects were observed with methylethanolamine and dimethylethanolamine, but not choline. The enhancement of AO efflux by ethanolamine was independent of extrasynaptosomal calcium (in contrast to KCl-induced AO efflux), was unaffected by tetrodotoxin and did not involve depolarization of the synaptosomal plasma membrane. KCl was unable to release AO from synaptosomes following exposure to ethanolamine, however ethanolamine and other amino alcohols were found to enhance both basal and KCl-evoked release of [^{3}H]-D-aspartic acid from synaptosomes. Using isolated synaptic vesicles we demonstrate that amino alcohols are able to 1) abolish the ATP-dependent intravesicular proton concentration (i.e. stimulate efflux of AO) in a similar way to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2) increase the ATP-supported transvesicular membrane potential (i.e. quench oxonol V fluorescence) in contrast to CCCP and 3) enhance intravesicular uptake of [^{3}H]-D-aspartic acid. These results suggest that positively charged, membrane impermeant amino alcohol species are generated within synaptic vesicles as they sequester protons. Cationic forms of these amino alcohols boost the transvesicular electrical potential which increases transmitter uptake into synaptic vesicles and facilitates enhancement of basal and evoked release of transmitter. Our data suggest a potential role for ethanolamine and related amino alcohols in the regulation of synaptic vesicle filling. These findings may also have relevance to neuropathophysiological states involving altered production of ethanolamine.
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Keywords: Ethanolamine; Acridine orange; Oxonol V; [^{3}H]-D-aspartate; Synaptosome; Synaptic vesicle; Mammalian brain

1. Introduction

The resting levels of ethanolamine in mammalian brain are known to be in the low millimolar range (Ellison et al., 1987) and, despite a considerable amount of research, the role that this amino alcohol plays in normal brain function and neuropathological states remains to be fully elucidated. Studies indicate that ethanolamine facilitates both amino acid and cholinergic neurotransmission in the brain. For example, Wolfensberger et al. (1982) found that ethanolamine augments glutamate-dependent excitation and GABA-dependent inhibition of avian tectal neurons. The enhancement of GABA-induced depression by ethanolamine in these experiments was suggested to be related to the capacity of this amino alcohol to reduce GABA breakdown *via* inhibition of GABA aminotransferase (Loscher, 1983). Ethanolamine markedly increases the levels of the amino acid neurotransmitters aspartic acid and glutamic acid in microdialysates from the anterior hippocampus (Buratta et al., 1998). Similarly, ethanolamine and other amino alcohols were found to selectively stimulate K^+-evoked acetylcholine release from hippocampal slices, which was attributed to activation of calcium entry through L-type calcium channels (Bostwick et al., 1992, 1993).

In other studies, high affinity uptake of ethanolamine has been demonstrated in the retina (Pu and Anderson, 1984) and in cultured cerebrocortical neurons (Massarelli et al., 1982) observations which accord with ethanolamine's function as an

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doi:10.1016/j.ejphar.2007.03.020

important metabolic precursor molecule for acetylcholine and phosphatidylcholine (Corazzi et al., 1986) and the involvement of ethanolamine in phospholipid metabolism (Porcellati et al., 1971).

Several investigations show that ethanolamine release in a number of brain regions is associated with electrical or chemical depolarization. For example, the levels of ethanolamine rise in perfusates of the avian optic tectum as a result of electrical stimulation (Wolfensberger et al., 1982), and release of this substance from rat pontine nuclei can be electrically evoked (Pershak et al., 1986). Enhanced levels of ethanolamine were also demonstrated in dialysates of rat striatum during challenge with an elevated concentration of K^+ (Korf and Venema, 1985), however, the same depolarizing treatment failed to increase ethanolamine levels in perfusates from rat *substantia nigra* (Van Der Heyden et al., 1979). In our laboratory we found that synaptosomes and synaptoneurosomes release [^{3}H]ethanolamine during superfusion in a calcium-dependent fashion in response to a KCl challenge (Liao and Nicholson, 2005) suggesting that depolarization-evoked release of [^{3}H]ethanolamine from the nerve ending may occur *via* classical exocytosis. Associated experiments using the pH-sensitive fluorescent dye acridine orange (AO) demonstrated that ethanolamine rapidly accesses synaptic vesicles within the nerve ending. AO has found particular utility in investigations of exocytotic function in synaptosomes and synaptic vesicle proton content. We also employed another fluoroprobe, oxonol V, which allowed ethanolamine-induced changes to the membrane potential of synaptic vesicles to be followed. We now report on these and subsequent observations using [^{3}H]-D-aspartic acid, which led us to develop and test the hypothesis that ethanolamine modifies presynaptic release of this marker of L-glutamic acid by affecting synaptic vesicle function.

2. Materials and methods

2.1. Chemicals and radiochemicals

Ethanolamine, methylethanolamine, dimethylethanolamine, choline, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), veratridine, AO and tetrodotoxin were obtained from Sigma-Aldrich Canada. Rhodamine 6G was from Eastman Kodak, Rochester, NY, USA. Oxonol V was supplied by Molecular Probes Inc., Eugene, OR, USA. [^{3}H]-D-aspartic acid (specific activity 23.9 Ci/mmol) was from Perkin Elmer:NEN, Boston, MA, USA.

2.2. Animal care

Investigations into the effects of amino alcohols on synaptosomal function were performed using CD1 mice (male; 20–25 g) purchased from the University of British Columbia, BC, Canada. Mice were housed in group cages at the Simon Fraser University Animal Care Facility in a standardized environment (21 °C; 55% relative humidity; 12 h light/dark cycle) and were allowed *ad libitum* access to food and water. Mice were rapidly killed by cervical dislocation and decapitation. All animal procedures adhered to the Canadian Council on Animal Care guidelines and were approved by the Simon Fraser University Animal Care Committee.

2.3. Isolation of synaptosomes from mouse brain

The whole brain material from three mice (acridine orange experiments) or one mouse ([^{3}H]-D-aspartic acid release experiments) was cooled rapidly in ice-cold 0.32 M sucrose (adjusted to pH 7.4 with Tris base), and then chopped into small pieces. The purified synaptosomal fraction was isolated according to the method of Hajos (1975) with minor modifications. Synaptosomes are obtained at close to 90% purity by this method as assessed by electron microscopy (Hajos, 1975). Brain tissue was first homogenized in ice-cold buffered 0.32 M sucrose (20 ml) to generate synaptosomes using 6 excursions of a motor driven pestle. The homogenate was centrifuged (1500 ×*g*; 10 min) and the supernatant retained on ice. The pellet was dispersed in sucrose and centrifuged again. Supernatants were combined, centrifuged (9000 ×*g*; 20 min) and the crude synaptosomal pellet (P_2) was then gently resuspended in 0.32 M sucrose (5 ml). The P_2 fraction was then divided equally and each portion carefully run onto the surface of 0.8 M sucrose (20 ml. pH 7.4) in a centrifuge tube. The two tubes were then centrifuged (9000 ×*g*; 26 min). Material in each 0.8 M layer was removed and diluted (over 30 min with continuous mixing) to the equivalent of 0.32 M with ice-cold distilled water. After centrifugation (9000 ×*g*; 20 min) the purified synaptosomal pellet was suspended in calcium-free saline (NaCl 128 mM, KCl 5 mM, $Na_2HPO_4{\cdot}7H_2O$ 1 mM, $MgCl_2{\cdot}7H_2O$ 1.2 mM, EGTA 100 μM, glucose 14 mM and HEPES 20 mM buffered to pH 7.4 with Tris base) and held on ice.

2.4. Experiments using synaptosomes and the pH-sensitive dye AO

Experiments with the pH-sensitive fluorescent indicator AO were conducted according to published methods (Zoccarato et al., 1999; Melnik et al., 2001). A 200 μl aliquot of purified synaptosomes (0.47±0.03 mg protein) was added to 2.8 ml calcium-free saline containing BSA (1 mg/ml) and AO (5 μM final concentration) and then incubated at 35 °C with gentle shaking for 20 min. The suspension of AO-loaded synaptosomes was then transferred to a stirred quartz fluorescence cuvette thermostated at 35 °C, and, using an excitation wavelength of 490 nm, the fluorescence emission intensity was measured continuously at 530 nm in a Perkin–Elmer LS-50 fluorescence spectrophotometer. Slit widths were each 3 nm. Immediately after starting the recording Ca^{++} (1.2 mM) or tetrodotoxin (10 μM) was added if required, additions of study compounds were then made from approximately 100 s onwards and assays were normally terminated at 400 s. Ethanolamine, methylethanolamine, dimethylethanolamine, and choline were added in saline (10 μl). Veratridine and CCCP were added in DMSO (2 μl). Neither addition of control carriers to AO-loaded synaptosomes nor addition of the study compounds to saline

containing AO (in the absence of biological material), had any effect on the level AO fluorescence.

2.5. Isolation of synaptoneurosomes and their use in the depolarization assay

The synaptoneurosomal fraction, isolated from the whole brain of a single CD1 mouse using the method of Harris and Allen (1985) was used for plasma membrane potential measurements. All fractionation procedures were carried out between 1 and 4 °C and the final synaptoneurosomal pellet was suspended in saline (1 ml) and held on ice. Aliquots of this suspension were used directly for assay. The membrane potential of synaptoneurosomes was measured based on the fluorescence output of the voltage-sensitive indicator rhodamine 6G, as we have outlined in a previous report (Nicholson et al., 2003). We found that synaptoneurosomes maintain stable resting potentials and respond well to depolarizing treatments. Synaptoneurosomes therefore represent an ideal preparation for examining the effects of amino alcohols on the neuronal plasma membrane potential.

2.6. Release of [^{3}H]-D-aspartic acid from superfused synaptosomes

Synaptosomes were incubated for 5 min at 32 °C in the presence of 5 μCi ([^{3}H]-D-aspartic acid (specific activity 23.9 Ci/mmol) in saline (NaCl 128 mM, KCl 5 mM, $Na_2HPO_4 \cdot 7H_2O$ 1 mM, $CaCl_2 \cdot 2H_2O$ 0.8 mM, $MgCl_2 \cdot 7H_2O$ 1.2 mM, glucose 14 mM and HEPES 20 mM buffered to pH 7.4 with Tris; total volume 1500 μl). Loading was terminated by transferring synaptosomes to ice and centrifugation. Synaptosomes were resuspended in 800 μl of ice-cold saline and placed in each of ten Swinnex filtration units (Millipore) containing Whatman GF/B filters. Superfusions were carried out as we have described previously (Verdon et al., 2000). Individual filter units were constantly supplied with saline from a 30 ml syringe barrel. Saline was then routed to an LKB 2070 Ultrorac fraction collector adapted to collect 10 superfusates simultaneously. A constant 1 ml/min flow rate was maintained using a 10 channel peristaltic pump (Watson Marlow 503S) set up between the filtration units and the fraction collector. The effects of amino alcohols were examined after nerve preparations had been superfused with 40 ml control saline to establish consistent rates of release. From this point onwards, ten 3 ml fractions were collected from each filtration unit and total radioactivity in each tube measured by liquid scintillation counting. Amino alcohols were dissolved directly in saline. The depolarizing (35 mM KCl) treatment (with the [NaCl] reduced by an equivalent amount to maintain isoosmotic conditions) was routinely added at fraction 4 and maintained until the end of superfusion. The release profiles were constructed by expressing ^{3}H collected in each fraction as a percentage of ^{3}H present in the first fraction. Percentage increases in resting and evoked release of [^{3}H]-D-aspartate were calculated by adding the percentage increases for each amino alcohol alone or with KCl and expressing these values as a percentage of the summed basal or KCl alone values. All superfusions were conducted between 22 and 25 °C.

2.7. Preparation of synaptic vesicles

Synaptic vesicles were prepared from bovine brain using procedures described in the literature (Shioi et al., 1989; Roz and Rehavi, 2003). A brain from a freshly killed bovine was obtained from a local abattoir and transported to the laboratory on ice. All the following fractionation procedures were performed at 1–4 °C. After removing as much white matter as possible, cerebral cortex tissue was chopped, homogenized (10% w/v) in 0.32 M sucrose (containing 1 mM $NaHCO_3$, 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$) using a motor driven homogenizer (6 up and down strokes) and synaptosomes isolated according to Hajos (1975). The synaptosomal pellets were gently dispersed in the sucrose solution (0.3 ml) and then synaptosomes were osmotically disrupted by the addition of 10 ml of 5 mM Tris–HCl buffer solution followed by incubation on ice for 45 min. Suspensions were centrifuged at 20,000 ×*g* for 20 min and the supernatants then centrifuged at 62,000 ×*g* for 40 min to yield the synaptic vesicle pellets, which were suspended in 0.32 M sucrose, 1 mM $NaHCO_3$ at protein concentration of 7.5 mg/ml and stored at −80 °C.

2.8. Measurement of the effects of amino alcohols on proton levels within synaptic vesicles

The effects of ethanolamine, methylethanolamine, dimethylethanolamine and CCCP on ATP-dependent proton accumulation into synaptic vesicles were studied using the AO method

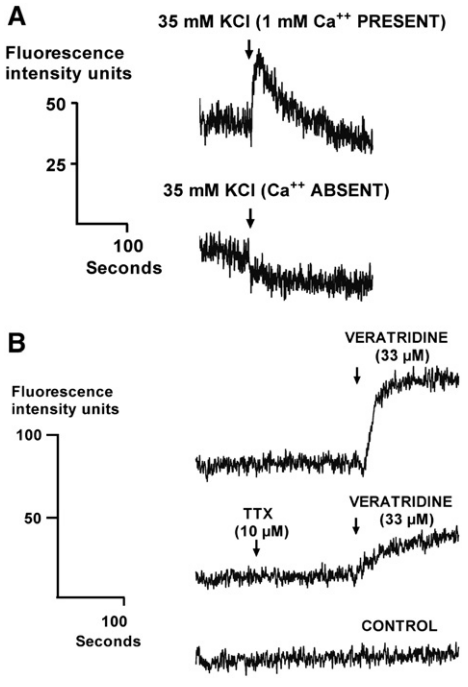

Fig. 1. (A) Depolarization-induced exocytosis observed as an AO fluorescence increase arising from exposure of synaptosomes to 35 mM KCl (above). KCl cannot initiate exocytosis in the absence of Ca^{++} (below). In (B) the release of AO occurring after challenge with 33 μM veratridine (above), inhibition of veratridine's effect by tetrodotoxin (TTX; middle) and a control profile (lower) are displayed. Traces are representative of results from 3–5 independent experiments. Ca^{++} was present at 1 mM except where otherwise indicated.

(Roz and Rehavi, 2003). Recordings were carried out with a Perkin–Elmer LS-50 fluorescence spectrophotometer. The excitation wavelength was 493 nm excitation (slit width: 3 nm) and the emission signal was sampled at 530 nm (slit width: 3 nm). Synaptic vesicles (200 μg of protein) were first incubated in 2 ml buffer (150 mM KCl, 4 mM $MgSO_4$, 10 mM HEPES, pH 7.4) containing AO (1.5 μM) for 1 min at 30 °C. ATP (1 mM final concentration) was then added followed by the study compounds. Fluorescence changes were monitored for 340 s.

2.9. Measurement of amino alcohol-induced changes to the electrical (membrane) potential of synaptic vesicles

The effects of ethanolamine and other compounds on ATP-induced polarization of synaptic vesicles were monitored by recording fluorescence changes of the lipophilic anionic dye oxonol V as described by Tabb et al. (1992). These experiments were performed at 30 °C using the Perkin–Elmer LS-50 with the excitation wavelength set at 617 nm (slit width: 5 nm) and an emission wavelength of 643 nm (slit width:10 nm). Synaptic vesicles (300 μg protein) were allowed to equilibrate with oxonol V (0.65 μM final concentration) in 2 ml buffer (0.25 M sucrose, 4 mM KCl, 1 mM $MgSO_4$, 25 mM Tris/maleate adjusted to pH 7.4) over a 1 min period. ATP (1.5 mM final concentration) was then introduced which allowed synaptic vesicles to polarize. This was followed by addition of the amino alcohols followed by CCCP as required. Fluorescence recordings were run for a total of 300 s.

2.10. The effect of amino alcohols on the uptake of [^{3}H]-D-aspartic acid by synaptic vesicles

Synaptic vesicles were equilibrated at 0 °C for at least an hour in a 20-fold excess of buffer (0.25 M sucrose, 1 mM $MgSO_4$, 4 mM KCl, 35 mM Tris/maleic acid adjusted to pH 7.4). Polarization of synaptic vesicles was achieved by warming to 30 °C, and incubating for 5 min with ATP (2 mM) with stirring. To 200 μl synaptic vesicles (approximately 70 μg protein) in buffer, D-aspartate (0.8 μCi [^{3}H]-D-aspartate; final concentration 38 μM) was added with or without ethanolamine, methylethanolamine or dimethylethanolamine, as appropriate, and incubations continued for 5 min at 30 °C. Uptake was terminated by the addition of ice-cold buffer (2 ml) containing ATP and the appropriate amino alcohol and, after vortexing, the synaptic vesicles were quickly filtered under vacuum through a nylon filter (pore size < 0.45 μm). The synaptic vesicles retained on the filters were then washed twice with 2 ml of the same ice-cold same solution. The filters were then placed in 1 ml 10% sodium dodecyl sulfate to release radioactivity from synaptic vesicles. Radioactivity was quantitated using liquid scintillation counting. Uptake of [^{3}H]-D-aspartic acid was temperature-sensitive and all uptake values at 30 °C were corrected by

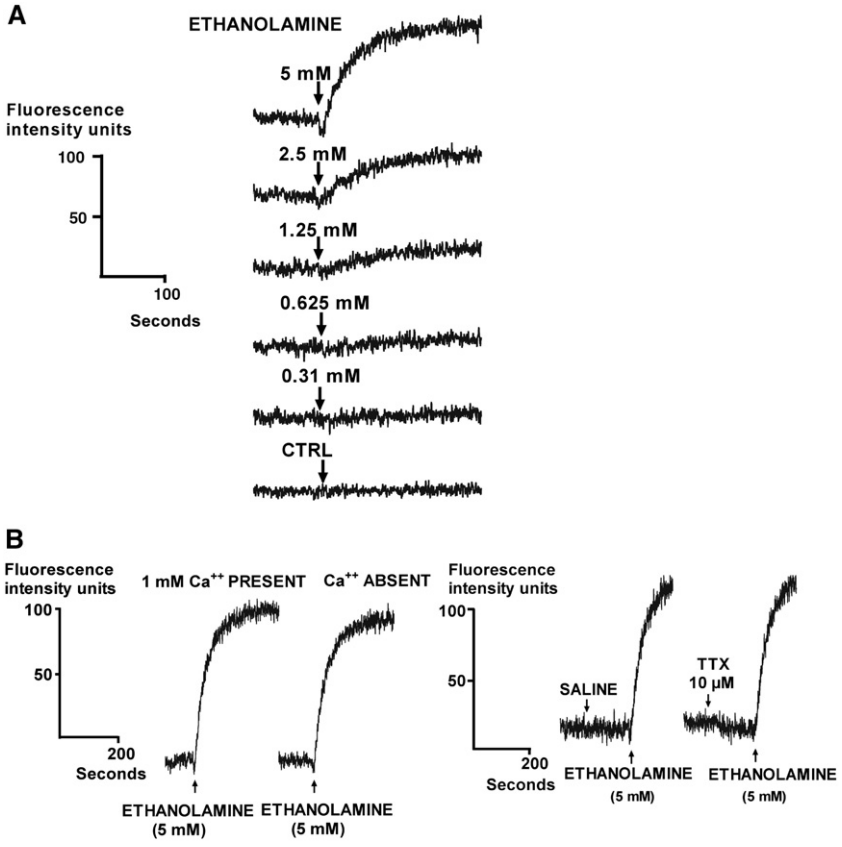

Fig. 2. Showing (A) increases in AO fluorescence intensity in synaptosomal suspensions exposed to ethanolamine concentrations between 0.31 and 5 mM and (B) the lack of effect on ethanolamine's response when Ca^{++} is absent from the saline (left) or when synaptosomes are pretreated with 10 μM tetrodotoxin (TTX; right). Profiles are typical of three experiments and Ca^{++} was included in the saline at 1 mM except where indicated otherwise.

subtracting the radioactivity non-specifically associating with synaptic vesicles at 0 °C.

2.11. Protein assay

Protein levels were measured using to the method of Lowry as adapted by Peterson (1977).

2.12. Analysis of results

Where appropriate results are expressed as mean±standard error (S.E.M.) with the level replication stated. Fluorescence profiles were constructed and statistical analysis (Student's *t* test) performed using Prism 4 software (Graphpad, CA, USA).

3. Results

3.1. Effects of ethanolamine and other amino alcohols on acridine orange fluorescence in synaptosomes

In synaptosomal preparations 35 mM KCl produced a distinct rise in AO fluorescence above steady state control levels which only occurred in the presence of Ca^{++} (Fig. 1A) in accordance with previous reports (Zoccarato et al., 1999; Melnik et al., 2001). The sodium channel activator veratridine (33 μM) also enhanced AO fluorescence and this response was reduced substantially by 10 μM tetrodotoxin (Fig. 1B). Ethanolamine (5 mM) caused a marked and sustained increase in the intensity of AO fluorescence (Fig. 2A). Responses to ethanolamine were concentration-related and threshold increases were detected at 0.31 mM. The increase in AO fluorescence produced by ethanolamine was independent of Ca^{++} in the saline and was unaffected by prior treatment of synaptosomes with 10 μM tetrodotoxin (Fig. 2B). Other amino alcohols methylethanolamine and dimethylethanolamine at 5 mM, caused similar rises in AO fluorescence, however choline was devoid of any effect (Fig. 3). In Fig. 4A, a typical profile for 5 mM ethanolamine is shown together with responses to 35 mM KCl, the magnitude of which is unaffected by time of challenge. From the time at which the increase in AO fluorescence produced by 5 mM ethanolamine nears its peak and onwards 35 mM KCl becomes progressively less able to increase AO fluorescence (Fig. 4B).

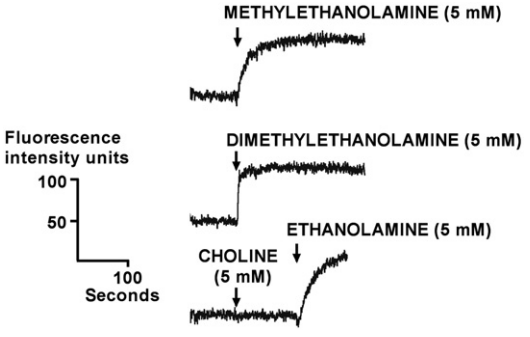

Fig. 3. Typical recordings of changes in AO fluorescence in synaptosomal suspensions exposed to methylethanolamine and dimethylethanolamine. Note the base choline produced no change in fluorescence, however following choline application, the preparation was still responsive to ethanolamine. Study compounds were added at 5 mM and Ca^{++} was present at 1 mM throughout. Results are representative of 3 experiments.

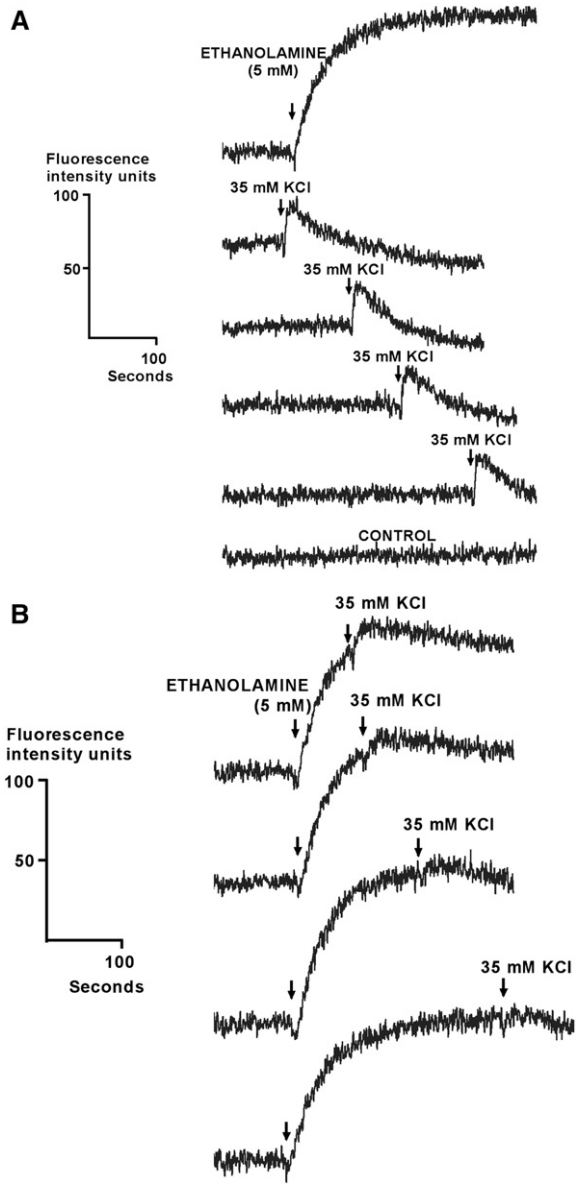

Fig. 4. (A) Control responses to 5 mM ethanolamine and 35 mM KCl, the latter applied at various times after approximately 100 s. In (B) traces show the diminishing ability of 35 mM KCl to add to the increase in AO fluorescence induced by 5 mM ethanolamine over time. Ca^{++} (1 mM) was included throughout. All traces are typical of 3 experiments.

3.2. Inability of ethanolamine to influence the plasma membrane potential

Measurements of synaptoneurosomal plasma membrane potential using the fluorescent voltage-sensitive probe rhodamine 6G demonstrated that ethanolamine and its methyl and dimethyl forms do not depolarize or hyperpolarize the nerve membrane (Table 1). It is also apparent from our results that the neuronal plasma membrane can still be depolarized by KCl in the presence of each amino alcohol.

Table 1
Inability of ethanolamine, methylethanolamine and dimethylethanolamine (all at 5 mM) to modify the membrane potential of synaptoneurosomes

Treatment	Membrane potentials (mV) 100 s after exposing synaptoneurosomes to various treatments	Membrane potentials (mV) produced by 35 mM KCl after each amino alcohol treatment
Control ($n=4$)	−78.40±1.57	–
Ethanolamine ($n=6$)	−81.88±0.51 [a]	−45.28±4.89
Methylethanolamine ($n=5$)	−79.10±0.74 [a]	−31.72±0.98
Dimethylethanolamine ($n=5$)	−78.84±1.01 [a]	−30.71±1.95
KCl ($n=5$)	−38.43±4.27 [b]	–
Veratridine ($n=4$)	−15.21±8.16 [b]	–

Membrane potentials were quantitated using the voltage-sensitive fluorescent dye rhodamine 6G as described in Materials and methods. Responses to standard depolarizing treatments (35 mM KCl and 33 μM veratridine) are provided for comparison. In addition the response to 35 mM KCl added after each amino alcohol challenge is displayed. Results are means±S.E.; n values are given in brackets.

[a] Not significantly different from control, $P>0.05$.

[b] Significantly different from control, $P<0.01$.

3.3. Release of [^{3}H]-D-aspartic acid from synaptosomes

The effects of amino alcohols (all at 5 mM) on the basal and 35 mM KCl-evoked release of [^{3}H]-D-aspartic acid from superfused synaptosomes are displayed in Fig. 5. Ethanolamine, methylethanolamine and dimethylethanolamine gradually increased basal release of [^{3}H]-D-aspartic acid which overall amounted to increases of 65, 68 and 65% respectively. Amino alcohol-induced increases in the level of resting release were in the main sustained for the remainder of the exposure period. Ethanolamine, methylethanolamine and dimethylethanolamine also enhanced 35 mM KCl-evoked release of [^{3}H]-D-aspartic acid from synaptosomes (by 52, 50, and 50% respectively) and these amino alcohol-enhanced [^{3}H]-D-aspartic acid release profiles generally shadowed that of KCl alone. No attempt was made to examine potential returns to basal release levels upon termination of KCl stimulation.

3.4. The effects of ethanolamine, methylethanolamine and dimethylethanolamine on the ATP-activated increase in intravesicular proton levels

Experiments to directly measure the effects amino alcohols on the H^+-ATPase-activated increase in intravesicular proton concentration were conducted with a synaptic vesicle preparation from bovine cortex. ATP caused the typical end point decrease in acridine orange fluorescence (Roz and Rehavi, 2003), consistent with increased pumping of protons into the interior of synaptic vesicles by the H^+-ATPase (Fig. 6). Subsequent exposure to ethanolamine, methylethanolamine and dimethylethanolamine (all 5 mM) caused an immediate drop in intravesicular proton concentration which reduced to a level lower than that observed before the ATP addition (Fig. 6A, B, C). The protonophore CCCP also brought about a rapid decline in intravesicular acidification (Fig. 6D) and produced negligible (ethanolamine and methylethanolamine) or slight (dimethylethanolamine) enhancement of amino alcohol-induced proton depletions (Fig. 6 A, B, C).

3.5. The effects of ethanolamine, methylethanolamine and dimethylethanolamine on the transmembrane potential of synaptic vesicles

Changes to the electrical transmembrane potential of synaptic vesicles are conveniently measured using the anionic fluoroprobe oxonol V. Addition of ATP to synaptic vesicles led to rapid formation of the membrane potential, as evidenced by a decline in fluorescence (due to increased association of free oxonol with synaptic vesicles; Fig. 7A, B, C, D). Ethanolamine, methylethanolamine and dimethylethanolamine (at 5 mM) strongly increased the extent of synaptic vesicle polarization (Fig. 7A, B, C). CCCP strongly depolarized synaptic vesicles in

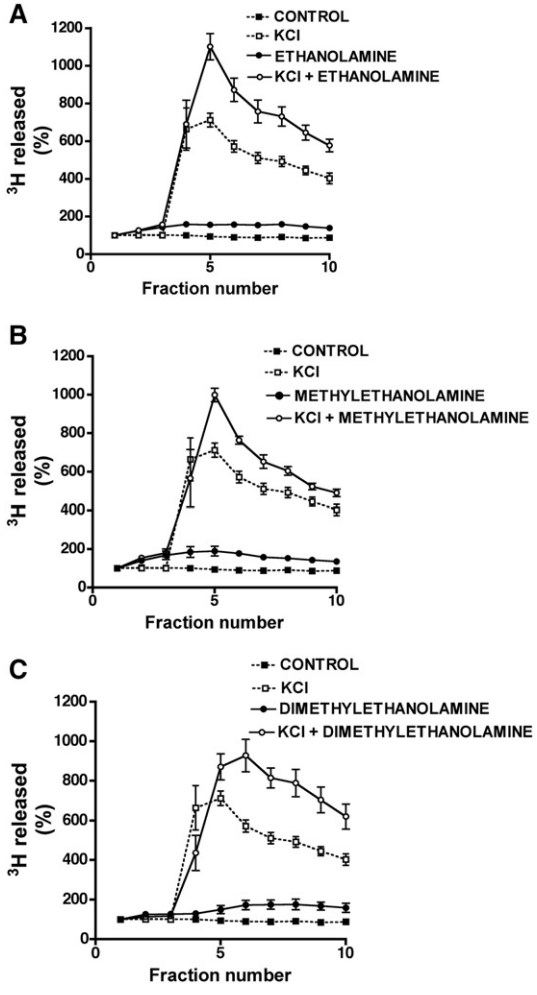

Fig. 5. The effect of (A) ethanolamine, (B) methylethanolamine and (C) dimethylethanolamine on the resting (basal) and KCl-evoked release of [^{3}H]-D-aspartic acid from mouse brain synaptosomes. Values represent ^{3}H released as a percentage of ^{3}H present in fraction 1. Data points represent means and vertical bars the S.E. of 3–6 experiments. Amino alcohols (5 mM) were applied just after the start of sample collection and saline containing KCl (35 mM) was routinely introduced at fraction 4.

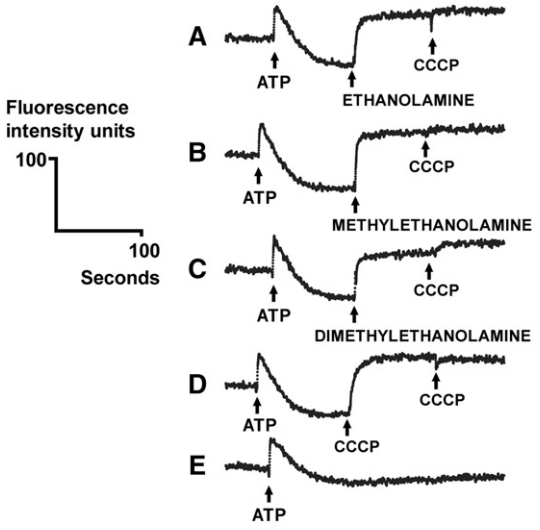

Fig. 6. The effect of (A) ethanolamine, (B) methylethanolamine, (C) dimethylethanolamine and (D) CCCP (7.5 μM) on ATP-dependent quenching of AO fluorescence in synaptic vesicle preparations. Recording (E) shows the baseline after ATP addition. Amino alcohols were added at 5 mM. Each trace is representative of at least 3 experiments. Note that the upward deflections produced by study compounds equate to a loss of intravesicular protons and that the ability of CCCP to reduce proton levels in the interior of synaptic vesicles beyond that of the amino alcohols was negligible or weak (traces A–C).

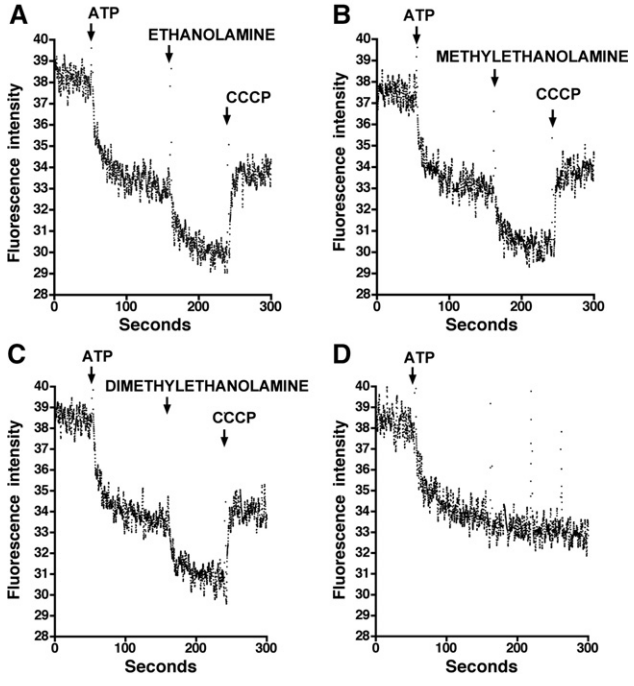

Fig. 7. The effect of (A) ethanolamine, (B) methylethanolamine and (C) dimethylethanolamine on the ATP-supported (H^+-ATPase-dependent) electrical potential of synaptic vesicles as determined using oxonol V. Downward deflections represent polarization and upward deflections signify depolarization of the synaptic vesicular membrane. Amino alcohols were added at 5 mM. CCCP fully reverses the membrane polarizing influence of all amino alcohols (A–C). Recording (D) is the baseline (saline/solvent control). Results for amino alcohols are typical of 3 or more experiments.

Table 2
The effect of ethanolamine, methylethanolamine and dimethylethanolamine (all at 5 mM) on the uptake of [^{3}H]-D-aspartic acid into synaptic vesicles prepared from bovine cortex

Treatment	Uptake of [^{3}H]-D-aspartic acid into synaptic vesicles (nmol/mg protein/min)
Control	0.56±0.01
Ethanolamine	0.99±0.05 [a]
Methylethanolamine	0.91±0.08 [b]
Dimethylethanolamine	1.37±0.15 [a]

Values represent means±S.E. of 3–4 experiments. See Materials and methods for details of the assay.
[a] Significantly different from control $P<0.01$.
[b] Significantly different from control $P<0.05$.

the presence of ethanolamine, methylethanolamine and dimethylethanolamine (Fig. 7A, B, C).

3.6. The effects of amino alcohols on the uptake of [^{3}H]-D-aspartate into synaptic vesicles

Each amino alcohol was examined for their ability to influence the accumulation of [^{3}H]-D-aspartic acid by synaptic vesicles in the presence of ATP (Table 2). The uptake of [^{3}H]-D-aspartic acid by synaptic vesicles was significantly ($P<0.05$) increased in the presence of ethanolamine, methylethanolamine and dimethylethanolamine by amounts which averaged 76, 62 and 145% respectively.

4. Discussion

The quenching of AO fluorescence upon addition of this probe to synaptosomal suspensions arises from its ability to become protonated when sequestered within the acidic interior of synaptic vesicles, whereas high [K^+]-induced increases in fluorescence above steady state are Ca^{++}-dependent and reflect release of AO-rich synaptic vesicle contents by exocytosis (Zoccarato et al., 1999; Melnik et al., 2001). In support of the specificity of AO for synaptic vesicles, bafilomycin A_1, which selectively blocks H^+ translocation by vacuolar H^+-ATPases (Floor et al., 1990; Moriyama and Futai, 1990), eliminates both initial quenching of AO fluorescence by synaptosomes and markedly stimulates loss of AO from synaptosomes previously loaded with probe (Zoccarato et al., 1999). Inorganic weak bases also elicit similar responses. For instance, ammonium chloride prevents the loading of synaptic vesicles with AO and activates the efflux of AO from synaptic vesicles previously equilibrated with fluoroprobe by sequestration of protons (Melnik et al., 2001). The sodium channel-independent components of AO efflux observed with organic bases such as 4-aminopyridine (Zoccarato et al., 1999) and veratridine (this study), likely also occur through a similar mechanism. Ethanolamine and the other amino alcohols used in this investigation also possess weakly basic properties.

In the present experiments we observed profound stimulatory effects of ethanolamine, methylethanolamine and dimethylethanolamine on the fluorescence signal of synaptosomes pre-equilibrated with AO suggesting that these amino alcohols

either enhance the exocytosis of synaptic vesicles or reduce the level of protons in the synaptic vesicle interior. In parallel assays, the depolarizing agents KCl and veratridine produced calcium-dependent and tetrodotoxin-sensitive efflux of AO respectively. However, unlike KCl which directly depolarizes synaptosomes and opens voltage-gated calcium channels (Blaustein and Goldring, 1975; Nachshen and Blaustein, 1980), ethanolamine does not affect the synaptosomal plasma membrane potential and its AO fluorescence response is not affected when Ca^{++} is absent from the external saline. These observations suggest that these amino alcohols do not act by opening voltage-gated calcium channels in the plasma membrane. Our findings therefore invoke a different mechanism to that suggested by Bostwick et al. (1993), who proposed that amino alcohols enhance calcium entry into nerve terminals after activation of L-type calcium channels. Voltage-gated sodium channel activation cannot be of relevance to the actions of ethanolamine either, since in addition to the failure of ethanolamine to depolarize the plasma membrane, ethanolamine-induced release of AO from synaptosomes was unaffected by tetrodotoxin. Additionally, our results demonstrate that ethanolamine eliminates the ability of 35 mM K^+ to release AO from synaptosomes, clearly suggesting that amino alcohols also discharge the K^+-sensitive synaptic vesicle pool labelled with AO. However, we found that ethanolamine, methylethanolamine and dimethylethanolamine enhance both basal and KCl-evoked release of [^{3}H]-D-aspartic acid from synaptosomes. Bostwick et al. (1993), reported that ethanolamine and other amino alcohols (R-alaninol and R-prolinol) enhance KCl-induced release of acetylcholine from hippocampal slices. While our observations on the release of [^{3}H]-D-aspartate from synaptosomes showed good agreement with those of Bostwick et al., our data also presented somewhat of a paradox since it has also been shown that bafilomycin A_1, which reduces the proton concentration of neurite vesicles (Cousin and Nicholls, 1997) also reduces KCl-evoked release of glutamic acid from synaptosomes (Pocock et al., 1995).

To explore this apparent anomaly in more depth we conducted investigations on synaptic vesicles isolated from bovine brain. Experiments with AO provided direct confirmation that ethanolamine as well as the mono- and dimethyl amino alcohols dramatically reduce proton levels in the synaptic vesicle interior. In these assays, clear parallels with CCCP were evident. The pumping of protons from the cytoplasm into synaptic vesicles by V-H^+-ATPases generates the main electrochemical gradient powering vesicular accumulation of neurotransmitters (Morel, 2003). Carbonylcyanidehalophenylhydrazones are known to dissipate proton gradients in synaptic vesicles (Roz and Rehavi, 2003). However, our oxonol V experiments also revealed that in marked contrast to CCCP, amino alcohols markedly increase the electrical transmembrane potential of synaptic vesicles and this latter observation correlates well their ability to enhance the uptake of [^{3}H]-D-aspartic acid into synaptic vesicles. Taken together, our data strongly imply that the reduction in intravesicular proton levels seen with amino alcohols cannot be due to efflux of protons from synaptic vesicles. Given the basic (proton accepting) nature of amino alcohols, ethanolamine most likely acts by rapidly accessing the interior of synaptic vesicles where it immediately sequesters protons. Protonation can occur on the amine and possibly also on the hydroxyl group yielding positively charged species which, as the oxonol V experiments predict, will contribute more to the overall electrochemical potential than proton equivalents. Comparable hyperpolarizing actions of nigericin have been observed, although this substance facilitates K^+ entry into the intravesicular compartment coupled to H^+ removal (Tabb et al., 1992). Since the protonated forms of amino alcohols are unable to traverse membranes easily, any decline in the higher transvesicular membrane potential should be limited. Additionally, the oxonol V data suggest that by reversing amino alcohol-induced hyperpolarization, CCCP shifts the equilibrium in favor of proton dissociation from amino alcohols and allows proton efflux from the synaptic vesicle interior to proceed. In summary, therefore, we propose that enhanced filling of synaptic vesicles with [^{3}H]-D-aspartate from the cytoplasmic compartment explains the increased basal and evoked amino acid release we observed with amino alcohols in our synaptosome experiments. Since [^{3}H]-D-aspartic acid is not metabolized, the quantities originally taken up into synaptosomes should be sufficient to sustain the increased amino alcohol-dependent transfer to synaptic vesicles.

In the synaptosomal assays we found that choline had no effect on AO fluorescence. Since choline contains a quaternary nitrogen it is unable to traverse membranes and is also considerably less susceptible to protonation. Moreover, the ability of choline to access synaptic vesicles will be very limited because although choline would be expected to be transported efficiently into the cytoplasmic compartment of cholinergic nerve endings, it must undergo metabolism to acetylcholine which is then pumped into synaptic vesicles (Sha et al., 2004).

The physiological relevance of our findings needs placing in proper perspective. Our concentration-response data for ethanolamine demonstrate threshold effects on AO fluorescence close to 0.31 mM (18.9 μg/ml), while well-defined increases in fluorescence occur up to 5 mM (305.4 μg/ml). The concentrations of ethanolamine required to influence AO fluorescence align well with a report (Ellison et al., 1987), which established that resting levels of this amino alcohol in various regions of mammalian brain lie between 197–870 nmol/g wet wt. (12.03–53.1 μg/ml equivalent). In other tissues, for example liver, a similar resting concentration of 0.54 mM (32.9 μg/ml) ethanolamine has been reported (Houweling et al., 1992). Therefore the concentrations of ethanolamine we employed in this research are close to physiological. A report by Andriamampandry et al. (1989) indicates that the steady state levels of methyl- and dimethyl ethanolamine may be lower than that of ethanolamine. Although it is generally assumed that ethanolamine exists mostly in the protonated form in tissues, it remains to be established whether intracellular enzymes for example, serine decarboxylase or those involved in base exchange reactions, initially release the unprotonated species and, indeed, if such reactions can occur in synaptic vesicles to the extent that might permit amino alcohol-dependent regulation of synaptic vesicle filling. The mechanism we outline in this report may

also have relevance to the development of certain neuropathophysiological states where ethanolamine concentrations are known to change. For example, ethanolamine levels in brain tissues from epilepsy patients are elevated five-fold compared to non-epileptogenic samples and considerable intracellular build-up of ethanolamine has been reported in epileptiform brain (Hamberger et al., 1991, 1993). In addition, ischemic episodes are thought to trigger the release of ethanolamine by activation of base exchange reactions in the brain (Buratta et al., 1998). In both disease states, increased release of L-glutamate (for which [^{3}H]-D-aspartate acts as a surrogate) has been implicated (Meldrum, 1994; Choi and Rothman, 1990).

The central finding of this investigation is that at concentrations close to those found in normal brain tissue, ethanolamine and closely related amino alcohols have the ability to positively modulate the vesicular membrane potential, enhancing the capacity of both synaptic vesicles to take up [^{3}H]-D-aspartate and synaptosomes to release this amino acid. Since [^{3}H]-D-aspartate is an exogenously applied mimic of L-glutamate, we are now considering the relevance of this mechanism to the dynamics of endogenous neurotransmitters such as glutamate and GABA. We are also interested in how changes in neuronal activity might influence the concentrations of amino alcohols such as ethanolamine within the nerve ending. Finally, in view of reports suggesting ethanolamine release may be more important in some areas of the brain than others (Korf and Venema, 1985; Van Der Heyden et al., 1979) regiospecific effects might also be anticipated.

Acknowledgement

This research was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to R.A.N.

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