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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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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.

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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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