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Modulation of Aspartate Release by Ascorbic Acid and Endobain E, an Endogenous Na^+, K^+-ATPase Inhibitor

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The isolation of a soluble brain fraction which behaves as an *endo*genous oua*bain*-like substance, termed *endobain E*, has been described. Endobain E contains two Na^+, K^+-ATPase inhibitors, one of them identical to ascorbic acid. Neurotransmitter release in the presence of endobain E and ascorbic acid was studied in non-depolarizing (0 mM KCl) and depolarizing (40 mM KCl) conditions. Synaptosomes were isolated from cerebral cortex of male Wistar rats by differential centrifugation and Percoll gradient. Synaptosomes were preincubated in HEPES-saline buffer with 1 mM D-[^{3}H]aspartate (15 min at 37°C), centrifuged, washed, incubated in the presence of additions (60 s at 37°C) and spun down; radioactivity in the supernatants was quantified. In the presence of 0.5–5.0 mM ascorbic acid, D-[^{3}H]aspartate release was roughly 135–215% or 110–150%, with or without 40 mM KCl, respectively. The endogenous Na^+, K^+-ATPase inhibitor endobain E dose-dependently increased neurotransmitter release, with values even higher in the presence of KCl, reaching 11-times control values. In the absence of KCl, addition of 0.5–10.0 mM commercial ouabain enhanced roughly 100% D-[^{3}H]aspartate release; with 40 mM KCl a trend to increase was recorded with the lowest ouabain concentrations to achieve statistically significant difference vs. KCl above 4 mM ouabain. Experiments were performed in the presence of glutamate receptor antagonists. It was observed that MPEP (selective for mGluR5 subtype), failed to decrease endobain E response but reduced 50–60% ouabain effect; LY-367385 (selective for mGluR1 subtype) and dizocilpine (for ionotropic NMDA glutamate receptor) did not reduce endobain E or ouabain effects. These findings lead to suggest that endobain E effect on release is independent of metabotropic or ionotropic glutamate receptors, whereas that of ouabain involves mGluR5 but not mGluR1 receptor subtype. Assays performed at different temperatures indicated that in endobain E effect both exocytosis and transporter reversion are involved. It is concluded that endobain E and ascorbic acid, one of its components, due to their ability to inhibit Na^+, K^+-ATPase, may well modulate neurotransmitter release at synapses.

KEY WORDS: Endobain E; ascorbic acid; aspartate release; neurotransmitter release; Na^+, K^+-ATPase.

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INTRODUCTION

Evidence from the literature point to a relationship between the levels of excitatory amino acids and ascorbic acid in brain tissue and a link between the release of glutamate and extracellular ascorbate concentration has been advanced (1). Electrophysiological studies carried out in striatal neurons indicate that ascorbic acid potentiates excitation induced by glutamate, the main excit-

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atory amino acid in mammalian CNS, though depending on dose and application period employed, ascorbic acid attenuates glutamate response instead (2).

Although glutamate catabolism involves several enzymatic pathways, after release to the synaptic cleft, its physiological action is stopped by uptake through the presynaptic and astrocyte membranes (3). On assaying glutamate reuptake, findings recorded suggest that this process, which is coupled to ascorbic acid exchange, is both neuronal and glial in nature; excitatory amino acid receptors seem independent in the glutamate-elicited ascorbate efflux (4).

Ascorbic acid is highly concentrated in extra as well as intracellular brain compartments, and seems to undergo dynamic changes in response to diverse physiological and pathophysiological conditions; significative ascorbic changes have been demonstrated in hypoxia, spreading depression and seizure activity (5).

On assaying extracellular fluid levels of ascorbic acid, it has been shown by *in vivo* electrochemistry that ascorbate signals are directly related to the amounts of microinjected glutamate and ascorbate release from brain cells has been associated with activation of glutamatergic neurons, mainly by glutamate–ascorbate heteroexchange across cell membranes of neurons or glial cells (4,6). The uptake blocker L-*trans* pyrrolidine 2,4-dicarboxylate reduces the response, suggesting a neuroprotective function for this coupling of ascorbic acid and glutamate release (7).

In previous work, a soluble brain fraction (peak II) was separated (8), which besides inhibiting Na^+, K^+-ATPase activity also shares other properties with ouabain (9), thus suggesting the term *endobain* (10). Fractionation of peak II by ionic exchange HPLC led to a more purified Na^+, K^+-ATPase inhibitor, II-E (11), which is structurally different from the plant-origin cardiac glycoside (12); however, it is also able to block high affinity [^{3}H]-ouabain binding (13), to induce neurotransmitter release (14), and to enhance phosphoinositide hydrolysis (15), justifying its denomination as *endobain E* (16). Phosphoinositide hydrolysis stimulation by endobain E involves metabotrobic glutamate receptors (17), attributable to glutamate release enhancement. Further HPLC analysis in an Aminex column of endobain E disclosed the presence of two components, one eluting with retention time and UV spectrum indistinguishable from those of ascorbic acid and a second, as yet unidentified, both exerting Na^+, K^+-ATPase inhibition (18).

In the present study, we analyzed endobain E and ascorbic acid effect on neurotransmitter release from cerebral cortex synaptosomes preloaded with D-[^{3}H]aspartate.

EXPERIMENTAL PROCEDURE

Animals and Drugs. Male Wistar rats, 80–100 g and 130–160 g weight were used respectively to prepare synaptosomes and endobain E. All studies described were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, USA. Reagents were analytical grade. Ouabain, ascorbic acid, Percoll and Sephadex G-10 were from Sigma Chemical Co. (St. Louis, MO, USA); 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and (+)-2-methyl-4-carboxyphenylglycine (LY-367385) were obtained from Tocris (Ballwin, MO, USA); Sephadex G-50 (fine grade) was from Pharmacia Fine Chemicals (Uppsala, Sweden). D-[^{3}H]aspartate, with a specific radioactivity of 666 GBq/mol $\times 10^{-3}$, was purchased from New England Nuclear, Du Pont (Boston, MA, USA); BCS scintillation cocktail was from Amersham Biosciences (Uppsala, Sweden).

Preparation of Synaptosomes. A technique based on previous reports (19,20) was employed. For each preparation, cerebral cortices from 2 rats were harvested and dropped into cold 0.32 M sucrose (neutralized to pH 7 with Tris base). Tissues were homogenized at 10% (w/v, original tissue) for two 1-min periods in 0.32 M sucrose using a Teflon glass homogenizer of the Potter-Elvehjem type, and centrifuged at $900 \times g$ for 10 min with one washing; resultant supernatants were pooled and centrifuged at $11{,}500 \times g$ for 20 min to obtain a pellet containing synaptosomes, mitochondria and myelin fragments. This pellet was resuspended in 0.32 M sucrose, layered on top of a Percoll gradient (8 and 18%) prepared in isoosmotic sucrose and centrifuged at $60{,}000 \times g$ for 30 min in a SW25 rotor of a L8-Beckman ultracentrifuge. The fraction laying at 18% Percoll (synaptosomes) was separated and diluted with HEPES buffer saline solution (HBSS) containing, in mM: HEPES 27; NaCl 133; KCl 2.4; $MgSO_4$ 1.2; KH_2PO_4 1.2; glucose 12, $CaCl_2$ 1.0, adjusted to pH 7.4 with Tris base. The sample was spun down at $100{,}000 \times g$ for 40 min in a 70.1 Ti rotor of a L8-Beckman ultracentrifuge, and immediately used for neurotransmitter release.

Preparation of II-E Fraction (Endobain E). Peak I and II fractions from rat cerebral cortex were prepared as previously described (8,11). Thus, for each preparation, cerebral cortices from 5 rats were pooled, homogenized at 25% (w/v, original tissue) in redistilled water, and centrifuged at $100{,}000 \times g$ for 30 min at 4°C in a 70.1 Ti rotor of a L8-Beckman ultracentrifuge. A 5-ml supernatant sample (brain soluble fraction) was taken to pH 7.4 with 0.1 M NH_4HCO_3, loaded on a Sephadex G-10 column (1×20 cm) and a single 11-ml fraction collected. Then, this filtrate was applied to a column packed with Sephadex G-50 (1.8×25 cm). For gel equilibration and elution, 0.01 M NH_4HCO_3 was used. Fractions of 1.4 ml each at a flow rate of 0.3 ml per min were collected in a Gilson Fraction Collector 202. The absorbance profile was recorded at 280 nm. Peaks I and II were made up with the fractions presenting maximal UV absor-

bance. Peak I was made up with fractions 19–23 but not used in this study; peak II was made up with fractions 48–52. Peak II was adjusted to pH 2.0 with 2 M HCl, lyophilized and stored at −20°C. The following day, peak II samples were processed by anionic exchange HPLC on a Synchropak AX-300 column, 4.6 × 250 mm (Synchrom Inc., Lafayette, IN, USA), and eluted at a flow rate of 0.5 ml per min with a 20-min gradient from 0.001 to 0.010 M NH_4HCO_3 to separate fractions II-A to II-H. Fractions were collected by monitoring of absorbance curves at 230 nm; II-E was collected at 12–13 min, then lyophilized, and used within 20 days (11). Lyophilized II-E samples were dissolved in 0.006 M HCl and immediately prior to assay neutralized with 0.2 M Tris base solution. Hereafter, II-E fraction is termed *endobain E*.

D-[^{3}H]Aspartate Release from Synaptosomes. The release was performed in fresh synaptosomes following a technique previously described (21), with modifications. Synaptosome pellets were resuspended (at 6 mg protein per ml) and preincubated at 37°C for 15 min with 1 mM D-[^{3}H]aspartate (specific activity 18 mCi/mmol) in HBSS and centrifuged at 16,000 × *g* for 1 min at 4°C; supernatants were discarded and pellets were washed four times in HBSS by succesive resuspension and centrifugation as above. Final pellets were resuspended in HBSS (0.6 mg protein per ml) and incubated at 37°C for 60 s in the absence or presence of KCl, ascorbic acid, endobain E or ouabain at 1 ml final volume. When indicated, incubation proceeded at 25°C or 18°C. To test the effect of glutamate receptor antagonists, drugs were included during synaptosomal sample preincubation at 37°C for 15 min with 1 mM D-[^{3}H]aspartate before addition of endobain E or ouabain. Radioactivity in pellets and supernatants was separately quantified by liquid scintillation with BCS scintillation cocktail. Results are presented as means (± SD) from 3 to 7 experiments performed with at least three different synaptosomal preparations. D-[^{3}H]aspartate release was calculated as a percentage of release recorded in the absence of additions.

Protein Measurement. Protein was determined by the method of Lowry et al. (22) using bovine serum albumin as standard.

RESULTS

Neurotransmitter release was studied in cerebral cortex synaptosomes preloaded with D-[^{3}H]aspartate under several experimental conditions. Basal D-[^{3}H]aspartate release ranged between 2 and 4 nmol per minute per mg synaptosome protein.

On assaying the effect of 0.5–5.0 mM ascorbic acid on the release, approximately 10–50% or 35–115% enhancement was recorded, whether the medium lacked or contained 40 mM KCl, respectively (Fig. 1). Data in the presence of KCl vs. those in the absence of KCl attained statisticallly significant differences in most experimental points.

The endogenous Na^+, K^+-ATPase inhibitor endobain E dose-dependently increased neurotransmitter release; with 1–10 µl endobain E (1 µl equivalent to 0.9 mg original tissue) aspartate release increase ranged from 100 to 270% with values slightly higher if the medium contained 40 mM KCl (Fig. 2a). With higher endobain E concentration, the release increased markedly, with values even higher in the presence of KCl, reaching 11-times control values with 40 µl endobain E (1 µl equivalent to 9 mg original tissue) (Fig. 2b).

Ouabain at 0.5–10.0 mM concentration, in the absence of KCl, enhanced roughly 100% D-[^{3}H]aspartate release, an effect which was no-dose dependent. With 40 mM KCl, a trend to increase was recorded with the lowest ouabain concentrations; values achieved statistically significant differences vs. KCl above 4 mM ouabain (Fig. 3).

In order to analyze potential participation of glutamate receptors in endobain E and ouabain effects on the release, experiments were performed in the presence of specific antagonists. D-[^{3}H]aspartate release was analyzed in the presence of MPEP or LY-367385, respectively antagonists for mGluR5 and mGluR1 metabotropic glutamatergic receptor subtypes. Experiments were carried out after synaptosomal preincubation with the antagonists. It was observed that MPEP or LY-367385 failed to decrease D-[^{3}H]aspartate release enhancement by 1–10 µl endobain E (data not shown). The effect of 1 mM ouabain was unaffected by LY-367385 but was reduced 40–60% with 0.5–10.0 µM MPEP (Fig. 4). Control assays to test the effect of antagonists alone were performed, to observe that percentage D-[^{3}H]aspartate release remained unaltered with MPEP or LY-367385 (Fig. 4, inset).

The presence of dizocilpine, a glutamate ionotropic antagonist, failed to alter D-[^{3}H]aspartate release stimulated either by KCl, endobain E or 1 mM ouabain; the antagonist also failed to alter basal release (Table I).

To test whether D-[^{3}H]aspartate release enhancement by endobain E or ouabain were due to effects on exocytosis or to reversion of neurotransmitter transporter, experiments were run at three incubation temperatures. In the presence of endobain E plus 40 mM KCl, release mean values at 37, 25 and 18°C were respectively 370, 296 and 261 (Fig. 5a). In the presence of 1 mM ouabain plus 40 mM KCl, data values at 37, 25 and 18°C were respectively 270, 192 and 132 (Fig. 5b).

DISCUSSION

Herein excitatory amino acid release from cerebral cortex synaptosomes was studied in the presence

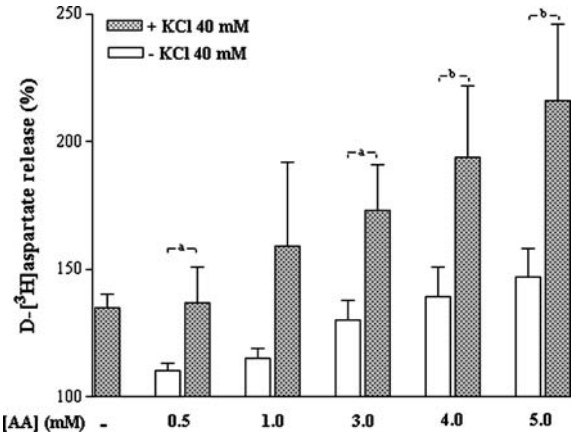

Fig. 1. The effect of commercial ascorbic acid on D-[^{3}H]aspartate release from cerebral cortex synaptosomes. Fresh synaptosomes were preloaded with D-[^{3}H]aspartate in HBSS at 37°C for 15 min, centrifuged, resuspended and incubated for 60 s at 37°C in the absence or presence of several ascorbic acid concentrations with or without 40 mM KCl. Results are presented as means (±SD) from 3 to 5 experiments performed with at least three different synaptosomal preparations. Values are shown as percentage taking as 100% data obtained in the absence of ascorbic acid. AA, ascorbic acid. $^aP < 0.05$; $^bP < 0.01$ vs. value recorded in the absence of KCl, by two-tailed Student's *t*-test.

of endobain E, which contains two Na^+, K^+-ATPase inhibitors, one of them identical to ascorbic acid. It was observed that both endobain E and commercial ascorbic acid enhance D-[^{3}H]aspartate release concentration-dependently and more markedly in depolarized synaptosomes.

Experimental evidence indicate that physiological stimulation increases both glutamate and aspartate in the extracellular compartment of the brain and that these amino acids show Ca^{2+}-dependent K^+-evoked release (3). With this background, we resorted to the employ of D-aspartate because: (a) aspartate is comparatively less vulnerable than glutamate to enzymatic catabolism; (b) aspartate release is taken as indicative of excitatory amino acid release (23); and (c) the release of endogenous aspartate is similar to that of endogenous glutamate under oxidative stress, hypoxia, and ischemia-like conditions in cultured retinal cells (24).

Since the concentration–response plot for endobain E on D-[^{3}H]aspartate release differed from that of ouabain, the involvement of distinct mechanisms is suggested. In fact, the comparison between ouabain and endobain E inhibition on Na^+, K^+-ATPase activity has indicated that they most likely bind to neighbouring sites rather than to the same site (13).

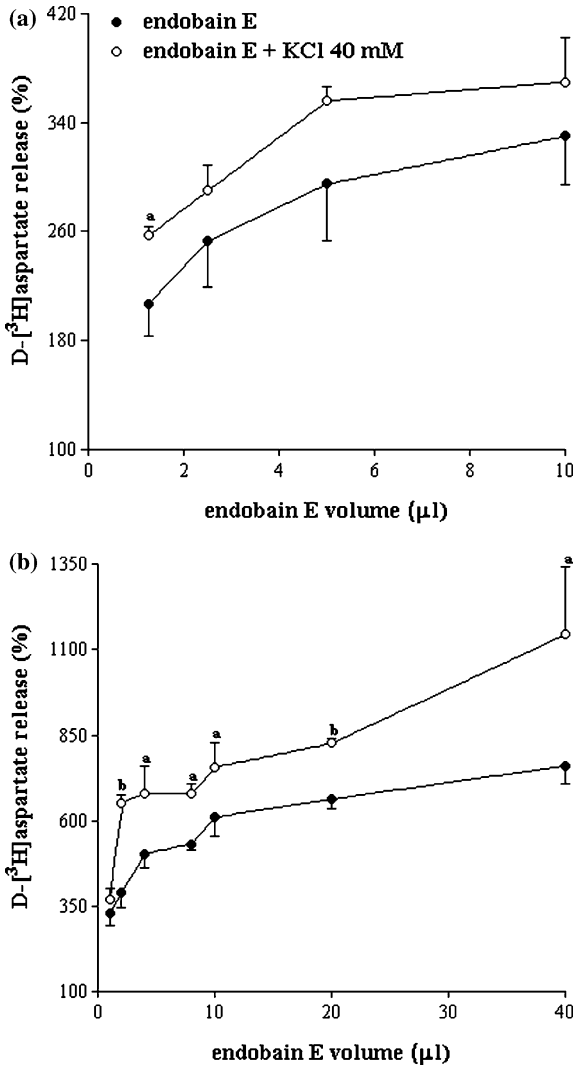

Fig. 2. The effect of endogenous Na^+, K^+-ATPase inhibitor endobain E on D-[^{3}H]aspartate release from cerebral cortex synaptosomes. Fresh synaptosomes were preloaded with D-[^{3}H]aspartate in HBSS at 37°C for 15 min, centrifuged, resuspended and incubated at 37°C for 60 s in the absence or presence of several endobain E concentrations with or without 40 mM KCl. Results are presented as means (±SD) from experiments performed with at least three different synaptosomal preparations. Values are shown as percentage taking as 100% data obtained in the absence of endobain E. (a) with 1–10 µl endobain E (1 µl equivalent to 0.9 mg original tissue); $n = 5$–13. (b) with 1–40 µl endobain E (1 µl equivalent to 9 mg original tissue); $n = 3$–13. $^aP < 0.05$; $^bP < 0.01$ vs. value recorded in the absence of KCl, by two-tailed Student's *t*-test.

The finding that only glutamate is stored in synaptic vesicles implies that glutamate is the excitatory transmitter. Released glutamate is taken up by both neurons and glia through specific transporters, and uptake of glutamate is involved in diverse

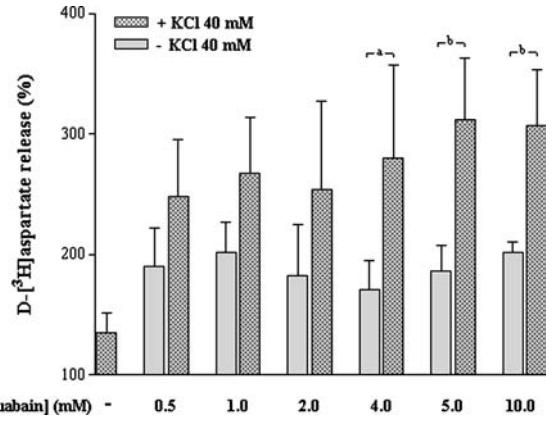

Fig. 3. The effect of ouabain on D-[^{3}H]aspartate release from cerebral cortex synaptosomes. Fresh synaptosomes were preloaded with D-[^{3}H]aspartate in HBSS at 37°C for 15 min, centrifuged, resuspended and incubated at 37°C for 60 s in the absence or presence of 40 mM KCl and 0.5–10.0 mM ouabain. Results are presented as means (± SD) from 3 to 4 experiments performed with at least three different synaptosomal preparations. Values are shown as percentage taking as 100% data obtained in the absence of additions. [a]$P < 0.05$; [b]$P < 0.01$ vs. value recorded in the absence of KCl, by two-tailed Student's *t*-test.

functions, including the release of ascorbate from neurons and glia. It is known that reversal of the glutamate transporter accounts for the parallel release of glutamate and aspartate from the cytoplasmic compartment (25).

In order to test whether metabotropic glutamate receptors were involved in endobain E and ouabain effect, assays were performed in the presence of specific antagonists. Since activation of mGluR5 receptor facilitates glutamate release (26,27), experiments were run with MPEP, a selective antagonist for this receptor. It was observed that MPEP failed to reduce endobain E effect whereas decreased 40–60% ouabain response. LY-367385, selective antagonist for mGluR1 receptor, did not decrease endobain E effect or ouabain effect. These findings lead to suggest that endobain E effect on release is independent of metabotropic glutamate receptors, whereas that of ouabain involves mGluR5 but not mGluR1 receptor subtype. Dizocilpine, a ionotropic NMDA glutamatergic receptor antagonist, failed to diminish endobain E or ouabain effect on the release.

Present results which show neurotransmitter release enhancement by endobain E and commercial ascorbic acid may be explained through modulation of Na^+, K^+-ATPase activity. In fact, in several experimental models, the inhibition of such enzyme activity leads to an increase in neurotransmitter release (28), and the ability of endobain E and commercial ascorbic acid to inhibit Na^+, K^+-ATPase has been documented (18).

Neurotransmitter release is influenced by incubation temperature, an effect dependent on both the neurotransmitter under study and the release stimulus. It has been postulated that lowering incubation temperature, it is possible to discern whether a given

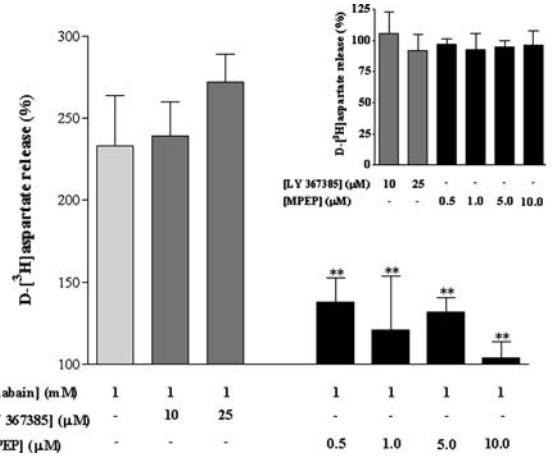

Fig. 4. The effect of mGluR antagonists LY-367385 and MPEP on D-[^{3}H]aspartate release in the presence of 1 mM ouabain. Fresh synaptosomes were preloaded with D-[^{3}H]aspartate in HBSS at 37°C for 15 min in the absence or presence of the antagonist, centrifuged, resuspended and incubated at **37°C** for 60 sec. Results are presented as means (± SD) from experiments performed with at least three different synaptosomal preparations. Values are shown as percentage taking as 100% data obtained in the absence of additions; $n = 3$–5. Inset: control experiments in the single presence of antagonists; $n = 3$–4. [**]$P < 0.01$ vs. value recorded in the presence of 1 mM ouabain, by two-tailed Student's *t*-test.

Table I. D-[^{3}H]Aspartate Release from Cerebral Cortex Synaptosomes in the Presence of KCl, Endobain E or Ouabain and Dizocilpine, an Antagonist for NMDA Ionotropic Glutamate Receptor

Addition	D-[^{3}H]-Aspartate release (% of basal)
With KCl 40 mM	135 ± 16 (9)
+ Dizocilpine 200 μM	155 ± 33 (3)
With endobain E (2 μl)	376 ± 61 (5)
+ Dizocilpine 200 μM	394 ± 61 (4)
With endobain E (10 μl)	471 ± 83 (5)
+ Dizocilpine 200 μM	422 ± 92 (4)
With ouabain 1 mM	261 ± 39 (6)
+ Dizocilpine 200 μM	247 ± 23 (4)
Dizocilpine 200 μM	103 ± 20 (8)

Synaptosome samples from rat cerebral cortex were processed for D-[^{3}H]aspartate release in the presence of KCl, endobain E or ouabain, with or without NMDA glutamatergic receptor antagonist dizocilpine. One microliter endobain E was equivalent to 9 mg original tissue. Results are expressed as means (± SD) taking as 100% values obtained in the absence of additions; between parenthesis the number of experiments is indicated.

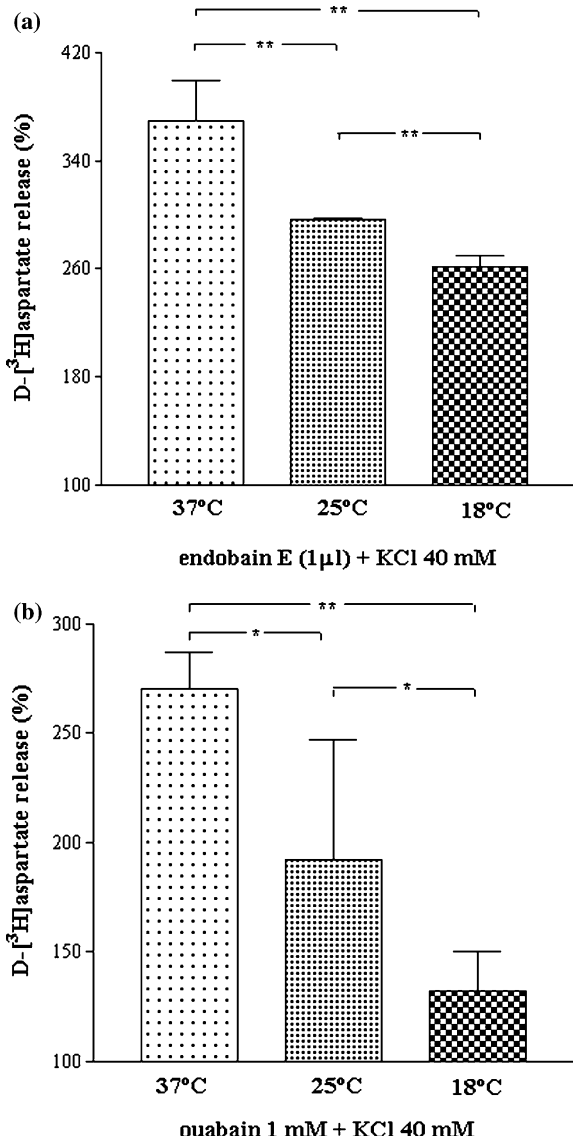

Fig. 5. The effect of endogenous Na^+, K^+-ATPase inhibitor endobain E or ouabain on D-[^{3}H]aspartate release from cerebral cortex synaptosomes at different incubation temperature. Fresh synaptosomes were preloaded with D-[^{3}H]aspartate in HBSS at 37°C for 15 min, centrifuged, resuspended and incubated at 37, 25 or 18°C for 60 s with 40 mM KCl, in the absence or presence of endobain E or ouabain. Results are presented as means (±SD) from experiments performed with at least three different synaptosomal preparations. Values are shown as percentage taking as 100% data obtained in the absence of additions. (a) with 1 µl endobain E (equivalent to 9 mg original tissue); $n = 4$. (b) with 1 mM ouabain; $n = 4$–7. $^*P < 0.05$; $^{**}P < 0.01$, by two-tailed Student's t-test.

effect on neurotransmitter release occurs through the exocytotic process or through reversion of plasma membrane transporter (29). For some neurotransmitters such as catecholamines noradrenaline and dopamine, lowering incubation temperature increases electrically induced release (29), whereas completely inhibits veratridine-evoked noradrenaline release (30). The release of GABA induced by Na^+, K^+-ATPase inhibition with ouabain decreases by reducing incubation temperature, an effect most likely due to reversion of neurotransmitter transporter (29,31). Since present results showing aspartate release enhancement by endobain E resemble ouabain action on GABA release, it is suggested that this effect, which is temperature-sensitive, occurs through reversal of the neurotransmitter transporter.

It has been postulated that glutamate action at the synapse is terminated by rapid uptake by the glutamate transporter protein which exchanges ascorbate for glutamate. Therefore, immediately following synapse activation by exocytotic glutamate release, the synaptic cleft becomes flooded with ascorbate. In the brain, the main antioxidant in the extracellular compartment is ascorbate, whereas in the presynaptic terminal such role is played by glutathione, whose synthesis depends on cysteine uptake into the terminal (32). The hypothesis that growth or pruning of synaptic spines are controlled in part by a balance in the synapse between neurodestructive prooxidant and neuroprotective antioxidant substances has been advanced and, interestingly, ascorbate is included in the latter group (33) and present results showing that it enhances aspartate release suggest its involvement in glutamatergic neurotransmission modulation.

A neuromodulatory or neuroprotective role in the brain was suggested for ascorbic acid (6). Regarding its effect on neurotransmission, whereas it fails to alter either spontaneous or stimulated dopamine or GABA release (34), present results showed that it enhances aspartate release from synaptosomes.

It is known that schizophrenia is associated with a deficit in cerebral glutathione, which leaves the brain susceptible to oxidants. It is known that rodents are able to compensate for glutathione deficit by ascorbic acid production. Studies performed in a mutant rat, which like humans, cannot synthesize ascorbic acid, indicate that under dopamine uptake blockade, low brain glutathione and ascorbic acid levels occur; the proposal that such alteration is involved in the development of some cognitive deficits affecting schizophrenic patients has been advanced (35).

To summarize, present results show D-[^{3}H]aspartate release from cerebral cortex synaptosomes by endogenous Na^+, K^+-ATPase inhibitor endobain E and ascorbic acid, suggesting their modulatory role in neurotransmission.

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