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An Endogenous Na^+, K^+-ATPase Inhibitor Enhances Phosphoinositide Hydrolysis in Neonatal but Not in Adult Rat Brain Cortex

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The effect of an endogenous Na^+, K^+-ATPase inhibitor, termed endobain E, on phosphoinositide hydrolysis was studied in rat brain cortical prisms and compared with that of ouabain. As already shown for ouabain, a transient effect was obtained with endobain E; maximal accumulation of inositol phosphates induced by endobain E was 604 ± 138% and 186 ± 48% of basal values in neonatal and adult rats, respectively. The concentration-response plot for the interaction between endobain E and phosphoinositide turnover differed from that of ouabain, thus suggesting the involvement of distinct mechanisms. In the presence of endobain E plus ouabain at saturating concentrations, no additive effect was recorded, suggesting that both substances share at least a common step in their activation mechanism of inositol phosphates metabolism or that they enhance phosphatidylinositol 4,5-biphosphate breakdown from the same membrane precursor pool, until its exhaustion. Experiments with benzamil, a potent blocker of Na^+/Ca^{2+} exchanger, showed that it partially and dose-dependently inhibited endobain E effect. These results indicate that the endogenous Na^+, K^+-ATPase inhibitor endobain E, like ouabain, is able to stimulate phosphoinositide turnover transiently during postnatal brain development.

KEY WORDS: Inositol phosphate; Na^+, K^+-ATPase; ouabain-like substance; neonatal brain cortex; Na^+/Ca^{2+} exchanger.

INTRODUCTION

Na^+, K^+-ATPase is a critical enzyme in neurons for the regulation of membrane potential, cell volume (1) and transmembrane fluxes of Ca^{2+} and excitatory amino acids whose transport is linked to that of sodium (2). It also seems to be crucial in the normal cell cycle and differentiation of the nervous system (3), during which its activity increases due to enzyme accumulation (4,5).

Searching for putative Na^+, K^+-ATPase endogenous regulators, several substances displaying ouabain-like properties have been described (6–8). In previous work, a soluble brain fraction (peak II) was separated (9), which besides inhibiting Na^+, K^+-ATPase activity it also shares several properties with ouabain (7), thus suggesting the term *endobain* (10). Fractionation of peak II by ionic exchange HPLC led to a more purified Na^+, K^+-ATPase inhibitory fraction, II-E (11), which is able to block high affinity [^{3}H]-ouabain binding (12) and to induce neurotransmitter release (13), justifying its denomination as *endobain E*.

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On studying rat brain Na^+, K^+-ATPase modulators at different postnatal stages, we have observed that such II-E factor is already present at 4 days after birth, at which developmental stage it probably exerts a greater effect as Na^+, K^+-ATPase inhibitor than at adult stages (14).

Activation of several brain neurotransmitter receptors enhances the metabolism of membrane phosphoinositides. The products of phosphoinositide hydrolysis by phospholipase C are inositol phosphates and diacylglycerol, the intracellular second messengers involved in calcium mobilization and stimulation of protein kinase C (PKC), respectively (15–17). It is widely accepted that this receptor-effector coupling takes place through G proteins (18,19). However, increased intracellular Ca^{2+} concentration induced by Ca^{2+} ionophores, depolarization, disruption of the Na^+/Ca^{2+} exchange system or receptor gating stimulate phospholipase C (PLC) as well (20), suggesting that phosphoinositide hydrolysis activation through G proteins and Ca^{2+}-mediated activation involve independent mechanisms (21,22). Interestingly, phosphoinositide hydrolysis may be stimulated by ouabain, the Na^+, K^+-ATPase inhibitor, and such effect is significantly higher in neonatal (7 day-old) than in adult rat cerebral cortex (23).

Taking into account the above mentioned findings, the purpose of this study was to determine the possible activity of endobain E, as compared to commercial ouabain, on membrane phosphoinositide-derived cell signaling system. By employing brain prisms prelabelled with [^{3}H]*myo*inositol, we observed that phosphoinositide hydrolysis remains unaltered by endobain E in adult brain but is highly stimulated in neonatal brain, a process that partially involves the Na^+/Ca^{2+} exchanger.

EXPERIMENTAL PROCEDURE

Materials. Reagents were analytical grade. Ouabain, benzamil and Sephadex G-10 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-50 (fine grade) was from Pharmacia Fine Chemicals (Uppsala, Sweden). A Dowex anion exchange resin (AG 1-X8, 100-200 mesh, formate form) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). OptiPhase "Hisafe" 3 was purchased from Wallac Oy (Turku, Finland). [^{3}H]*myo*inositol (20 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.).

Animals. Wistar neonatal rats (6–9 day-old) of either sex and male adult rats (35–40 day-old or 65–70 day-old) were used, considering "day 0" the day of birth.

Preparation of II-E Fraction. Adult male rats (35–40 day-old) were used. Peak I and II fractions from rat cerebral cortex were prepared as previously described (9,11). Thus, for each preparation, cerebral cortices from 5 rats (3 g fresh tissue) were pooled, homogenized at 25% (wt/vol) in bidistilled water, and centrifuged at 100,000 *g* for 30 min in a 70.1 rotor of an L8-Beckman ultracentrifuge. A 5 ml supernatant sample (brain soluble fraction) was taken to pH 7.4 with 0.1 M NH_4HCO_3 and loaded on a Sephadex G-10 column (1 × 20 cm) and a single 11 ml fraction collected. This filtrate was then 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 Model 202 Fraction Collector. The absorbance profile was recorded at 280 nm.

Peaks I and II were made up with fractions presenting maximal UV absorbance. 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 with 2 N 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, 250 × 4.6 mm (Synchrom Inc., Lafayette, IN, U.S.A.), and eluted at a flow rate of 0.5 ml per min with a 20 min gradient from 1 to 10 mM NH_4HCO_3 to separate fractions II-A to II-H. Fractions were collected by monitoring absorbance at 230 nm; II-E was collected at 12–13 min, then lyophilized and stored at −20°C. Lyophilized II-E samples were dissolved in 0.006 N HCl at a suitable concentration and kept up to 20 days at −20°C. Hereafter, II-E fraction is termed *endobain E*.

Determination of [^{3}H]Inositol Phosphates (IPs). A procedure based on described methods (24,25) with modifications, was performed. After removing adult (65 to 70 day-old) and neonatal (6 to 9 day-old) rat cerebral cortex and placing on ice on a Petri dish with gassed Krebs-Henseleit buffer containing (mM): NaCl, 120; KCl, 4.7; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25 and glucose, 11.7, equilibrated to pH 7.4 with O_2/CO_2 (95:5), the tissue was lightly minced (13) and prisms dispersed at 10% (wt/vol) in the same buffer. Samples were incubated in bulk at 37°C for 1 h under gentle shaking with an intermediate change of buffer, followed by a 60 min incubation with [^{3}H]*myo*inositol (6 μCi/ml; final concentration 3×10^{-7} M) and four washes with fresh buffer replaced every 5 min. Fifty μl of packed prisms (with 1.34 ± 0.25 and 1.94 ± 0.56 mg protein in immature and adult rats, respectively, means ± SD, $n = 7$) were transferred to tubes containing 0.24 ml of the same buffer with the addition of LiCl (7.5 mM final concentration, with NaCl iso-osmotically reduced), and the indicated drugs to 0.3 ml final volume. Tubes were then gassed (O_2/CO_2; 95:5), capped and shaken at 37°C for 60 min. Incubations were stopped by the addition of 940 μl chloroform: methanol (2:1), followed by chloroform (310 μl) and bidistilled water (310 μl) to separate phases. Tubes were vortex-mixed vigorously, then centrifuged at 1,000 *g* for 10 minutes to facilitate phase separation. Radiolabeled IPs were separated from inositol by ion-exchange chromatography using small columns containing 0.5 ml of a 50% slurry AG 1-X8 resin in the formate form. Upper phase aliquots (750 μl) diluted to 3 ml with bidistilled water were added to the resin suspension, centrifuged and washed 4 times with 3 ml of *myo*inositol (5 mM). [^{3}H]IPs were eluted with 1 ml of 1 M ammonium formate/0.1 M formic acid and 800 μl of this eluate added to 10 ml of OptiPhase "Hisafe" 3 and counted in a Tracor Analytic (Model 6892) scintillation spectrometer with 30% efficiency.

The relatively long time of incubation (60 min) did not allow to measure the accumulation of the more polar hydrolysis products inositol-1,4-diphosphate and inositol-1,4,5-triphosphate, since changes in these compounds are detectable at very short times after addition of receptor agonists to the incubation buffer (for a review, see 26). However, since the concentration of ammonium formate employed

allows the recovery of all IPs, the term IPs instead of IP_1 (inositol monophosphate) was used.

Radioactivity in the lipid fraction was monitored by counting aliquots from the lower organic phase (200 μl) after drying overnight at room temperature and adding PPO-toluene solution (0.4% wt/vol, 10 ml) with 45% efficiency.

In all experiments, aliquots containing the same amount of brain tissue (that is, similar intraexperimental protein content) were employed, which allowed the comparison between basal condition and treatment in the same sample. Accumulation of [^{3}H]IPs was thus expressed as the percentage of basal value (without additions).

Protein Measurement. Protein was determined by the method of Lowry et al. (27) using bovine serum albumin as standard.

RESULTS

Labeling of inositol phosphates (IPs) and inositol lipids in brain prisms of neonatal and adult rats was studied after incubation with [^{3}H]*myo*inositol in the absence or presence of commercial ouabain and endobain E.

A dose-dependent curve in the accumulation of IPs in neonatal brain prisms was obtained with ouabain, attaining roughly 1100% of basal value with 5×10^{-3} M concentration. Estimated EC_{50} value for ouabain-induced IPs accumulation in neonatal brain prisms was 1×10^{-5} M (Fig. 1A). No further stimulation of inositol phospholipid hydrolysis was achieved by increasing ouabain concentration up to 1×10^{-2} M (1136 ± 376%). In adult animals, the accumulation induced by 5×10^{-3} M ouabain was roughly 250% of basal value. Significance between neonatal and adult ouabain values was already reached at 1×10^{-5} M ouabain (Fig. 1A). Absolute values for IPs accumulation from a single representative experiment were as follows: 19,795 *vs* 1,892 dpm per mg protein in neonatal tissue and 4,168 *vs* 1,667 dpm per mg protein in adult tissue, in the presence or absence of 5×10^{-3} M ouabain, respectively.

Phospholipid labeling in adult cerebral cortex prisms remained unaltered in the presence of ouabain, but almost all ouabain concentrations tested significantly inhibited labeling (10–30%) in neonatal tissue (Fig. 1B), thus showing an inverted plot of that observed in IPs labeling (Fig. 1A).

Endobain E, 0.125–1 mg original fresh tissue/μl, dose-dependently enhanced neonatal IPs accumulation, with significance between developmental stages reached at 0.5 mg original fresh tissue/μl. Maximal accumulation induced by an 8-fold endobain E concentration was roughly 600% of basal value in neonatal rats, whereas in the adults no significant stimulation was recorded. When a higher concentration of endobain E

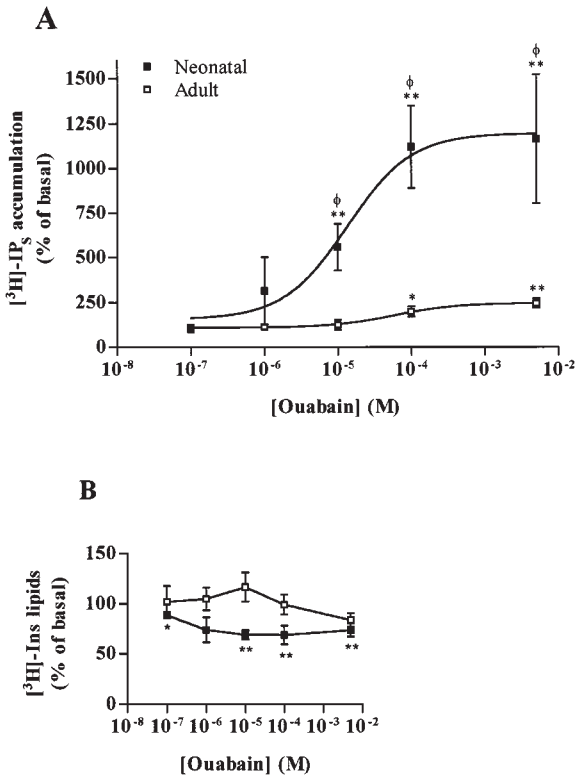

Fig. 1. Concentration-response curve for ouabain-stimulated IPs accumulation (**A**) and phospholipid labeling (**B**) in cerebral cortex prisms. Results are mean values from 4 (neonatal) or 3 (adult) experiments performed in triplicate (± SD), and are expressed as percentages of labeling taking as 100% values in the absence of ouabain. Basal accumulation of [^{3}H]IPs was 2944 ± 567 dpm (adult) and 2773 ± 588 dpm (neonatal) (mean ± SD; n = 3). [^{3}H]IPs, inositol phosphates, [^{3}H]Ins lipids, [^{3}H]Inositol lipids. ϕ denotes $P < 0.01$ for the ouabain effect on neonatal *versus* adult prisms, by Student's *t* test. * denotes $P < 0.05$ and ** $P < 0.01$ *versus* control without ouabain (basal condition), by Student's *t* test.

was employed in the assay (2 mg original fresh tissue/μl), no saturation was observed and a significantly lower value of IPs stimulation was obtained, instead. Estimated EC_{50} value for endobain E-induced IPs accumulation in neonatal brain prisms was 0.25 mg original fresh tissue/μl (Fig. 2A). Absolute values for IPs accumulation from a single representative experiment were as follows: 11,791 *vs* 2,184 dpm per mg protein in neonatal tissue and 2,345 *vs* 1,224 dpm per mg protein in adult tissue, in the presence or absence of 1 mg original tissue per μl of endobain E, respectively.

Phospholipid labeling in adult cerebral cortex prisms was only decreased with the addition of endobain E equivalent to 0.5 mg original fresh tissue/μl, whereas in neonatal tissue a reduction of 10–25% of the label was recorded in the presence of most en-

dobain E concentrations tested (Fig. 2B), showing again an inverted plot of that observed in IPs labeling (Fig. 2A).

In order to exclude non-specific effects, an HPLC elution control was processed in which the 12–13 min fraction was collected from the column previous to biological sample injection. Such fraction was processed as endobain E to observe no effect on phosphoinositide turnover at all.

In both neonatal and adult rat prisms, total amount of [^{3}H]*myo*inositol incorporation into phospholipids was calculated (radiolabel in the lipid plus the soluble IPs fraction) and expressed as dpm per mg protein. Although a lower [^{3}H]inositol incorporation in adult *vs* neonatal tissue was found, no difference was obtained between basal condition and ouabain or endobain E treatment (Table I).

Table I. Total [^{3}H]*Myo*inositol Incorporation into Neonatal and Adult Rat Brain Prisms

Addition	Neonatal dpm. mg prot.$^{-1}$	Adult (dpm. mg prot.$^{-1}$)
—	57,983 ± 4,307	26,076 ± 5,846
Ouabain 5×10^{-3} M	62,865 ± 2,504	24,169 ± 4,557
—	43,474 ± 8,404	23,718 ± 2,655
Endobain E (1 mg/μl)	44,412 ± 10,336	20,334 ± 3,514

Total [^{3}H]*myo*inositol incorporation into phospholipids was calculated by summing the radioactivity in the lipid plus in the soluble IPs fraction (corresponding to a protein content of 1.34 ± 0.25 and 1.94 ± 0.56 mg in neonatal and adult rats, respectively; $n = 7$). Results are mean values from 3 to 7 experiments performed in triplicate (± SD). No significant differences were obtained from values in basal condition *versus* ouabain or endobain E treatment.

In order to further explore ouabain and endobain E effect on phosphoinositide hydrolysis, parallel assays in neonatal brain prisms were carried out for both agents, either added alone or jointly at saturating concentrations. It was found that the stimulatory effect of joint addition differ from the theoretical value corresponding to strict additivity (Fig. 3).

Since the involvement of Na^+/Ca^{2+} exchange in inositol phospholipid turnover has been reported (28),

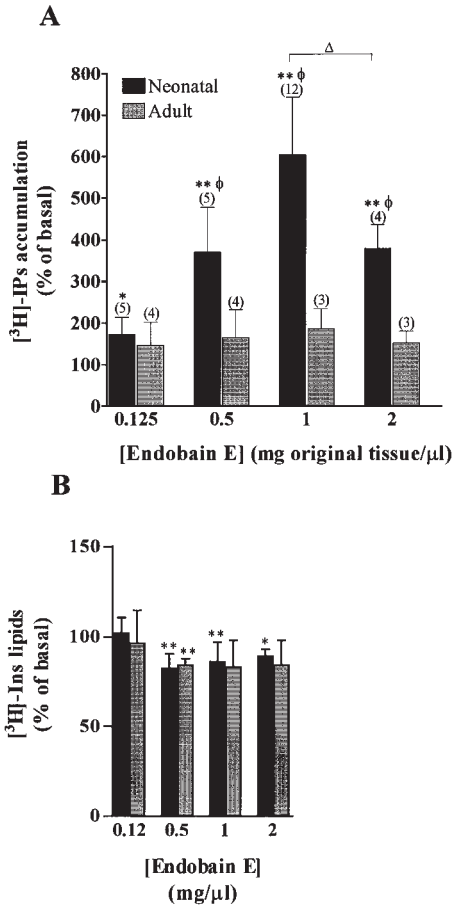

Fig. 2. Concentration-response plot for endobain E stimulated IPs accumulation (**A**) and phospholipid labeling (**B**) in cerebral cortex prisms. Results are expressed as percentages of labeling taking as 100% values in the absence of endobain E (means ± SD). Figures at the top of bars indicate the number of experiments performed in triplicate. [^{3}H]IPs, inositol phosphates, [^{3}H]Ins lipids, [^{3}H]Inositol lipids. ϕ denotes $P < 0.01$ for the endobain E effect on neonatal *versus* adult prisms, by Student's *t* test. * denotes $P < 0.02$ and ** $P < 0.01$ *versus* control without endobain E (basal condition), by Student's *t* test. Δ denotes a significant difference ($P < 0.02$) calculated intraexperiment, by Student's *t* test.

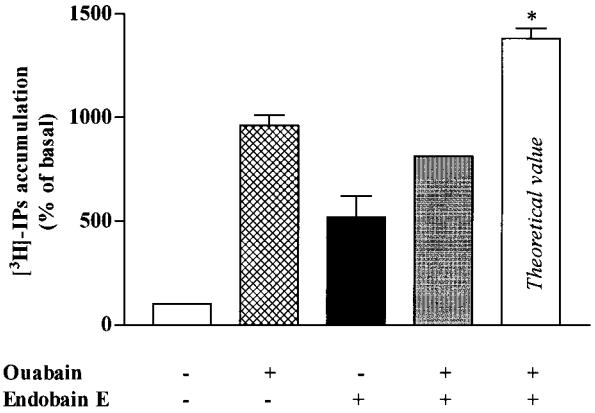

Fig. 3. Effect of the simultaneous presence of ouabain and endobain E on IPs accumulation. Saturating concentrations for inositol phosphates (IPs) stimulation were 1×10^{-4} M and 1 mg original fresh tissue/μl for ouabain and endobain E, respectively. An additional bar showing the theoretical value that would have been obtained if there were strict additivity is included. Total [^{3}H]inositol in the experiment (≅ 59,000 dpm) greatly exceeded the theoretical additive value (≅ 30,000 dpm). Data are mean values (± SD) from four runs performed with two different endobain E preparations (SD of the third bar is too small to be visible). * denotes $P < 0.01$ *versus* ouabain and endobain E joint addition, by Student's *t* test.

we performed experiments with benzamil, a potent blocker of the exchanger. It failed to affect basal IPs accumulation but partially and dose-dependently inhibited endobain E effect by 28 and 66%, at 1×10^{-4} and 5×10^{-4} M concentration, respectively (Fig. 4). IPs accumulation induced by carbachol or glutamate was also diminished by benzamil, though full blockade was not recorded in either case (Fig. 4).

DISCUSSION

In this study we evaluated the effect of an endogenous Na^+, K^+-ATPase inhibitor, termed *endobain E*, on phosphoinositide hydrolysis in brain prisms and compared it with commercial ouabain. Results showed that endobain E produces a transient enhancement in phosphoinositide hydrolysis along postnatal brain development, which differs from that of ouabain.

Regarding total [^{3}H]inositol incorporation expressed with respect to protein content, we found that it was lower in adult than in neonatal brain tissue, in agreement with values previously obtained in cat visual cortex (29) and rat cerebral cortex (30). In addition, since endobain E or ouabain treatment did not affect total [^{3}H]inositol incorporation whatever the stage of development assayed, both endobain E- or ouabain-induced IPs accumulation was calculated as the percentage of basal accumulation without additions in each stage of development, respectively, as an expression of phosphoinositide hydrolysis (24).

Ouabain and endobain E plots for neonatal IPs accumulation showed that the former displayed a dose-dependent pattern which reaches a plateau and levels off, as previously reported by Balduini and Costa (23), while the latter rendered a bell-shaped dose-response pattern, i.e., at the highest concentration used, the response diminished in comparison with values recorded at lower concentrations. Since the concentration-response plot for the interaction between endobain E and phosphoinositide turnover 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 act by binding to neighbouring sites rather than to the same site (12). In addition, experiments performed in the presence of endobain E plus ouabain at saturating concentrations showed no additive effect, probably indicating that both substances share at least a common step in their activation mechanism of IPs metabolism or that they enhance phosphatidylinositol 4,5-biphosphate breakdown from the same membrane precursor pool, until its exhaustion.

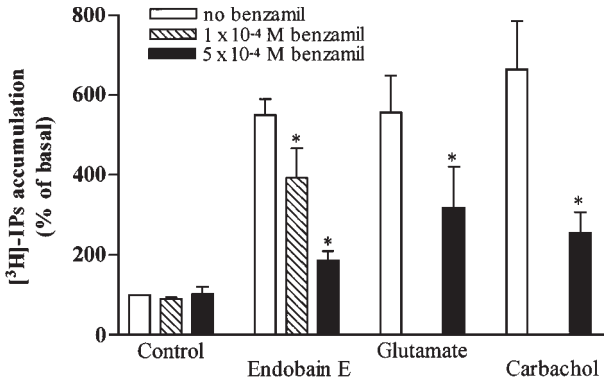

Fig. 4. Effect of benzamil on basal and endobain E-, glutamate- and carbachol-induced formation of inositol phosphates (IPs). Endobain E concentration was equivalent to 1 mg original fresh tissue/μl and 1×10^{-3} M was used for glutamate and carbachol stimulation. Data are mean values (± SD) from 2 to 5 individual experiments performed in triplicate. * denotes $P < 0.01$ *versus* control without benzamil (basal condition), by Student's *t* test.

As regards the chemical nature of the endogenous Na^+, K^+-ATPase inhibitor, endobain E, it seems to consist of a highly hydrophilic low molecular weight compound, neither peptidic nor lipidic in nature (11), which differs from ouabain in UV spectrum, chromatographic behavior and alkaline stability (31) and probably involves an ascorbic acid derivative (32). In this context, the above mentioned differences between endobain E and ouabain may well explain dissimilar behavior on phosphoinositide hydrolysis. In fact, other endogenous ligands of Na^+, K^+-ATPase have shown different physiological profiles from ouabain in various assays, such as a factor from bovine hypothalamus (hypothalamic inhibitory factor, HIF) (33).

The possible implications of the differential sensitivity to an endogenous Na^+, K^+-ATPase inhibitor in neonatal and adult rats are not clear at the moment. However, it is worth noting that among different neurotransmitters, only glutamate and muscarinic agonists have been shown to transiently stimulate phosphoinositide hydrolysis in rat brain cortex during discrete periods of brain development (30,34–37). Besides, it has been suggested that receptor-mediated increased phosphoinositide turnover is directly implicated in the cellular mechanism responsible for neuronal neocortical plasticity (29,38,39).

Present results showed that effective concentrations (EC_{50}) of endobain E and ouabain for phosphoinositide hydrolysis enhancement in brain prisms are greater or equal than that required to inhibit synaptosomal membrane Na^+, K^+-ATPase activity, that is, an IC_{50} value of 19 μg original fresh tissue/μl for endobain E (14) and 3×10^{-6} M for ouabain (12). Thus,

taking into account that drug accessibility to target sites in synaptosomal membranes is expected to be higher than that in brain prisms, these findings suggest that Na^+, K^+-ATPase inhibition and phosphoinositide hydrolysis enhancement are related events.

The bell-shaped dose-response pattern for neonatal IPs accumulation displayed by endobain E may be attributable to negative feedback control of a given protein, i. e. PKC, forwardly activated by this intracellular signalling cascade. In support, Balduini and Costa (23) showed the same profile of the ouabain effect as reported herein and a dose-dependent inhibition on the ouabain accumulation of IPs when phorbol 12-myristate 13-acetate was employed in the assay. In this respect, a reasonable hypothesis is that reduction of Na^+, K^+-ATPase activity by endobain E stimulates phosphoinositide hydrolysis and activates PKC which, in turn, phosphorylates the pump, or an associated regulatory protein, resulting in the normalization of decreased Na^+, K^+-ATPase activity. However, activation of Na^+, K^+-ATPase by its phosphorylation has only been reported in peripheral nerves and aortic preparations (40–42). Interestingly, PKC stimulation seems to represent a classical negative regulation step of PLC-coupled receptors (43, 44) and a differential regulation of metabotropic glutamate receptors activities by PKC seems to be partially responsible for the developmental profiles of inositol phosphate formation stimulated through such receptor activation (45).

A possibility concerning endobain E mechanism of action is the activation of the Na^+/Ca^{2+} exchanger in the reverse mode, occurring after Na^+, K^+-ATPase inhibition (46). In fact, the involvement of Na^+/Ca^{2+} exchange in inositol phospholipid hydrolysis stimulation has been reported (28) and amiloride partially blocks ouabain effect on phosphoinositide hydrolysis while antagonists of muscarinic and glutamate receptors were ineffective in blocking this response (23). In this context, our results showed that benzamil, a more specific Na^+/Ca^{2+} exchanger blocker than amiloride, partially antagonizes endobain E effect with a IC_{50} value within the range to that previously reported (47). However, benzamil not only inhibits endobain E- but also glutamate- and carbachol-elicited responses, as previously shown with amiloride (48), thus appearing as a non-specific inhibitor which could not account for differences in endobain E mechanisms of action *versus* glutamate or carbachol.

To sum up, our results indicate that the endogenous Na^+, K^+-ATPase inhibitor endobain E, like ouabain, is able to stimulate phosphoinositide turnover transiently during postnatal brain development, though most likely through dissimilar mechanisms.

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