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Endobain E, a brain Na^+, K^+-ATPase inhibitor, decreases norepinephrine uptake in rat hypothalamus

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Abstract

The ability of an endogenous brain Na^+, K^+-ATPase inhibitor, termed endobain E, to increase [^{3}H]norepinephrine release in rat hypothalamus was previously reported. Endobain E effect on neurotransmitter uptake was studied by assaying [^{3}H]norepinephrine uptake in rat hypothalamus preparations, to observe uptake inhibition, which reached 60% with endobain E equivalent to 100 mg fresh cerebral cortex, an effect achieved with 40 or 400 μM ouabain. Results support the proposal that endobain E behaves as an ouabain-like substance. Taken jointly results obtained on neurotransmitter release and uptake, the suggestion that endobain E may enhance norepinephrine availability in the synaptic gap and thus lead to an increase in noradrenergic activity is advanced.

Keywords: Norepinephrine uptake; Hypothalamus; Na^+, K^+-ATPase inhibitor; Ouabain; Ouabain-like factor; Endobain

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Introduction

The sodium pump is involved in several physiological functions such as regulation of the cell volume, cell differentiation and maintenance of sodium and potassium equilibrium through biological membranes; the enzymatic version of such pump is sodium plus potassium activated ATPase (Na^+, K^+-ATPase) (Albers and Siegel, 1999), an enzyme concentrated at nerve ending membranes (Rodríguez de Lores Arnaiz et al., 1967) where sodium exit and potassium entry occur during neurotransmission.

A close relationship between Na^+, K^+-ATPase activity and neurotransmitter release has been demonstrated, suggesting that this enzyme could play a role in the mechanism of neurotransmission modulation (Paton et al., 1971). Kainic acid treatment, known to induce neuronal hyperexcitability, increases neurotransmitter release and enhances ATPase mRNA (Anderson and Stahl, 1997). Ouabain, a selective and specific Na^+, K^+-ATPase inhibitor, increases acetylcholine, 5-hydroxytryptamine and catecholamine release; on the other hand, conditions that activate the enzyme, concomitantly decrease neurotransmitter release (see Vizi and Oberfrank, 1992). The ability of endogenous ouabain-like compounds to enhance neurotransmitter release has been described (Vizi, 1978).

We have reported that catecholamines norepinephrine (NE) and dopamine modify neuronal Na^+, K^+-ATPase activity, respectively stimulating or inhibiting the enzyme in the presence or absence of a brain soluble fraction in the assay medium; from such fraction, by gel filtration through Sephadex G-50, two fractions, termed peaks I and II, were separated and found to stimulate and inhibit Na^+, K^+-ATPase activity, respectively (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1986). Besides inhibiting enzyme activity, peak II shares several properties with ouabain, including that of inducing neurotransmitter release contained in pineal nerve synaptic vesicles. The mentioned findings support the notion that peak II contains an endogenous ouabain-like factor, so that the term *endobain* was suggested (see Rodríguez de Lores Arnaiz, 1993, 2000). Further purification of peak II by anionic exchange high performance liquid chromatography (HPLC) in a Synchropack AX-300 column allowed the separation of a subfraction, II-E, which highly inhibits Na^+, K^+-ATPase activity (Rodríguez de Lores Arnaiz and Peña, 1995) and blocks high affinity [^{3}H]ouabain binding (Rodríguez de Lores Arnaiz et al., 1998) thus being denominated *endobain E*.

We have previously reported that in rat hypothalamus endobain E enhances NE release in a concentration-response fashion, reaching 200%, equivalent to the effect achieved with 400 μM ouabain (Vatta et al., 1999). Neuronal NE uptake arrests the activity of released NE, through a mechanism mediated by an active transport system dependent on Na^+, K^+-ATPase activity (Paton, 1976). Taken jointly, such background supports the hypothesis that endobain E could also modulate neuronal NE uptake. Since this catecholamine is highly concentrated in brain regions as the hypothalamus, here we studied the effect of endobain E on neurotransmitter uptake in rat hypothalamus preparations after incubation with [^{3}H]-NE.

Materials and methods

Male Wistar rats were housed in steel cages, kept in a controlled room at 22–24°C with a 12 h light-dark cycle (light from 7.00 a.m. to 7.00 p.m.) and allowed free access to tap water and food. Rats weighing 100–150 g and 250–300 g were used to harvest cerebral cortex and hypothalamic tissues,

respectively. 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.

The following reagents were used in the experiments: norepinephrine hydrocloride, DL-(7-^{3}H (NE)) with 15 Ci-mmol specific activity (New England Nuclear, Boston, MA, USA), hydrocortisone, pargyline, ouabain, Sephadex G-10 and tropolone (Sigma, St Louis, MO, USA), Sephadex G-50, fine grade (Pharmacia Fine Chemicals, Uppsala Sweden) and EcoLite, a liquid scintillation cocktail (ICN Pharmaceuticals Inc., CA., USA).

Experimental procedures previously described were used to obtain peaks I and II, and II-E fractions from rat cerebral cortex (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1986; Rodríguez de Lores Arnaiz and Peña, 1995). Briefly, for each preparation, cerebral cortices from five rats were pooled, homogenized at 25% (w/v) 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); a single fraction was collected and then applied to a column packed with Sephadex G-50 (1.8 × 25 cm). For gel equilibration and elution, 10 mM NH_4HCO_3 was used. Fractions of 1.4 ml each at a flow rate of 0.3 ml/min were collected in a Gilson equipment. The absorbance profile was recorded at 280 nm.

Peaks I and II were made up with the fractions presenting maximal ultraviolet absorbance. Peak I was made up with fractions 19–23 but was not used in this study; peak II was made up with fractions 48–52. Peak II was taken to pH 2.0 with 2 N HCl, lyophilized and stored at −20°C overnight; peak II samples, each equivalent to 0.6 g fresh cerebral cortex were dissolved in 1 mM NH_4HCO_3 and processed by anionic exchange HPLC performed on a Synchropak AX-300 column, 250 × 4.5 mm (Synchrom Inc., Lafayette, IN, USA), and eluted at a flow rate of 0.5 ml/min with a 20 min gradient from 1–10 mM NH_4HCO_3 to separate fractions II-A to II-H. Fractions were collected by visual inspection of absorbance curves at 230 nm; II-E, hereafter termed *endobain E*, was collected at 12–13 min, then lyophilized and stored at −20°C. Before use, samples were dissolved in 0.006 N HCl at a concentration of 2 mg original tissue/μl.

To obtain hypothalamic tissue, rats were killed by decapitation between 10.00 and 12.00 a.m. to avoid circadian changes. Brains were quickly removed and hypothalami immediately dissected, cooled and weighed. Hypothalami were lightly minced and then transferred into a glass tube with a nylon mesh fitted at the bottom to allow free interchange with the medium. All experiments were carried out in vitro.

As incubation medium, standard Krebs bicarbonate solution was used. Tissues were placed in 2-ml incubation medium in a Dubnoff incubator and preincubated at 37°C for 30 min, with continuous shaking and bubbling with 95% O_2 + 5% CO_2, pH 7.4, and later transferred to fresh medium containing 1.25 μCi/ml of [^{3}H]-NE; incubation proceeded for 30 min in the absence (control group) or in the presence of endobain E (10, 15, 20, 30, 40, 50 and 100 μl/tube) or 4, 40 and 400 μM ouabain. Pargyline, tropolone and hydrocortisone were each present at 100 μM concentration throughout preincubation and incubation periods to inhibit respectively monoamineoxidase (MAO) activity, catecholamine-O-methyltransferase (COMT) activity and extraneuronal NE uptake. After incubation, tissues were washed for 5 min with 2 ml of cooled original Krebs bicarbonate solution and then homogenized with 2.5 ml of 10% trichloroacetic acid and centrifuged at 27,000 × g (4°C) for 15 min (Sorvall Superspeed RC2-B). Sample tritium activity was measured by scintillation counting using a Packard-PRIAS 240CL/D. Neuronal NE uptake is expressed as dpm per g fresh tissue as mean values ± SEM. One way analysis of variance (ANOVA) and the Student-Newman-Keuls test were used for statistical analysis, taking p values < 0.05 as statistically significant.

Results and discussion

Neuronal NE uptake was quantified after incubating rat hypothalamic tissue in the presence of [^{3}H]-NE. It was observed that the uptake remained unaltered with 10 μl endobain E (1 μl equivalent to 2 mg original tissue), decreased roughly 35–40% with 15–40 μl; with 50 μl and 100 μl endobain E, decrease attained 50% and 61%, respectively. For comparison, experiments with commercial ouabain were run, to observe approximately 50% and 60% decrease with 40 and 400 μM concentration, respectively, whereas only a trend with 4 μM concentration (Fig. 1).

Present results indicated that the endogenous ouabain-like substance, endobain E, similar to ouabain, decreases neuronal NE uptake to hypothalamus tissue.

It should be recalled that ouabain concentrations employed herein are within the range used by other authors to study catecholamine uptake in tissue slices of mouse caudate-putamen (Jones et al., 1999) and cultured astrocytes (Inazu et al., 2003).

In our experimental model, the possibility that tritium measurements could correspond to extraneuronal NE uptake and/or metabolites of NE is ruled out since hydrocortisone and inhibitors

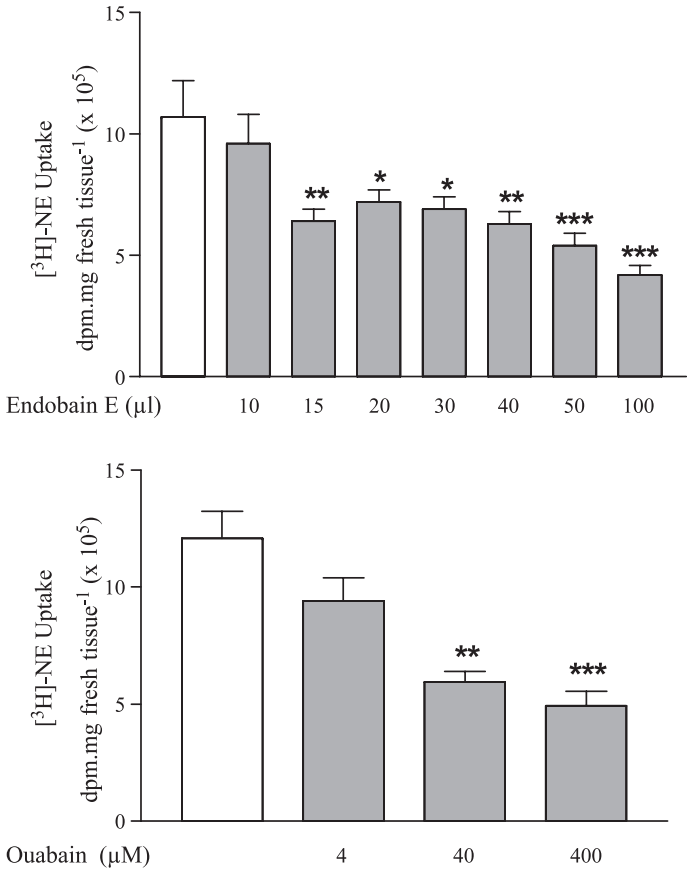

Fig. 1. Effect of endobain E (upper panel) and ouabain (lower panel) on neuronal [^{3}H]-norepinephrine (NE) uptake in rat hypothalamus. Data are expressed as dpm [^{3}H]-NE uptaken.mg fresh tissue^{-1} ($\times 10^5$) and are means ± SEM from 4–9 experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as compared with control values.

for MAO and COMT, were included in the assay. Therefore, present measurements allowed quantification of NE uptake from presynaptic endings (Vatta et al., 1996).

Neuronal uptake at presynaptic terminals is the major step through which extracellular NE concentration is regulated, a mechanism responsible for the termination of NE action at the synaptic gap (Blakely et al., 1994). Decreased NE uptake is generally associated with an increase in neurotransmitter release, thus indicating central NE overactivity. Therefore, our results of NE uptake agree with those of NE release (Vatta et al., 1999) and support the potential enhancement of central noradrenergic neurotransmission induced by endobain E.

Neuronal NE reuptake carrier (cocaine sensitive) allows catecholamine inactivation, a mechanism mediated by an active transport system dependent on Na^+, K^+-ATPase activity, which is sensitive to both temperature and extracellular Na^+ concentration (Blakely et al., 1994). Na^+, K^+-ATPase inhibition is most likely the mechanism involved in neuronal NE uptake decrease induced by ouabain and endobain E.

Available evidence regarding factors which share biological properties with ouabain has been reviewed (Goto et al., 1992; Rodríguez de Lores Arnaiz, 1992; 2000); such factors, isolated from CNS, adrenal glands and biological fluids, are grouped into either identical or closely resembling ouabain or else non-steroidal compounds of diverse chemical structure. Some of these factors are able to increase neurotransmitter release, as endobain E does. The latter is a highly hydrophilic anionic compound of low molecular weight, neither peptidic nor lipidic in nature, sensitive to acid or alkali treatment (Rodríguez de Lores Arnaiz and Peña, 1995), differing from ouabain on the basis of ultraviolet spectra, chromatographic behaviour and alkaline stability (Peña and Rodríguez de Lores Arnaiz, 1997). HPLC analysis demonstrated the presence of ascorbic acid in endobain E samples, as well as that of a second Na^+, K^+-ATPase inhibitor, distinct to ascorbic acid and most likely chemically related to ascorbic acid (Rodríguez de Lores Arnaiz et al., 2003).

A correlation between CNS ouabain content with diverse pathophysiological conditions has been found. Brain ouabain-like activity is increased in spontaneously hypertensive and in the Dahl-sensitive rats, and such increase appears to be responsible for decreased sympathoinhibition, increased sympathoexcitation and the development of hypertension (Leenen et al., 1994; Huang and Leenen, 1995). Furthermore, there is an association between the development of congestive heart failure with marked increases in both, peripheral and brain ouabain-like activity, most likely mediating the increase in resting sympathetic tone and the enhancement of sympathoexcitatory responses to stress (Leenen et al., 1995).

Hypothalamic and brain catecholamines are closely involved in the regulation of several biological functions such as cardiocirculatory activity, water and electrolyte balance, endocrine and neuroendocrine secretions, as well as thirst and sodium appetite required to restore water and sodium losses, among others. In the light of our findings, endobain E may well be involved in the regulation of the above functions.

Neuronal NE uptake (cocaine sensitive) leads to catecholamine inactivation, a mechanism mediated by Na^+, K^+-ATPase dependent active transport system whose activity is modulated by extracellular sodium concentration and incubation temperature. Taking into account results obtained, it would be of interest to explore whether endobain E diminishes the activity and/or the availability of the carrier, or alternatively, it alters membrane potential and therefore the driving force for Na^+ coupling.

In conclusion, present results, showing a decrease in NE uptake by endobain E are in line with those indicating enhancement of NE release (Vatta et al., 1999). Taken jointly, these findings suggest that, similar to ouabain, endobain E inhibits NE uptake and enhances NE release in hypothalamic neurons;

therefore, endobain E may increase catecholamine availability in the synaptic gap (and protect amine from MAO degradation) and in turn enhance noradrenergic activity. This enhancement is compatible with the hypothesis that endobain E is related to the development and/or maintenance of several pathophysiological states of the cardiovascular system.

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