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Neurotensin inhibits neuronal Na^+, K^+-ATPase activity through high affinity peptide receptor

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Abstract

Neurotensin is a peptide present in mammalian CNS and peripheral tissues, which may play a major role in neurotransmission or neuromodulation, subserving diverse physiological functions. We studied the effect of added neurotensin on ATPase activities in synaptosomal membranes isolated from rat cerebral cortex. Neurotensin at 3×10^{-8}–3×10^{-6} M concentration decreased 20–44% Na^+, K^+-ATPase activity but failed to modify Mg^{2+}-ATPase activity; lower neurotensin concentrations (3×10^{-14}–3×10^{-10} M) had no effect on enzyme activities. This inhibitory effect was abolished by neurotensin heating, by enzyme preincubation with neurotensin during periods exceeding 10 min, or by adding 1×10^{-6} M SR 48692, a high affinity neurotensin receptor antagonist. Levocabastine, which blocks low affinity neurotensin receptor, failed to alter enzyme inhibition by the peptide. It is suggested that the sodium pump may be a target for neurotensin effects at neuronal level involving the participation of high affinity neurotensin receptor.

Keywords: Neurotensin effect; Synaptosomal membrane enzymes; ATPases; Na^+, K^+-ATPase; Neurotensin receptor

1. Introduction

Neurotensin is a tridecapeptide present in mammalian central nervous system (CNS) and peripheral tissues, whose widespread distribution in cell bodies and nerve terminals in the brain suggests that it may play a major role in neurotransmission or neuromodulation, subserving diverse physiological CNS functions [16].

In the brain, neurotensin acts as a neuromodulator, particularly of dopaminergic transmission in nigrostriatal and mesocorticolimbic systems [4], thus implying its involvement in dopamine-associated neurodegenerative and neuropsychiatric disorders [21].

Besides, neurotensin acts as a neurotransmitter, and its effects are mediated by specific membrane receptors, as neurotensin can bind to both the high (NT_1) and low (NT_2) affinity binding sites [31]. A specific neurotensin antagonist, SR 48692, should prove to be useful in evaluating the physiology of neurotensin and the characterization of receptors mediating the various biologic peptide effects [13].

Neurotensin is known to exert a wide variety of actions, including pain relief and changes in nonvascular smooth muscle contractility, as well as growth stimulation of colon and pancreatic cancer in humans [16]. Neurotensin stimulates ventral tegmental area neurons by activating a guanosine nucleotide binding protein that is insentive to pertussis toxin, leading to a reduction in membrane K^+ conductance, whose relative contribution varies from cell to cell [15]. Neurophysiological evidence suggest that neurotensin, acting at presynaptic receptors, is able to modulate excitatory synaptic transmission as shown in the parabrachial nucleus [27].

The main active transport system in neurons, as in most other animal cells, is the sodium pump or Na^+, K^+-ATPase that extrudes 3 Na^+ and accumulates 2 K^+ per molecule of ATP hydrolyzed [2]. As such enzymatic activity may be modified by certain neurotransmitters, including noradrenaline and dopamine [23], the purpose of this study was to evaluate the effect of neurotensin on synaptosomal membrane ATPase activity obtained from rat cerebral cortex. Synaptosomal membrane enzyme activity was determined by measuring the hydrolysis of ATP both in the absence and presence of ouabain. Present results showed that neuroten-

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sin inhibits Na^+, K^+-ATPase activity, an effect prevented by SR 48692, suggesting the involvement of high affinity neurotensin receptor in enzyme inhibition.

2. Methods

2.1. Animals and drugs

Adult male Wistar rats weighing 100–120 g were used. Reagents were analytical grade. Ouabain, disodium ATP (grade I, prepared by phosphorylation of adenosine), phosphoramidon (N-(α-rhamno-pyranosyloxy-hydroxyphosphinyl) leucyl-tryptophan), and neurotensin acetate were from Sigma Chemical Co. (St. Louis, MO, USA); neurotensin trifluoroacetate was purchased from RBI (Natick, MA, USA). Peptide solutions in bidistilled water were freshly prepared for each experiment. SR 48692 {2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl) pyrazol 3-yl) carbonylamino] tricyclo (3.3.1.1.^{3,7}) decan-2-carboxylic acid} and lebocabastine were provided by Dr Danielle Gully of Sanofi Recherche, France, and Dr Miguel A. Larroca of Jansen-Cilag, Argentina, respectively.

2.2. Preparation of synaptosomal membranes

Synaptosomal membranes were prepared according to the method developed in our laboratory [24]. For each preparation, cerebral cortices from five rats were pooled and homogenized at 10% (w/v) in 0.32 M sucrose (neutralized to pH 7 with Tris base) using a Teflon glass homogenizer of the Potter-Elvehjem type. The homogenate was processed by differential centrifugation to separate the nuclear and mitochondrial fractions. The crude mitochondrial pellet was resuspended at 10% (w/v, original tissue) in water (pH 7 with Tris base) and homogenized during 2 min for osmotic shock. A pellet containing mitochondria, nerve ending membranes, and myelin was separated by centrifugation at 20 000 $\times$ *g* for 30 min, then resuspended in 0.32 M sucrose and layered on top of a gradient containing 0.8, 0.9, 1.0, and 1.2 M sucrose. The gradient was centrifuged at 50 000 $\times$ *g* for 2 h in a SW 28 rotor of an L8 Beckman ultracentrifuge; the fraction at 1.0 M sucrose level was separated, diluted with 0.16 M sucrose, and spun down at 100 000 $\times$ *g* for 30 min.

The pellet was stored at -70°C and, before enzyme assay, resuspended by brief homogenization in bidistilled water to reach a final concentration of 8.5–10.5 mg protein per ml, stored frozen, and used for 3 weeks without significant change in enzyme activities.

2.3. Enzyme assay

ATPase activity was measured as described by Albers et al. [1]. Total ATPase activity was assayed in a medium containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.16 M Tris-HCl (pH 7.4), and 4 mM ATP. Mg^{2+}-ATPase activity was determined in a similar medium with no added Na^+ and K^+ and containing 1 mM ouabain. The difference between activities was taken to correspond to Na^+, K^+-ATPase.

Before performing ATPase assay, synaptosomal membrane samples were preincubated with 0.16 M Tris-HCl buffer (pH 7.4) or neurotensin at 37°C for 10 min; incubation volume (μl) was 5:40 for membranes:neurotensin, unless otherwise stated. When indicated, the preincubation period lasted 5–60 min.

Aliquots of preincubated fractions (3 μl) were distributed in two series of microtubes containing the respective medium (40 μl) for the assay of total- and Mg^{2+}-ATPase activities, and incubated at 37°C for 30 min. The reaction was stopped with 30% (w/v) TCA solution. ATPase activity was monitored by colorimetric determination of orthophosphate released [19]. In all enzyme assays, tubes containing enzyme preparations and assay media maintained at 0°C throughout the incubation period were used as blanks.

Where indicated, neurotensin solutions were placed in a boiling water bath and maintained for 10 min, then cooled on ice and added to enzyme samples previously incubated at 37°C for 10 min. As control, unheated neurotensin solutions were used. In this experiment, neurotensin final concentration during enzyme assay was 3.0×10^{-6} M.

To test the effect of a peptidase inhibitor, previous to neurotensin addition, synaptosomal membranes were preincubated at 37°C for 15 min with 1.0×10^{-6} M phosphoramidon and then proceeded as described above.

To ascertain antagonist effect, either SR 48692 dissolved in DMSO 10% (v/v) or levocabastine dissolved in bidistilled water, were preincubated with the enzyme at 37°C for 5 min, when neurotensin was added for a further 5 min period**.**

Protein content in synaptosomal membrane was determined by the method of Lowry et al. [20], using bovine serum albumin (BSA) as standard.

Results are expressed as μmoles Pi released per mg protein per hour or as percentage enzyme activity taking as 100% values obtained with no additions. Comparison between experimental data obtained with control and drug treated membranes was made by Student's one sample *t* test, taking *P* values < 0.05 as significant.

3. Results

Synaptosomal membrane ATPase activity in the absence and presence of neurotensin was studied. In the absence of neurotensin, values of 49.2 $\pm$ 6.0 and 12.1 $\pm$ 2.6 μmoles Pi released per mg protein per h were respectively found for Na^+, K^+-, and Mg^{2+}-ATPase activities (means $\pm$ SD, $n = 6$). Neurotensin at the range of 3.0×10^{-8}–3.0×10^{-6} M concentration inhibited 20–44% Na^+, K^+-ATPase activity but failed to modify Mg^{2+}-ATPase activity. Lower neuro-

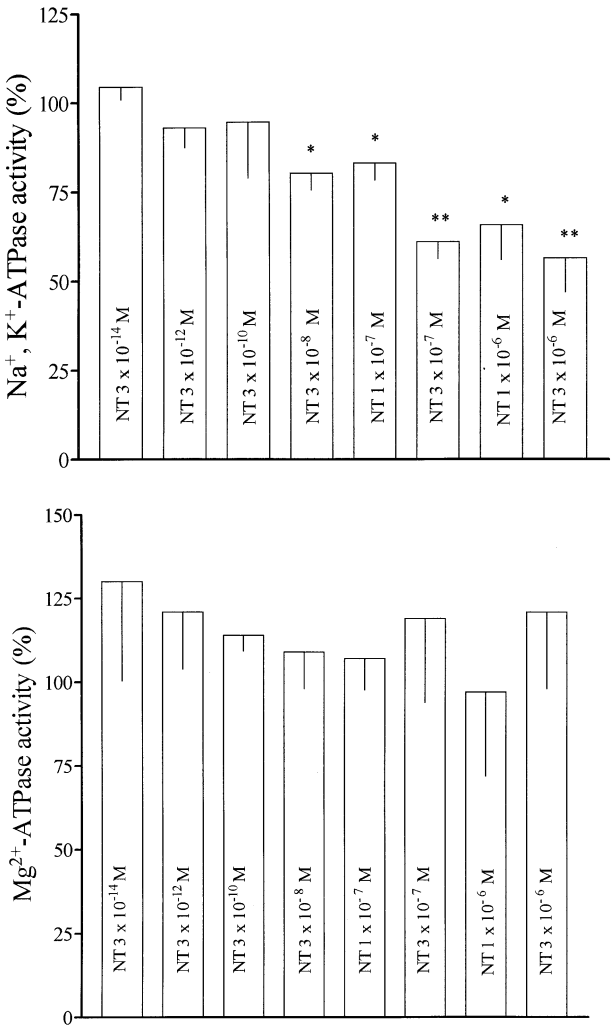

Fig. 1. Effect of neurotensin on synaptosomal membrane Na^+, K^+- and Mg^{2+}-ATPase activities. Synaptosomal membranes from rat cerebral cortex were preincubated for 10 min in the absence and presence of neurotensin (NT) and assayed for ATPases. Results are expressed as percentage enzyme activity taking as 100% values obtained for each experiment in the absence of neurotensin. SD of mean values from 4 to 6 experiments performed in triplicate and neurotensin concentrations are indicated within bars. $*P < 0.05$; $**P < 0.01$ with respect to the control without neurotensin, by Student's *t* test.

tensin concentrations (3.0×10^{-14}–3.0×10^{-10} M) had no effect on enzyme activities (Fig. 1).

Control assays performed with Sigma neurotensin acetate versus RBI neurotensin trifluoroacetate failed to show differences in peptide effect according to commercial source. To ascertain whether neurotensin effect was due to acetate moeity, Na^+, K^+-ATPase activity was determined in the presence of 3.6×10^{-6} M acetate, to find no significant difference versus control without acetate.

To test whether the above findings were attributable to the peptide itself or to a possible drug contaminant, additional ATPase assays were run in the presence of neurotensin solution preheated at 100°C for 10 min**,** a treatment that reduced absorbance 77% at 280 nm. It was observed that neurotensin at

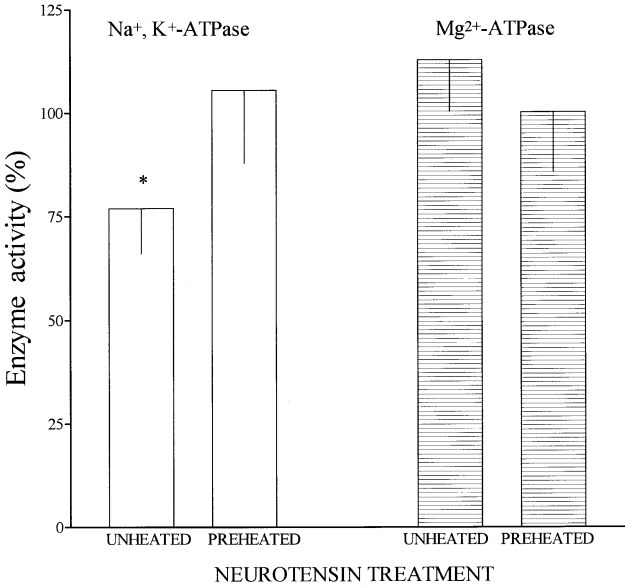

Fig. 2. Effect of heat treatment on neurotensin inhibitory action on Na^+, K^+- ATPase activity. Synaptosomal membranes were assayed for ATPase activities in the presence of unheated or preheated neurotensin at 3.0×10^{-6} M final concentration. Results are expressed as percentage enzyme activities taking as 100% values obtained for each experiment in the absence of neurotensin. SD of mean values from four experiments performed in triplicate are indicated within bars. $*P < 0.05$ with respect to the control without neurotensin, by Student's *t* test.

3.0×10^{-6} M final concentration decreased 23% or failed to modify Na^+, K^+-ATPase when unheated or heated solutions were used, respectively. Here again, Mg^{2+}-ATPase activity was unchanged in either condition (Fig. 2).

In the presence of 3.5×10^{-6} M neurotensin, 5 and 10 min enzyme preincubation led respectively to 24 and 47% decrease in Na^+, K^+-ATPase activity, whereas the enzyme activity remained unaltered after 20–60 min preincubation (Fig. 3).

To prevent neurotensin enzymatic degradation during ATPase assay, synaptosomal membranes were preincubated at 37°C for 15 min with 1×10^{-6} M phosphoramidon. Neurotensin was then added and the above procedure followed for ATPase assay. Fig. 4 shows that before membrane incubation with phosphoramidon, enzyme activity was reduced 49% by 3.5×10^{-6} M neurotensin, and that after membrane incubation with phosphoramidon, a similar Na^+, K^+-ATPase inhibition by neurotensin was found. Membrane incubation with phosphoramidon alone failed to modify basal Na^+, K^+-ATPase activity (Fig. 4).

To determine whether enzyme inhibition by neurotensin was due to interaction with neurotensin receptors, experiments were performed including SR 48692 or levocabastine. A 25% enzyme inhibition was observed after 5 min preincubation with 3.5×10^{-6} M neurotensin, an effect fully prevented by 1.0×10^{-6} M SR 48692, an antagonist concentration unable to modify enzyme activity per se (Fig. 5). Additional experiments performed in the presence of 3.5×10^{-6} M neurotensin with or without DMSO 10% (v/v) showed that this solvent failed to alter either neuro-

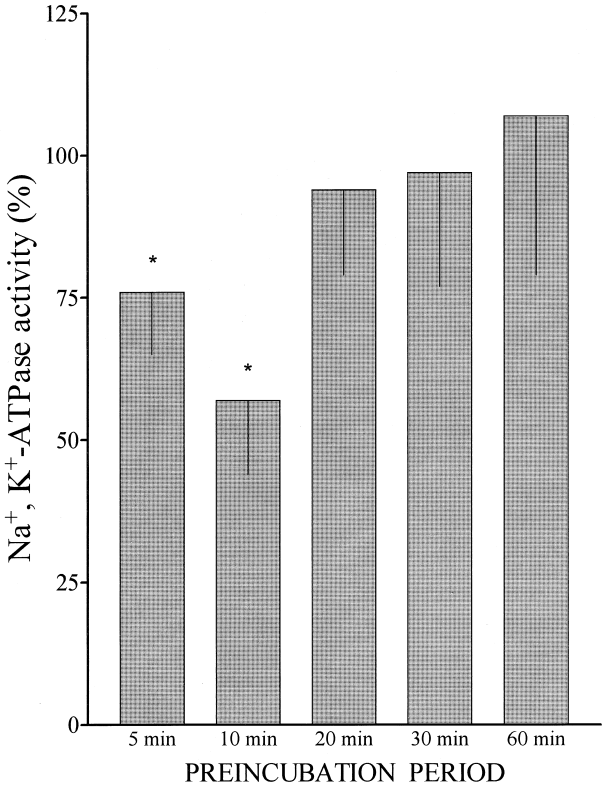

Fig. 3. Effect of preincubation period on Na^+, K^+-ATPase inhibition by neurotensin. Synaptosomal membranes were preincubated with 3.5×10^{-6} M neurotensin 5–60 min and then assayed for ATPase activity. Results are expressed as percentage enzyme activity taking as 100% values obtained in the absence of neurotensin. SD of mean values from five experiments, each performed in triplicate, are indicated within bars. $*P < 0.05$ with respect to the control without neurotensin, by Student's *t* test.

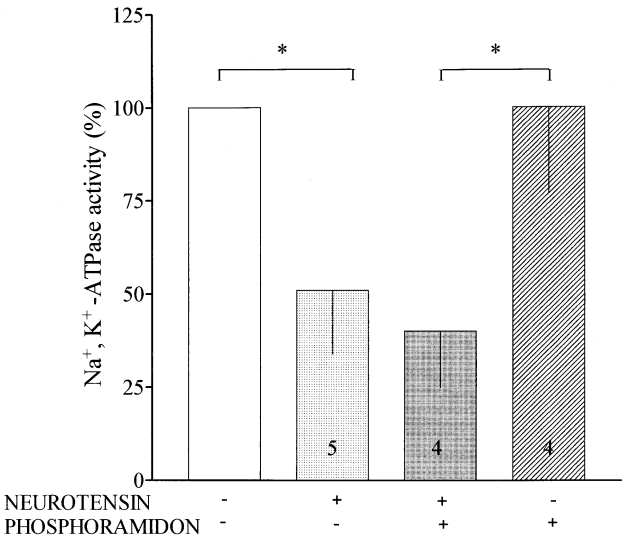

Fig. 4. Preincubation of synaptosomal membranes with phosphoramidon, a 24-11 endopeptidase inhibitor, does not modify Na^+, K^+-ATPase inhibition by neurotensin. Synaptosomal membranes were preincubated for 15 min with or without 1.0×10^{-6} M phosphoramidon, followed by 3.5×10^{-6} M neurotensin for a further 10 min, and assayed for ATPases. Results are expressed as percentage enzyme activity taking as 100% values obtained for each experiment with no additions. SD of mean values and the number of experiments performed in triplicate are indicated within bars. $*P < 0.05$ with respect to the control without additions, by Student's *t* test.

tensin effect on Na^+, K^+-ATPase, or basal enzyme activity (data not shown).

The extent of enzyme inhibition by 3.5×10^{-6} M neurotensin remained unchanged by the presence of levocabastine, a low affinity neurotensin receptor antagonist at 1.0×10^{-7} M or 1.0×10^{-6} M concentration, which in control assays, respectively, failed to modify or decreased 15% basal enzyme activity (Fig. 6).

4. Discussion

Results presented herein show that neurotensin addition to synaptosomal membranes inhibits Na^+, K^+-ATPase activity but fails to modify Mg^{2+}-ATPase. The inhibitory effect on Na^+, K^+-ATPase activity was abolished by neurotensin heating, by enzyme preincubation with neurotensin for periods exceeding 10 min, or by adding SR 48692.

Present results suggest a certain degree of specificity because Mg^{2+}-ATPase activity remains unaltered, unlike Na^+, K^+-ATPase activity.

Although neurotensin totally resists hydrolysis by aminopeptidases, the activity of endopeptidases leads to the formation of various inactive neurotensin catabolites [32]. A loss of neurotensin inhibitory effect on Na^+, K^+-ATPase activity was observed after preincubation times exceeding 20 min, which finds a ready explanation in peptide degradation by metalloendopeptidases, shown to be present in rat brain synaptosomal membranes [9]. To test whether neurotensin effect on ATPase activity was disminished by enzymatic peptide degradation, experiments were performed with synaptosomal membranes pretreated with phosphoramidon, a 24-11 endopeptidase inhibitor, to observe that the final neurotensin effect remained unaltered.

Similar enzyme inhibition was found when RBI neurotensin trifluoroacetate instead of Sigma neurotensin acetate was used as substrate. Furthermore, peptide denaturation by neurotensin heating abolished its inhibitory effect on the enzyme. Thus, enzyme inhibition by neurotensin can hardly be attributed to potential substrate contaminants.

It has been reported that neurotensin applied to supraoptic nucleus neurons causes membrane depolarization and reversibly attenuates after hyperpolarization, which follows current-evoked spike trains, most likely due to neurotensin receptor site occupation [14]. Such effects were achieved with neurotensin at 1.0×10^{-9}–3.0×10^{-6} M concentration, not dissimilar to those inhibiting Na^+, K^+-ATPase activity (present results). Likewise, in the nanomolar range (3×10^{-9}–1×10^{-8} M) neurotensin in vitro markedly reduces the affinity of dopamine agonist binding to subcortical limbic sites [3] by intramembrane interaction between the peptide and dopamine D_2 receptor [35]. Neurotensin concentrations required for such neurophysiological effects

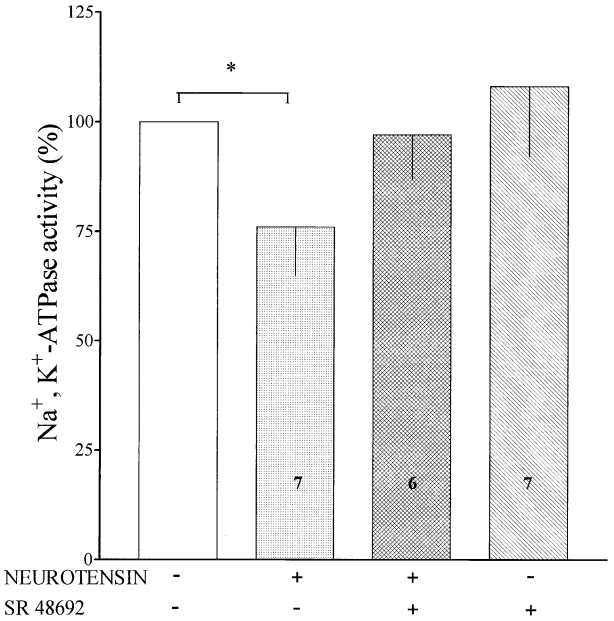

Fig. 5. Effect of antagonist SR 48692 on Na^+, K^+- ATPase inhibition by the peptide. Synaptosomal membranes were preincubated for 5 min with or without 1.0×10^{-6} M SR 48692 dissolved in 10% DMSO, followed by 3.5×10^{-6} M neurotensin for a further 5 min. Results are expressed as percentage enzyme activity taking as 100% values obtained for each experiment with no additions. SD of mean values and the number of experiments performed in triplicate are indicated within bars. $^*P < 0.05$ with respect to the control without additions, by Student's *t* test.

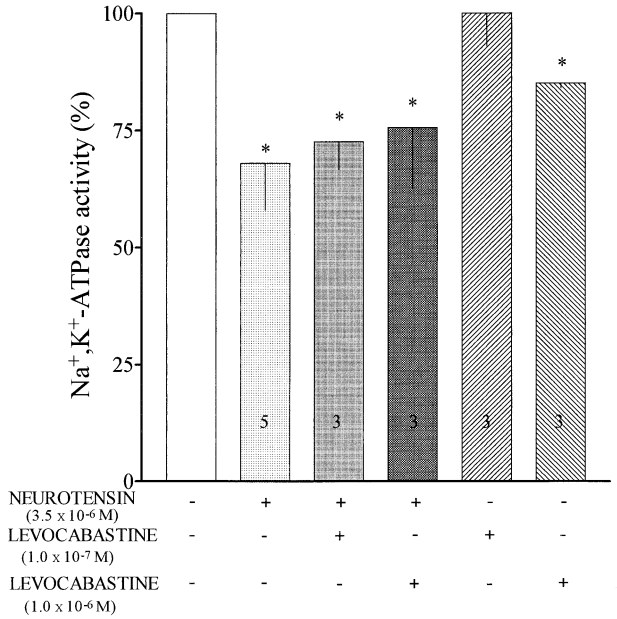

Fig. 6. Effect of levocabastine on Na^+, K^+-ATPase activity. Synaptosomal membranes were preincubated for 5 min with or without 1.0×10^{-7}–1.0×10^{-6} M levocabastine, followed by 3.5×10^{-6} M neurotensin for a further 5 min. Results are expressed as percentage enzyme activities taking as 100% values obtained for each experiment with no additions. SD of mean values and the number of experiments performed in triplicate are indicated within bars. $^*P < 0.05$ with respect to the control without additions, by Student's *t* test.

are lower than those used to inhibit Na^+, K^+-ATPase activity, which range from 3×10^{-8} to 3×10^{-6} M.

Several studies support the coexistence and corelease of neurotensin and dopamine in several brain regions [6]. Central dopaminergic neurons are modulated by neurotensin [21] and there is considerable evidence for a functional interaction between neurotensin and dopamine systems, including depolarization of substantia nigra dopaminergic neurons [22] and marked effects on dopaminergic neurons of ventro-tegmental [28] and midbrain [29] areas. Neurotensin selectively increases spontaneous activity of ventrotegmental area (A10) dopamine cells, mimicking clozapine effect [17]. The neuroleptic-like action of neurotensin has been attributed to membrane interaction between neurotensin and dopamine D_2 receptors [12]. Besides, the ability of neurotensin to modulate autoreceptor mediated dopamine effects in midbrain has been advanced [30]. Neurotensin induces a nonselective conductance in midbrain dopaminergic neurons, which is not Ca^{2+}-activated and is independent of ligand-gated channels [11].

In previous studies we have shown that dopamine is able to inhibit synaptosomal membrane Na^+, K^+-ATPase activity [23], and here, after a similar experimental procedure, we show that neurotensin likewise modifies such enzyme activity. These findings lead us to suggest that, besides the well known interaction between dopamine and neurotensin with their respective receptors, both neuroactive substances may also be involved in regulation of central neurotransmission through their ability to inhibit membrane Na^+, K^+-ATPase. Interestingly, a CNS peptide, angiotensin-(1–7), with excitatory properties [5], behaves as a Na^+, K^+-ATPase synaptosomal membrane stimulator [18].

As one of the nonopioid peptides capable of interfering with pain sensation [8], neurotensin interacts positively with ethanol [10] eliciting hypothermia, altered locomotor activity, and analgesia besides inhibiting Na^+, K^+-ATPase activity (present findings). Most of these properties are shared with peptide calcitonin, which similarly inhibits synaptosomal membrane Na^+, K^+-ATPase activity [25].

Neurotensin has been shown to induce physiological and pharmacological effects, some mediated by the high affinity NT_1 neurotensin receptor [26]. On the other hand, a low affinity NT_2 neurotensin receptor has been molecularly cloned from rat, mouse, and human brain using a strategy based on sequence homology with the known NT_1 neurotensin receptor [33]. SR 48692 binds to both NT_1 and NT_2 receptor subtypes, behaving as antagonist and agonist, respectively [36]. Remarkably enough, neurotensin behaves as a NT_2 neurotensin receptor antagonist [34], although no signal transduction pathway has so far been identified.

The participation of NT_1 receptor in enzyme inhibition is supported by our finding that Na^+, K^+-ATPase inhibition by neurotensin is fully prevented by SR 48692. At variance, the NT_2 receptor antagonist levocabastine, failed to alter enzyme inhibition by neurotensin, ruling out the involve-

ment of the low affinity neurotensin receptor in enzyme inhibition. On extending the study of neurotensin effect on Na^+, K^+-ATPase activity of synaptosomal membranes from areas other than cerebral cortex, we found no effect on membranes isolated from cerebellum (data not shown). In this connection, studies on NT_1 receptor subtypes regional distribution performed with SR 48692 have shown that only a very low density of binding sites is present in the gray matter of the cerebellum [7]. Therefore, our findings support the contention that neurotensin effect is rather specific for NT_1 subtype receptor.

To sum up, neurotensin effects on neurotransmission involve Na^+, K^+-ATPase inhibition at synaptic level through the high affinity peptide receptor.

Acknowledgments

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