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Original Communication

High affinity receptors, and not the low affinity receptors of neurotensin are involved in neuronal Na^+, K^+-ATPase inhibition by the peptide

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ABSTRACT

Neurotensin acts as a neuromodulator or as a neurotransmitter that binds to a group of receptors. Two of the receptors, namely NTS1 and NTS2, bind to neurotensin with high affinity and low affinity, respectively. Neurotensin added *in vitro* inhibits synaptosomal membrane Na^+, K^+-ATPase activity. This effect seems to be mediated by NTS1 receptor because it is fully blocked by antagonist SR 48692. Herein neurotensin effect was assayed after administration of SR 48692 and levocabastine which are antagonists for NTS1 and NTS2 receptors, respectively. Male Wistar rats were administered by i.p. injection, with 150 μg/kg SR 48692 (Sanofi-Aventis U.S., Inc.) suspended in the vehicle (0.01% Tween 80 in saline solution), 50 μg/kg levocabastine (disolved in saline solution) and the corresponding vehicle solutions. Thirty minutes later, the animals were sacrificed, cerebral cortices removed, separately pooled, and processed to obtain synaptosomal membranes. In membrane samples, Na^+, K^+- and Mg^{2+}-ATPase activities were determined in the absence and presence of 3.5×10^{-6} M neurotensin. Basal Na^+, K^+-ATPase activity in membranes isolated from control rats (vehicle injected) decreased roughly by 60% by the peptide. This effect was entirely prevented by the administration of NTS1 antagonist SR 48692. Administration of levocabastine, which enhanced basal Na^+, K^+-ATPase activity, failed to prevent neurotensin inhibitory effect on this enzyme activity. Mg^{2+}-ATPase activity remained unaltered in all conditions tested. It is concluded that Na^+, K^+-ATPase inhibition by neurotensin seems mediated only by NTS1 receptor because the administration of NTS1 antagonist SR 48692, and not the NTS2 antagonist levocabastine prevented the effect of the peptide.

KEYWORDS: CNS, cerebral cortex, neurotensin, Na^+, K^+-ATPase, SR 48692, synaptosomal membranes, levocabastine, NTS1 receptor, NTS2 receptor

ABBREVIATIONS

High affinity neurotensin receptor (NTS1), Low affinity neurotensin receptor (NTS2)

INTRODUCTION

Neurotensin is a tridecapeptide widely distributed in the brain and peripheral tissues of several mammalian species as well as of humans [1, 2]. At the central nervous system, neurotensin acts as a neurotransmitter or neuromodulator and is

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involved in several processes which include locomotion, modulation of stress, pain and profound analgesia, among others [3].

Neurotensin binds to a group of receptors [1, 4]. Two of them, namely NTS1 and NTS2, are seven-transmembrane domain receptors coupled to G proteins, which bind neurotensin with high affinity and low affinity, respectively [4]. Another two neurotensin receptor types are mainly intracellular receptors and are termed NTS3/ sortilin and nts4/SorLA [5].

During the passage of the nerve impulses, the movement of sodium and potassium ions through neuronal membranes occurs. Maintenance and restoration of ionic equilibria take place with the participation of the sodium pump, whose enzymatic version is the Na^+, K^+-ATPase. This enzyme activity concentrates in the surrounding membranes of nerve terminals, a vital region for neurotransmission [6].

Previous work from our laboratory showed the ability of neurotensin to inhibit synaptosomal membrane Na^+, K^+-ATPase activity. Results suggested the involvement of high affinity neurotensin receptor (NTS1) [7, 8] because the effect is entirely blocked by SR 48692, a non-peptide antagonist for the receptor [9].

To study further the neurotensin interaction with Na^+, K^+-ATPase, cerebral cortex membranes obtained from rats injected with SR 48692 and levocabastine, antagonists for NTS1 and NTS2 neurotensin receptors, respectively, were employed.

MATERIALS AND METHODS

Animals and drugs

Adult male Wistar rats weighing 100-150 g were used. 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 reagents were analytical grade. Ouabain and neurotensin acetate were from Sigma Chemical Co., St. Louis, MO, USA. Peptide solutions in bidistilled water were freshly prepared for each experiment. SR 48692 {2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxy phenyl) pyrazol 3-yl) carbonylamino]tricyclo $(3.3.1.1^{3.7})$ decan-2-carboxylic acid} and levocabastine clorhidrate were kindly provided by Sanofi-Aventis, U.S., Inc., and Jansen-Cilag, Argentina, respectively.

Administration of the drugs

Six rats were used in each experiment. Half of the animals received the drug i.p. and the other half received the corresponding vehicle. SR 48692 suspended in saline solution with 0.01% Tween 80 (vehicle) was administered at a dose of 150 µg/kg [10, 11]. Levocabastine chlorhidrate dissolved in saline solution was administered at a dose of 50 µg/kg [12]. Thirty minutes later, animals were killed by decapitation, skulls opened and cerebral cortices harvested. This protocol was repeated at least three times.

Preparation of synaptosomal membrane fractions

Synaptosomal membranes from cerebral cortex were isolated by differential and sucrose gradient centrifugation as previously described [13]. In each experiment, tissues from 3 rats were homogenized at 10% (w/v) in cold 0.32 M sucrose (neutralized to pH 7.0 with 0.2 M Tris base) using a Teflon glass homogenizer of the Potter-Elvehjem type. The homogenate was subjected to differential centrifugation to separate the nuclear and mitochondrial fractions. The crude mitochondrial pellet was resuspended in redistilled water (pH 7.0 with 0.2 M Tris base) for the osmotic shock. A pellet containing mitochondria, synaptosomal membranes and myelin was separated by centrifugation at 20,000 g for 30 min, then resuspended in 0.32 M sucrose, layered on top of a gradient containing 0.8, 0.9, 1.0, and 1.2 M sucrose and centrifuged at 50,000 g for two hours in a SW 28 rotor of an L8 Beckman ultracentrifuge. The fraction at the level of 1.0 M sucrose was collected, diluted with 0.16 M sucrose and spun down at 100,000 g for 30 min to obtain the synaptosomal membrane fraction. Pellets were stored at -70 ºC and, prior to enzyme assay, resuspended by brief homogenization in redistilled water and used for three weeks without appreciable change in enzyme activities.

Enzyme assays

ATPase activity was measured as already described [14]. Total ATPase activity was assayed in a medium containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.20 M Tris-HCl buffer (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 the activities was taken to correspond to Na^+, K^+-ATPase. Before performing ATPase assay, samples of synaptosomal membrane fractions were preincubated with 0.20 M Tris-HCl buffer (pH 7.4) or 3.5 x 10^{-6} M neurotensin solution at 37 ºC for 10 min; incubation volume (µl) was 35:5 for buffer and membranes. 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 ºC for 30 min. The reaction was stopped with 30% (w/v) trichloroacetic acid solution. ATPase activity was monitored by colorimetric determination of orthophosphate released [15]. In all enzyme assays, tubes containing enzyme preparations and assay media maintained at 0 ºC throughout the incubation period were used as blanks.

Protein measurement

Protein was determined using bovine serum albumin as standard [16].

Data analysis

Data are presented as mean values ± SD of *n* experiments. To compare each value versus corresponding control without addition, Student's *t*-test was employed. The probability level indicative of statistical significance was set at $p < 0.05$.

RESULTS

ATPase activities were assayed in synaptosomal membranes isolated from cerebral cortex of rats injected with antagonists SR 48692, levocavastine or the corresponding vehicles (controls).

Basal Na^+, K^+-ATPase activity in control membranes was roughly 35 µmol Pi. mg. $prot^{-1}$. h^{-1}, a value which decreased by 62% in the presence of 3.5 x 10^{-6} M neurotensin. After administration of SR 48692 the basal Na^+, K^+-ATPase activity failed to differ statistically from that recorded in vehicle injected rats. Na^+, K^+-ATPase activity in these membranes was not modified by the presence of neurotensin in the enzyme assay (Fig. 1A).

In another series of experiments, rats were administered with NTS2 antagonist levocabastine and the neurotensin effect on Na^+, K^+-ATPase activity was tested. Basal Na^+, K^+-ATPase activity in control membranes was roughly 34 µmol Pi. mg. $prot^{-1}$. h^{-1}, a value which decreased by 51% in the presence of 3.5 x 10^{-6} M neurotensin.

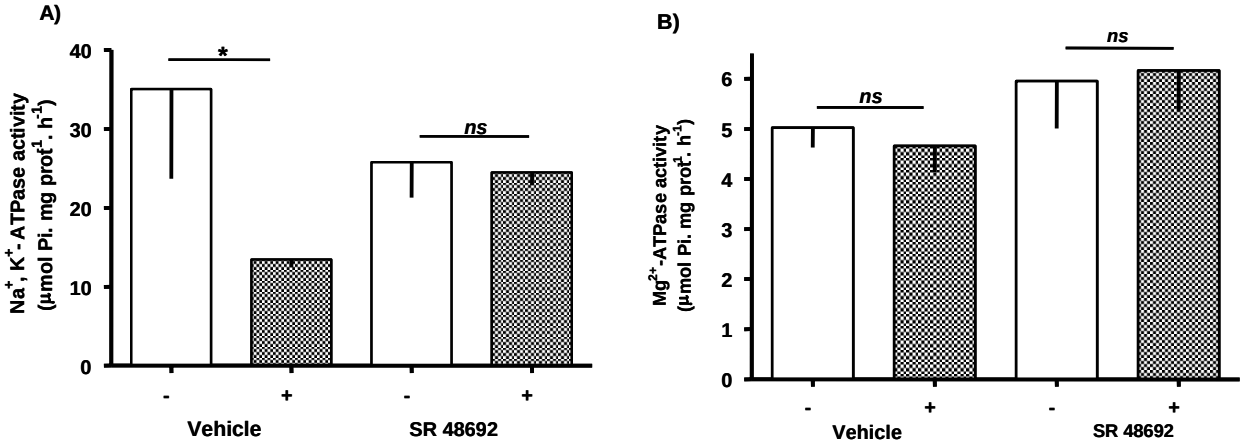

Fig. 1. Neurotensin effect on ATPase activities in rat cerebral cortex after SR 48692 administration. Rats received i.p. a single dose of SR 48692 or vehicle and 30 minutes later, were decapitated, tissues harvested and subjected to subcellular fractionation to obtain synaptosomal membranes. Membrane samples were preincubated in the absence or presence of 3.5 x 10^{-6} M neurotensin (NT) and assayed for ATPase activities. Results are expressed as specific enzyme activity. SD of 3-5 experiments performed per triplicate is indicated within the bars. White bars, without neurotensin; dotted bars, with neurotensin. (A) Na^+, K^+- ATPase activity; (B) Mg^{2+}-ATPase activity. *$p < 0.05$ with respect to the values recorded without neurotensin (Student´s *t* test); ns, non-significant difference.

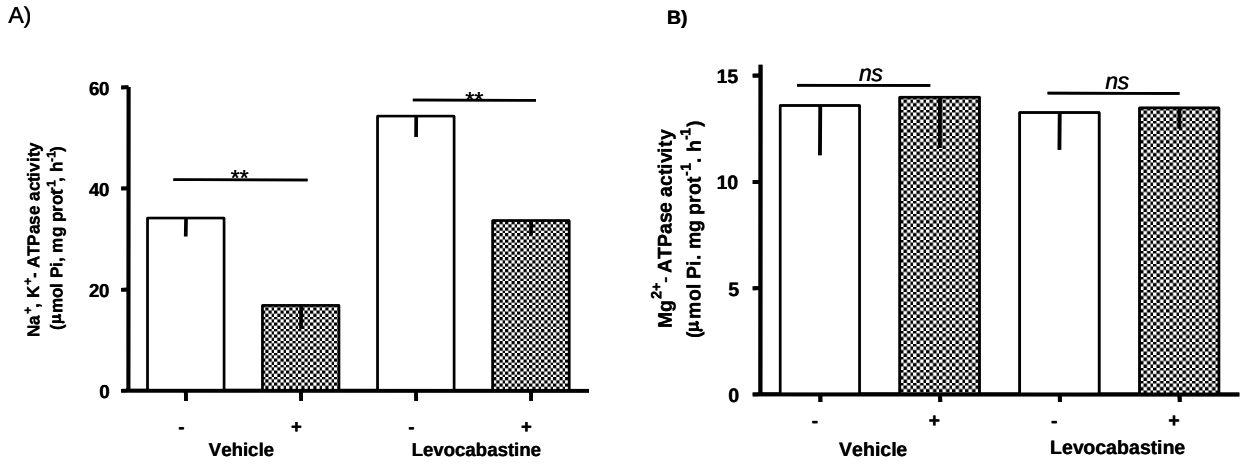

Fig. 2. Neurotensin effect on ATPase activities in rat cerebral cortex after levocabastine administration. Rats received i.p. a single dose of levocabastine or vehicle and 30 minutes later, were decapitated, tissues harvested and subjected to subcellular fractionation to obtain synaptosomal membranes. Membrane samples were preincubated in the absence or presence of 3.5 x 10^{-6} M neurotensin (NT) and assayed for ATPase activities. Results are expressed as specific enzyme activity. SD of 6 experiments performed per triplicate is indicated within the bars. White bars, without neurotensin; dotted bars, with neurotensin. (A) Na^+, K^+-ATPase activity; (B) Mg^{2+}-ATPase activity. $^{**}p < 0.001$ with respect to the values recorded without neurotensin (Student´s *t* test); ns, non-significant difference.

After administration of levocabastine the basal Na^+, K^+-ATPase activity was roughly 55 µmol Pi. mg. $prot^{-1}$. h^{-1} ($p < 0.05$ *versus* vehicle injected). The value dropped by 38% by the presence of 3.5 x 10^{-6} M neurotensin in the enzyme assay (Fig. 2A).

Basal synaptosomal membrane Mg^{2+}-ATPase activity was not modified by the treatments or by the presence of 3.5 x 10^{-6} M neurotensin (Figs. 1B and 2B).

DISCUSSION

In the present study, neurotensin effect on synaptosomal membrane Na^+, K^+-ATPase activity after administration of neurotensin receptor antagonists to rats was assayed. Results indicated that neurotensin inhibitory effect on Na^+, K^+-ATPase activity was entirely prevented by the administration of NTS1 antagonist SR 48692 whereas it remained unaltered by the administration of NTS2 antagonist levocabastine.

Neurotensin works as a neurotransmitter or a neuromodulator in the brain, where it exerts multiple actions. Like many other neuropeptides, neurotensin is a messenger of intracellular communication [2, 17], whose actions occur through specific peptide receptors (see Introduction). Antagonists are currently used for verifying whether such effects are mediated by specific subtype receptors [18].

Previous work showed that neurotensin inhibits Na^+, K^+-ATPase activity, an effect which is blocked by *in vitro* addition of NTS1 receptor antagonist SR 48692 during brain membrane enzyme assay [7, 8]. To explore further the involvement of neurotensin receptors in Na^+, K^+-ATPase inhibition by the peptide, herein experiments *ex vivo* were run in rat brain after administration of SR 48692 and levocabastine, the respective antagonists for NTS1 and NTS2 receptors.

Acute treatment with SR 48692 failed to alter basal synaptosomal membrane Na^+, K^+-ATPase activity but entirely prevented enzyme inhibition by neurotensin. These findings favoured the view that NTS1 receptor is most likely involved in the peptide effect on Na^+, K^+-ATPase activity.

Neurotensin acts as an agonist for all NTS1-mediated pathways, whereas it may exert either agonist or antagonist activities, according to the NTS2 mediated pathway involved [19]. Therefore, it was of interest to explore likewise

potential involvement of NTS2 receptor in neurotensin effect on Na^+, K^+-ATPase activity. For this reason, the H1 antihistaminic drug levocabastine was employed to block NTS2 receptors. With this drug, the findings show that neurotensin internalizes in nerve terminals via an endocytic pathway, which is mechanistically distinct from that responsible for neurotensin internalization in nerve cell bodies [20]. Acute administration of levocabastine enhanced basal Na^+, K^+-ATPase activity in synaptosomal membranes but failed to impair further neurotensin effect on Na^+, K^+-ATPase activity.

Na^+, K^+-ATPase catalyses the hydrolysis of ATP in a two-step process: a phosphorylation step and a dephosphorylation step; the latter proved to be K^+-dependent and sensitive to ouabain [21]. Neurotensin exerts inhibitory effects *in vitro* when ATP hydrolysis by synaptosomal membranes and ligand [^{3}H]-ouabain binding to cerebral cortex membranes are assayed. The mechanism involved in each case markedly differed, because *in vitro* addition of SR 48692 blocks the former but not the latter, suggesting that [^{3}H]-ouabain binding inhibition by neurotensin hardly involves NTS1 receptor. Curious enough, *in vitro* SR 48692 itself fails to alter basal Na^+, K^+-ATPase activity but it decreases ligand [^{3}H]-ouabain binding to membranes [7, 8, 22].

Present findings indicated that levocabastine enhanced basal Na^+, K^+-ATPase activity in cerebral cortex synaptosomal membranes. A possible explanation for this finding might involve a drug effect on catecholaminergic neurotransmission on the basis of cocaine-like activity of antihistaminic drugs [23]. Regarding this point, it may be mentioned that administration of desipramine, which is known to inhibit catecholamine uptake, enhances Na^+, K^+-ATPase activity at central nervous system level [24, 25].

In summary, the present results indicate that acute administration to rats of SR 48692, which fails to alter basal Na^+, K^+-ATPase activity, prevented the inhibitory effect of neurotensin on Na^+, K^+-ATPase activity. At variance, administration of NTS2 antagonist levocabastine enhanced basal Na^+, K^+-ATPase activity but failed to modify the extent of Na^+, K^+-ATPase inhibition by neurotensin addition. It is suggested that NTS1 receptor, and not the NTS2 receptor is involved in neuronal Na^+, K^+-ATPase inhibition by neurotensin.

CONCLUSION

Neurotensin effect on synaptosomal membrane Na^+, K^+-ATPase was studied after administration of SR 48692 and levocabastine, the respective antagonists for NTS1 and NTS2 neurotensin receptors. Basal Na^+, K^+-ATPase activity in membranes isolated from control rats (vehicle injected) decreased roughly by 60% by the peptide. This effect was entirely prevented by administration of SR 48692 but remained unaltered by administration of levocabastine. Present results gave additional evidence that Na^+, K^+-ATPase inhibition by neurotensin seems to be mediated by NTS1 receptor, but not by NTS2 receptor.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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