FEBS 17008

FEBS Letters 386 (1996) 91–94

Molecular cloning of a levocabastine-sensitive neurotensin binding site

Pascale Chalon, Natalio Vita, Mourad Kaghad, Marie Guillemot, Jacques Bonnin, Bruno Delpech, Gerard Le Fur, Pascual Ferrara, Daniel Caput*

Sanofi Recherche, BP 137, 31676 Labège Cedex, France

Received 4 April 1996

Abstract **A search for sequences homologous to the neurotensin receptor cDNA in a rat hypothalamic library has identified a novel neurotensin receptor (NTR-2). The 1539 bp cDNA encodes a 416 amino acid protein and shows highest homology to the previously cloned neurotensin receptor (NTR-1) (64% homology and 43% identity). Binding and pharmacological studies demonstrate that NTR-2 expressed in COS cells recognizes neurotensin (NT) with high affinity as well as several other agonists and antagonists. However, a fundamental difference was found; unlike NTR-1, NTR-2 recognizes, with high affinity, levocabastine, a histamine H1 receptor antagonist previously shown to compete with NT for low-affinity binding sites in brain.**

Key words: Neurotensin receptor; Molecular cloning

1. Introduction

Neurotensin (NT) is a tridecapeptide involved in intercellular communication in the central nervous system and peripheral organs [1]. In the brain, NT acts as a neurotransmitter/neuromodulator [2,3]. In particular, it modulates dopamine transmission in the nigrostrial and mesolimbic pathways [4,5]. Additionally, NT plays a role in nociception, hypothermia, control of pituitary hormone secretion and muscle relaxation [6]. In adult rat brain, NT binds at two distinct sites distinguishable by (i) their sensitivity to levocabastine, a histamine H1 receptor antagonist and (ii) their differential affinity to neurotensin [7]. The levocabastin-sensitive NTR appears after birth and exhibits a distribution in the brain different from the high-affinity NTR [8].

High-affinity levocabastine-insensitive NTR has been cloned from rat [9] and human [10,11]. This receptor represents less than 30% of the NT binding sites in the rat adult brain; and is found mainly in the substantia nigra and the ventral tegmental area. The cloned rat NTR is a 424 amino acid protein that belongs to the G-protein-coupled superfamily of receptors. The biochemical and pharmacological properties of this receptor have been extensively studied (for a review see [6]). It has been shown that upon activation the receptor modulates intracellular levels of cGMP [12], cAMP [13,14] and inositol phosphates [15-19]. The recently discovered non-peptide NT antagonist, SR 48692A inhibits the binding of NT to the cloned receptor [20]. However, a recent study shows that in mouse and rat, NT-induced hypothermia and analgesia are insensitive to SR48692 [21], suggesting that NT can act through different receptor subtypes. SR48692 has also been shown to differentially affect NT-induced behavior and changes in dopaminergic transmission [22,23]. Besides pharmacological arguments in favor of NTR subtypes, a recent study comparing the distributions of NTR and NTR transcripts in rat brain strongly suggested the existence of another NTR [24,25]. NTR transcripts are detected in several regions of the brain, but not in the subfornical organ, though NT binding sites were clearly associated with the cell bodies in this region [24]. These findings prompted us to undertake a search for other NTRs using a strategy based on sequence homology with the known NTR. We describe here the molecular cloning of a novel NTR sharing 64% homology with the previously described receptor. We also report on the binding and pharmacological properties of this receptor expressed in COS cells.

2. Materials and methods

2.1. Drugs and peptides

^{125}I-labeled [Tyr^3]neurotensin and unlabeled neurotensin were purchased from NEN and Sigma, respectively. Dulbecco's modified essential medium (DMEM), fetal calf serum (FCS) and phosphate-buffered saline (PBS) were from Gibco (Paisley, UK). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF) and 1,10-phenanthroline were from Sigma (St. Louis, MO). A selective, non-peptide antagonist SR 48692 was synthesized at Sanofi Recherche, Montpellier, France [20]. The anti-histamine agent, levocabastine, was obtained from Janssen Pharmaceutica (Geel, Belgium).

2.2. RNA extraction and analysis

Total RNA was extracted from rat hypothalamus using the acid guanidinium isothiocyanate phenol chloroform method [26]. Poly(A) containing RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as described by Aviv and Leder [27]. RNA was size-fractionated by electrophoresis on 1% agarose/formaldehyde gels then blotted to nitrocellulose membranes [28]. Hybridization with a ^{32}P-labeled NTR cDNA was performed under low stringency conditions.

2.3. cDNA library construction and DNA sequencing

The cDNA library was constructed using the primer-adapter procedure [29] and the pT3-T7 vector (Pharmacia). Nucleotide DNA sequences were determined by the dideoxy method of Sanger et al. [30].

2.4. Transfection of COS cells and binding experiments

The pharmacological characterization was performed using COS-3 cells transfected with pSVL-NTR2 recombinant vector. 3 days after transfection, cell monolayers in 6-well plates were washed twice with 50 mM Tris-HCl pH 7.5, 0.1% BSA, 0.1% NaN_3, 1 mM 1,10-phenanthroline (binding buffer) before the binding of radioligand.

Saturation experiments were performed in 1 ml of binding buffer containing ^{125}I-neurotensin over the range from 0.05 to 14.5 nM. After 1 h incubation at room temperature, the medium was aspirated and cell monolayers were washed twice with incubation buffer. Finally, cells were solubilized with 1 ml of 1 N NaOH and bound radioactivity was evaluated. Non-specific binding was defined as binding in the presence of a 500-fold excess of unlabeled ligand and under these experimental conditions was less than 1% of the total counts.

Displacement experiments were conducted in an analogous manner using 0.2 nM ^{125}I-neurotensin as radioligand. Neurotensin, neuromedin N, SR 48692 and levocabastine were used as competitors.

*Corresponding author. Fax: (33) (61) 004119.

PII S0014-5793(96)00397-3

Binding data derived from saturation and competition experiments were analysed by using the computerized nonlinear curve-fitting software (Prim, GraphPad, San Diego, CA).

3. Results

3.1. Cloning and sequencing of protein related to the NT receptor

Data from pharmacological studies consistently suggest that more than one neurotensin receptor might exist. Based on the assumption that NTR subtypes could be structurally related, we screened by homology a cDNA library made from rat hypothalamus. Approximately half a million recombinant bacteria were plated at high density on filters. Hybridization under low stringency conditions with a labeled rat NTR cDNA probe yielded several positive clones which were subsequently isolated. Systematic DNA sequencing analysis showed that only one of them shared substantial homology with the NTR cDNA. This 1539 bp long cDNA contained a large open-reading frame encoding a 416 amino acid protein. The putative NTR-2 protein, which shows 43% identity and 64% homology with the NTR-1, has the structural features of a G-protein-coupled receptor (Fig. 1). Interestingly, although the lengths of both proteins are similar, the new receptor has a shorter N-terminal extracellular region, and a longer cytoplasmic loop between the fifth and sixth putative transmembrane regions. It should also be noted that the cytoplasmic C-terminal region shows the lowest homology to NTR-1.

3.2. Pharmacological characterization of cloned receptor

We have examined the binding properties of NT to the new receptor expressed in COS-3 cells after transfection of the cloned cDNA. This receptor is able to bind ^{125}I-neurotensin in a specific and saturable manner. Radiolabeled NT did not

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NTR-2   1  .........................METS......SPWPPRPSPSAGLS    18
                                           ||        |   | |
NTR-1   1  MHLNSSVPQGTPGEPDAQPFSGPQSEMEATFLALSLSNGSGNTSESDTAG   50
                .         .         .         .         .
          19  LEARLGVDTRLWAKVLFTALYSLIFAFGTAGNALSVHVVLKARA..GRPG   66
                | | |    ||| || |   |  || ||
          51  PNSDLDVNTDIYSKVLVTAIYLALFVVGTVGNSVTAFTLARKKSLQSLQS  100
                .         .         .         .         .
          67  RLRYHVLSLALSALLLLLVSMPMELYNFVWSHYPWVFGDLGCRGYYFVRE  116
                ||  |||||  ||  ||  ||  |||||  |  |  ||  |||  |||||||  |
         101  TVHYHLGSLALSDLLILLLAMPVELYNFIWVHHPWAFGDAGCRGYYFLRD  150
                .         .         .         .         .
         117  LCAYATVLSVASLSAERCLAVCQPLRARRLLTPRRTRRLLSLVWVASLGL  166
                | |||  |  |||||  ||  ||  |  |  |  |    ||     |  |||  |
         151  ACTYATALNVASLSVERYLAICHPFKAKTLMSRSRTKKFISAIWLASALL  200
                .         .         .         .         .
         167  ALPMAVIMGQKHEVESADGEPEPASRVCTVLVSRATLQVFIQVNVLVSFA  216
                |  ||   ||             |   |||   |  ||  |  ||||   ||
         201  AIPMLFTMGLQ...NRSGDGTHPGGLVCTPIVDTATVKVVIQVNTFMSFL  247
                .         .         .         .         .
         217  LPLALTAFLNGITVNHLMALYSQVPSASAQVSSIPSRLELLSEEGLLGFI  266
                |       ||    |  |       |                |
         248  FPMLVISILNTVIANKLTVMVHQAAE.........QGRVCTVGTHNGLEHS  289
                .         .         .         .         .
         267  TWRKTLSLGVQASLVRHKDASQIRSLQHSAQVLRAIVAVYVICWLPYHAR  316
                |   |                  |  |   ||||  |   |  ||||||  |
         290  TFNMTI.............EPGRVQALRHGVLVLRAVVIAFVVCWLPYHVR  327
                .         .         .         .         .
         317  RLMYCYIPDDGWTNELYDFYHYFYMVTNTLFYVSSAVTPILYNAVSSSFR  366
                |||  |||  |   ||   |  ||||||||  ||  |||||||   |||||  ||   ||
         328  RLMFCYISDEQWTTFLFDFYHYFYMLTNALFYVSSAINPILYNLVSANFR  377
                .         .         .         .         .
         367  KLFLESLGSLCGE...QHSLVPLPQEAPESTTSTYSFRLWGSPRNPSLGEIQV 416
                ||  |  ||          |    |  |  |   |
         378  QVFLSTLACLCPGWRHRRKKRPTFSRKPNSMSSNHAFSTSATRETLY      424
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Fig. 1. Alignment of the rNTR-2 to the rNTR-1. The complete rNTR-2 and rNTR-1 sequences are compared; gaps for alignment are indicated by dots and identical residues indicated by vertical lines. The solid lines above the sequences indicate the proposed transmembrane domains.

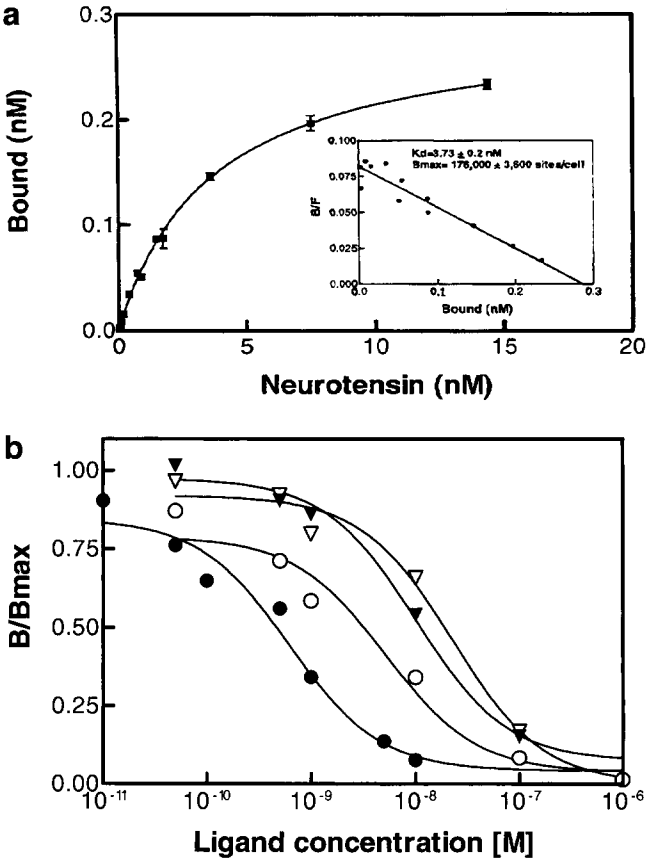

Fig. 2. (a) Saturation isotherm and Scatchard plot (inset) of the specific binding of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin to COS-7 cells transfected with the rNTR-2 cDNA. Each point represents the mean of triplicates. (b) Inhibition of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin binding to COS-7 cells transfected with the rNTR-2 cDNA by unlabeled neurotensin (●), neuromedin N (○), SR 48692 (▽) and levocabastine (▼). Each point represents the mean of triplicates.

bind to untransfected cells, or to cells transfected with the vector DNA alone (not shown). Scatchard plot analysis of ^{125}I-neurotensin binding (Fig. 2a) showed a single high-affinity population of receptors with a dissociation constant (K_d) of 3.7 ± 0.2 nM and a binding capacity (B_{max}) of $180\,000 \pm 3000$ sites/cell. Fig. 2b shows competition curves of various ligands with labeled neurotensin. Agonists and antagonists competed for the binding of ^{125}I-neurotensin with an order of potency similar to that for the NTR-1 previously described with the exception of the high affinity of levocabastine [10]. Neurotensin is the most potent competitor, with an apparent half-maximal inhibiting concentration (IC_{50}) of 0.77 nM, followed by neuromedin N, IC_{50} 5.1 nM, and levocabastine, IC_{50} 10.0 nM. The recently described non-peptide antagonist, SR 48692, showed an IC_{50} of 22.5 nM.

3.3. mRNA tissue distribution

The tissue distribution of NTR-2 mRNA was examined by northern blot analysis. As shown Fig. 3, poly(A) containing RNA from cortex and hypothalamus gave a strong signal when hybridized with labeled NTR-2 cDNA. Fainter signals were also obtained with RNA from heart and intestine. Two transcripts were systematically detected, with sizes of 1.6 and

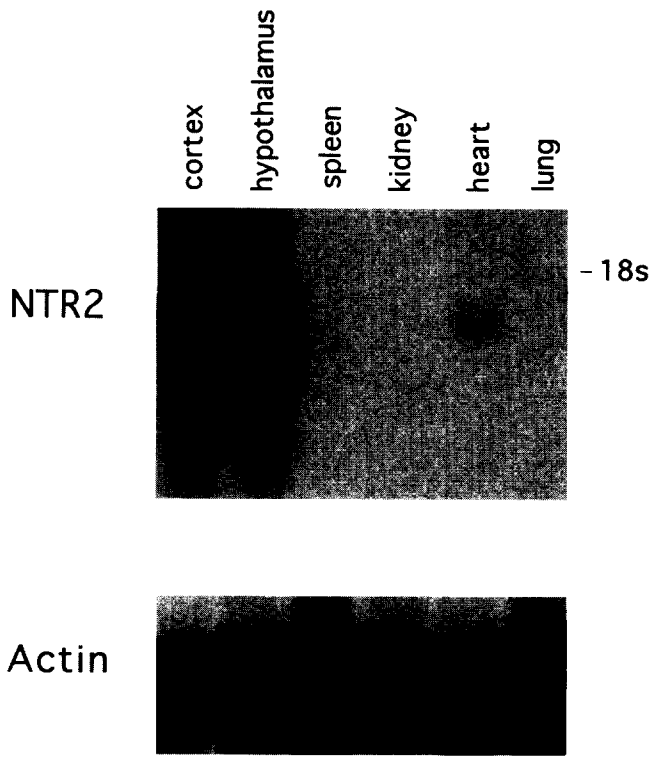

Fig. 3. Northern blot analysis. Northern blot analyses were conducted using 10 μg of poly(A) RNA per lane and employing as labeled probes cDNAs encoding NTR-2 or actin.

1.4 kb. The shorter transcript is a splice variant encoding a C-truncated receptor (results not shown).

No transcript was detected in pituitary gland, spleen and kidney even using RT-PCR analysis.

4. Discussion

The discovery, almost a decade ago, of levocabastine-sensitive NT sites in rat [8] suggested the existence of NTR subtypes. The recent molecular cloning of a neurotensin receptor in rat [9] and human [10,11] (NTR-1), and the discovery of selective non-peptide antagonists of the NTR [20] have provided further support for the existence of receptor subtypes. SR48692, for example, inhibits NT binding to NTR-1 but only antagonizes some of the effects of NT in vivo [21], suggesting that some of these effects might be mediated through an SR 48692-insensitive NTR. In addition, recent findings using a combination of high resolution in situ techniques demonstrated that NTR could be encoded by different mRNAs [24]. These observations constitute evidence for different types of NTR and prompted us to search for homologous receptors. We have identified a novel NTR (NTR-2) from rat hypothalamus which shares structural homologies (43% identity and 64% similarity) with NTR-1. Interestingly, the lowest homologies between NTR-1 and NTR-2 are in their third cytoplasmic loops and C-terminal domains, two regions bearing coupling specificity to G-protein. Binding studies show that the affinity of NT for NTR-2 is slightly lower than for NTR-1 and that, importantly, levocabastine can efficiently compete with NT for occupancy of this site.

NTR-2 seems to correspond to the levocabastine-sensitive NT site previously described [7]. NTR-2 transcripts are abundant in cortex and hypothalamus (Fig. 3) (100 more than NTR-1 mRNA), and absent in pituitary gland and brain embryos (results not shown). NTR-2 transcripts are also found in heart and gut, suggesting a possible implication of a NT/NTR-2 pathway in muscle function. However, the functions of NTR-2 are presently unknown. Until now, levocabastine-sensitive NT binding sites have been considered as recognition sites for NT, devoid of signaling activity and hence named acceptor sites [31]. It will be surprising, considering the G-protein-coupled receptor structure of NTR-2, if this receptor does not signal to the cells. Nonetheless, the molecular cloning of NTR-2 will allow the testing of this assumption, and biochemical and pharmacological studies of NTR-2 which will certainly shed light on the biology of this receptor.

In conclusion, it must be pointed out that the sensitivity of NTR-2 to SR48692 makes its implication in SR-insensitive NT-induced effects such as hypothermia and analgesia unlikely, and so suggests other(s) NTR subtype(s).

Finally, the availability of rat NTR-2 cDNA probe will enable us to answer the important question of the existence of an homologous receptor in man.

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