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Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor

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A human neurotensin receptor (hNTR) cDNA was cloned from the colonic adenocarcinoma cell line HT29. The cloned cDNA encodes a putative peptide of 418 amino acids with 7 transmembrane domains. The amino acid sequence of the hNTR is 84% identical to the rat NTR [Neuron, 4 (1990) 847–854]. Transfection of this cDNA into COS cells results in the expression of receptors with pharmacological properties similar to those found with HT29 cells. Northern blot analysis using the hNTR cDNA probe indicated a single transcript of 4 kb in the brain, the small intestine and blood mononuclear cells.

Neurotensin; Receptor; Cloning; Binding; HT 29 cell

1. INTRODUCTION

Neurotensin (NT) is a tridecapeptide originally isolated from calf hypothalamus [1]. Thereafter, the presence of high concentrations of neurotensin was found in numerous areas of the central nervous system (CNS) and gastro-intestinal tissues [2]. In these tissues, neurotensin produces a diversity of pharmacological effects [3]. Evidence of a close connection between the NT and dopaminergic systems has recently increased the interest for NT-containing circuits considering their potential implications in abnormal behavior and neuropsychiatric disorders [4].

The pharmacological effects displayed by neurotensin are mediated by specific membrane receptors. The biochemical and pharmacological properties of these binding sites have been extensively studied using mammalian brain homogenates as well as membrane preparations from neuronal and certain non-neuronal cell lines [5,6]. It has been shown that the interaction with these receptors modulates intracellular levels of cGMP, cAMP and inositol phosphates [7,8].

Different groups were able to solubilize and purify to homogeneity NT receptors (NTRs) from bovine [9], rat [10] and mouse brains [11,12], and more recently, Tanaka et al. [13] reported the molecular cloning of the rat neurotensin receptor.

In the present study, a cDNA encoding the human neurotensin receptor was isolated by screening a human colon adenocarcinoma cell line (HT-29) cDNA expression library using a radioligand-binding strategy. This cDNA encodes a 418 amino-acid protein with a transmembrane topology similar to that of other G-protein coupled receptors. The properties of the cloned binding site expressed in COS cells are identical to those found in HT29 cells. In addition, besides the small intestine and brain, the expression of neurotensin receptor mRNAs was also detected in peripheral blood mononuclear cells (PBMC).

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2. MATERIALS AND METHODS

2.1. *Drugs and peptides*

Labeled (^{125}I-labeled [monoiodo-Tyr^3]neurotensin, 2,000 Ci/mmol) and unlabeled neurotensin were purchased from Amersham (Buckinghamshire, UK) and Bachem (Bubendorf, Switzerland), respectively. Dulbecco's modified essential medium (DMEM), fetal calf serum (FCS) and phosphate-buffered saline (PBS) were from Gibco (Paisley, Scotland). Bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF) and 1,10-orthophenanthroline were from Sigma (St. Louis, MO).

A selective, non-peptide antagonist SR 48692 was synthesized at Sanofi Recherche, Montpellier, France [14]. The anti-histamine agent, levocabastine, was obtained from Janssens Pharmaceutica (Geel, Belgium).

2.2. *Cell culture*

The human colonic adenocarcinoma HT29 cell line (kindly provided by Dr. P. Kitabgi) and the COS-7 cell line were cultured as previously described [8].

2.3. *RNA extraction and analysis*

Total RNA was extracted from HT29 cells by using the acid–guanidinium isothiocyanate–phenol–chloroform method [15]. Poly(A)

RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as described by Aviv and Leder [16]. RNA analysis was performed by electrophoresis on 1% agarose/formadehyde gels then transferred to nitrocellulose and probed with a ^{32}P-labeled hNTR cDNA as described [17].

2.4. *cDNA library construction and DNA sequencing*

The cDNA library was constructed using the primer-adapter procedure [18] and the pSVL vector (Pharmacia). Approximately 5×10^5 primary recombinants were produced and organized in pools of 1,000 clones. DNA sequences were determined by the method of Sanger [19].

2.5. *Transfection of COS cells and radiolabeled ligand detection*

Preparation of recombinant plasmids and the transfection of COS-7 cells by the DEAE dextran procedure were as previously described

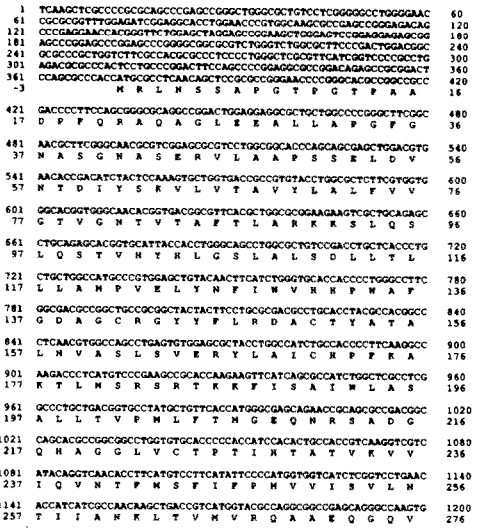

Fig. 1. Nucleotide and predicted amino acid sequences of the hNTR cDNA.

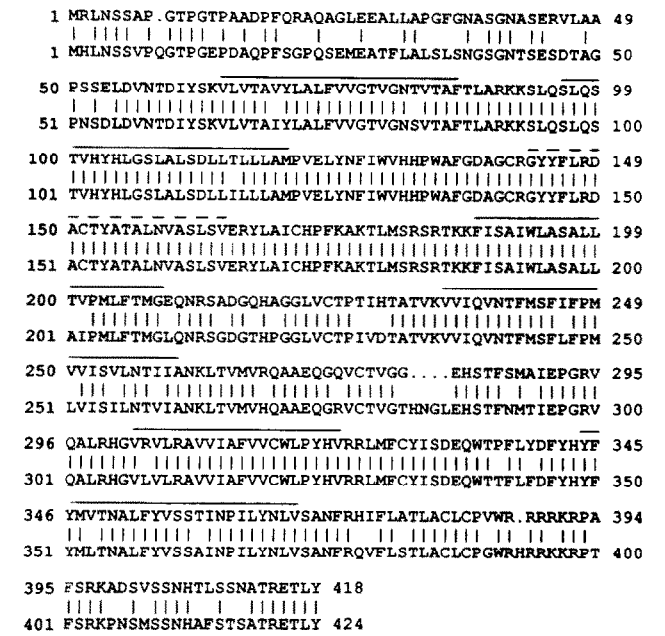

Fig. 2. Alignment of the hNTR to the rNTR. The complete hNTR and rNTR sequences are compared; gaps for alignment are indicated by dots and identical residues indicated by vertical lines. The solid lines above the sequences indicate the proposed transmembrane domains

[20]. Briefly, 5×10^5 cells were plated in Slides Flasks (Nunc) and grown in DMEM containing 5% FCS in 5% CO_2 at 37°C. After an overnight incubation, the cells were transfected with 3 μg of extracted DNA from pools in 2 ml of DMEM containing 400 mg of DEAE dextran (Pharmacia) and 200 μM of chloroquine diphosphate. After 5 h at 37°C, the transfecting medium was removed and the cells were treated with 2 ml of PBS containing 10% DMSO for 1 min, rinsed twice with PBS and incubated in DMEM containing 1% FCS for 3 days. Receptor-expressing pools were detected by binding using ^{125}I-labeled [monoiodo-Tyr^3]neurotensin (see below) as a probe followed by autoradiography using a Phospholmager device (Molecular Dynamics, Sunnyvale, CA).

2.6. *Binding experiments*

The pharmacological and functional characterization of the cloned receptor was performed using COS-transfected cells seeded in 6-well plates (Falcon). Three days after transfection, cell monolayers were washed twice with 50 mM Tris-HCl pH 7.5, 0.2% BSA, 0.1% NaN_3, 1 mM 1,10-*ortho*-phenanthroline (binding buffer).

Saturation experiments were done in 1.5 ml of binding buffer containing ^{125}I-labeled [monoiodo-Tyr^3]neurotensin over a range from 0.05 to 2 nM in the absence or in the presence of a 500-fold excess of unlabeled neurotensin. After 1 h incubation at room temperature, plates were aspirated and cell monolayers were washed twice with incubation buffer. Finally, 2 ml of 1 N NaOH were added and bound radioactivity was quantified after cell solubilisation. Non-specific binding was defined as binding in the presence of a 500-fold excess of unlabeled ligand and under these experimental conditions was < 1% of the total counts.

Displacement experiments were done in an analogous manner using 0.2 nM of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin as radioligand. The following unlabeled ligands were used as competitors: neurotensin, neuromedin N, SR 48692 and levocabastine.

Binding data derived from saturation and competition experiments were analysed by using the computerized nonlinear curve fitting described by Munson and Rodbard [21].

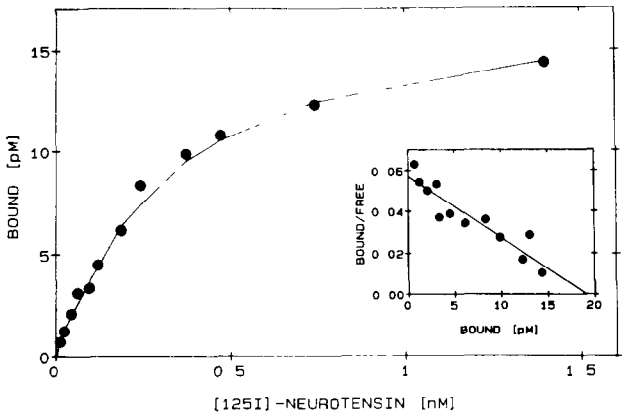

Fig. 3. Saturation isotherm and Scatchard plot (inset) of the specific binding of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin to COS-7 cells transfected with the hNTR cDNA. Each point represents the mean of triplicates.

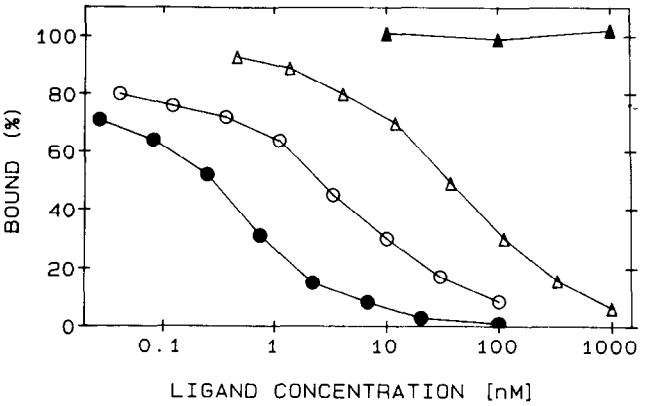

Fig. 4. Inhibition of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin binding to COS-7 cells transfected with the hNTR cDNA by unlabeled neurotensin (●), neuromedin N (○), SR 48692 (△) and levocabastine (▲). Each point represents the mean of triplicates.

3. RESULTS AND DISCUSSION

3.1. *Cloning and sequencing of hNTR*

A cDNA expression library containing 5×10^5 recombinant clones was constructed and divided into pools of 10^3 clones. Plasmid DNA from each pool was introduced into COS cells and the binding of neurotensin was detected with a labeled ligand. Only one of the first hundred pools tested showed a significant binding activity. Stepwise fractionations of this neurotensin receptor cDNA-containing pool identified a single recombinant plasmid able to confer a neurotensin binding activity to COS cells.

Fig. 1 shows the 4,140-nucleotide sequence of hNTR cDNA. Considering the determined size of the NTR mRNA (Fig. 5), hNTR cDNA seems to represent a full length transcript. The longest open reading frame found encodes a protein of 418 residues with a theoretical molecular weight of 46,288 kDa. The protein shows several structural features of G-protein coupled receptors and a high degree of identity (84%) and similarity (92%) with the rat neurotensin receptor [13] (Fig. 2).

3.2. *Pharmacological and functional characterization of cloned hNTR*

We have examined the binding properties of the human neurotensin receptor expressed in COS cells after transfection of the cloned cDNA. Little or no

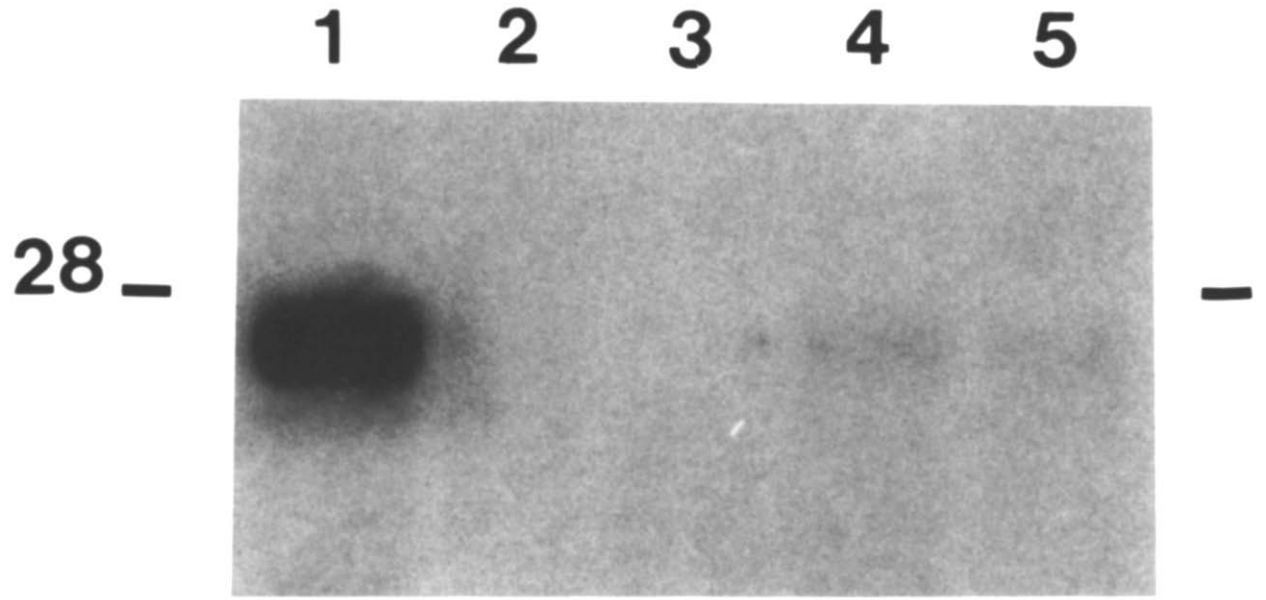

Fig. 5. Northern analysis of poly(A) RNA (1μg) from cells and tissues hybridized to ^{32}P-labeled hNTR cDNA. Lane 1, HT29 cells; lane 2, human brain (rear part); lane 3, human brain (front part); lane 4, intestine; lane 5, PBMC.

binding was detected with untransfected cells or cells transfected with the vector DNA alone (not shown). The expressed receptor is able to bind in a specific and saturable manner ^{125}I-labeled [monoiodo-Tyr^3]neurotensin (Fig. 3). Scatchard plot analysis of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin binding (Fig. 3, inset) showed a single high affinity population of receptors with a dissociation constant (K_d) of 0.56 ± 0.1 nM and a binding capacity (B_{max}) of 30,000 ± 3,000 sites/cell.

Fig. 4 shows competition curves of various ligands with ^{125}I-labeled [monoiodo-Tyr^3]neurotensin. Agonists and antagonists competed for the binding of [monoiodo-Tyr^3]neurotensin with an order of potency identical to the neurotensin receptor expressed in the HT29 cell line [6] and other tissues [14]: (i) neurotensin is the most potent competitor, followed by neuromedin N and SR 48692; (ii) the apparent half maximal concentrations for an inhibition (IC_{50}) derived from the competition curves were 0.3, 2.6 and 38 nM, respectively; and (iii) levocabastine, an anti-histamine agent described as a ligand for the low affinity neurotensin binding site [22] does not compete (using concentrations up to 10 μM) with ^{125}I-labeled [monoiodo-Tyr^3] neurotensin for the binding to COS cells, demonstrating that the transfected cDNA encodes a high affinity NTR.

3.3. *Tissue distribution of hNTR mRNA*

The tissue distribution of hNTR mRNA was examined by Northern blot analysis. As shown in Fig. 5, ^{32}P-labeled hNTR cDNA hybridized to poly (A) RNAs isolated from HT29 cells, brain, colon and peripheral blood mononuclear cells (PBMC) gave rise to a single band although it is barely detectable in the brain RNAs (lanes 2 and 3). This result was confirmed by polymerase chain reaction (PCR) experiments (not shown).

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