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Neurotensin is an antagonist of the human neurotensin NT_2 receptor expressed in Chinese hamster ovary cells

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Abstract

The human levocabastine-sensitive neurotensin NT_2 receptor was cloned from a cortex cDNA library and stably expressed in Chinese hamster ovary (CHO) cells in order to study its binding and signalling characteristics. The receptor binds neurotensin as well as several other ligands already described for neurotensin NT_1 receptor. It also binds levocabastine, a histamine H_1 receptor antagonist that is not recognised by neurotensin NT_1 receptor. Neurotensin binding to recombinant neurotensin NT_2 receptor expressed in CHO cells does not elicit a biological response as determined by second messenger measurements. Levocabastine, and the peptides neuromedin N and xenin were also ineffective on neurotensin NT_2 receptor activation. Experiments with the neurotensin NT_1 receptor antagonists SR48692 and SR142948A, resulted in the unanticipated discovery that both molecules are potent agonists on neurotensin NT_2 receptor. Both compounds, following binding to neurotensin NT_2 receptor, enhance inositol phosphates (IP) formation with a subsequent $[Ca^{2+}]_i$ mobilisation; induce arachidonic acid release; and stimulate mitogen-activated protein kinase (MAPK) activity. Interestingly, these activities are antagonised by neurotensin and levocabastine in a concentration-dependent manner. These activities suggest that the human neurotensin NT_2 receptor may be of physiological importance and that a natural agonist for the receptor may exist.

Keywords: Neurotensin; G-protein; Receptor; Levocabastine; SR142948A; SR48692

1. Introduction

Neurotensin is a 13-amino acid peptide released by internal cleavage at flanking dibasic residues from a 17-kDa precursor protein synthesised in the brain and gut of several species including man (Carraway and Leeman, 1973). A wide variety of biological activities have been described for neurotensin in peripheral tissues (Kitabgi et al., 1985) and in the central nervous system (Carraway et al., 1982). In the brain there is a general consensus that one of its major functions is the regulation of dopaminergic neurotransmissions (Nemeroff, 1980), which makes neurotensin receptors attractive targets for antipsychotic drugs. Early binding studies showed the existence of low and high affinity neurotensin receptors ($K_d \cong 2.5$ and $\cong$ 0.5 nM, respectively) (Vincent, 1995). Interestingly, it was also shown that the postnatal ontogeny of these two classes of sites is different; the low affinity sites, undetectable in newborn mouse brain, are expressed at the age of 2 weeks and persist throughout life, while the high affinity class is present in newborn animals, the expression peaking during the 1st week and then declining (Zsurger et al., 1992). Levocabastine, a histamine H_1 receptor antagonist structurally unrelated to neurotensin, selectively inhibits neurotensin binding to the low affinity site providing a convenient tool for differentiating the sites (Schotte et al., 1986). Further observations led to the suggestion that this site was an acceptor or recognition site without physiological function (Schotte and Leysen, 1989).

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A high affinity receptor insensitive to levocabastine (neurotensin NT_1 receptor) has been cloned from rat brain (Tanaka et al., 1990) and from the human cell line HT-29 (Vita et al., 1993). Both receptors belong to the G protein-coupled receptor family. The high homology in the amino acid sequences of the two receptors strongly suggests that they represent species variants of a unique receptor type.

Recently, we cloned a second neurotensin receptor sensitive to levocabastine (neurotensin NT_2 receptor) from a rat brain cDNA library (Chalon et al., 1996), and a mouse homologue was also recently reported (Mazella et al., 1996). Neurotensin NT_2 receptor also belongs to the G protein-coupled receptor family, and shares $\cong 60\%$ homology with neurotensin NT_1 receptor. Interestingly, a recent report describes the association of the levocabastine-sensitive neurotensin receptor to a sub-population of glial cells and a different mode of regulation when compared with the neurotensin NT_1 receptor present in neurones (Nouel et al., 1997). These observations, which suggested a physiological role for neurotensin NT_2 receptor, prompted us to look for a neurotensin NT_2 receptor in the human brain despite contradictory reports about the existence of a levocabastine-sensitive neurotensin receptor in the brain of species other than rat and mouse (Schotte et al., 1986; Zsurger et al., 1992).

We describe here the molecular cloning of a new human receptor that belongs to the family of G protein-coupled receptors and shares nearly 80% identity with the rat and mouse levocabastine-sensitive neurotensin binding site. We report the binding, pharmacological, and functional characterisation of the recombinant receptor expressed in Chinese hamster ovary (CHO) cells, studies which reveal a surprising contrast with neurotensin NT_1 receptor expressed in the same cells.

2. Materials and methods

2.1. Drugs and peptides and cell lines

Labelled ^{125}I [monoiodo-Tyr^3]neurotensin, (2000 Ci/mmol) and unlabelled 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-*ortho*-phenanthroline were from Sigma (St. Louis, MO).

The non-peptide neurotensin NT_1 receptor antagonists SR48692 and SR142948A were synthesised at Sanofi Recherche, Montpellier, France (Gully et al., 1992, 1997). The anti-histamine agent, levocabastine, was obtained from Janssen Pharmaceutica (Geel, Belgium).

CHO cells expressing high levels of neurotensin NT_1 or NT_2 receptors were obtained and cultured as previously described (Oury-Donat et al., 1995).

2.2. cDNA library construction, isolation of cDNAs and sequence analysis

A human cortex cDNA library was constructed as described elsewhere (Chalon et al., 1996). Human neurotensin NT_2 receptor cDNAs were identified in a hybridisation screen using labelled rat neurotensin NT_2 receptor cDNA as a probe as previously described (Chalon et al., 1996). The human cDNA sequence for the neurotensin NT_2 receptor has been submitted to the GenBank/EMBL Data Bank (accession number Y10148).

2.3. RNA extraction and analysis

Total RNA was extracted from different areas of a human brain and from peripheral organs using the acid guanidinium isothiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Poly(A) RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as described (Aviv and Leder, 1972). RNA analysis was performed by electrophoresis on 1% agarose/formaldehyde gels then transferred to nitro-cellulose and probed with a ^{32}P-labelled human neurotensin NT_2 receptor cDNA as described (Sambrook et al., 1989).

2.4. Binding experiments

The pharmacological and functional characterisation of the cloned receptor was performed on CHO cells stably transfected with the human neurotensin NT_2 receptor (CHO-NT-2R). The cells were seeded in 6-well plates (Falcon) and 3 days later were washed twice with binding buffer comprising 50 mM Tris–HCl pH 7.5, 0.2% BSA, 0.1% NaN_3 and 1 mM 1,10-*ortho*-phenanthroline.

Saturation experiments were done in 1.5 ml of binding buffer containing ^{125}I-labelled [monoiodo-Tyr^3] neurotensin over a range from 0.05 to 2 nM as previously described (Vita et al., 1993). Non-specific binding was defined as binding in the presence of a 500-fold excess of unlabelled 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-labelled [monoiodo-Tyr^3] neurotensin as radioligand. The following unlabelled ligands were used as competitors: neurotensin, xenin, SR48692, SR142948A and levocabastine.

Binding data derived from saturation and competition experiments were analysed by using GraphPad Prism 2.01 (GraphPad Software, San Diego, CA)

2.5. Measurement of inositol phosphate (IP) accumulation

Cells (10^6) in 6-well plates were labelled for 24 h with 5 μCi/ml [^{3}H]*myo*-inositol. LiCl (20 mM) was added 15 min before and during the addition of drugs. The stimulation was performed for 30 min with neurotensin or with SR48692 (10^{-10} to 10^{-5} M) or SR142948A (10^{-11} to

10^{-5} M). After 30 min stimulation, the reaction was stopped by aspiration of the medium and rapid addition of 1 ml cold methanol–0.1 M HCl (50:50, v/v). The aqueous phase was removed and applied to 1 ml Dowex columns and IP were eluted with 0.2 M ammonium formate/0.1 M formic acid. Ninety-five percent of the radioactivity, quantified by liquid scintillation counting was inositol monophosphate (IP1).

2.6. Calcium mobilisation

Fluorescent $[Ca^{2+}]_i$ measurements were made on single isolated cells using Fura-2/acetomethyl ester (Molecular Probes, Interchim, France) as previously described (Gully et al., 1997). Cells were perfused after a stable basal period for 1 min with the drug to be tested. Cellular fluorescence was filtered by a 490–530 nm bandpass filter (Nikon) and measured with a Darkstar-800 CCD camera (Photonic Sciences, Millham, UK). Ratiometric Ca^{2+} images were generated at 5-s intervals. Every cell in the field of the digitised image was quantified. Background compensation was performed by subtracting the illumination from an area of the image which contained no cells. The $[Ca^{2+}]_i$ values were calculated from the 350/380-nm fluorescence ratios as previous described (Grynkiewicz et al., 1985), using R_{min} and R_{max} values of 0.4 and 6.0, respectively; the K_d of Ca^{2+} for Fura-2 was assumed to be 224 nM (Grynkiewicz et al., 1985).

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                1                                                    50
hNT2-R  METSSPRPPR PSSNPGLSLD ARLGVDTRLW AKVLFTALYA LIWALGAAGN
rNT2-R  METSSPWPPR PSPSAGLSLE ARLGVDTRLW AKVLFTALYS LIFAFGTAGN
mNT2-R  METSSLWPPR PSPSAGLSLE ARLGVDTRLW AKVLFTALYS LIFALGTAGN

               51                                                   100
ALSVHVVLKA RAGRAGRLRH HVLSLALAGL LLLLVGVPVE LYSFVWFHYP
ALSVHVVLKA RAGRPGRLRY HVLSLALSAL LLLLVSMPME LYNFVWSHYP
ALSVHVVLKA RTGRPGRLRY HVLSLALSAL LLLLISVPME LYNFVWSHYP

              101     *                                              150
WVFGDLGCRG YYFVHELCAY ATVLSVAGLS AERCLAVCQP LRARSLLTPR
WVFGDLGCRG YYFVRELCAY ATVLSVASLS AERCLAVCQP LRARRLLTPR
WVFGDLGCRG YYFVRELCAY ATVLSVASLS AERCLAVCQP LRARRLLTPR

              151                                              *   200
RTRWLVALSW AASLGLALPM AVIMGQKHEL ETADGEPEPA SRVCTVLVSR
RTRRLLSLVW VASLGLALPM AVIMGQKHEV ESADGEPEPA SRVCTVLVSR
RTCRLLSLVW VASLGLALPM AVIMGQKHEM ERADGEPEPA SRVCTVLVSR

              201                                                  250
TALQVFIQVN VLVSFVLPL. ALTAFLNGVT VSHLLALCSQ VPSTSTPGSS
ATLQVFIQVN VLVSFALPL. ALTAFLNGIT VNHLMALYSQ VPSASAQVSS
ASSRSTFQVK RAGLLRSPLW ELTAILNGIT VNHLVALYSQ VPSASAQVNS

              251                                                  300
TPSRLELLSE EGLLSFIVWK KTFIQGGQVS LVRHKDVRRI RSLQRSVQVL
IPSRLELLSE EGLLGFITWR KTLSLGVQAS LVRHKDASQI RSLQHSAQVL
IPSRLELLSE EGLLGFITWR KTLSLGVQAS LVRHKDASQI RSLQHSAQVL

              301                    *                             350
RAIVVMYVIC WLPYHARRLM YCYVPDDAWT DPLYNFYHYF YMVTNTLFYV
RAIVAVYVIC WLPYHARRLM YCYIPDDGWT NELYDFYHYF YMVTNTLFYV
RAIVAVYVIC WLPYHARRLM YCYIPDDGWT DELYDFYHYF YMVTNTLFYV

              351                                                  400
SSAVTPLLYN AVSSSFRKLF LEAVSSLCGE HHPMKRLPPK .PQSPTLMDT
SSAVTPILYN AVSSSFRKLF LESLGSLCGE QHSLVPLPQE APESTTSTYS
SSAVTPVLYN AVSSSFRKLF LESLSSLCGE QRSVVPLPQE APESTTSTYS

              401           417
ASGFGDPPET RT~~~~~
FRLWGSPRNP SLGEIQV
FRLWGSPRNP SLGEIQV
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Fig. 1. Alignment of the human, rat, and mouse neurotensin NT_2 receptor. The sequences are compared; gaps for alignment are indicated by dots. The proposed transmembrane domains are boxed, and conserved extracellular cysteines are indicated by asterisks.

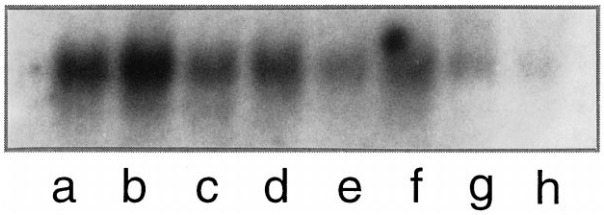

Fig. 2. Northern analysis of poly(A) RNA (1 μg) from human tissues hybridized to ^{32}P-labelled human neurotensin NT_2 receptor cDNA. Lanes: (a) amygdala; (b) caudate nucleus; (c) corpus callosum; (d) hippocampus; (e) whole brain; (f) substantia nigra; (g) subthalamic nucleus; and (h) thalamus.

2.7. Measurement of [^{3}H]arachidonic acid release

Cells were plated at a density of 10^6 cells/well on 6-well plates and labelled for 24 h with 0.25 μCi/ml [^{3}H]arachidonic acid. After labelling, cells were washed three times at intervals of 5 min with 1 ml MEM containing 0.1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4 and 0.2% BSA. Cells were then incubated for 15 min with neurotensin or levocabastine or in the presence of various concentrations of SR48692 (10^{-10} to 10^{-6} M) or SR142948A (10^{-10} to 10^{-6} M). At the end of the stimulation, the reaction was stopped on ice, the medium was collected, centrifuged and radioactivity determined by liquid scintillation counting.

2.8. Mitogen-activated protein kinase (MAPK) activity

MAPK activity was measured with the BIOTRAK™ MAP Kinase assay from Amersham. Briefly, cells were washed and lysed in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiotreitol, 1 mM *ortho*-vanadate, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptine, 10 μg/ml aprotinin and 1% (v/v) Triton X-100, pH 7.4 at 4°C. The solubilised cell extracts were clarified by centrifugation at 14,000 × *g* for 30 min and then incubated at 30°C with substrate buffer and magnesium [γ^{32}P]ATP buffer (1 μCi/sample, 3000 Ci/mmol). The reaction was stopped by spotting samples on Whatman P-81 filter papers, washed four times in each of 75 mM *ortho*-phosphoric acid and distilled water and the radioactivity determined by liquid scintillation counting.

2.9. Measurement of cAMP levels

Cells cultured in 12-well-plates were incubated for 20 min in 2.5 mM HEPES/Tris (pH 7.5), containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.2% BSA, 4.5 g/l glucose and 1 mM 3-isobutyl-1-methylxanthine. Cells were

then incubated 10 min in the presence of neurotensin or SR48692 or SR142948A (10^{-10} to 10^{-6} M). The reaction was stopped by aspiration of the medium and rapid addition of 1 ml ethanol. After extraction, cAMP content was measured using cAMP assay kits from Amersham (Les Ulis, France).

2.10. Data analysis

Data were expressed as percent above control or as a percent of 10^{-8} M SR48692 or SR142948A-induced IP1 formation or arachidonic acid release. EC_{50} and IC_{50} values were analysed by using GraphPad Prism 2.01.

3. Results

3.1. Cloning of the human neurotensin NT_2 receptor cDNA and tissue distribution of the corresponding mRNA

A 1575-bp long cDNA encoding human neurotensin NT_2 receptor was identified in a hybridisation screen of a human cortex cDNA library using the rat neurotensin NT_2 receptor cDNA (Chalon et al., 1996). The open reading frame (nucleotides 47 to 1264) defined a polypeptide of 410 amino acids. The hydropathicity profile analysis of the putative protein sequence indicated that it belonged to the large family of G-protein coupled receptors (Fig. 1).

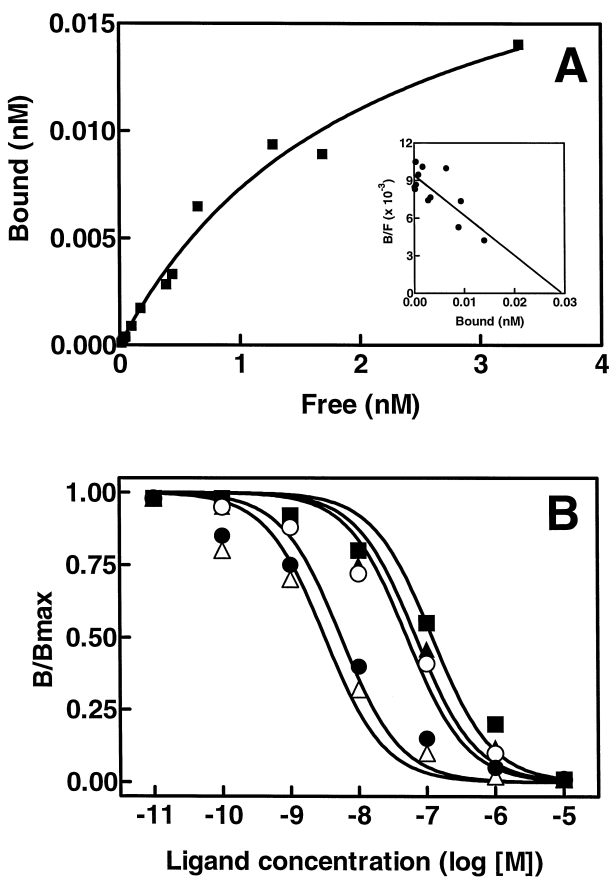

Fig. 3. (A) Saturation isotherm and Scatchard plot (inset) of the specific binding of ^{125}I-labelled [monoiodo-Tyr^3] neurotensin to stable transformed CHO-hNT-2R cells. Each point represents the mean of triplicates. (B) Inhibition of ^{125}I-labelled [monoiodo-Tyr^3] neurotensin binding to stable transformed CHO-hNT-2R cells by unlabelled neurotensin (●), xenin (△), SR142948A (○), SR48692 (▲) and levocabastine (■). Values represent the mean of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

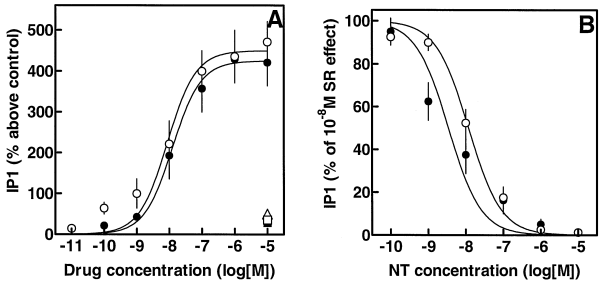

Fig. 4. Accumulation of IP1 following activation of human neurotensin NT_2 receptor expressed in CHO cells. (A) CHO cells expressing neurotensin NT_2 receptor were incubated with neurotensin (□), neuromedin N (△), levocabastine (■), SR48692 (●), or SR142948A (○) and IP1 determined as described in Section 2. (B) Effect of increasing doses of neurotensin on the IP1 accumulation of CHO-hNT-2R cells in response to 100 nM of SR48692 (●) or SR142948A (○). Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

The homologies between the human, rat and mouse neurotensin NT_2 receptors are extensive (79% identity and 87% similarity and 75% identity and 77% similarity, respectively) (Fig. 1), and to a lesser extent with the human and rat neurotensin NT_1 receptors (38% identity and 62% similarity and 40% identity and 49% similarity, respectively) (not shown).

A close inspection of the neurotensin NT_2 receptor sequence reveals a short cytoplasmic N-terminal sequence with no clear potential glycosylation site and a cysteine residue in each of the extracellular loops, also conserved in the mouse and rat neurotensin NT_2 receptors. The homology between the human and rat neurotensin NT_2 receptors is the lowest in the cytoplasmic C-terminal region. However, almost all the threonine and serine residues present in this region, which could be targets for phosphorylation, are conserved, as well as a cysteine which could be a candidate for palmitoylation, thereby anchoring the region to the plasma membrane.

It is noteworthy that although the length of neurotensin NT_1 and NT_2 receptors are similar, the neurotensin NT_2 receptor has a shorter N-terminal cytoplasmic region, and a longer cytoplasmic loop between the fifth and sixth putative transmembrane regions.

The tissue distribution of human neurotensin NT_2 receptor mRNA was examined by Northern blot analysis. As shown in Fig. 2, high levels of transcripts were detected in different regions of adult human brain. In addition, human neurotensin NT_2 receptor transcripts were also detected by polymerase chain reaction (PCR) in cerebellum, kidney, uterus, heart and lung tissues (results not shown).

3.2. Expression and characterisation of the human neurotensin NT_2 receptor

The ectopic expression of neurotensin NT_2 receptor in CHO cells generated specific neurotensin binding sites. Scatchard analysis of the saturation curve showed a single component site with a K_d value of 2.6 ± 0.5 nM and a maximal binding capacity of 28 ± 2 pM (corresponding to 4×10^4 receptors/cell) (Fig. 3A). The affinity displayed by neurotensin for the recombinant human neurotensin NT_2 receptor is slightly lower than that displayed for the human neurotensin NT_1 receptor, K_d of 0.7 ± 0.2 nM (Vita et al., 1993).

Studies on neurotensin NT_2 receptor-expressing CHO cells (Fig. 3B), demonstrate that neurotensin was effective in inhibiting binding of labelled neurotensin (IC_{50} of 5.8 ± 1 nM). Importantly, two previously described non-peptide neurotensin NT_1 receptor antagonists, SR142948A (Gully et al., 1997) and SR48692 (Gully et al., 1992) also competed with labelled neurotensin (IC_{50} of 49 ± 4 and 67 ± 5

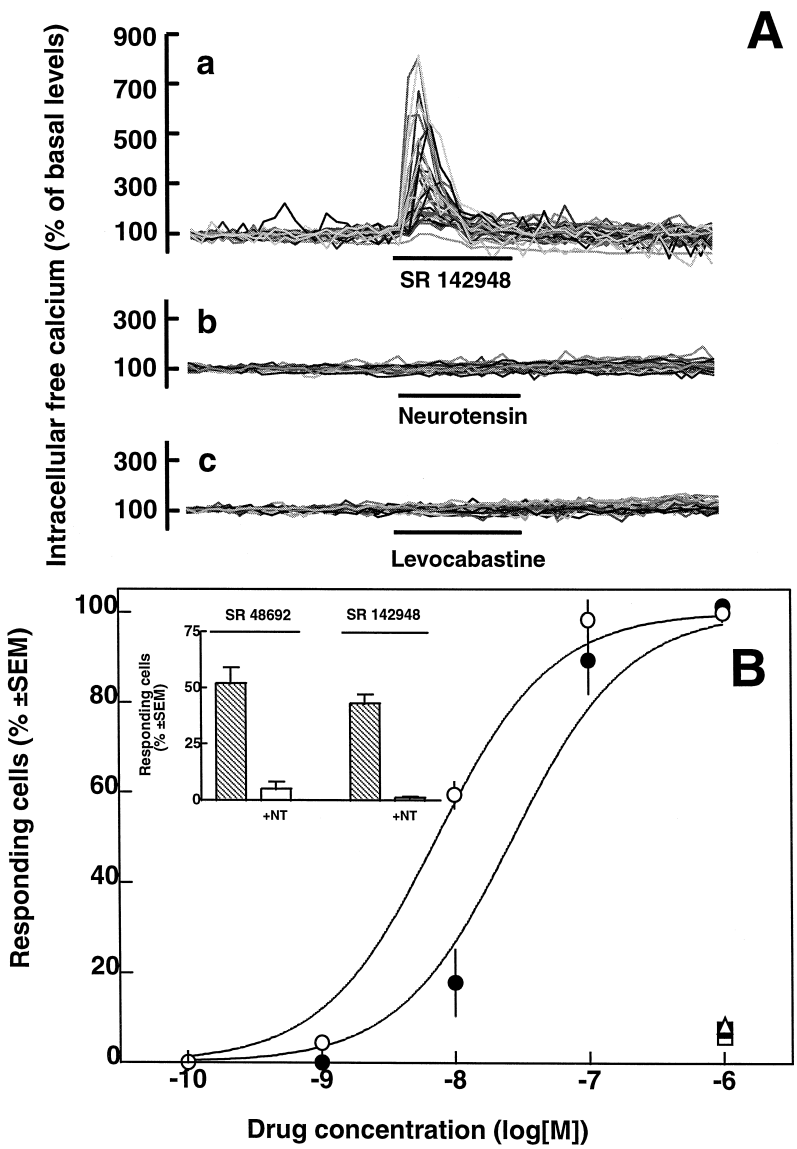

Fig. 5. Intracellular Ca^{2+} mobilisation following activation of human neurotensin NT_2 receptor expressed in CHO cells. (A) Effect of 1 μM SR142948A (a), neurotensin (b) and levocabastine (c) on free $[Ca^{2+}]_i$ on CHO-hNT-2R cells. Basal values were 128 ± 8 nM ($n = 34$ cells), 170 ± 9 nM ($n = 31$ cells) and 154 ± 7 nM ($n = 32$ cells), respectively. (B) CHO cells expressing human neurotensin NT_2 receptor were incubated with neurotensin (□), neuromedin N (△), levocabastine (■), SR48692 (●), or SR142948A (○) and intracellular Ca^{2+} mobilisation determined as described in Section 2. Insert: antagonism by 1 μM neurotensin of the intracellular calcium mobilisation induced by 100 nM SR142948A or SR48692 on CHO-hNT-2R cells. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

nM, respectively) whereas levocabastine showed an IC_{50} of 91 ± 8 nM.

3.3. Signal transduction analysis of recombinant human neurotensin NT_2 receptor expressed in CHO cells

Since the major signal transduction pathway described for neurotensin NT_1 receptor is the G protein-dependent stimulation of phospholipase C leading to Ca^{2+} mobilisation (Amar et al., 1986), we measured the accumulation of IP after stimulation of CHO-hNT-2R cells with several ligands. As shown in Fig. 4A, neurotensin, neuromedin N and levocabastine were unable to induce IP1 accumulation at any of the concentrations tested. In parallel experiments, and as previously reported (Oury-Donat et al., 1995), on CHO cells expressing the neurotensin NT_1 receptor, neurotensin induced IP1 accumulation with an EC_{50} value of 3.6 nM, and this activity was fully antagonised by SR48692 or SR142948A (Oury-Donat et al., 1995). Incubation of CHO-hNT-2R cells with SR48692 or SR142948A resulted in stimulation of polyphosphoinositide hydrolysis. The EC_{50} value for SR48692 and SR142948A-mediated IP1 production was 12 and 8.6 nM, respectively (Fig. 4A). The effects of SR48692 and SR142948A were fully antagonised by neurotensin with IC_{50} of 3.4 and 12 nM, respectively, as shown in Fig. 4B. Levocabastine also antagonised the activities of SR48692 and SR142948A with IC_{50} of 120 and 10 nM (not shown).

One of the known consequences of an enhanced phosphoinositide turnover is a release of calcium from inositol triphosphate-sensitive intracellular stores. We therefore investigated the effect of SR48692 and SR142948A compounds on calcium mobilisation after neurotensin NT_2 receptor activation. As shown on Fig. 5A, perfusion of CHO-NT-2R cells with 1 μM of SR142948A induced an elevation of free intracellular calcium ($[Ca^{2+}]_i$) to 302 ± 27% above basal levels in 97% of cells ($n = 34$ cells) whereas at the same concentration, neurotensin ($n = 31$ cells) and levocabastine ($n = 32$ cells) did not modify $[Ca^{2+}]_i$. Concentration-effect comparison (Fig. 5B) revealed that SR142948A and SR48692 stimulate $[Ca^{2+}]_i$ with similar potency with EC_{50} of 7.9 and 28 nM, respectively. Under the same conditions neurotensin, neuromedin N and levocabastine were unable to increase free $[Ca^{2+}]_i$ up to 1 μM but reversed the effect of 100 nM of SR142948A or SR48692 (insert of Fig. 5B). The antagonism of neurotensin on the SR142948A induced effect was more important than the reversal produced by levocabastine at the same concentration (1 μM) (not shown).

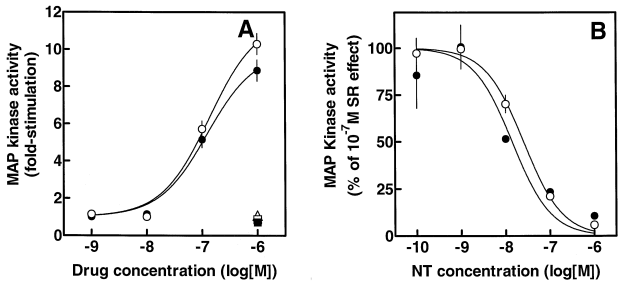

Fig. 7. MAPK activity following activation of human neurotensin NT_2 receptor expressed in CHO cells. (A) CHO cells expressing human neurotensin NT_2 receptor were incubated with neurotensin (□), neuromedin N (△), levocabastine (■), SR48692 (●), or SR142948A (○) and MAPK activity determined as described in Section 2. (B) Effect of increasing doses of neurotensin on the MAPK activity of CHO-NT-2R cells in response to 100 nM of SR48692 (●) or SR142948A (○). Results are expressed as a percentage of the maximal SR-response. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

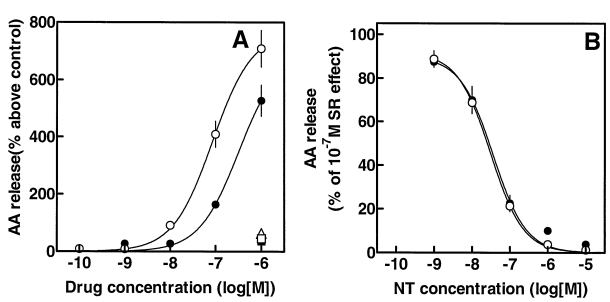

Fig. 6. Arachidonic acid release following activation of human neurotensin NT_2 receptor expressed in CHO cells. (A) CHO cells expressing human neurotensin NT_2 receptor were incubated with neurotensin (□), neuromedin N (△), levocabastine (■), SR48692 (●), or SR142948A (○) and arachidonic acid release determined as described in Section 2. (B) Effect of increasing doses of neurotensin on the arachidonic acid release of CHO-hNT-2R cells in response to 100 nM of SR48692 (●) or SR142948A (○). Results are expressed as a percentage of the maximal SR-response. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three further experiments.

Similar results were obtained when the arachidonic acid production by activated phospholipase A_2 was measured. As shown in Fig. 6A, SR48692 and SR142948A were able to activate phospholipase A_2 with EC_{50} values of 1.6×10^{-7} and 9×10^{-8} M, respectively. Neurotensin, neuromedin N and levocabastine binding to the receptor did not resulted in arachidonic acid production (Fig. 6A), but as described for IP1 accumulation, neurotensin antagonised the activation with IC_{50} values of 2.8×10^{-8} M against SR48692 and of 2.3×10^{-8} M against SR142948A, as shown in Fig. 6B. Levocabastine also antagonised SR48692 and SR142948A activity with IC_{50} values of 4.8×10^{-7} and 7.6×10^{-7} M, respectively (not shown).

Since it has been previously reported that neurotensin binding to neurotensin NT_1 receptor increases MAPK activity as well as cAMP production (Poinot-Chazel et al., 1996), we investigated these signal transduction pathways on CHO-NT-2R cells.

Fig. 7A shows that SR48692 and SR142948A induced a concentration-dependent ($EC_{50} \cong 10^{-7}$ M) increase in MAPK activity on CHO-hNT-2R cells, whereas under the same conditions neurotensin, neuromedin N and levocabas-

tine were ineffective. As for the other intracellular responses, SR48692- and SR142948A-induced effects at 100 nM were reversed by neurotensin (IC_{50} values of 1.1×10^{-7} and 2.2×10^{-8} M, respectively) (Fig. 7B) and levocabastine (not shown).

Interestingly, we were unable to detect changes in cAMP formation following binding of SR142948A, SR48692, neurotensin, or levocabastine to CHO-hNT-2R cells (results not shown), whereas it has been shown that neurotensin induced cAMP accumulation in CHO cells expressing neurotensin NT1 receptors (Oury-Donat et al., 1995)

4. Discussion

We describe here the cloning and characterisation of a human homologue of the recently described rat and mouse neurotensin NT_2 receptors. The receptor is a 410-amino acid protein that belongs to the G protein-coupled receptor family, and its mRNA is mainly expressed in the brain, cerebellum and to a lesser extent in the heart, kidney, lung and uterus. Further work with the described sequence should allow the identification of the cells in the brain that express the receptor which have been recently suggested to be different from those expressing neurotensin NT_1 receptor (Nouel et al., 1997).

The receptor binds neurotensin as well as several other ligands already described for neurotensin NT_1 receptor. It also binds levocabastine, showing at the molecular level that a levocabastine-sensitive NT_2 receptor is present in humans. However, it should be noted that the human neurotensin NT_2 receptor binds levocabastine with lower affinity than the mouse and rat receptors which may explain a previous report that indicated that only a high concentration of levocabastine was able to displace a fraction of the total neurotensin binding from the human cortex (Schotte et al., 1986).

Several features, other than the affinity to levocabastine, markedly differentiate neurotensin NT_2 receptor from neurotensin NT_1 receptor. Neurotensin binds to recombinant neurotensin NT_2 receptor expressed in CHO cells but does not elicit a second messenger response. Levocabastine behaves very much as neurotensin. Experiments with SR48692 and SR142948A, non-peptide antagonists for neurotensin NT_1 receptor, resulted in the unanticipated discovery that these molecules are potent agonists on neurotensin NT_2 receptor. Second messenger measurements with the SR compounds show that upon activation neurotensin NT_2 receptor is able to couple to G proteins that activate phospholipases C and A_2, and MAPK, but not adenylyl cyclase. Further work showed that these induced activities can be antagonised by neurotensin, neuromedin N and levocabastine.

Human neurotensin NT_2 receptor behaves very much as rat neurotensin NT_2 receptor, but different results were recently described for mouse neurotensin NT_2 receptor. The recombinant mouse neurotensin NT_2 receptor when expressed in oocytes have been reported to activate ion channels in the presence of 200 nM neurotensin or levocabastine and SR48692 was unable to antagonise neurotensin activity (Mazella et al., 1996). Several reasons may explain these discrepancies. For example, the differences may be related to the species, or more important, to the different experimental approach used to evaluate the activity of neurotensin NT_2 receptor, in one case oocytes, in the other transfected mammalian cells. In fact, recently the same group reported that the mouse neurotensin NT_2 receptor when expressed in the human embryonic kidney (HEK) 293 cell line does not respond to neurotensin (Botto et al., 1998). It should be noted also that it has been recently reported that in the oocyte expression system the well-known neurotensin NT_1 receptor antagonist SR48692 is ineffective in blocking neurotensin-induced electrophysiological activity via the neurotensin NT_1 receptor even at concentrations of SR48692 which completely displace neurotensin from the receptor (Botto et al., 1997). This suggests that further work should be done to fully understand the behaviour of recombinant neurotensin receptors in oocytes.

We have observed the agonistic activity of SR48692 and SR142948A, activities antagonised by neurotensin, not only in transfected CHO cells as described here, but also in COS cells transiently expressing human neurotensin NT_2 receptor. However, our results cannot exclude the possibility that in certain cell environments the receptor may be activated by neurotensin.

The physiological role of the levocabastine-sensitive NT_2 receptor has been seriously questioned mainly because: (i) no neurotensin activity could be associated with this receptor; (ii) no signal transduction pathway was identified following neurotensin binding; and (iii) neurotensin binding to neurotensin NT_2 receptor was insensitive to GTP. Our results showing that neurotensin behaves as an antagonist on neurotensin NT_2 receptor provide an explanation for those observations. Furthermore, our results also show that neurotensin NT_2 receptor is able to transduce a signal to the cell upon activation with either SR48692 or SR142948A, suggesting that an endogenous natural agonist for this receptor may exist, and work is in progress in our laboratory to identify a putative natural ligand different from neurotensin.

Different ligands for different subtypes of receptors have been previously described. For example urocortin has been recently described as a ligand for corticotrophin releasing factor CRF_2 receptor (Donaldson et al., 1996), a receptor that was cloned by homology to corticotrophin releasing factor CRF_1 receptor, for which the neuropeptide corticotrophin releasing factor is the accepted natural ligand (Perrin et al., 1995). But a neuropeptide acting as an agonist on one subtype of receptor and as antagonist on other has not been described, and, if this is the case for

neurotensin in vivo, the regulatory role of this neuropeptide may be more complex than previously thought. Further work should help to define the physiological role of neurotensin NT_2 receptor, which may help in understanding some of the biological responses following neurotensin injection in the central nervous system, such as hypothermia and analgesia, which are probably not associated with neurotensin NT_1 receptor (Dubuc et al., 1994).

Finally, the availability of the cloned neurotensin NT_1 and NT_2 receptors will greatly facilitate the development of new specific ligands for both receptors to better address the in vivo pharmacology and physiology of neurotensin.

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