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Stable expression of high affinity NK_1 (substance P) and NK_2 (neurokinin A) receptors but low affinity NK_3 (neurokinin B) receptors in transfected CHO cells

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Stable CHO cell clones which selectively express all three rat tachykinin receptors were established by transfection. The binding of radiolabeled substance P and neurokinin A (substance K) to CHO clones expressing the NK_1 and NK_2 receptors, respectively, were saturatable and of high affinity (K_d=0.17 nM (NK_1); 3.4 nM (NK_2)). Scatchard analysis of the binding data indicated for both receptors binding to a single population of binding sites, and competition binding studies showed that the binding specificities of the receptors corresponded to those of classical NK_1 and NK_2 receptors. In contrast, the binding of eledoisin to the NK_3 receptor expressed in the transfected CHO cells was of low affinity (IC_{50}=240 nM) compared to the high affinity of the receptor found when it was transiently expressed in COS-7 cells (IC_{50}=8 nM). However, in both cases the receptor exhibited the specificity of a classical NK_3 receptor. The established cell clones may provide an important tool for further analysis of the molecular mechanisms involved in binding, activation, and coupling of receptors for tachykinin peptides.

Tachykinin receptor; Transfection; CHO cell; COS-7 cell; G protein

1. INTRODUCTION

The mammalian tachykinins, substance P, neurokinin A (substance K) and neurokinin B (neuromedin K) are characterized by the common carboxy-terminal sequence Phe-X-Gly-Leu-Met-NH_2. They are widely distributed neuropeptides in both the central and peripheral nervous system and involved in multiple biological actions including modulation of sensory afferent transmission, intestinal motility, stimulation of salivation, vasodilation and activation of cells of the immune system (for reviews see refs. [1–4]).

The tachykinins act on at least three distinct receptors called NK_1, NK_2 and NK_3 which preferentially bind substance P, neurokinin A, and neurokinin B, respectively [5–7]. Recently Nakanishi and co-workers have isolated all three cDNA's encoding these receptors by use of a *Xenopus* expression system [8–10]. The cloning of the NK_1 receptor was also recently reported by Hershey and Krause 1990 [11]. The predicted amino acid sequences of the receptors revealed homologous structures typical for G protein coupled receptors characterized by the presence of seven putative transmembrane domains [12]. Only a few neuropeptide receptors have been cloned until now and the tachykinin family was the first family of neuropeptides for which a series of different receptor subtypes have been cloned [12]. Hence, the tachykinin receptors constitute an optimal system for analyzing structure–function aspects of the binding and activation of receptors for neuropeptides. For example, it gives the opportunity to construct chimeric receptors between the receptor subtypes, an approach which has proven very usefull with respect to the adrenergic receptors [13].

In the present study we have established stable, transfected clones of CHO cells which selectively express the rat NK_1, NK_2 and NK_3 receptors. The binding properties of the NK_1 and NK_2 receptors corresponded to those of the physiological, classical receptors. In contrast, the NK_3 receptors expressed in CHO cells were of low affinity as compared to NK_3 receptors transiently expressed in COS-7 cells from the same plasmid.

2. EXPERIMENTAL

2.1. *Peptides*

Substance P, neurokinin A, neurokinin B and eledoisin were purchased from Peninsula Laboratories.

2.2. *Expression vectors, transfection and cell culture*

The cDNA encoding the NK_1, NK_2 and NK_3 receptors were generously provided by Dr. S. Nakanishi, Kyoto University, Dept. of Medicine, Kyoto, Japan. The NK_1 receptor cDNA was excised from the original plasmid prTKR2 [9] as a 2049 bp *Hin*dIII–*Eco*RI fragment, the NK_2 receptor cDNA was excised from prTKR1-1 [14] as a 1,816 bp *Eco*RI–*Bam*HI fragment and the NK_3 receptor cDNA was excised from prTKR3 [10] as a 2,159 bp *Eco*RI–*Eco*RI fragment. These fragments were cloned into the pTEJ-8 expression vector under control of the ubiquitin promotor and the SV40 polyadenylation signal. The

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expression vector also contains the neomycin phosphotransferase gene [15]. For stable expression the resulting plasmids, pTEJ8-NK1, pTEJ8-NK2, and pTEJ8-NK3 were transfected into CHO cells by the calcium phosphate precipitation method as described [15,16]. For transient expression in COS-7 cells, 10^6 cells were seeded in a 90 mm culture plate and grown for 24 h before transfection by the calcium phosphate precipitation method using 20 μg of DNA. After 24 h the cells were harvested, seeded in a 12-well culture plate and grown for another 24 h before analyzing. CHO cells and COS-7 cells were grown as described [15].

2.3. *Preparation and purification of radioligands*

Monoiodinated ^{125}I-Bolton Hunter substance P (^{125}I-BH-SP), ^{125}I-Bolton Hunter neurokinin A (^{125}I-BH-NKA) and ^{125}I-Bolton Hunter eledoisin (^{125}I-BH-ELE) were prepared according to earlier described methods [6]. The reaction mixture was subjected to reverse-phase HPLC as described [17]. The fractions containing the labeled peptides were pooled and stored at −20°C in acid ethanol containing 0.2% mercaptoethanol. Under these conditions the radioligands were stable for 2–3 months. As the separation of labeled and unlabeled peptide was complete, the specific activity of the radioligands were assumed to be equal to the specific activity of the Bolton Hunter reagent (4,400 cpm/fmol) after correction for decay and counter efficiency.

2.4. *Binding assay*

Cells. Transfected CHO-cells or COS-7 cells were transferred to either 12-well culture plates ($0.3–1.0 \times 10^5$ cells) or 6-well culture plates ($1.0–1.5 \times 10^6$ cells) (Costar) 24 h before performing the binding assay. Cells were washed twice in phosphate-buffered saline, pH 7.4, (PBS) followed by incubation for 3 h at 4°C with 25–50 pM radioligand and varying concentrations of cold peptides in 0.5 ml or 1.0 ml of 150 mM NaCl, 5 mM $MnCl_2$ in 50 mM HEPES, pH 7.4, supplemented with 0.1% bovine serum albumin (Sigma), 100 μg/μl bacitracin (Sigma), 5 μg/μl leupeptin (Sigma) and 10 μg/μl chymostatin (Sigma). The incubation media were removed and the cells were washed twice in ice-cold binding buffer. The cells were lysed in 1 ml lysis buffer (3 M acetic acid, 8 M urea and 2% Nonidet P-40) before counting. All determinations were performed in triplicate. The unspecific binding was determined as the binding of radioligand in the presence of 1 μM cold peptide. The specific binding constituted more than 90% of total bound on the NK_1 receptor, more than 75% of total bound on the NK_2 receptor and more than 50% of total bound on the NK_3 receptor (CHO cells). In selected experiments the stability of the ^{125}I-Bolton Hunter labeled peptides during incubations was checked by analyzing 100 μl aliquots of the incubation media on an HPLC system as described above. After 3 h of incubation more than 85% of the radioactivity co-migrated with the radiolabeled peptides.

Membranes. The cells were harvested in 5 mM ice-cold EDTA in PBS, pelleted by centrifugation at 200 × *g* and homogenized using an Ultraturrax homogenizer in 25 mM Tris-HCl, pH 7.4, containing 100 μg/μl bacitracin, 4 μg/μl leupeptin, 10 μg/μl chymostatin and 1 mM phenylmethylsulfonyl fluorid (PMSF). The homogenate was centrifuged for 1 h at 25,000 × *g* and the pelleted membranes were resuspended in the binding buffer. The membranes (200 μg protein) were incubated for 1.5 h at 20°C with 50 pM ^{125}I-BH-SP and various concentrations of cold peptide in 0.5 ml of a 25 mM HEPES buffer, pH 7.4, supplemented with 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $MnCl_2$, 1% BSA and the above mentioned protease inhibitors. The incubation was stopped by the addition of 0.5 ml ice-cold binding buffer before centrifugation at 3,500 × *g* for 10 min. The pellet was washed in buffer, repelleted and counted. All binding data were analyzed by computerized non-linear regression analysis using GraphPAD, ISI Software, Philadelphia, PA.

3. RESULTS

The stable NK_1 receptor transfectants bound 28±7% (mean ± S.E.M., n=17; range 2.0–81%) of the added radioligand (^{125}I-BH-SP), the NK_2 receptor transfectant bound 0.9±0.5% (mean ± S.E.M., n=6; range 0.05–2.2%) (^{125}I-BH-NKA) and the NK_3 receptor transfectants bound 1.3±0.1% (mean ± S.E.M., n=25; range 0.3–2.8%)(^{125}I-BH-ELE) when assaying 1.0×10^6 cells in the presence of 50 pM radioligand. No specific binding could be determined in either untransfected CHO cells or in CHO cells transfected with the expression vector alone. The NK_1 receptor clone with the highest level of radioligand binding (81%), CHO-NK1-11, and a clone with an intermediate level of radioligand binding (24%), CHO-NK1-5, were selected for further characterization. To characterize the NK_2 receptor we chose the clone, CHO-NK2-1, which bound 2.1% of the radioligand and to characterize the NK_3 receptor we chose CHO-NK3-7 which bound 2.8% of the radioligand.

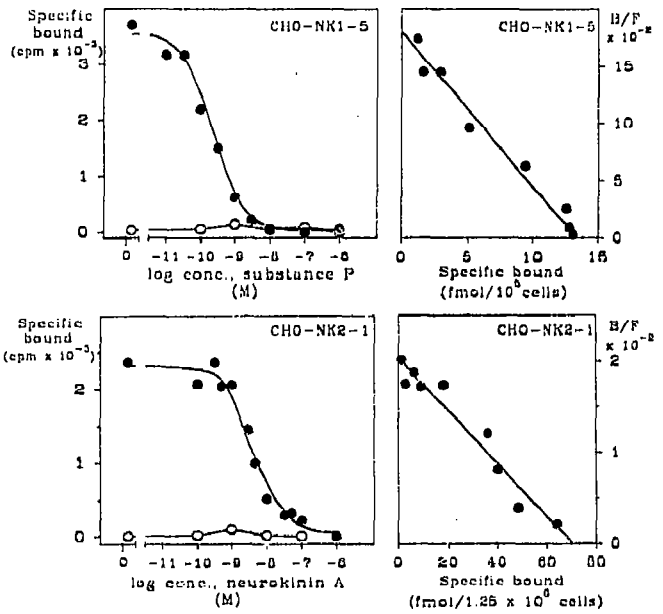

Fig. 1. *Upper left panel:* specific binding of ^{125}I-BH-SP to CHO-NK1-5. The cells (CHO-NK1-5) were incubated with ^{125}I-BH-SP (25 pM) and increasing concentrations of substance P as indicated. Data are expressed as the specific binding in cpm (●) and are from a representative experiment of three. The binding of ^{125}I-BH-SP to CHO cells transfected with the pTEJ-8 expression vector alone is indicated (○). Scatchard analysis of the binding data are illustrated in *upper right panel. Lower left panel:* the specific binding of ^{125}I-BH-NKA to CHO-NK2-1. The cells (CHO-NK2-1), were incubated with ^{125}I-BH-NKA (50 pM) and increasing concentrations of neurokinin A as indicated. Data are expressed as specific binding in cpm (●) and are from a representative experiment of three. The binding of ^{125}I-BH-NKA to CHO cells transfected with the pTEJ-8 expression vector alone is indicated (○). In *lower right panel* Scatchard analysis of the binding data is shown.

The specific binding of ^{125}I-BH-SP to the NK_1 receptor reached equilibrium after 2.5 h (not shown). The displacement of ^{125}I-BH-SP with increasing concentrations of 'cold' substance P showed that the specific binding was of high affinity (Fig. 1, upper panel). Scatchard

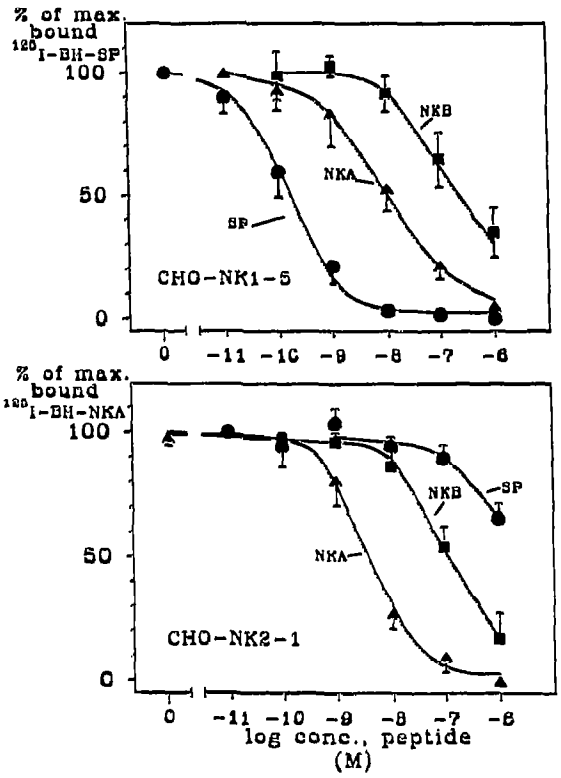

Fig. 2. Binding specificity of the NK_1 and NK_2 receptor expressed in CHO cells. *Upper panel*: inhibition of ^{125}I-BH-SP binding to CHO-NK1-5 by substance P (●) (n=12) (K_i = 1.8 × 10^{-10} M), neurokinin A (▲) (n=6) (K_i = 9.5 × 10^{-9} M) and neurokinin B (■) (n=6) (K_i = 4.0 × 10^{-7}). *Lower panel*: inhibition of ^{125}I-BH-NKA binding to CHO-NK2-1 by neurokinin A (▲) (n=7) (K_i = 3.6 × 10^{-9} M), neurokinin B (■) (n=3) (K_i = 1.3 × 10^{-7} M) and substance P (●) (n=3) (K_i = 7.6 × 10^{-6} M). All the data are expressed as percent of maximum bound radioligand (mean±S.D.).

analysis of the binding data showed that the CHO-NK1-5 clone expressed a single high-affinity binding site with a K_d of 0.19±0.03 nM and a B_{max} corresponding to approx. 80,000 binding sites per cell (Fig. 1, upper panel and Table 1). The CHO-NK1-11 cell clone expressed, like CHO-NK1-5, a single high-affinity binding site as

Table 1

Binding properties of the rat NK_1 and NK_2 receptors stably expressed in transfected CHO cells

Cell clone	$K_{d,\ cells}$ (nM)	$K_{d,\ membranes}$ (nM)	B_{max} (fmol/10^5 cells)	Binding sites per cell
CHO-NK1-5	0.19 ± 0.03	N.D.	13.7 ± 0.2	80,000
CHO-NK1-11	0.14 ± 0.01	0.2 ± 0.1	52 ± 3	300,000
CHO-NK2-1	3.4 ± 0.3	N.D.	3.6 ± 1.3	20,000

The K_d and B_{max} values are calculated from Scatchard analysis of binding data from 'cold' saturation binding experiments as described in Section 2. Data are expressed as mean ± S.E.M. of three experiments. N.D. = not determined.

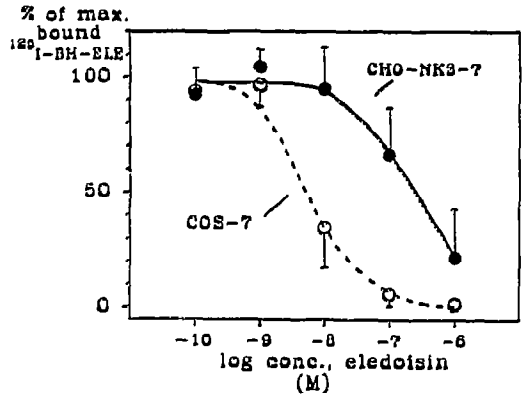

Fig. 3. Specific binding of ^{125}I-BH-ELE to the NK_3 receptor stably expressed in CHO cells (●—●) and transiently expressed in COS-7 cells (○···○) The cells were incubated with 50 pM ^{125}I-BH-ELE and indicated concentrations of eledoisin. Data are expressed as percent of max. bound ^{125}I-BH-ELE (mean ± S.E.M., n=3).

determined both on intact cells and cell membranes, Table I (not shown).

The binding of ^{125}I-BH-NKA to the NK_2 receptor also reached equilibrium after 2.5 h (not shown). The binding was saturatable, of high affinity and to a single population of binding sites (approx. 20,000 per cell) (Fig. 1, lower panel and Table I).

The specificity of the binding of ^{125}I-BH-SP and ^{125}I-BH-NKA to the NK_1 and NK_2 receptors expressed in CHO-cells was analyzed by competition binding studies. As illustrated in Fig. 2, upper panel, substance P most potently displaced ^{125}I-BH-SP from the NK_1 receptor followed by neurokinin A and nerokinin B. On the NK_2 receptor, neurokinin A most potently displaced ^{125}I-BH-NKA followed by neurokinin B and substance P (Fig. 2, lower panel).

In contrast to the results obtained on the NK_1 and NK_2 receptors, we found that binding to the NK_3 receptor transfectants was apparently of low affinity (Fig. 3). Thus, using ^{125}I-BH-ELE as radioligand IC_{50} for eledoisin was 240 nM±120 nM (mean ± S.E.M., n=3). However, when the NK_3 receptor was transiently expressed in COS-7 cells, we obtained high-affinity binding in the nanomolar range (IC_{50} = 8±2 nM, mean ± S.E.M., n=3) in accordance with previously published results [10]. Transient expression of the NK_1 and NK_2 receptor in COS-7 cells also resulted in high-affinity binding (not shown). The specificity of the NK_3 receptor transiently expressed in COS-7 cells corresponded likewise to previous results [10] as the binding of ^{125}I-BH-ELE was inhibited most potently by neurokinin B (IC_{50} = 1.7 × 10^{-9} M) followed by eledoisin (IC_{50} = 8 × 10^{-9} M), neurokinin A (IC_{50} = 3.2 × 10^{-8} M) and substance P (IC_{50} = 1.8 × 10^{-7} M) (data not shown). A similar rank order of potency was obtained for the NK_3 receptor stably expressed in the CHO cells although the IC_{50} values were approx. 30–50-fold higher.

4. DISCUSSION

The construction of cell lines which selectively express a specific receptor subtype offers the advantage of a controlled system for structural and functional characterization of the receptors. Of the tachykinin receptors, the rat NK_1 and NK_3 receptors have previously been transiently expressed in COS-7 cells and the bovine NK_2 receptor has recently been stably expressed in mouse B82 fibroblasts [18,19]. In the present study we have established stable CHO cell clones which selectively express all three rat tachykinin receptors. The NK_1 receptor and the NK_2 receptor demonstrated both high affinity and the expected specificity [4–7]. In contrast, the NK_3 receptor exhibited low-affinity binding when expressed in CHO cells compared to the high affinity of the receptor when it was transiently expressed in COS-7 cells. The high affinity of the NK_3 receptor when transiently expressed in COS-7 cells has also been demonstrated in previous studies [10]. It is a generally accepted hypothesis that high-affinity binding of agonists to G protein-coupled receptors is dependent on the formation of a ternary complex between the agonist, the receptor and at least the corresponding G protein α-subunit [20]. Thus, an explanation for the expression of low affinity NK_3 receptors in CHO cells would be that these cells do not express the correct G protein α-subunit, or at least not in a functional form. If the optimal α-subunit is absent or non-functional, the receptor will interact with other α-subunits present, however, with less efficiency [21]. This could explain why we at all observe binding to the NK_3 receptor expressed in the CHO cells. In general, it is not expected to be possible to detect binding of an agonist radioligand to a receptor which has been uncoupled from its corresponding G protein due to a too low agonist affinity [20].

It is known that the CHO cells lack at least the $G_{\alpha 0}$ α-subunit (P.H. Andersen, personal communication) but it is unclear to which G protein α-subunits the tachykinin receptors couple. The NK_1, NK_2, and NK_3 receptors are generally believed to couple to phosphoinositide turnover via a pertussis toxin-insensitive mechanism [3] and it is likely that this involves an α-subunit belonging to the so-called G_q class of α-subunits (for review see ref. [22]). However, the NK_3 receptor has also been shown to inhibit cAMP accumulation through a pertussis toxin-sensitive mechanism which indicates that the NK_3 receptor may couple to an α-subunit belonging to the G_i/G_o class [23]. Hence, the fact that the NK_3 receptor is not expressed in its high-affinity form in CHO cells may offer the opportunity to characterize the conversion of the receptor from its low-affinity form to its high-affinity form by e.g. complementation through co-expression of different G protein α-subunits in these cells.

An alternative explanation for the low affinity state of the NK_3 receptor in CHO cells could possibly be found in the 71 amino acid-long, extracellular amino terminus of the NK_3 receptor which contains four glycosylation sites compared to only two glycosylation sites in the 31–32 amino acid-long amino terminus of the NK_1 and NK_2 receptors [9,10,14]. If CHO cells and COS-7 cells exhibit different glycosylation patterns, the glycosylated amino terminus of the NK_3 receptor could possibly interfere with ligand binding only in CHO cells. This should also be elucidated in the future by analyzing a NK_3 receptor with a truncated amino terminus.

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