Evidence That the Proposed Novel Human “Neurokinin-4” Receptor Is Pharmacologically Similar to the Human Neurokinin-3 Receptor but Is Not of Human Origin

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ABSTRACT

There have been proposals that the tachykinin receptor classification should be extended to include a novel receptor, the “neurokinin-4” receptor (NK-4R), which has a close homology with the human NK-3 receptor (hNK-3R). We compared the pharmacological and molecular biological characteristics of the hNK-3R and NK-4R. Binding experiments, with [125I][MePhe7]-NKB binding to HEK 293 cell membranes transiently expressing the hNK-3R (HEK 293-hNK-3R) or NK-4R (HEK 293-NK-4R), and functional studies (Ca2+ mobilization in the same cells) revealed a similar profile of sensitivity to tachykinin agonists and antagonists for both receptors; i.e., in binding studies with the hNK-3R, MePhe7-NKB > NKB > senktide > NKA = Substance P; with the NK-4R, MePhe7-NKB > NKB > senktide > Substance P = NKA; and with antagonists, SB 223412 = SR 142801 > SB 222200 > SR 48968 > CP 9994 for both hNK-3R and NK-4R. Thus, the pharmacology of the two receptors was nearly identical. However, attempts to isolate or identify the NK-4R gene by using various molecular biological techniques were unsuccessful. Procedures, including nested polymerase chain reaction studies, that used products with restriction endonuclease sites specific for either hNK-3R or NK-4R, failed to demonstrate the presence of NK-4R in genomic DNA from human, monkey, mouse, rat, hamster, or guinea pig, and in cDNA libraries from human lung, brain, or heart, whereas the hNK-3R was detectable in the latter libraries. In view of the failure to demonstrate the presence of the putative NK-4R it is thought to be premature to extend the current tachykinin receptor classification.

The mammalian tachykinins, or neurokinins (NKS), are a family of small peptides, notably, Substance P, NKA, and NKB that share the common carboxy-terminal region Phe-X-Gly-Leu-Met-NH2 (Maggio, 1988; Maggi et al., 1993). The tachykinins are localized in both the central and peripheral nervous systems, in particular in capsaicin-sensitive primary afferent neurons (unmyelinated C fibers) that innervate many regions, including the airways, gastrointestinal and urinary tracts, and the skin (Otsuka and Yoshioka, 1993; Maggi et al., 1995; Maggi, 1996). The biological effects of the tachykinins are mediated via three tachykinin receptor subtypes, NK-1, NK-2, and NK-3, which are members of the superfamily of G-protein-coupled, seven transmembrane (TM)-spanning receptors (Maggio, 1988; Nakanishi, 1991; Maggi et al., 1993). The human variants of the three tachykinin receptors have been cloned and expressed (Gerard et al., 1990; Buell et al., 1992; Huang et al., 1992), and in the past few years potent and selective nonpeptide antagonists for the individual receptors have been identified (Snider et al., 1991; Maggi et al., 1993; McLean et al., 1993; Emonds-Alt et al., 1995; Maggi, 1996; Sarau et al., 1997; Gao and Peet, 1999).

In 1992 Xie et al. (1992), in an expression-cloning search for the κ-opioid receptor, reported on the cloning and expression of a novel human orphan receptor that was highly homologous (81% sequence identity at the amino acid level) to the human NK-3 receptor (hNK-3R). Despite this close ho-
mology, no specific binding of the peptide tachykinin agonist [3H]leuketakin was detected in Cos-7 cells transfected with the receptor, and it was concluded to be an atypical opiate receptor (Xie et al., 1992). In a subsequent study, NKB, the natural ligand with the highest affinity for the hNK-3R (Maggio, 1988; Nakanishi, 1991; Maggi et al., 1993), produced a concentration-dependent response in Xenopus oocytes, and also in clonally selected NIH 3T3 fibroblasts, expressing this orphan receptor (Donaldson et al., 1996). The sensitivity of the orphan receptor, which was designated “NK-4”, to tachykinin agonists was similar to that of the hNK-3R, except that the latter did not respond to Substance P (1 µM). Northern blot analysis revealed low-level expression of the NK-4R in some, but not all, human tissues examined (Donaldson et al., 1996), with a pattern of expression reportedly different from that observed previously for the hNK-3R (Buell et al., 1992). More extensive pharmacological characterization of the hNK-3R and the putative NK-4R, was reported later by using radioligand binding and arachidonic mobilization studies in Chinese hamster ovary cells stably expressing the two receptors. The authors concluded that the pharmacological profiles of the receptors were very similar in many respects, and it was proposed that the novel receptor may represent an NK-3R homolog or an NK-4R (Krause et al., 1997). Unfortunately, in neither of these studies were the effects of tachykinin receptor agonists observed previously for the hNK-3R (Buell et al., 1992). More extensive pharmacological characterization of the hNK-3R and the putative NK-4R, was reported later by using radioligand binding and arachidonic mobilization studies in Chinese hamster ovary cells stably expressing the two receptors. The authors concluded that the pharmacological profiles of the receptors were very similar in many respects, and it was proposed that the novel receptor may represent an NK-3R homolog or an NK-4R (Krause et al., 1997). Unfortunately, in neither of these studies were the effects of tachykinin receptor antagonists, in particular hNK-3R antagonists, explored. The major goals of this study were as follows: 1) to compare the pharmacological profiles of the hNK-3R and the NK-4R by using a standard binding (inhibition of [125I]-[MePhe7]-NKB in cell membranes) assay and an intact cellular functional assay (Ca2+ mobilization), with several tachykinin agonists and also tachykinin receptor-selective antagonists; and 2) to gain additional information about the expression and molecular biological characteristics of the NK-4R; a comparison was made with the hNK-3R.

**Experimental Procedures**

**Materials.** [125I]-[MePhe7]-NKB (specific activity, 2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). NKA, NKB, Substance P, and [MePhe7]-NKB were purchased from Peninsula Laboratories (Belmont, CA) and senktide (succinyl-[Asp9 MePhe6]-SP(6-13)) from California Peptide Research, Inc. (Napa, CA). Taq polymerase and buffer were purchased from Perkin-Elmer (Branchburg, NJ). Restriction enzymes and buffers were obtained from Promega (Madison, WI). SB 222200, SB 223412, SR 142801, SR 48964, and CP 99994 were synthesized in the Department of Medicinal Chemistry, SmithKline Beecham S.p.A, Milan, Italy. Restriction enzymes and buffers were obtained from Promega (Madison, WI). SB 222200, SB 223412, SR 142801, SR 48964, and CP 99994 were synthesized in the Department of Medicinal Chemistry, SmithKline Beecham S.p.A, Milan, Italy.

**Receptor Cloning and Expression.** The cDNA encoding the putative NK-4R was kindly provided by Dr. G.-X. Xie. Polymerase chain reaction (PCR) primers were designed according to the published sequence (Xie et al., 1992): 5′-GGGGCTCCGGGCACTC-3′ and 5′-GACCCCAGAGAGAAATCAAGAGCC-3′. The GX coding region was amplified by PCR in standard buffer conditions with 1.5 mM MgCl2 and 4% dimethyl sulfoxide by using 35 cycles of 94, 54, and 72°C at 1 min/step. The 1.4-kb PCR product was initially subcloned into an expression vector pCI (Invitrogen, Carlsbad, CA) and subsequently subcloned into the expression vector pCDNA3 (Invitrogen). Correct orientation of the cDNA insert encoding the GX receptor was confirmed by restriction analysis.

The sequences of the PCR amplicon and coding region of the GX plasmid obtained from Dr. Xie were shown to be identical by automated DNA sequencing but differed from the published sequence (Xie et al., 1992). Three silent point mutations were noted in the codons for Leu-22, Gly-68, and Ile-184; however, two nucleotide changes results in the conversion of Ala to Arg at position 59. These differences are not in the binding domains of the receptor and are not in the restriction sites or regions amplified in subsequent PCR studies (see below).

The GX/pCDNA3 plasmid was purified (Qiagen Maxiprep; Qiagen Inc., Valencia, CA) and used to transfect human embryonic kidney cells (HEK 293 cells) with a calcium phosphate transfection method as described in Lee et al. (1994). Cells were harvested 48 h after transfection and used for binding or functional assays.

**hNK-3R and NK-4R PCR.** Nested PCR primers corresponding to regions of nucleotide identity between hNK-3R and NK-4R within exons 1 and 4 were designed to amplify intervening regions that contain receptor-specific restriction endonuclease sites (Fig. 1). Primers exon 1: forward (1) 5′-GGGGCTCCGGGCACTC-3′; forward (2) 5′-GGGGCTCCGGGCACTC-3′; reverse (1) 5′-GGGGCTCCGGGCACTC-3′; reverse (2) 5′-GGGGCTCCGGGCACTC-3′; forward (1) 5′-GGGGCTCCGGGCACTC-3′; forward (2) 5′-GGGGCTCCGGGCACTC-3′; reverse (1) 5′-GGGGCTCCGGGCACTC-3′; reverse (2) 5′-GGGGCTCCGGGCACTC-3′. PCR reactions were carried out in a Perkin-Elmer 2400. All templates were amplified in standard buffer conditions with 1.5 mM MgCl2 and 4% dimethyl sulfoxide by using 35 cycles of 94, 54, and 72°C at 1 min/step. For genomic PCR, 100 ng of CsCl-purified cellular DNA was used as the initial template in a reaction volume of 100 µl. One microliter from the initial PCR reaction was used as template for the nested reaction. For the cDNA library PCR, 10° colony-forming units or plaque-forming units from the indicated plasmid or phage libraries were screened in the initial reaction. One microliter from the initial reaction was used for the nested reaction; all reaction volumes were 100 µl. Products from the nested PCR reaction were purified by agarose gel electrophoresis (5% native gel). Human cDNA for the hNK-3R, with sequence identical to published reports, was isolated from human placenta poly(A)+ RNA using reverse transcription-PCR technology and site-directed mutagenesis.

**Radioligand Binding Assays.** Receptor binding assays were performed with membranes from HEK 293 cells transiently expressing the hNK-3 receptors (HEK 293-hNK-3R) or the NK-4R (HEK 293-NK-4R), as detailed previously (Sarau et al., 1997). [125I]-[MePhe7]-NKB competition binding studies with HEK 293-hNK-3R or HEK 293-NK-4R membranes were conducted with approximately 15 µg of membrane protein and 0.15 nM 125I-[MePhe7]-NKB in a total of 150 µl of 50 mM Tris, pH 7.4, 4 mM MnCl2, 1 µM phosphoramidon, and 0.1% ovalbumin, with or without various concentrations of antagonist, for 90 min at 25°C. Incubations were stopped by rapid addition of ice-cold buffer.
filtration through Whatman GF/C filters that were presoaked for 60 min in 0.5% BSA, with a Brandell tissue harvestor (Gaithersburg, MD). Membranes were washed with 10 ml of ice-cold 20 mM Tris, pH 7.4, containing 0.1% BSA and then placed in vials with 10 ml of Beckman Ready Safe and counted in a liquid scintillation counter. Concentration-response curves for each compound were run with duplicate samples in at least three independent experiments. Non-specific binding was assessed as the binding in the presence of 0.5 μM cold MePhe7-NKB. The IC50 for ligands, defined as the concentration required to inhibit 50% of the specific binding, was determined from concentration-response curves. Values presented are the apparent inhibition constant (Ki), which was calculated from the IC50 as described by Cheng and Prusoff (1973).

**Ca2+ Mobilization Assay.** Ca2+ mobilization experiments in HEK 293-hNK-3R or HEK 293-NK-4R cells were conducted with Fluo 3-loaded cells and a Fluorescence Imaging Plate Reader ( Molecular Devices, Sunnyvale, CA) as outlined previously (Sarau et al., 1999). Briefly, cells (approximately 80% confluent) were harvested and plated in 96-well black wall/clear bottom plates (Becton Dickinson Labs, Bedford, MA) at approximately 40,000 cells/well and grown in the incubator for 18 to 24 h. On the day of assay the medium was aspirated and replaced with 100 μl of Earls’ minimal essential medium with Earls’ salts, L-glutamine 0.1% BSA, 4 mM Fluo 3 acetoxymethyl ester (Fluo 3 AM; Molecular Probes, Eugene, OR), and 2.5 mM probenecid. Plates were incubated for 60 min at 37°C, and then the medium was aspirated and replaced with the same medium without Fluo-3 AM, and incubated for 10 min at 37°C in 100 μl of buffer [120 mM NaCl, 4.6 mM KCl, 103 mM KH2PO4, 25 mM NaHCO3, 1.0 mM CaCl2, 11 mM glucose, 20 mM HEPES (pH 7.4) with 2.5 mM probenecid]. Plates were placed into a Fluorescence Imaging Plate Reader where cells were exposed to excitation (488 nm) from a 6-W argon laser. Fluorescence was monitored at 566-nm emission for all 96 wells simultaneously, and data points were collected every second. The maximal change in emission after agonist addition was quantitated. The percentage of maximal NKB-induced Ca2+ mobilization was determined for each concentration of antagonist and the IC50, defined as the concentration of test compound that inhibits 50% of the maximal response induced by 1 nM NKB, assessed. Values presented are generally the mean IC50 ± S.E. of three individual experiments unless stated otherwise.

### Results

#### Pharmacological Characterization

**Binding Experiments.** Initially, studies were performed to assess the binding characteristics of 125I-[MePhe7]-NKB to membranes prepared from transiently expressed HEK 293-NK-3R cells. Binding of the radioligand was saturable, specific, and of high affinity; the Kd and Bmax were determined to be 1.0 ± 0.2 nM and 2.1 ± 0.7 pmol/mg, respectively (n = 3; data not shown). The binding of 125I-[MePhe7]-NKB to HEK 293-hNK-3R cell membranes was saturable, specific, and of high affinity; the Kd and Bmax values were 0.9 ± 0.2 nM and 0.6 ± 0.1 pmol/mg, respectively (n = 3; data not shown).

A comparison was made of the effects of tachykinin receptor agonists and antagonists on the binding of 125I-[MePhe7]-NKB to HEK 293-hNK-3R or HEK 293-NK-4R cell membranes; the results are summarized in Fig. 2 and Table 1. The

**Fig. 2.** Competition binding of 125I-[MePhe7]-NKB to (A, C) HEK 293-hNK-4Rs and (B, D) HEK 293-NK-3Rs by tachykinin agonists (A, B) senktide (●), Substance P (F), NKB (E), [MePhe7]-NKB (f), or NKA (e), and antagonists (C, D) SB 223412 (f), SB 222200 (Œ), SR 142801 (E), SR 48968 (e), or CP 99994 (F) as described under Experimental Procedures. Values presented are the mean of two or three experiments. Standard errors are omitted for clarity, but are given for the EC50 values (agonists) and IC50 values (antagonists) in Table 1.
Comparison of Human NK-3 and NK-4 Receptors

natural ligand with the highest affinity for the NK-3 receptor, NKB, and the NK-3R-selective agonists senktide and [MePhe7]-NKB (0.1 nM-0.1 μM) produced concentration-dependent inhibition of 125I-[MePhe7]-NKB binding to HEK 293-NK-4R cell membranes, with respective IC50 values of 18.3 ± 3.3, 15.1 ± 2.6, and 3.3 ± 0.5 nM (n = 3) (Fig. 2A; Table 1). In contrast, Substance P (NK-1R-prefering natural ligand) and NKA (NK-2R-prefering natural ligand) demonstrated much lower affinity for 125I-[MePhe7]-NKB binding to HEK 293-NK-4R cell membranes with IC50 values of 2130 ± 540 and 3530 ± 180 nM, respectively (n = 3) (Fig. 2A; Table 1). Thus, the relative rank order potency was [MePhe7]-NKB > NKB = senktide > Substance P = NKA. The NK-3R-selective antagonists SB 223412 (Sarau et al., 1997), SB 222200 (Sarau et al., 2000), and SR 142801 (Emonds-Alt et al., 1995) also produced potent, concentration-dependent inhibition of 125I-[MePhe7]-NKB binding to HEK 293-NK-4R cell membranes with respective IC50 values of 4.1 ± 0.7, 11.0 ± 2.7, and 2.6 ± 0.6 nM (n = 3; Table 1). The NK-1R-selective antagonist CP 99994 (McLean et al., 1993) was a weak competitor, with an IC50 of 8500 ± 2400 nM, whereas the NK-2R antagonist SR 48968 (Emonds-Alt et al., 1992) showed significant inhibition with an IC50 of 711 ± 120 nM (n = 3) (Fig. 2C; Table 1).

The rank order for the agonist displacement of 125I-[MePhe7]-NKB binding to HEK 293-hNK-3R cell membranes was not very different from that obtained with the NK-4R membranes: [MePhe7]-NKB > NKB > senktide > NKA = Substance P (Fig. 2B; Table 1). Furthermore, the antagonists also showed a similar rank order potency for inhibition of 125I-[MePhe7]-NKB binding to HEK 293-hNK-3R cell membranes: SB 223412 = SR 142801 > SB 222200 > SR 48968 > CP 99994 (Fig. 2D; Table 1).

**Ca2+ Mobilization Studies.** Cellular functional activity of agonists and antagonists was determined by assessment of their effects on Ca2+ mobilization in HEK 293-NK-4R or HEK 293-hNK-3R cells (Fig. 3). In HEK 293-NK-4R cells, the rank order potencies of agonists was senktide = NKB = [MePhe7]-NKB > NKA > Substance P. All agonists demonstrated the same efficacy, eliciting a similar maximum response (Fig. 3A). A similar rank order of potencies for the agonists was demonstrated for their ability to produce Ca2+ mobilization in HEK 293-hNK-3R. However, senktide was more potent (3-fold) than NKB or [MePhe7]-NKB in HEK 293-hNK-3R cells (Fig. 3B).

In studies examining the effects of antagonists against NKB-induced Ca2+ mobilization in HEK 293-hNK-3R, SR 48968 possessed similar potencies for inhibition of NKB (1 nM)-induced Ca2+ mobilization in HEK 293-NK-4R cells, with IC50 values of 50 ± 34 nM (n = 3), 105 ± 52 nM (n = 3), and 254 ± 180 nM (n = 3), respectively. SR 48968 and CP 99994 had IC50 values of 1212 ± 660 nM (n = 3) and >10 μM (n = 3), respectively (Fig. 3C). A similar profile for the antagonists was evident against NKB-induced Ca2+ mobilization in the HEK 293-hNK-3R cells (Fig. 3D). Thus, SB 223412, SR 142801, and SB 222200 had similar potencies, with respective IC50 values = 125 ± 46 nM (n = 3), 231 ± 110 nM (n = 3), and 264 ± 73 nM (n = 3). Significant inhibition was demonstrated with SR 48968 (IC50 = 14,600 nM; n = 2), but not with CP 99994, in concentrations up to 10 μM (n = 2) (Fig. 3D).

### Molecular Biological Characterization

**Isolation and Expression of NK-4R.** A comparison of the alignment of the human tachykinin receptor family, including NK-4R, is indicated in Fig. 4. The predicted organization of the NK-4R is based on the sequence alignment. PCR and nested PCR procedures were used to isolate the NK-4R cDNA from human lung, brain, or heart cDNA libraries and to detect the NK-4R gene with multiple primers directed to the 5’- or 3’-untranslated region (UTR) and internal-coding regions of NK-4R. No evidence for an NK-4R-specific signal was detected from human genomic DNA and cDNA libraries that are abundant in NK-4R mRNA (G.-X. Xie, personal communication), although multiple primer sets were used under a variety of PCR reaction conditions (data not shown). In addition, the NK-4R gene was not identified in Northern or Southern blot analysis by using 5’-UTR- or

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**TABLE 1**

| Effects of tachykinin receptor ligands on 125I-[MePhe7]-NKB binding and Ca2+ mobilization in (hNK-4R) and hNK-3R cells
<table>
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<tr>
<td><strong>Binding (Inhibition of 125I-[MePhe7]-NKB) nM</strong></td>
<td><strong>Ca2+ Mobilization</strong></td>
</tr>
<tr>
<td>hNK-4R IC50</td>
<td>hNK-3R IC50</td>
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<tr>
<td><strong>Agonists</strong></td>
<td></td>
</tr>
<tr>
<td>NKB</td>
<td>18.3 ± 3.3</td>
</tr>
<tr>
<td>[MePhe7]-NKB</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Senktide</td>
<td>15.1 ± 2.6</td>
</tr>
<tr>
<td>NKA</td>
<td>3,530 ± 180</td>
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<tr>
<td>Substance P</td>
<td>2,130 ± 540</td>
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**Antagonists**

<table>
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<th>Inhibition of NKB (1 nM) IC50</th>
<th>Inhibition of NKB (1 nM) IC50 nM</th>
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<tbody>
<tr>
<td>SB 223412</td>
<td>4.1 ± 0.7</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>SR 142801</td>
<td>2.6 ± 0.6</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>SR 222200</td>
<td>11.0 ± 2.7</td>
<td>18.1 ± 5.4</td>
</tr>
<tr>
<td>SR 48968</td>
<td>711 ± 120</td>
<td>1,250 ± 160</td>
</tr>
<tr>
<td>CP 99994</td>
<td>8,500 ± 2,400</td>
<td>23,000 ± 4,100</td>
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3'-UTR hNK-4R-specific probes derived from either GX/ pCDNA3 or the published sequence (Xie et al. 1992; data not shown). These unexpected results are inconsistent with the reported human origins of NK-4R (Xie et al., 1992).

Nested PCR and Restriction Site Analysis of hNK-3R and NK-4R. In view of the unsuccessful isolation and detection of the NK-4R cDNA, a series of experiments, with nested PCR and restriction endonucleases specific for the hNK-3R

Fig. 3. Tachykinin agonist-induced Ca²⁺ mobilization (A, B) and effects of tachykinin receptor antagonists SB 223412, SB 222200, SR 142801, SR 48968, and CP 99994 on NKB-induced Ca²⁺ mobilization (C, D) in HEK 293-NK-4R (A, C) or HEK 293- hNK-3R cells (B, D). A and B, Fluo-3-loaded HEK 293-hNK-3 or HEK 293-NK-4R cells were stimulated with increasing concentrations of senktide (●), Substance P (○), NKB (△), MePhe⁷-NKB (▲), or NKA (■). The results are expressed as Ca²⁺-mobilized (optical units) and are the mean for three experiments. Standard errors are omitted for clarity but given for the EC₅₀ values in Table 1. C and D, Fluo-3 loaded HEK 293-hNK-3 or HEK 293-NK-4R cells were stimulated with NKB after the addition of increasing concentrations of SB 223412 (●), SB 222200 (○), SR 142801 (△), SR 48968 (▲), or CP 99994 (■). The results are expressed as a percentage of the maximum Ca²⁺ concentrations elicited by 1 nM NKB in the absence of any antagonist. Values presented are the mean of three experiments. Standard errors are omitted for clarity but given for the IC₅₀ values in Table 1.

Fig. 4. Alignment of human tachykinin receptor family. The predicted organization of the sequence of NK-4R is based on the sequence alignment.
and NK-4R, were performed to verify that the NK-4R is a human gene. Specifically, nested PCR primers were designed to amplify a DNA fragment from exon 1 (containing TM1–TM3) and separate primers to amplify a DNA portion from exon 4 (containing TM6 and TM7) (Fig. 1); see Experimental Procedures for the primers used. The PCR primers were designed to simultaneously amplify both hNK-3R and NK-4R sequences from regions of 100% nucleotide identity, but contained hNK-3R- or NK-4R-specific restriction endonuclease sites within the intervening sequences (Fig. 1). Therefore, if both NK-3R or NK-4R genes are present in either human genomic DNA or cDNA libraries, the characteristic restriction patterns for both genes should be observed. The results of these studies are summarized in Fig. 5. With the exon 1 amplicon (Fig. 5) and the exon 4 amplicon (data not shown) only NK-3R-specific restriction patterns were identified in PCR products derived from human genomic DNA for both exon 1 and exon 4 amplicons (Fig. 5) and cDNA libraries from human placenta, lung, heart, or brain, tissues reported to contain NK-4R mRNA (Xie et al. 1992; G.-X. Xie, personal communication). For example, the results from placenta cDNA library and exon 1 fragments (lanes 9–11) indicate that a single band was detected when no endonuclease digest was used (lane 9), two bands were obtained by using the hNK-3R-specific digest (PvuII; lane 10), and only one band was demonstrated by using the NK-4R-specific digest (MboI; lane 11). These data indicate the presence of hNK-3R, but not NK-4R, in this cDNA library. There was no evidence for the presence of either hNK-3R or NK-4R in human liver or skeletal muscle cDNA libraries because no band was demonstrated in lanes 22 and 23, respectively.

To investigate whether the putative NK-4R may be a non-human gene product, the same PCR amplification and restriction digest experiments were conducted with mouse, rat, hamster, guinea pig, and monkey (Cos cell) genomic DNA. Results similar to that obtained from human genomic DNA (Fig. 5) were obtained; there was no evidence of an NK-4R gene in any of the species tested (data not shown). Furthermore, no positive hybridization was detected, by using a synthetic oligonucleotide specific to the 3’-UTR of NK-4R, in a genomic DNA blot from Clontech (Palo Alto, CA), which includes human, rat, dog, rabbit, yeast, monkey, mouse, bovine, and chicken (data not shown).

**Discussion**

The existence of a novel tachykinin receptor, designated NK-4, which has a close structural and functional homology with the hNK-3R, has been proposed (Donaldson et al., 1996; Krause et al., 1997). The major goals of this study were, first, to conduct a comprehensive comparison of the pharmacological profiles of the hNK-3R and the NK-4R, with radioligand binding and Ca$^{2+}$ mobilization experiments. For the first time the effects of tachykinin receptor antagonists in NK-4R were explored. These studies were performed with the hNK-3R and NK-4R transiently expressed in HEK 293 cells. This should normalize the responses of the receptor-expressing cells and avoid differential expression of stable clonal cell lines. Second, attempts were made to compare the distribution and molecular biological characteristics of both receptors. Binding and functional studies confirmed the similar pharmacological characteristics of the hNK-3R and NK-4R,
although some small differences were apparent, e.g., senkhtide appeared to have lower affinity for hNK-3R than NK-4R, but this was not observed in the functional analysis where the three hNK-3R-selective ligands showed equal potency. Surprisingly, despite the use of several independent approaches, no evidence was obtained that the putative NK-4R is a gene product of human or many nonhuman species.

It has been demonstrated that high primary sequence identity exists within TM regions of hNK-3R and NK-4R, ranging from 83 to 100% with an average of 92% identity. It is known that the residues that are important for the interaction of tachykinin receptors (in particular, NK-1R) with potent and selective antagonists are located in TM regions (Fong et al., 1993; Gether et al., 1993a,b). This information, in addition to the very similar pharmacological profile of the hNK-3R and NK-4R to various tachykinin ligands, including NK-3R-selective agonists (Donaldson et al., 1996; Krause et al., 1997; current study) and NK-3R antagonists (current study) highlights the importance of determining whether this putative hNK-3R-like receptor is in fact a human tachykinin receptor subtype or is a species variant of the hNK-3R. To address this question several approaches were used: Northern and Southern analysis with NK-4R-specific probes, PCR cloning from various human tissues reported to express NK-4R, and restriction analysis of PCR amplicon derived from genomic and cDNA libraries. Overall, these experiments did not provide evidence that NK-4R is a human gene product; furthermore, it does not appear to be a gene product of the several nonhuman species investigated: mouse, rat, monkey, bovine, dog, chicken, yeast, rabbit, or guinea pig.

To investigate whether the NK-4R sequence corresponds to a human gene, regions of either exon 1 (containing TM1–TM3) or exon 4 (containing TM6 and TM7) from NK-3R and NK-4R were simultaneously amplified by nested PCR and then differentiated by the presence of either hNK-3R- or NK-4R-specific restriction sites. PCR amplification with human genomic DNA and various human cDNA libraries revealed amplicons with restriction endonuclease sites corresponding to the hNK-3R but not the NK-4R; the tissues explored included placenta, from which the NK-4R was originally cloned (Xie et al., 1992). It is unlikely that the PCR primers selectively amplify the hNK-3R amplicon over the NK-4R amplicon because each primer set corresponds to a region of nucleotide identity between the two receptor cDNAs and thus serves as internal PCR control. It is possible that the PCR primers may have failed to amplify NK-4R from the human genomic DNA if the intron-exon organization of the NK-4R gene differs from that of the hNK-3R gene. However, this appears unlikely because the NK-4R and hNK-3R sequences are very similar (75 and 82% sequence identity at the nucleotide and amino acid level, respectively) and the gene organization of the known tachykinin receptors NK-1, NK-2, and NK-3 are identical (Fig. 4; Donaldson et al., 1996). These studies are consistent with the failure to amplify an NK-4R fragment by using various sets of PCR primers, PCR conditions, and cDNA synthesis conditions from human placenta RNA, and the inability to amplify an NK-4R fragment of the 3'-UTR from human genomic DNA with various primer sets and PCR conditions. Collectively, the results from the PCR analysis of human cDNA and genomic DNA suggest strongly that the NK-4R is not represented in the human genome.

The current results contrast with those from a previous study in which NK-4R transcripts were found in skeletal muscle, liver, lung, and heart (Donaldson et al., 1996); the reason(s) for this discrepancy is not apparent. However, given the 75% nucleotide identity between hNK-3R and NK-4R the specificity of a hybridization probe generated from a full-length NK-4R cDNA may be in question; in contrast, the PCR technique used in this study does not depend on the generation of a specific hybridization probe.

Finally, attempts were made to associate the novel receptor to a nonhuman species. However, 3'-UTR probes synthesized from the published sequence (Xie et al., 1992) or cloned from the plasmid provided by Dr. Xie failed to hybridize with Southern blots of human, rat, dog, rabbit, yeast, monkey, mouse, bovine, and chicken genomic DNAs. In addition, no NK-4R amplification was observed when PCR analysis of mouse, rat, monkey, guinea pig, and hamster genomic DNAs was undertaken. Thus, the NK-4R cDNA does not appear to originate from a number of common laboratory species.

In summary, based on binding and functional studies with tachykinin ligands, including NK-3R-selective agonists and antagonists, the reported hNK-3R-like receptor (putative NK-4R) has pharmacological characteristics that are very similar to those of the hNK-3R. This similarity in profiles is not unexpected, given the close homology between the two receptors (overall, 82% at the amino acid level). Unfortunately, attempts to identify and localize the NK-4R, by using genomic and cDNA libraries from human and nonhuman species and standard molecular biological techniques, were unsuccessful. The results suggest that the NK-4R is not represented in the human genome, and highlight the caution that should be exercised in using this gene product in studies related to characterization of tachykinin receptors. Furthermore, it would appear to be inappropriate and premature to extend the current human tachykinin receptor classification beyond the present NK-1R, NK-2R, and NK-3Rs. Additional experiments, with libraries from species not used in the current study, are required to explore further whether this receptor is a species variant of the hNK-3R.

Acknowledgments

We thank John Adamou, Bob Ames, Mary Brawner, Nabil Elshourbagy, and Parvathi Nuthulaganti for the cloning and expression efforts; Mario Grugni, Roberto Rigolio, and Karl F. Erhard for the synthesis of SR 142801; Luca F. Raveglia for the synthesis of CP 99994; and Mark Luttmann for assistance in the preparation of the manuscript.

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