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Isolation and Pharmacological Characterization of a Hamster Urinary Bladder Neurokinin A Receptor cDNA

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SUMMARY

Functional cDNA clones for hamster neurokinin-2 receptor (NK-2R) were isolated from hamster urinary bladder using a polymerase chain reaction-based methodology. The hamster NK-2R consists of 384 amino acids with a relative molecular weight of 43,418. Hamster NK-2R shares significant amino acid sequence homology with other tachykinin receptors, particularly with rat, bovine, and human NK-2R (94.3, 84.4, and 86.5%, respectively). To examine the pharmacology of cloned hamster NK-2R, we transfected mouse erythroleukemia cells with this receptor, prepared high speed membranes, and studied the receptor properties utilizing the ligand [4,5-^{3}H-Leu^9]NKA in a receptor-binding assay. For pharmacological comparison, we also transfected the human NK-2R into mouse erythroleukemia cells. [^{3}H]NKA bound to hamster NK-2R receptor in a protein-dependent, high affinity (K_{d1} = 4.14 ± 0.31 nM), saturable (B_{max1} = 679 ± 26 fmol/mg of protein), and highly specific manner (89 ± 2%). A smaller population (10% density) of lower affinity receptors (K_{d2} = 150 ± 92 nM), was also observed in competition experiments. [^{3}H]NKA bound to the human receptor with significantly higher affinity and overall greater receptor density (K_{d1} = 0.37 ± 0.11 nM, B_{max1} = 234 ± 175 fmol/mg of protein; K_{d2} = 9.0 ± 2 nM, B_{max2} = 1989 + 990 fmol/mg of protein). [^{3}H]NKA binding to both hamster and human receptors was enhanced greatly by divalent cations, whereas GTP analogs weakly inhibited binding to hamster receptor, but potently inhibited binding to the human receptor. Competition experiments with agonists demonstrated binding to high and low affinity states of NK-2 receptors, with identical order of potency in hamster or human NK-2R; NKA > [Nle^{10}]NKA(4–10) > [β-Ala^8]NKA(4–10) ≫ substance P ⋙ Senktide. However, remarkable differences were observed in studies with selective NK-2 antagonists (hamster, SR48,968 > L659,877 > R396 ≫ MEN10,376 versus human, SR48,968 > MEN10,376 > L659,877 > R396). The rank order of antagonist affinity is consistent with the observation of NK-2 receptor pharmacology in the native tissues.

NKA, a decapeptide that belongs to a class of biologically active peptides known as tachykinins (1), has been shown to be widely distributed in the central nervous system as well as in peripheral organs and tissues, where it binds to the NK-2 receptors, causing contraction of airway, vascular, and nonvascular smooth muscle (2–5).

After the cloning and characterization of the bovine NK-2 receptor (6), several groups have cloned the NK-2 receptor from rat and human and expressed them in functionally active forms in various cell types (7–10). These studies have demonstrated that NK-2 receptors possess seven putative transmembrane domains, suggesting that they are coupled to G proteins, and they provided understanding, at the molecular level, of the differences between NK-1, NK-2, and NK-3 tachykinin receptors.

More recently, chimeric receptors have been constructed to delineate the critical binding domains that distinguish agonist selectivity for binding and activation of NK-1 versus NK-2 receptors (11). Mutagenesis experiments have been conducted to determine the specific sites of agonist and antagonist binding in the human NK-1 receptor (12). Additional site-directed mutagenesis studies have identified some critical residues within the NK-1 receptor that are involved in antagonist binding, and also residues that may contribute to the species selectivity between human and rat NK-1 receptors of specific nonpeptide antagonists (13, 14).

However, none of the former studies investigated the species differences between NK-2 receptors. Recent studies by Maggi *et al.* (15, 16) using peptide antagonists suggested the existence of NK-2A and NK-2B receptor "subtypes." Based on these studies, they classified NK-2 receptors in guinea pig and rabbit vascular and airway smooth muscles as NK-2A, and receptors in hamster urinary bladder and trachea were classified as NK-2B. We have demonstrated recently that the cloned human NK-2 receptor expressed in insect Sf-21 cells infected with recombinant baculovirus displays pharmacological selectivity

ABBREVIATIONS: NK, neurokinin; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LCR, locus control region; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; TMS, transmembrane segment.

compatible with guinea pig or rabbit NK-2 receptors and dissimilar to the hamster (17).

Moreover, utilizing the ligand [4,5-^{3}H-Leu^9]NKA and highly selective NK-2 receptor antagonists in receptor ligand binding assays, we provided further evidence that NK-2 receptors in hamster urinary bladder display pharmacological heterogeneity relative to human or guinea pig (18). In the present report we describe the isolation, expression, and pharmacological characterization of the NK-2 receptor cDNA from hamster urinary bladder. We also provide evidence, based on antagonist binding properties, that the hamster receptor is distinct from human and bovine NK-2 receptors and that the pharmacological differences are likely caused by different amino acids between the receptors from the species.

Materials and Methods

Isolation and characterization of cDNA. Total RNA was isolated (19) from freshly excised Syrian Golden hamster tissues. PCR-based methods (RACE-PCR) (20, 21) were used to isolate the 5′- and 3′-ends of the hamster NK-2 receptor cDNA as outlined in Fig. 1. The 3′-end was obtained as follows. First-strand cDNA was synthesized (cDNA kit, Boehringer Corp., Ltd., Indianapolis, IN) from 5 μg of hamster urinary bladder total RNA using the 58-mer R1R2(dT)17 primer (25 pmol) 5′d(AAGGATCCGTCGACATCGATAATACGACTCACTATAAGGGA (T)17). A primary PCR reaction was performed using one amplimer derived from the 5′-end of the RACE 58 mer, R1 = 5′d(AAGGATCCGTCGACATCGATAAT), and a gene-specific amplimer, GSA1 = 5′d(ACGGTGACCAACTACTTCATCGTC), which was derived from a consensus sequence in the second TMS of rat and bovine NK-2 receptor (6, 10).

PCR (22) was performed at 65° for 2 min, 72° for 2 min, and 92° for 2 min for 33 cycles using a Techne PHC-1 thermal cycler and *Taq* polymerase (Amplitaq) using conditions described by the manufacturers (Perkin-Elmer, Norwalk, CT). The products of this primary reaction (0.1%) were used for a secondary PCR with nested amplimers. One amplimer was a nested RACE amplimer, R2 = 5′d(GATAATACGACTCACTATAAGGGA), and the other was a nested gene-specific amplimer, GSA2 = 5′d(TGCTGGCATCTGGCTGGTGGCCCTGGC), which was chosen from a region in the fourth TMS. This region is identical between rat and bovine NK-2 receptor, but is quite different (especially at the 3′-end of the amplimer) in rat SP (NK-1) receptor and rat neuromedin K (NK-3) receptor. Secondary products were Southern blotted and hybridized to a human NK-2 receptor probe that had been labeled with [^{32}P]dCTP using a random primer labeling kit (Boehringer). A 1200-bp product was identified and directly sequenced on both strands using ^{32}P-end-labeled oligonucleotides and Sequenase (United States Biochem. Corp., LaJolla, CA) (23). The 5′-end was obtained by similar methods. First-strand cDNA was synthesized from 5 μg of hamster urinary bladder total RNA using a hamster gene-specific amplimer, HubGSA1 (25 pmol) = 5′d(CCAGGGGCAGCACCGGAAAGCAAG), and the products were purified by spin dialysis (Centricon 100, Amicon Corp., Beverly, MA). The cDNA was tailed at its 3′-end with A's using terminal transferase (GIBCO BRL, Gaithersburg, MD), and second-strand cDNA was synthesized with the RACE 58-mer R1R2(dT)17 using *Taq* polymerase. Primary PCR was performed using the RACE outer amplimer (R1) and HubGSA2 = 5′d(AACACCACCAGAACCATGGCCTTC). 0.1% of the primary products was used for secondary PCR with the nested

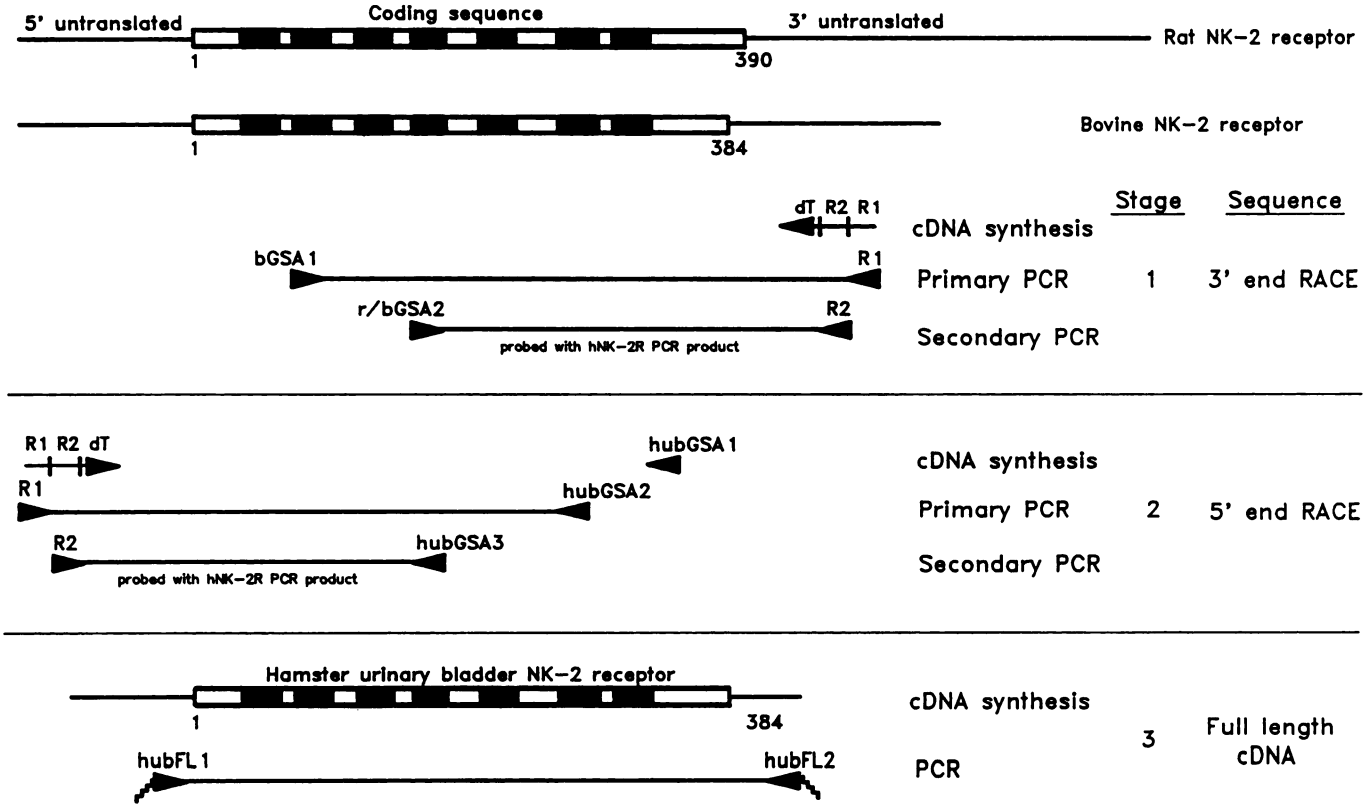

Fig. 1. Isolation of hamster NK-2 receptor cDNA. Schematic diagram of rat and bovine NK-2 receptor clones and the stages used to isolate hamster NK-2 receptor cDNA. The priming locations for oligonucleotides used in this study are shown, *arrowheads* indicate 3′-ends and *wavy lines* indicate non-NK-2 receptor sequences added for cloning. GSA, gene specific amplimer; b, bovine sequence, r/b, rat and bovine; hub, hamster urinary bladder. The 5′- and the 3′-end RACE products were obtained using nested amplimers (see Materials and Methods). Full length cDNA was then amplified and cloned into pMAMneoBlue.

RACE amplimer (R2) and a nested HubGSA3 = 5′d(GCCAAAGTACCAGATGTTGTG). A product of 700 bp was identified by hybridization with human NK-2 receptor cDNA probe and was directly sequenced on both strands. The sequences of the 5′- and 3′-RACE-PCR products overlapped.

Full length cDNA was obtained by using hamster amplimers HubFL1 = 5′d(GCGATATCTGTGTTCCTGGCCCAGAATCGGC) and HubFL2 = 5′d(CCGTCTAGAATGGAGTGTGGGACAAGCTATTAC), which are 25 bp upstream of the ATG initiation codon and 91 bp downstream of the stop codon, respectively. These contained *Eco*RV and *Xba*I restriction sites at their 5′-ends, respectively. First-strand cDNA was synthesized from hamster urinary bladder RNA using oligo(dT) as primer, and then PCR was performed. The correct size PCR product (1287 bp) was obtained and directly sequenced on both strands. This product was then cloned unidirectionally into pMAMneoBLUE (CLONTECH, Palo Alto, CA). Four recombinants were sequenced totally using [^{35}S]αATP and Sequenase, and three were found to contain no PCR errors.

Functional expression of hamster NK-2 receptor. mRNA was transcribed *in vitro* using T7 polymerase from a pMAMneoBlue-hamster NK-2 receptor clone that had been linearized by digestion with *Sal*I and then with *Xba*I. An *in vitro* transcription kit (Promega, Madison, WI) was used, and the mRNA was capped with M7GpppG (Boehringer). The synthesized mRNA (approximately 5 ng of mRNA/oocyte) was microinjected into *Xenopus* oocytes.

Electrophysiological recordings were made at least 24 hr after injection, using standard two-electrode voltage clamp techniques (24) with a holding clamp potential of −60 mV and stepping by ±20 mV.

Tissue expression of hamster NK-2 receptor. RNA-PCR was performed on total RNA prepared from hamster urinary bladder, kidney, lung, and trachea. Total RNA was electrophoresed on formaldehyde agarose gels and visualized by ethidium bromide staining. Each RNA contained intact rRNA, and equivalent amounts of RNA, based on the ethidium bromide staining, were used for cDNA synthesis. First strand cDNA was synthesized with oligo(dT) as primer. The hamster NK-2 receptor amplimers were 5′d(CTGAGCCTAACTCATTTCTGCTGG) and 5′d(TCACTTTGCAGCTCCTAGATTGGC). The primers are predicted to amplify a 1578-bp DNA fragment. The hamster GAPDH amplimers were 5′d(ATGGTGAAGGTCGGCGTGAACGGA) and 5′d(CTCCTTGGAGGCCATGTAGGCCAT), which are predicted to amplify a 999-bp DNA fragment. PCR was performed at 63° for 2 min, 72° for 2 min, and 93° for 2 min for 20, 25, 30, 35, and 40 cycles. Aliquots of each reaction were subjected to electrophoresis on a 1% agarose gel, and DNA was visualized by ethidium bromide staining. NK-2 receptor was also detected by Southern blot hybridization using a ^{32}P-labeled hamster NK-2 receptor cDNA probe.

Expression of human and hamster NK-2 receptors in the LCR/MEL cell system. Heterologous expression in the LCR/MEL cell system was performed essentially as described previously (25). Briefly, human NK-2 receptor cDNA (9) was digested with *Eco*O109 and *Bam*HI, and an adaptor containing a *Hind*III site was ligated to the *Eco*O109 site and cloned into the MEL cell shuttle vector, pMEG3. The entire *Cla*I-Asp718 cassette (containing the human β-globin promoter, part of β-globin exon 2, the second intron, third exon, poly(A) recognition site, 3′ β-globin-flanking DNA, and including the entire cDNA for human NK-2 receptor) was subcloned into the human globin LCR microlocus plasmid, pGSE1417 (26). A similar procedure was used to subclone the entire hamster NK-2 receptor from pMAMneoBLUE as an *Eco*RI-*Sal*I fragment, and recombinant vectors were used for transfection of MEL-C88 cells.

Transfection into MEL-C88 cell line was performed by electroporation, and directly after transfection, cells were diluted in culture medium to 10^4 to 10^5 cells/ml, and 1-ml aliquots were transferred to each well of a 24-well plate. G418 (GIBCO-BRL) was added to a concentration of 1 mg/ml 24 hr after the transfection to select for stable transfectants. Individual clones were picked 7 to 10 days after the addition of selective medium. For expression studies, cells were maintained in exponential growth for a period of 4 days, and then DMSO was added to a final concentration of 2% (v/v) to induce differentiation and hence expression. Samples were taken 96 hr after induction for NKA binding assays and for mRNA and protein analysis. The transfectant with the highest expression levels was grown and induced at 5-liter scale for pharmacological characterization.

Membrane preparation. Membranes from recombinant MEL cells were prepared as follows. Briefly, cells were homogenized at 4° (Brinkman PT-20 Polytron, setting 3, with one 15-sec burst on ice) in a buffer consisting of 50 mM Tris-HCl (pH 7.4), 5 mM KCl, 120 mM NaCl, 10 mM EDTA, and containing several protease inhibitors (1 mM phenylmethylsulfonyl fluoride; 0.1 mg/ml of soybean trypsin inhibitor, and 1 mM iodoacetamide). The homogenate was centrifuged at 1,200 × *g* for 10 min at 4° to remove cell debris. The supernatant was centrifuged at 48,000 × *g* for 45 min at 4°. The pellet was resuspended with a glass-Teflon motorized homogenizer in 30 volumes of ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The suspension was centrifuged at 48,000 × *g* for 30 min at 4° and then was resuspended to a concentration of 1 to 3 mg of protein/ml (n = 3) in assay buffer (20 mM HEPES, pH 7.4, containing 0.02% BSA, 0.1 mM thiorphan, 0.3 mM dithiothreitol, 30 mM KCl, 3 mM $MgCl_2$), and was flash frozen in liquid nitrogen. Samples were stored at −70° until ready for use. No deterioration of receptor binding activity (as evident by consistent K_d or B_{max} values) was observed for periods up to 3 to 4 months.

[^{3}H]NKA binding assays. Incubations were carried out in assay buffer containing membranes, tested competitors, and [^{3}H]NKA (1.0–1.5 nM). In competition experiments, mixtures (0.315 ml) containing various concentrations of competing agents (agonists, antagonists, or vehicle) were incubated at 25° for 30 min, with or without 1 μM NKA (to define nonspecific binding). Reactions were initiated by adding membranes (0.1–0.15 mg of protein/ml final concentration). Separation of receptor-bound from free ligand was accomplished by dilution with 1 ml of wash buffer (20 mM Tris-HCl, pH 7.5) followed immediately by vacuum filtration with a total volume of 10 ml of the wash buffer (utilizing a Brandel Cell Harvester MB-48R with Whatman GF/B filters that were presoaked in 0.1% polyethylenimine).

In saturation-kinetics experiments, incubation conditions were identical to the competition experiments except that the concentration of [^{3}H]NKA varied from 0.05 to 6 nM. Kinetic experiments were also identical to the competition experiments except for the time and ligand, which were varied as indicated in the text.

Data analysis. Computation of equilibrium binding constants (K_d and K_i), receptor density (B_{max}), and kinetic rate constants (K_{obs}, $t_{½}$, k_{-1} and k_1), as well as statistical analysis, were carried out as published previously (17) using GraphPad "InPlot" software (GraphPad Software, San Diego, CA). In addition, to test the possibility that the ligand binds to two receptor populations or subtypes, all equilibrium saturation data were also analyzed with AccuFit Saturation-Two Site software (27), based on the computation methods for ligand binding developed by Feldman (28). The differences between various binding models were tested with ANOVA, and changes were considered significant when $p < 0.05$.

Statistics. Differences between treated groups were compared to controls (GraphPad "Instat" software), and changes were considered significant when $p < 0.05$ using Student's paired t test.

Materials. The synthesis and chemical and pharmacological validation of [4,5-^{3}H-Leu^9]NKA ([^{3}H]NKA: specific activity 137 Ci/mmol) has been described (18). This ligand, NKA, tachykinins, tachykinin analogs, all other peptide hormones, and NKA antagonists (L659,877, R396, and MEN10,376) were purchased from Cambridge Research Biochemicals, (Cheshire, UK) and were >95% pure by HPLC analysis. Thiorphan, SR48,968, and (±)CP96,345, were supplied by the Medicinal Chemistry Department, ZENECA Pharmaceuticals Group (Wilmington, DE). Gpp(NH)p and GTP(γ)S were purchased from Boehringer. All the nonpeptide hormones and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). NKA was dissolved in assay buffer. All drugs and chemicals were dissolved in HPLC

grade DMSO or methanol. Final solvent concentration in the assay did not exceed 0.5% (v/v).

Results

Isolation of hamster NK-2 receptor cDNA. The scheme used to isolate the 5′- and 3′-ends of the hamster NK-2 receptor by PCR-based methods is shown in Fig. 1. These were DNA sequenced on both strands, and the sequences overlapped. The 5′-RACE-PCR product was from position (−)293 to 770, and the 3′-RACE product was from 482 to 1560 (Fig. 2). Once the 5′- and 3′-ends had been identified, the complete cDNA was isolated by PCR using amplimers 25 bp upstream of the ATG initiation codon and 91 bp downstream of the stop codon. Full length products from both a 30 and 40 cycle hamster urinary bladder PCR have been sequenced directly, and a 40-cycle product has been sequenced directly from two separate cDNA synthesis reactions. The sequence obtained agreed completely with the sequence from the overlapping 5′- and 3′-RACE product, and analysis has shown that the sequence is heterozygous at amino acid positions 144 (TCA or TCG; Ser) and at position 253 (GTT or GTA; Val). We have cloned and sequenced the PCR product and used a clone that is identical to that obtained from direct sequencing for further characterization.

The hamster NK-2 receptor consists of 384 amino acids with a relative molecular weight of 43,418 (Fig. 2). This protein shares a significant sequence similarity with other seven-helix

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                                                                                                    TGACTGTGGTGTGAAGGAGCGGAACCAGCGTGCTATCTATCAGGATGCCTCCT -241

GCTCTGAGATTGATTACTCCATCTCCACAAGATGGTTGAATGGTCTGACTGGAAGCCTGTTTTCTGCTGAGCCTAACTCATTTCTGCTGGAGCTGTAGGTGTGGCCCCCTGGCTACACGT -121

GTCTGCTTGTAAATTGTCCTCACTCCCATATATCTTCACACACCAAAGAGGACTTCTCTTCTGTAGCAATTCTAGAAAGAACTGCAGGCCAGACCCTGTGTTCCTGGCCCAGAATCGGCC   -1
 1                                                  *
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MetGlyGlyArgAlaIleValThrAspThrAsnIlePheSerGlyLeuGluSerAsnThrThrGlyValThrAlaPheSerMetProAlaTrpGlnLeuAlaLeuTrpAlaThrAlaTyr   40
ATGGGGGGCCGTGCCATTGTCACTGACACCAACATCTTTTCTGGCCTCGAGAGCAACACCACTGGTGTCACCGCCTTCTCCATGCCTGCCTGGCAGCTGGCACTGTGGGCCACAGCCTAC  120

_________I_____________________________________                                  _____________________________II_____________
LeuGlyLeuValLeuValAlaValThrGlyAsnAlaThrValIleTrpIleIleLeuAlaHisGluArgMetArgThrValThrAsnTyrPheIleIleAsnLeuAlaLeuAlaAspLeu   80
CTGGGTCTGGTGCTGGTCGCTGTAACAGGCAACGCCACAGTCATCTGGATCATTCTGGCCCACGAGAGAATGCGCACAGTCACCAACTATTTCATCATCAACCTGGCCTTGGCAGACCTC  240
_________                                                                                                  ____________III_____
CysMetAlaAlaPheAsnAlaThrPheAsnPheValTyrAlaSerHisAsnIleTrpTyrPheGlyArgAlaPheCysTyrPheGlnAsnLeuPheProIleThrAlaMetPheValSer  120
TGCATGGCAGCCTTCAATGCCACCTTCAACTTTGTCTACGCCAGTCACAACATCTGGTACTTTGGCCGCGCCTTCTGCTATTTCCAGAACCTCTTTCCCATCACAGCCATGTTCGTCAGC  360
_____________________________                                  ____________________________________IV_
IleTyrSerMetThrAlaIleAlaAlaAspArgTyrMetAlaIleValHisProPheGlnProArgLeuSerAlaProIleThrLysAlaThrIleAlaGlyIleTrpLeuValAlaLeu  160
ATCTACTCCATGACTGCCATCGCTGCCGACAGGTACATGGCCATCGTCCACCCCTTCCAGCCACGGCTCTCAGCCCCCATCACCAAGGCGACTATCGCGGGCATCTGGCTGGTAGCTCTG  480
_____________________________                                                                  _____________________
AlaLeuAlaSerProGlnCysPheTyrSerThrIleThrValAspGlnGlyAlaThrLysCysValValAlaTrpProAsnAspAsnGlyGlyLysMetLeuLeuLeuTyrHisLeuVal  200
GCTCTGGCCTCCCCACAGTGCTTCTACTCCACCATCACTGTGGACCAGGGAGCCACCAAGTGTGTGGTGGCCTGGCCTAATGACAACGGAGGCAAGATGCTCCTTCTGTATCATCTGGTG  600
_____________________V_____________________________________
ValPheValLeuValTyrPheLeuProLeuValValMetPheValAlaTyrSerValIleGlyLeuThrLeuTrpLysArgAlaValProArgHisGlnAlaHisGlyAlaAsnLeuArg  240
GTTTTTGTCCTCGTCTACTTCCTACCCCTTGTGGTGATGTTCGTGGCTTACAGTGTCATCGGCCTCACTCTGTGGAAACGCGCGGTCCCCAGACACCAGGCTCACGGTGCCAACCTGCGC  720
                                                  ____________________________________VI_____________________________
HisLeuHisAlaLysLysLysPheValLysAlaMetValLeuValValLeuThrPheAlaIleCysTrpLeuProTyrHisLeuTyrPheIleLeuGlySerPheGlnLysAspIleTyr  280
CATCTGCATGCCAAGAAGAAGTTTGTGAAGGCCATGGTTCTGGTGGTGTTGACATTTGCCATTTGCTGGCTGCCCTACCACCTCTACTTCATCCTGGGTAGCTTCCAGAAGGACATCTAC  840
   ____________________________________________VII_____________________________________________
TyrArgLysPheIleGlnGlnValTyrLeuAlaLeuPheTrpLeuAlaMetSerSerThrMetTyrAsnProIleIleTyrCysCysLeuAsnHisArgPheArgSerGlyPheArgLeu  320
TACCGCAAGTTCATCCAGCAGGTCTACCTGGCGCTCTTCTGGCTAGCCATGAGCTCCACCATGTACAATCCCATCATTTACTGCTGCCTCAACCACAGGTTTCGCTCTGGATTCAGGCTT  960

AlaPheArgCysCysProTrpValThrProThrGluGluAspArgLeuGluLeuThrArgThrProSerLeuSerArgArgValAsnArgCysHisThrLysGluThrLeuPheMetThr  360
GCTTTCCGGTGCTGCCCCTGGGTGACGCCCACTGAGGAAGACAGACTGGAGCTGACTCGCACTCCATCCCTCTCCAGGAGAGTCAACAGGTGTCACACAAAAGAGACTTTGTTCATGACT 1080

AlaAspMetThrHisSerGluAlaThrAsnGlyGlnValGlySerProGlnAspValGluProAlaAlaPro***
GCGGACATGACCCACTCTGAGGCTACCAATGGCCAGGTTGGGAGTCCCCAAGATGTGGAGCCTGCCGCACCCTGAAGTCTTGAGACTGTCTGCAGGGCCAGGCTTAGTCCCTTTGAGAAA 1200

CAGCTGATAGAAAAGGCCTGATGTAATAGCTTGTCCCACACTCCATTCTTCAACCACCAATAGAAAAACAAGATGGTGCCTGGAGCTCCACAGGCAATGCTAATACAAAGAGCTCACATA 1320

CCCACCATAAGCATTCCTGGGATGGAGGAAAGAGCCAGGAAGACAGGGAGCCAACACTTGGCCAATCTAGGAGCTGCAAAGTGAAATGTCTCCCTGCAGTATAAAGATGGATGGGAAAAC 1440

TGGATCTCATTAAAGATAACTGCAGGTGTCATGAGAAGAGGCAGTTGAAGGGATGATGAGATGGTAAAGGCACTTGCTGCCTAGCCTGGTGACCTGAGTTCAATTCCTTAGCCTACATGG 1560
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Fig. 2. Nucleotide sequence of the hamster NK-2 receptor cDNA. The DNA sequence of the hamster urinary bladder NK-2 receptor gene and the deduced amino acid sequence, beginning with the initiator methionine, is shown. The nucleotide residues are numbered with respect to the cDNA sequence with the A of the initiator methionine numbered as 1, and nucleotides on the 5′-side of nucleotide 1 being indicated by negative numbers. A postulated N-glycosylation site is indicated by an *asterisk*. Positions of the putative TMS I–VII of NK-2R are indicated above the amino acid sequence.

G protein-coupled receptors and, in particular, is highly homologous to the rat, bovine, and human NK-2 receptors (6, 9, 10) (94.3, 84.4, and 86.5%, respectively) particularly in the seven TMSs (Fig. 3). Significant variability occurs in the 5′- and 3′-regions of the coding sequences. Human and bovine receptors have two potential N-glycosylation sites at Asn residues 11 and 19 of the predicted proteins; however, hamster receptor has a single site at residue 19. The third cytoplasmic loop and the cytoplasmic C-terminal tail have several serine and threonine residues. These residues have the potential to be phosphorylated by serine/threonine protein kinases, thus suggesting that receptor function is regulated by protein kinases.

Electrophysiological characterization of hamster NK-2 receptor. Hamster NK-2R mRNA was transcribed *in vitro* from the pMAMneoBLUE construct, and this RNA was microinjected into *Xenopus* oocytes. Forty-eight hr after injection, the oocytes were used for electrophysiological measurement, and the hamster NK-2 receptor-injected oocytes showed marked increases in membrane conductance to applications of NKA peptide at 10^{-8} to 10^{-6} M (140 and 220 nA, respectively at 10^{-8} and 10^{-7} M NKA) (data not shown). Water-injected oocytes were used as controls, and the peptides were applied at the same concentrations as for hamster NK-2 receptor-injected oocytes. No responses were observed, even at 10^{-6} M NKA. These results confirm that the hamster NK-2 receptor sequence described here encodes a functional NKA receptor.

Tissue expression of hamster NK-2 receptor. The RNA-PCR technique was used to compare NK-2 receptor mRNA levels in hamster tissues. A product of the expected size (1578 bp) was visible and was most intense in urinary bladder (Fig. 4). Because of the faint signals with ethidium bromide, the gels were Southern blot hybridized with ^{32}P-labeled hamster NK-2 receptor cDNA probe. The results show that all four tissues examined express NK-2 receptors, and that urinary bladder has the highest, and kidney the lowest, levels of receptor. PCR was also performed using primers recognizing hamster GAPDH (29) as a control to demonstrate that equivalent amounts of tissue RNA were used for cDNA synthesis. A GAPDH product of 999 bp was visible in equivalent amounts in each of the RNA-PCRs, and products were clearly visible after 20 or 25 cycles, compared to 35 or 40 cycles for NK-2 receptor. No products were visible with negative controls performed in the absence of cDNA. Genomic DNA PCRs also failed to give any products with either NK-2 receptor or GAPDH primers. The gene structure of hamster NK-2 receptor and GAPDH has not been published; however, it is likely that both contain multiple exons, as this is the case for these gene sequences from all other species investigated. We have evidence that the hamster NK-2 receptor gene contains an intron in the position corresponding to intron I in other tachykinin receptors (unpublished observations). Control experiments have shown that the hamster genomic DNA was capable of amplification by PCR.

DNA sequencing of the entire hamster trachea PCR product has shown that this sequence is identical to hamster urinary bladder NK-2 receptor.

Characterization of the hamster NK-2R expressed in MEL cells. We utilized the ligand [4,5-^{3}H-Leu^9]NKA (18) to characterize the properties of the hamster NK-2R in a binding assay. Binding was highly specific (89 ± 2%, $n = 3$) and protein-dependent in a range of 0.1 to 1.0 mg of protein/ml. Similar specific binding in the same protein-concentrations range was observed with the human NK-2R (94 ± 3%, $n = 3$). In contrast, we did not detect meaningful binding in nontransfected (non-induced or induced) cells (<5% specific binding with <3 fmol of [^{3}H]NKA bound/mg of protein). Specific binding levels were enhanced in a concentration-dependent manner (in the range of 0.1–10 mM) by divalent cations ($Mg^{2+} > Ca^{2+}$) and nearly doubled at 3 mM $MgCl_2$. This effect was largely mediated by doubling the apparent site-density (i.e., B_{max}) without affecting ligand affinity (*i.e.*, K_d, data not shown).

Kinetic analysis of [^{3}H]NKA binding illustrates that binding was rapid, concentration-dependent, and reached equilibrium proportional to ligand concentration (Fig. 5A). There was a significant ($p < 0.003$) linear correlation ($r = 0.9977$) of Kobs versus ligand concentration (Fig. 5B). The kinetic rate constants calculated from the slope (k_1) and Y′ intercept (k_{-1})

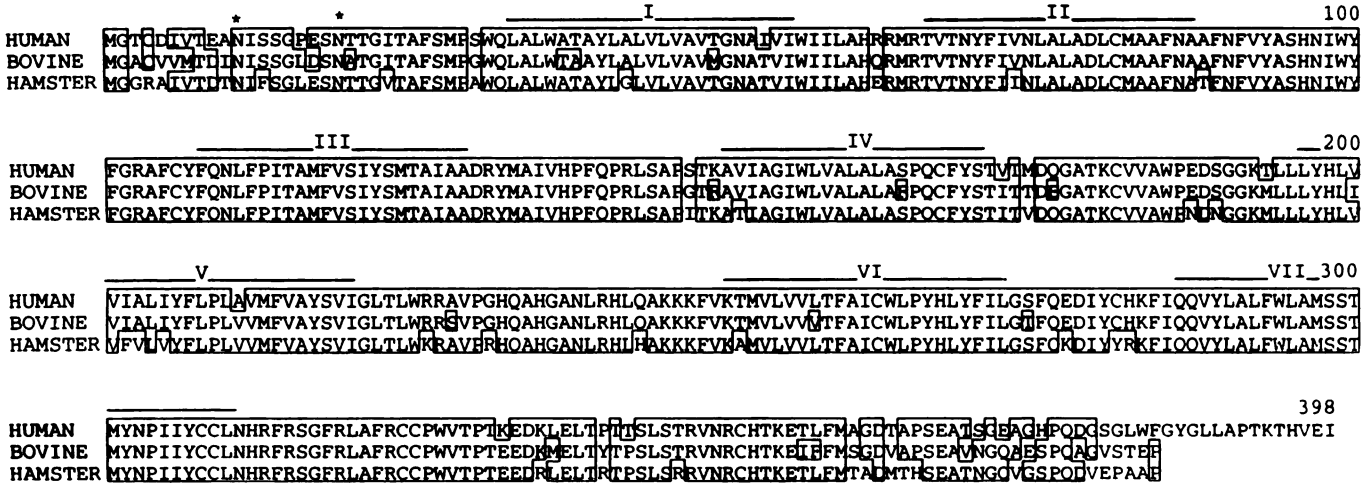

Fig. 3. Alignment of the amino acid sequences of human, bovine, and hamster NK-2 receptors. Human and bovine NK-2 receptors belong to the proposed NK-2 receptor subtype, NK2A, and hamster NK-2 receptor to the NK-2B subtype based on antagonist binding profiles. Aligned residues identical in at least two different receptors are shown *boxed*. The positions of the two potential glycosylation sites (Asn11 and Asn19) in human and bovine receptors are shown with *asterisks*. In hamster NK-2 receptor, only Asn19 has the potential to be glycosylated.

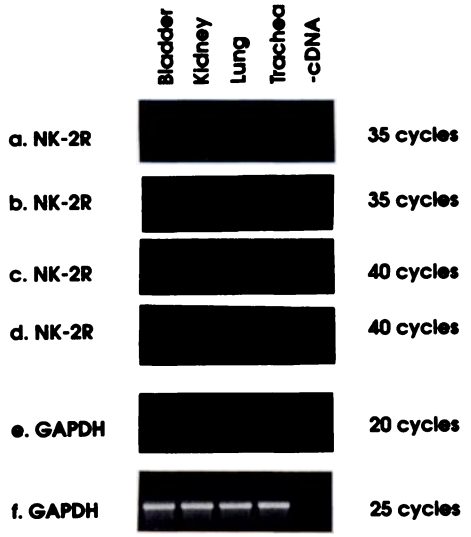

Fig. 4. Analysis of hamster NK-2 receptor expression. RNA was prepared from hamster urinary bladder, kidney, lung, and trachea and was subjected to RNA-PCR analysis using NK-2 receptor (NK-2R) or GAPDH oligonucleotide primers. The number of PCR cycles for respective lanes is indicated. PCR was also performed in the absence of cDNA (as a negative control). Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining (*a*, *c*, *e*, and *f*) or by Southern blot hybridization using a hamster NK-2 receptor cDNA probe (*b* and *d*). Both primer pairs generated a single product of the correct size; therefore, only this region of the agarose gel is presented.

data were: $k_1 = 0.0065$ nM^{-1} * min^{-1}; $k_{-1} = 0.0422$ min^{-1}, and $k_{-1}/k_1 = 6.5$ nM.

In parallel experiments with the human NK-2R (conducted in the same ligand concentration range), we observed the following kinetic rates: $k_1 = 0.048$ nM^{-1} * min^{-1}; $k_{-1} = 0.295$ min^{-1} ($r = 0.9971$, $p < 0.003$), which yielded an identical $k_{-1}/k_1 = 6.1$ nM. In both receptors, the data could only fit a mono-exponential rate equation, excluding the existence of a second site within the range tested (0.15–6 nM ligand).

[^{3}H]NKA binding to hamster NK-2 receptor was reversible (Fig. 6), in that NKA rapidly dissociated bound [^{3}H]NKA in a bi-exponential manner that could be resolved into fast and slow kinetic components (Table 1). Addition of Gpp(NH)p, together with either NKA or SR48,968, caused a faster dissociation than either ligand alone (Fig. 6), which was caused by shifting some of the binding sites to the faster dissociating component (53–55% with Gpp(NH)p versus 41–45% in control), with concomitant two-fold enhancement of the slow dissociation phase ($t_{½}$ = 25–29 min with Gpp(NH)p versus 46–54 min in control, Table 1). These data suggest that the hamster NK-2 receptors in MEL cells are coupled to G Proteins (see below) and can undergo GTP-induced transition from high to low affinity states.

The potent, nonpeptide NK-2 antagonist SR48,968 (30, 31), as well as R396 and L659,877 (the peptide and cyclic-peptide NK-2 selective antagonists) (32, 35), equally and rapidly dissociated bound ligand in a nearly indistinguishable manner (Fig. 6, Table 1). In contrast, at equal concentration (3 μM) to the other ligands, MEN10,376 (the peptide antagonist selective for rabbit and guinea pig NK-2 receptors) (16, 33) induced a much slower and incomplete dissociation ($t_{½}$ = 31 min). However, at >30 μM, this antagonist was able to dissociate bound ligand similar to the other antagonist, reflecting its weaker affinity to the hamster receptor (data not shown).

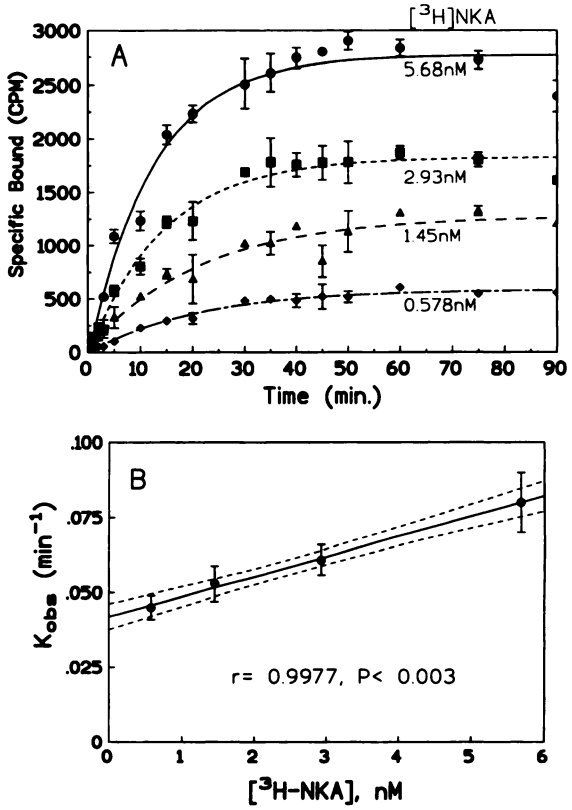

Fig. 5. Kinetics of [^{3}H]NKA association to hamster NK-2R-transfected MEL cells membranes. Membranes were incubated with the indicated concentrations of [^{3}H]NKA for 90 min at 25°. Nonspecific binding was defined with 1 μM NKA. A: Data were fit with a rate equation: Bt = Beq * $(1 - e^{(-K_{obs} * t)})$. B: Kobs determined from the kinetic data were plotted as a function of ligand concentration and were fit to the equation: $k_{obs} = k_1 * [L] - k_{-1}$, to obtain association ($k_1 = 0.0065$ nM^{-1} * min^{-1}) and dissociation ($k_{-1} = 0.0422$ min^{-1}) rate constants to yield kinetic $K_d(k_{-1}/k_1) = 6.5$ nM. Data are mean of triplicate from a typical experiment. A regression analysis demonstrated a significant linear correlation ($p < 0.003$, $r = 0.9977$). *Dashed lines* indicate 95% confidence limit.

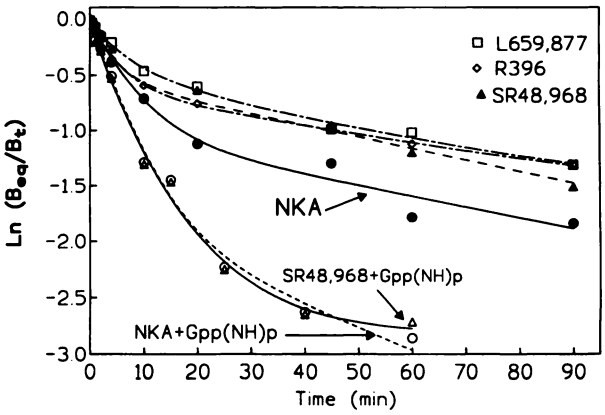

Fig. 6. Dissociation kinetics of [^{3}H]NKA from hamster NK-2R-transfected MEL cells membranes. Membranes were equilibrated for 30 min with approximately 2 nM [^{3}H]NKA before initiating dissociation with 3 μM of the indicated competitor. Data were collected for another 90 min and were fit with a bi-exponential rate equation: Bt = $Beq_1 * e^{(-K_{-1} * t)} + Beq_2 * e^{(-K-2 * t)}$. All dissociation data fit significantly better ($p < 0.05$, ANOVA) to a bi-exponential kinetic model. The data was linearly transformed and plotted as Ln (Bt/Beq) versus time (min) to better illustrate the two kinetic components. Data are mean of triplicate determination from a typical experiment.

TABLE 1

Kinetic analysis of [^{3}H]NKA dissociation from cloned hamster NK-2 receptors expressed in MEL cells

Results are mean ± standard error from two experiments (except for [SR-48,968 + Gpp(NH)p] and L659,877, where n = 1) in triplicate.

Compound	Fast component		Slow component	
	$t_{½}$	Sites	$t_{½}$	Sites
	min	%	*min*	%
NKA	2.7 ± 1.7	45	46 ± 18	55
NKA + Gpp(NH)p	2.4 ± 1.4	53	29 ± 13	47
SR48,968	2.1 ± 0.6	41	54 ± 8	59
SR48,968 + Gpp(NH)p	1.0 ± 0.2	55	25 ± 4	45
R396	2.9 ± 0.6	48	47 ± 9	52
L659,877	4.9 ± 1.3	38	75 ± 13	62

NKA and SR48,968 also completely and rapidly ($t_{½}$ = 2–4 min) dissociated bound [^{3}H]NKA from the human receptor, but unlike the hamster receptor, NKA-induced dissociation was mono-exponential from a single population of binding sites (data not shown).

The saturation analysis of the equilibrium [^{3}H]NKA binding to hamster NK-2 receptor illustrates binding to a single class of high affinity and saturable receptors (Fig. 7A). The binding constants obtained in three experiments were: K_d = 4.14 ± 0.31 nM; B_{max} = 679 ± 26 fmol/mg of protein (mean ± standard error). This equilibrium affinity value is in good agreement with the k_{-1}/k_1 = 6.1 nM derived from kinetic experiments. In these saturation experiments, a two-site model could not fit the data. However, in competition experiments conducted with a higher concentration of NKA, we did detect a small population (10 ± 1%) of lower affinity receptors (K_{d2} = 150 ± 92 nM).

Fig. 7B illustrates that [^{3}H]NKA bound in a saturable manner to human NK-2R. However, unlike the hamster receptor, Scatchard analyses (Fig. 7B, insert) illustrated that [^{3}H]NKA bound to low and high affinity populations of the human receptors: K_{d1}, = 0.59 ± 0.22 nM; B_{max1} = 815 ± 350 fmol/mg of protein and K_{d2}, = 13 ± 4.7 nM; B_{max2} = 2955 ± 1270 fmol/mg of protein. The data fit a two-site model better than a single site ($p < 0.05$ ANOVA, run test $p > 0.05$). The data from several independent experiments were: K_{d1}, 0.37 ± 0.11 nM; B_{max1} = 234 ± 175 fmol/mg of protein and K_{d2}, = 9.0 ± 1.9 nM; B_{max2} = 1989 ± 990 fmol/mg of protein (mean ± standard error, n = 5, $p < 0.05$ by simultaneous ANOVA of all data). These experiments also suggest that the human receptor was expressed at a higher level than the hamster receptor (judged by total density values of B_{max} = 2223 versus 679 fmol/mg of protein, respectively). The observations of two affinity states for the human NK-2R are similar to our findings with this receptor expressed in the baculovirus-infected Sf-21 insect cells (17).

Fig. 8 illustrates that under optimal conditions (3 mM $MgCl_2$), binding of [^{3}H]NKA to hamster (Fig. 8A) and human (Fig. 8B) receptors was inhibited by GTP analogs. However, Fig. 8 also illustrates that the extent and potency of inhibition were significantly greater in human NK-2R than observed with the hamster receptor (30–40% with 0.3 and 3 μM for GTP(γ)S and Gpp(NH)p, n = 2, respectively, in the hamster compared with IC_{50} = 0.7 and 7.5 nM for GTP (γ)S and Gpp(NH)p, n = 2, respectively, in the human). The greater inhibition of the human receptor by GTP analogs appears to be an intrinsic property of the human receptor itself and not caused by the host cell where it was transfected, because we observed near identical inhibition with human NK-2R expressed in the baculovirus-infected Sf-21 insect cells (17). The weak and incomplete inhibition by GTP analogs of [^{3}H]NKA binding to the cloned hamster receptor is similar to that observed in native tissue (18, 34).

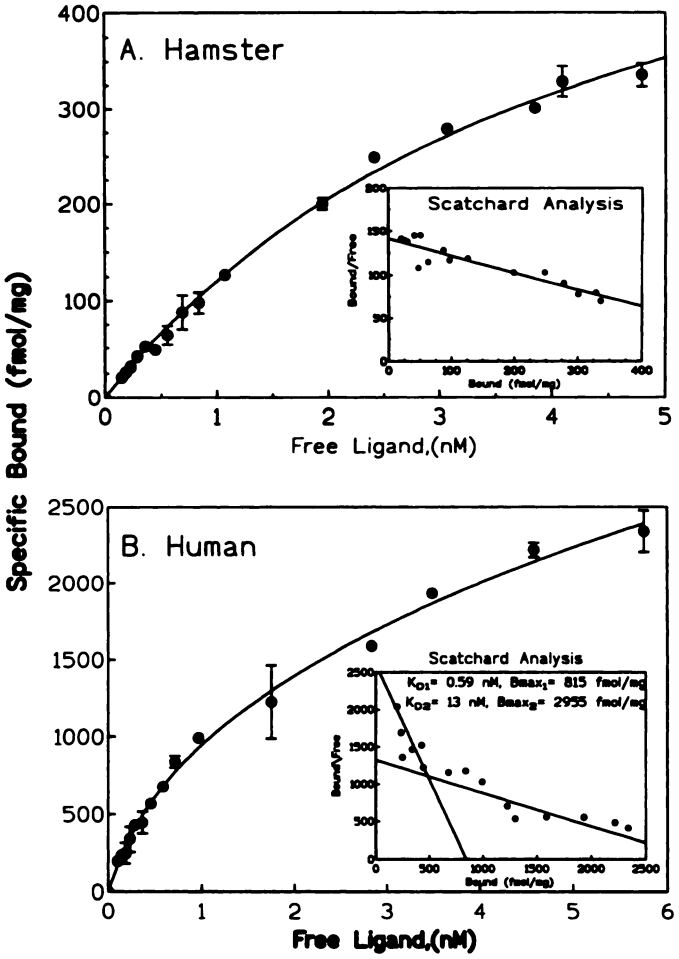

Fig. 7. Saturation analysis of [^{3}H]NKA binding to MEL cell membranes containing *A)* hamster or *B)* human NK-2 receptors. Membranes were incubated with [^{3}H]NKA (0.05–6.0 nM) under standard conditions. Data was fit to the saturation equation $B = B_{max1} * [F]/(K_{d1} + [F]) + B_{max2} * [F]/(K_{d2} + [F])$ and linearized according to the method of Scatchard (*insert*). In hamster (*A*) data did not fit a two-site model better than single site ($p > 0.05$), whereas in human (*B*) there was a significantly better fit ($p < 0.05$) to a two-site model. Data are mean of triplicate determination from a representative experiment.

The pharmacology of the hamster and human NK-2 receptors in MEL cells was explored with a series of selective NK-2 agonists and antagonists. Fig. 9 illustrates that tachykinin agonists potently inhibited [^{3}]NKA binding to hamster (Fig. 9A), or human (Fig. 9B) NK-2R, with a similar rank order of potency: NKA > [Nle^{10}]NKA(4–10) > [β-Ala^8)NKA(4–10) ≫ SP, and senktide = inactive. Table 2 illustrates that NKA bound with a greater proportion to the high affinity sites (90 ± 1%) and was also significantly ($p < 0.04$) more potent than the other NK-2 agonists (K_i = 1.35 ± 0.21, 6.18 ± 0.16, and 9.08 ± 3.83 nM, mean ± standard error, n = 3, for NKA, [Nle^{10}] NKA(4–10), and [β-Ala^8]NKA(4–10), respectively). A similar trend was observed in the human receptor (Table 2). NKA was marginally, but significantly ($p < 0.05$), more potent (2–3-fold)

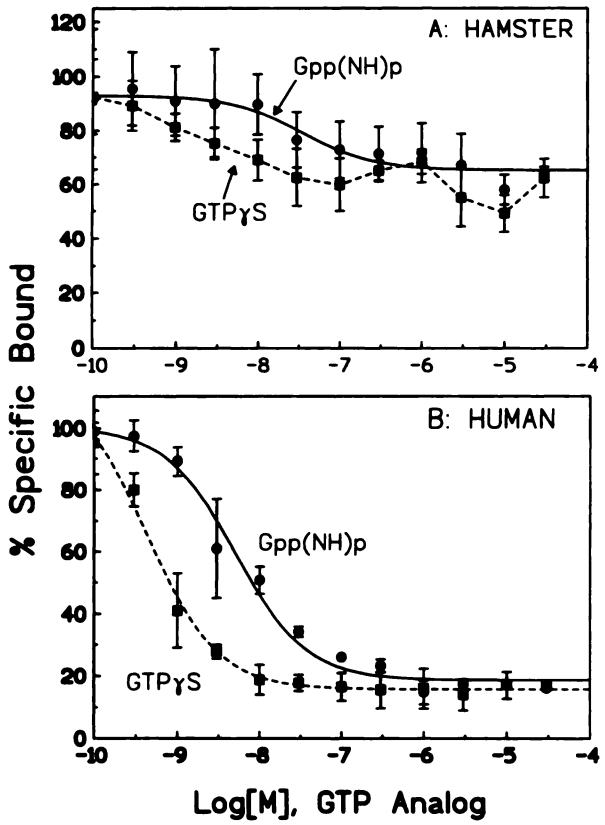

Fig. 8. Inhibition of [^{3}H]NKA binding to NK-2 receptors by GTP analogs. Membranes containing hamster (*A*) or human (*B*) NK-2 receptors were incubated with [^{3}H]NKA (1.0 nM) in Mg^{2+} (3 mM) containing HEPES buffer (20 mM. pH 7.4) for 30 min at 25° in the presence of increasing concentrations of GTP(γ)S or Gpp(NH)p. Specific binding (%) was calculated relative to controls in the absence of GTP analogs. Data are mean of quadruplicate from a representative experiment. In hamster, significant inhibition ($p < 0.05$) is observed with GTP analogs > 10 nM or 100 nM for GTP(γ)S or Gpp(NH)pt, respectively.

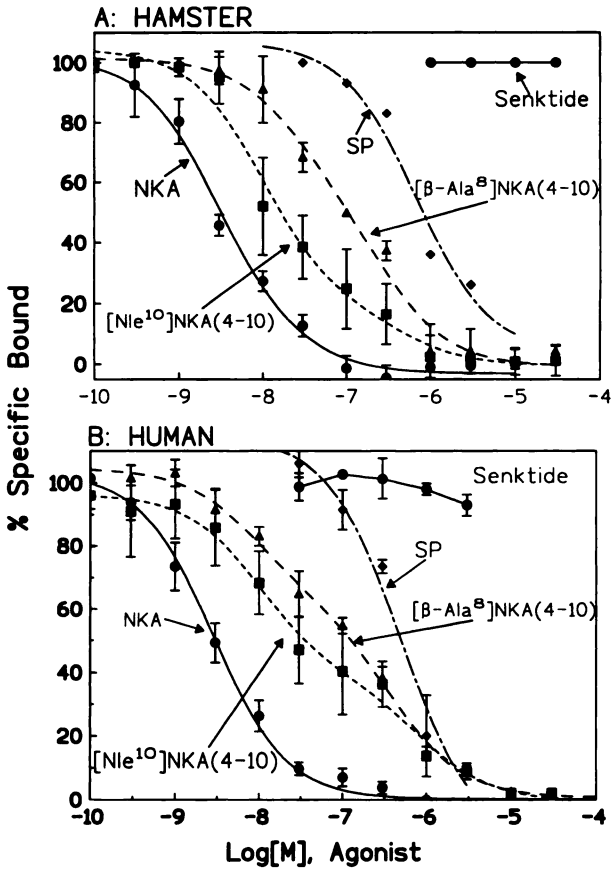

Fig. 9. Inhibition of [^{3}H]NKA binding to MEL cells membranes by tachykinin agonists. Membranes containing hamster (*A*) or human (*B*) NK2 receptors were incubated with 2 nM ligand in the presence of increasing concentrations of agonists. Nonspecific binding was determined with 1 μM NKA. With both receptors, data for NKA and selective NK-2 agonists fit significantly better a two-site binding model (see Table 1). SP data fit a single-site model. Data are expressed as percentage of control and are from a representative experiment determined in quadruplicate.

than the other agonists at the low affinity sites in both receptors (Table 2). Interestingly, unlike the preferential binding of NKA and [Nle^{10}]NKA(4–10) to the high affinity sites in hamster and human alike, [β-Ala^8]NKA(4–10) bound predominantly to the low affinity sites, similar to our previous observation with the human receptor expressed in the baculovirus Sf-21 cells. This preference may explain the apparent overall weaker potency of this agonist in competition experiments (Fig. 9), despite affinity constants (K_i) comparable to [Nle^{10}]NKA(4–10) at both binding sites.

In contrast to the NK-2 selective ligands, SP bound equipotently to both hamster and human receptors, demonstrating 300- to 600-fold less potency than NKA, whereas the selective NK-3 agonist senktide was inactive.

We further characterized the pharmacology of the hamster (and human) receptors with selective NK-2 antagonists. Fig. 10 illustrates that SR48,968 was the most potent antagonist in both hamster (Fig. 10A) and human (Fig. 10B) receptors. Results obtained in smooth muscle contraction assays have demonstrated 10- to 15-fold higher potency for SR48,968 in human bronchus versus hamster trachea or urinary bladder (pA_2 = 9.40 and 8.11, respectively) (31). In contrast, Table 2 illustrates that SR48,968 was equipotent (pK_i = 8.9) in both receptors. The potency observed in human receptor expressed in MEL cells was the same as that observed for the same receptor expressed in the baculovirus Sf-21 cells (9.01 ± 0.10 nM) (17).

In hamster NK-2 receptor, Fig. 10A (and Table 2) illustrates that R396 was a much more potent antagonist than MEN10,376 (126-fold), which is in excellent agreement with our observation in native hamster urinary bladder (172-fold) (18), In contrast, Fig. 10B illustrates an inverse order of potency in human NK-2R, where MEN10,376 was much more potent than R396 (55-fold, Table 2). Table 1 also illustrates that the cyclic-peptide antagonist L659,877 (35) was the most potent peptide antagonist of hamster NK-2 receptor. In contrast, L659,877 was 21-fold less potent in human than in hamster receptor (Table 2). Regardless of NK-2 receptor species or absolute potency, all antagonists' inhibition curves were compatible with binding to a single population of receptors.

Thus, the observed order of potency in the cloned hamster NK-2 receptor of SR48,968 = L659,877 > R396 ≫ MEN10,376 is in excellent agreement with our observations in the native hamster urinary bladder membranes, and is remarkably different from that observed with the human NK-2 receptor of SR48,968 ≫ MEN10,376 > L659,877 > R396 (Table 3). The order of affinities obtained in the present studies with the human NK-2 receptor are identical to those observed by us for the same cloned human NK-2 receptor expressed in Sf-21 cells (Table 3), demonstrating further that its pharmacological prop-

TABLE 2

Pharmacological analysis of cloned NK-2 receptors expressed in MEL cells

pK_i(-LogK_i) data are mean ± standard error from n = three to five experiments with cloned hamster or human receptors, determined in two to four replicates. When errors are not reported, n = 2. NA, not active up to 10 μM; ND, not detected.

Cloned NK-2R	Hamster				Human			
	Site 1		Site 2		Site 1		Site 2	
	pK_i	Sites	pK_i	Sites	pK_i	Sites	pK_i	Sites
		%		%		%		%
Agonists								
NKA	8.89 ± 0.07	90 ± 1	7.08 ± 0.38	10 ± 1	9.04 ± 0.16	91 ± 2	7.15 ± 0.33	9 ± 2
[Nle^{10}]NKA(4–10)	8.21 ± 0.01	75 ± 7	6.57 ± 0.03	26 ± 7	8.48 ± 0.13	68 ± 6	6.42 ± 0.15	32 ± 6
[β-Ala^8]NKA(4–10)	8.11 ± 0.17	49 ± 11	6.52 ± 0.17	51 ± 11	8.35 ± 0.16	46 ± 6	6.59 ± 0.15	54 ± 6
SP	ND	—	6.37	100	ND	—	6.18	100
Senktide	NA	—	—	—	NA	—	NA	—
Antagonists								
SR48,968	8.86 ± 0.08	100	ND	—	8.88 ± 0.12	100	ND	
L659,877	8.69 ± 0.13	100	ND	—	7.69 ± 0.03	100	ND	
R396	8.39 ± 0.08	100	ND	—	6.79 ± 0.05	100	ND	
MEN10,376	6.28 ± 0.08	100	ND	—	8.49 ± 0.02	100	ND	
CP96,345	—	—	NA	—	—	—	NA	—

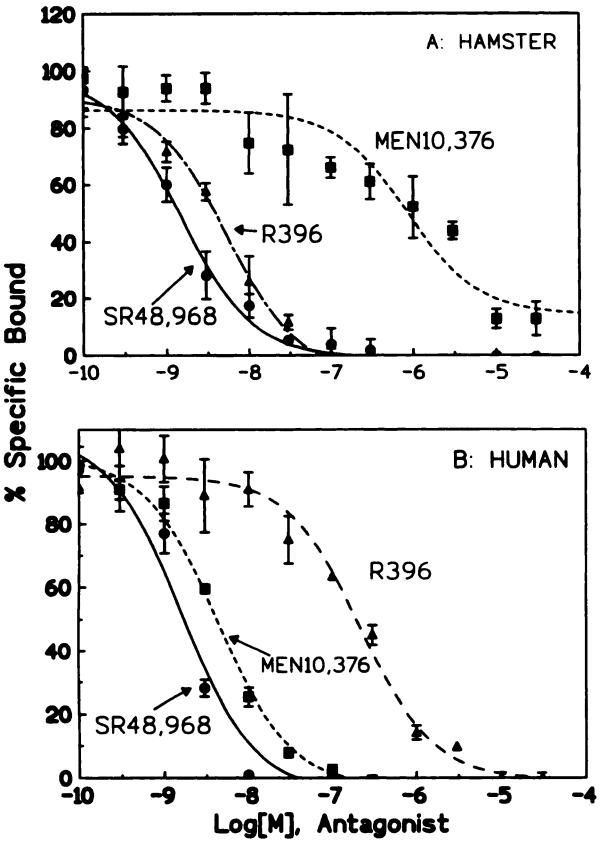

Fig. 10. Inhibition of [^{3}H]NKA binding to MEL cells membranes by selective NK-2 antagonists. Membranes containing hamster (*A*) or human (*B*) NK2 receptors were incubated with 2 nM ligand in the presence of increasing concentrations of antagonists. Nonspecific binding was determined with 1 μM NKA. Data are expressed as percentage of control and are from a representative experiment determined in quadruplicate.

erties were independent of the host cells. The rank order of antagonists' affinity in the cloned receptors is consistent with the proposed NK-2 receptor's pharmacological classification of NK-2A (human) and NK-2B (hamster) in native tissues.

Discussion

We report here the isolation of cDNA for hamster NK-2 receptor from urinary bladder and its functional expression in Xenopus oocytes. Heterologous expression and detailed pharmacological characterization has been done in the LCR/MEL cell system. In this system, the cDNA is inserted between the human β-globin promoter and the second intron of the β-globin gene. This expression cassette is then placed downstream of the human globin LCR and is then transfected into MEL cells. This system does not depend on gene amplification, is independent of the integration position of the expression cassette, and allows stable high level expression within a few weeks.

The pharmacology for the recombinant hamster NK-2 receptor expressed in MEL cells is very similar to that previously described for hamster urinary bladder membranes (18). As in the native tissue, the binding of [^{3}H]NKA to the cloned receptor appears to be stimulated by divalent cations and inhibited (albeit incompletely) by GTP analogs. This is in contrast to the human receptor expressed in either MEL cells or baculovirus Sf-21 cells (18), where GTP analogs potently inhibit agonist binding. However, in both receptors GTP analogs inhibited binding by shifting the majority of NK-2 receptors to a lower affinity state and increasing dissociation rates. The weak inhibition seen with the cloned hamster NK-2R is similar in its weak response in native tissue (17, 34), whereas the potent effects of GTP analogs on human receptor is observed in both insect Sf-21 (17) and mammalian (MEL) host cells. Thus, we propose that the major difference between these two NK-2 receptors is probably an intrinsic property of the receptors and does not appear to be caused by the heterologous expression.

NK-2 receptors are characterized pharmacologically in airway, vascular, and nonvascular smooth muscle in many peripheral tissues. We have studied the expression of NK-2 receptor mRNA in several hamster tissues by RNA-PCR. The NK-2 receptor reported here was isolated from urinary bladder and, therefore, we used the expression in this tissue as a positive control. We also examined the expression in airways (trachea and lung), which have been used extensively for pharmacology, and also in kidney. Hamster urinary bladder contained the highest levels of NK-2 receptor mRNA. Trachea and lung also expressed the receptor at lower levels than bladder, which agrees with pharmacological data for these tissues. Detectable levels were found in kidney, and NK-2 receptors have been reported previously in guinea pig renal pelvis (36). In other species (human, rabbit, and guinea pig), NK-2 receptors also

TABLE 3

Comparative pharmacological properties of selective NK-2 peptide antagonists in NK-2 receptors

c-hNK-2R-BVsf21, cloned human NK-2 receptor expressed in BVSf-21 cells; c-hNK-2R-MELs, cloned human NK-2 receptor expressed in MEL cells; c-HUBM-MELs, cloned hamster urinary bladder NK-2 receptor expressed in MEL cells; n-HUBM, native NK-2 receptor in hamster urinary bladder membranes.

Receptor	Rank order (pK_i)	Ref.
c-hNK-2R-BVsf21	SR48,968 (9.01) > MEN10,376 (7.97) > L659,877 (7.54) > R396 (6.59)	17
c-hNK-2R-MELs	SR48,968 (8.88) > MEN10,376 (8.49) > L659,877 (7.69) > R396 (6.79)	This work
c-HUBM-MELs	SR48,968 (8.86) > L659,877 (8.69) > R396 (8.39) > MEN10,376 (6.28)	This work
n-HUBM	SR48,968 (8.61) > L659,877 (8.13) > R396 (7.88) > MEN10,376 (5.84)	18

have been shown pharmacologically to be present in urinary bladder, trachea, and bronchus.

NKA and selective NK-2 agonist binding appears to invoke transition of affinity states, because we observed two populations of receptors with high and low affinity binding. Antagonists did not invoke this transition because they inhibited in a manner compatible with a single affinity state, regardless of chemical structure or absolute potency. This was a consistent observation for both receptors. Moreover, we consistently observed that [β-Ala^8]NKA(4–10) bound predominantly to the low affinity sites in both receptors, which may explain its apparent weaker binding compared with [Nle^{10}]NKA(4–10), despite nearly identical affinities at both high and low affinity sites at either receptor. In addition, both hamster and human low affinity NK-2 receptors appear to bind all NK-2 agonists, as well as SP, with a nearly uniform affinity with K_i = 100 to 300 nM. Overall, except for minor kinetic differences and the higher affinity for NKA displayed by the human receptor, agonists do not distinguish between these NK-2 receptor subtypes.

In contrast to studies with NK-2 agonists, a major difference in receptor pharmacology is observed with antagonists (Table 3). Although the nonpeptide antagonist SR48,968 displays similar affinity in human and hamster receptors, this is not the case with R396 or MEN10,376, which demonstrate great selectivity toward hamster and human receptors, respectively. The major difference in potency is conserved between native receptors in hamster urinary bladder and the cloned receptor expressed in MEL cells. Similarly, there is a consistent pattern of inhibition by antagonists observed with the human receptor, regardless of the host cell (Table 3).

The predicted protein sequence of hamster NK-2 receptor now can be compared to human and bovine NK-2 receptors, making it possible to identify amino acids that potentially may cause the pharmacological differences. Studies on G protein-coupled receptors, whose endogenous agonists are small nonpeptide molecules, have demonstrated that the binding sites for these agonists are located within the transmembrane domains (37, 38). Because the tachykinin peptides NKA, NKB, and SP are larger than these nonpeptide agonists, it seems likely that the extracellular domains of the tachykinin receptors comprise part of the peptide binding site. Recent studies support this hypothesis (11, 12). The NK-2 receptors from human, bovine, and hamster tissues are highly conserved, except at the N- and C-termini, where they show least homology (Fig. 3). These data further demonstrate that minor sequence differences between the receptors from different species can lead to pronounced pharmacological differences, in agreement with recent studies on 5-hydroxytryptamine$_{1B}$ and 12–15 lipoxygenase (39, 40). The results reported here provide the basis for further experiments using site-directed mutagenesis to determine the structural elements within the receptors that are responsible for conferring the differential pharmacology in specific species, which has been reported. Understanding the cause of the pharmacological difference observed for species variants of NK-2 receptors will not only clarify the structure/function of the receptor, but also provide a rational basis for the selection of suitable *in vivo* animal models for drug discovery.

Acknowledgments

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References

1. Maggio J. Tachykinins. *Ann. Rev. Neurosci.* **11**:11–28 (1988).
2. Regoli, D. and F. Nantel. Pharmacological of neurokinin receptors. *Biopolymers* **31**:777–783 (1991).
3. Regoli, D., G. Drapeau, S. Dion, and R. Couture. New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. *Trends Pharmacol. Sci.* **9**:290–295 (1988).
4. Buckner, C. K., S. V. Ghanekar, J. S. Kays, R. D. Krell, R. I. Fishleder, J. A. Will, and J. M. Vann. Pharmacological studies of tachykinin receptors mediating contraction of isolated airway smooth muscle. *Ann. N. Y. Acad. Sci.* **629**:340–358 (1991).
5. Frossard, N. and P. Barnes. Effect of tachykinins in small human airways. *Neuropeptides* **19**:157–161 (1991).
6. Masu, Y. K., Nakayama, H. Tamaki, Y. Harada, M. Kuno, and S. Nakanishi. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature (Lond)* **329**:836–838 (1987).
7. Gerard, N. P., R. L. Eddy, T. B. Show, and C. Gerard. The human neurokinin A (substance K) receptor. *J. Biol. Chem.* **265**:20455–20462 (1990).
8. Kris, R. M., V. South, A. Saltzman, S. Felder, G. A. Ricca, M. Jaye, K. Huebner, J. Kagan, C. M. Croce, and J. Schlessinger. Cloning and expression of human substance-K receptor and analysis of its role in mitogenesis. *Cell Growth Differ.* **2**:15–22 (1991).
9. Graham, A., B. Hopkins, S. J. Powell, P. Danks, and I. Briggs. Isolation and characterization of human lung NK-2 receptor gene using rapid amplification of cDNA ends. *Biochem. Biophys. Res. Commun.* **177**:8–16 (1991).
10. Sasai, Y. and S. Nakanishi. Molecular characterization of rat substance K receptor and its mRNAs. Biochem. Biophys. Res. Commun. **165**:695–702 (1989).
11. Yokota, Y., C. Akazawa, H. Ohkubo, and S. Nakanishi. Delineation of structural domains involved in the subtype specificity of tachykinin receptors through chimeric formation of substance P/substance K receptors. *EMBO J.* **11**:3585–3591 (1992).
12. Fong, T. M., R.-R.C. Huang, and C. D. Strader. Localization of agonist and antagonist binding domains of the human neurokinin 1 receptor. *J. Biol. Chem.* **267**:25664–25667 (1992).
13. Fong, T. M., H. Yu, and C. D. Strader. Molecular basis for the species selectivity of the neurokinin 1 receptor antagonists CP-96,345 and RP67580. *J. Biol. Chem.* **267**:25668–25671 (1972).
14. Sachais, B. S., R. M. Snider, J. A. Lowe III, and J. E. Krause. Molecular basis for the species selectivity of the substance P antagonist CP-96,345. *J. Biol. Chem.* **268**:2319–2323 (1993).
15. Maggi, C. A., R. Patacchini, S. Guiliani, P. Rovero, S. Dion, D. Regoli, A. Giachetti, and A. Meli. Comparative antagonists discriminate between NK-2 receptor subtypes. *Br. J. Pharmacol.* **100**:588–592 (1990).
16. Maggi, C. A., S. Giuliani, L. Ballati, A. Lecci, S. Manzini, R. Patacchini, R. Renzetti, P. Rovero, L. Quartara, and A. Giachetti. *In Vivo* evidence for tachykinergic transmission using a new NK-2 receptor-selective antagonist, MEN 10,376. *J. Pharmacol. Exp. Ther.* **257**:1172–1178 (1991).

17. Aharony, D., J. Little, S. Powell, B. Hopkins, K. R. Bundell, W. L. McPheat, R. Gordon, G. Hassall, R. Hockney, R. Griffin, and A. Graham. Pharmacological characterization of cloned human neurokinin A (NK-2) receptor expressed in a baculovirus-Sf21 insect cell system. *Mol. Pharmacol.* **44**:356–363 (1993).
18. Aharony, D., G. E. Conner, and D. P. Woodhouse. Pharmacologic characterization of binding of the novel ligand [4,5-^{3}H-Leu^9]-Neurokinin A to NK-2 receptors on hamster urinary bladder membranes. *Neuropeptides* **23**:121–130 (1992).
19. Chirgwin, J. M., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
20. Frohman, M. A., M. K. Dush, and G. R. Martin. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**:8998–9002 (1988).
21. Frohman, M. A. and G. R. Martin. Rapid amplification of cDNA ends using nested primers. *Technique* **1**:165–170 (1990).
22. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487–494 (1988).
23. Newton, C. R., N. Kalsheker, A. Graham, S. Powell, A. J. Gammack, J. Riley, and A. F. Markham. Diagnosis of a-1 antitrypsin deficiency by enzymatic amplification of human genomic DNA and direct sequencing of polymerase chain reaction products. *Nucleic Acids Res.* **16**:8233–8243 (1988).
24. Miledi, R. A. Calcium-dependent transient outward current in Xenopus laevis oocytes. *Proc. R. Soc. Lond. B Biol. Sci.* **215**:365–373 (1982).
25. Needham, M., C. Gooding, K. Hudson, M. Antoniou, F. Grosveld, and M. Hollis. LCR/MEL: A versatile system for high-level expression of heterologous proteins in erythroid cells. *Nucleic Acids Res.* **20**:997–1003 (1992).
26. Talbot, D., P. Collis, M. Antoniou, M. Vidal, F. Grosveld, and D. R. Greaves. A dominant control region from the human β-globin locus conferring integration site-independent gene expression. *Nature* **338**:352–355 (1989).
27. Hawkins, E. F. AccuFit Saturation-Two site: a new program for rapid and automated analysis of data from receptor saturation assays, Beckman Technical Information Bulletin: T-1666-NUC-89-4 (1989).
28. Feldman, H. A. Mathematical theory of complex ligand-binding systems of equilibrium: some methods for parameter fitting. *Anal. Biochem.* **48**:317–338 (1972).
29. Vincent, S. and P. Fort. Nucleotide sequence of hamster glyceraldehyde 3 phosphate dehydrogenase mRNA. *Nucleic Acids Res.* **18**:3054 (1990).
30. Emonds-Alt, X., P. Vilain, P. Goulaouic, V. Proietto, D. Van Broek, C. Advenier, E. Naline, G. Neliat, G. Le Fur, and J. C. Breliere. A potent and selective non-peptide antagonist of the neurokinin A (NK2) receptor. *Life Sci.* **50**:101–106 (1992).
31. Advenier, C., N. Roussi, Q. T. Nguyen, X. Emonds-Alt, J-C. Brelier, G. Neliat, E. Naline and D. Regoli. Neurokinin A (NK2) receptor revisited with SR 48,968, A potent non-peptide antagonist. *Biochem. Biophys. Res. Commun.* **184**:1418–1424 (1992).
32. Maggi, C. A., R. Patacchini, M. Astoli, P. Rovero, A. Giachetti, and P. L. M. Van Giersbergen. Affinity of R396, an NK-2 tachykinin receptor antagonist, for NK-2 receptors in preparation from different species. *Neuropeptides* **22**:93–98 (1992).
33. Maggi, C. A., A. Eglezos, L. Quartara, R. Patacchini, and A. Giachetti. Heterogeneity of NK-2 tachykinin receptors in hamster and rabbit smooth muscles. *Regul. Pept.* **37**:85–93 (1992).
34. Van Giersbergen, P. L. M., S. A. Shatzer, A. K. Henderson, J. Lai, S. Nakanishi, H. I. Yamamura, and S. H. Buck. Characterization of a tachykinin peptide receptor transfected into murine fibroblasts B82 cells. *Proc. Natl. Acad. Sci. USA* **88**:1661–1665 (1991).
35. McKnight, A. T., J. J. Maguire, A. E. Elliott, A. E. Fletcher, A. C. Foster, R. Tridgett, B. J. Williams, J. Longmore, and L. L. Iversen. Pharmacological specificity of novel, synthetic, cyclic peptides as antagonists at tachykinin receptors. *Br. J. Pharmacol.* **104**:355–360 (1991).
36. Eglezos, A., C. A. Maggi, R. Patacchini, L. Quartara, S. Guiliani, and A. Giachetti. NK-2 tachykinin receptors in the guinea pig renal pelvis. *Neuropeptides* **22**:15 (1992).
37. Strader, C. D., I. S. Sigal, and R. A. F. Dixon. Structural biases of β-adrenergic receptor function. *FASEB J.* **3**:1825–1832 (1992).
38. Khorana, H. G. Rhodopsin, photoreceptor of the rod cell. *J. Biol. Chem.* **267**:1–4 (1992).
39. Oksenberg, D., S. A. Marsters, B. F. O'Dowd, H. Jin, S. Havlik, S. J. Peroutka, and A. Ashkenazi. A single amino acid difference confers major pharmacological variation between human and rodent 5-HT 1B receptors. *Nature* **360**:161–163 (1992).
40. Sloane, D. L., R. Leung, C. S. Craik, and E. Sigal. A primary determinant for lipoxygenase positional specificity. *Nature* **354**:149–152 (1991).

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