0026-895X/93/020356-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:356-363

Pharmacological Characterization of Cloned Human NK-2 (Neurokinin A) Receptor Expressed in a Baculovirus/Sf-21 Insect Cell System

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Received December 29, 1992; Accepted May 7, 1993

SUMMARY

Using the novel ligand [4,5-^{3}H-Leu^9]neurokinin A ([4,5-^{3}H-Leu^9]NKA) in a receptor binding assay, we characterized the pharmacology of a cloned neurokinin NK-2 receptor from human lung (hNK-2R), expressed in baculovirus-infected Sf-21 insect cells. Functional hNK-2R cDNA clones were isolated from human lung using a polymerase chain reaction-based methodology. hNK-2R was cloned into pAcYM1, a vector designed to couple expression to the polyhedrin promoter, and the recombinant baculovirus was isolated and used to infect Sf-21 insect cells. hNK-2R expression levels were monitored by Northern blots and ^{125}I-NKA binding assays. Isolates demonstrating the highest specific binding of ^{125}I-NKA were grown and membrane preparations from high-speed centrifugations were prepared from both hNK-2R-expressing and wild-type virus-infected cells. [^{3}H]NKA bound in a protein-dependent, saturable (B_{max} = 820 ± 167 fmol/mg of protein), and highly specific (88 ± 5%) manner to hNK-2R, but not to membranes from cells infected with wild-type virus (14 ± 8%, 7 ± 10 fmol/mg of protein). [^{3}H]NKA binding was rapid (k_1 = 0.085 nM^{-1}·min^{-1}) and reversible ($t_{½}$ = 4–5 min). Equilibrium binding experiments demonstrated binding to a mixture of receptors in high and low affinity states (K_{d1} = 2.28 ± 0.26 nM and K_{d2} = 266 ± 91 nM). Binding to hNK-2R was greatly enhanced (400%-600%) by Ca^{2+} and Mg^{2+} (EC_{50} values of 30 μM and 140 μM, respectively), whereas guanosine-5′-*O*-(3′-thio)triphosphate and guanosine-5′-(β,γ-imido)diphosphate were inhibitory. Competition experiments with agonists also demonstrated binding to high and low affinity states, with the following order of potency: NKA > [Nle^{10}]NKA(4–10) > [β-Ala^8]NKA(4–10) ≫ substance P; Senktide and the NK-1 antagonist CP96,345 (10 μM) did not inhibit binding. Inhibition of binding by selective NK-2 antagonists was consistent with a single affinity state and demonstrated the following order of affinity: SR48,968 ≫ MEN10,376 > L659,877 > R396. These data suggest that infection of Sf-21 cells with baculovirus expression vector harboring the cDNA of hNK-2R resulted in expression of high affinity, G protein-coupled hNK-2R, with pharmacological selectivity compatible with the NK-2A receptor subtype.

NKA, a decapeptide discovered a decade ago (1, 2), belongs to a class of peptide hormones known as tachykinins, which share a common carboxyl-terminal sequence of Phe-X-Gly-Leu-Met-NH_2. The three mammalian tachykinins, SP, NKA, and NKB, possess an X substitution of phenylalanine, valine, and valine, respectively, in the common carboxyl terminus (3).

NKA has been shown to be widely distributed in the central nervous system as well as in peripheral organs and tissues, where it binds to NK-2 receptors, thereby causing contraction of airway, vascular, and nonvascular smooth muscle (3–7). These actions may implicate NKA (and other tachykinins) as primary candidates for causing the underlying hyper-responsiveness seen in asthmatic patients.

After the cloning and characterization of the bovine NK-2 receptor (8), several groups cloned the hNK-2R and expressed it in a functionally active form in various cell types (9–11). These studies have demonstrated that the hNK-2R possesses seven putative transmembrane domains, suggesting that it is coupled to G proteins, and that hNK-2R is selectively activated by NKA, compared with SP or NKB. These experiments provided an understanding, at the molecular level, of the differences between NK-2 and NK-1 or NK-3 tachykinin receptors.

However, none of the former studies investigated the interaction of the cloned human receptor with NK-2-selective agonists (versus NK-1- or NK-3-selective ligands), nor was the pharmacology of the recently described peptide (12–14) or

ABBREVIATIONS: NKA, neurokinin A; SP, substance P; NKB, neurokinin B; hNK-2R, human neurokinin-2 receptor(s); BV.Sf-21, baculovirus-infected Sf-21 insect cells; GTPγS, guanosine-5′-*O*-(3′-thio)triphosphate; Gpp(NH)p, guanosine-5′-(β,γ-imido)diphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EC_{50}, concentration causing 50% of maximal effect; IC_{50}, concentration causing 50% inhibition; AcNPV, *Autographica californica* nuclear polyhedrosis virus; ANOVA, analysis of variance; NK, neurokinin.

nonpeptide (15) NK-2 antagonists investigated in this system. Moreover, studies by Maggi *et al* (12,14), using peptide antagonists, strongly suggested the existence of two NK-2 receptor subtypes, NK-2A and NK-2B. More recently, using the NK-2 peptide antagonists MEN10,376 and R396 in functional, smooth muscle receptor assays, these investigators provided additional detailed evidence for the pharmacological heterogeneity of NK-2 receptors in various species, further supporting the original classification (22, 23). Based on these studies, NK-2 receptors in guinea pig and rabbit vascular and airway smooth muscle have been classified as NK-2A and receptors in hamster urinary bladder and trachea have been classified as NK-2B.

In contrast to these studies using functional receptor assays, no such evidence has yet been described from the molecular cloning and expression of recombinant rat, bovine, or human NK-2 receptors. Some of this discrepancy may arise from the different methodologies used in these studies. More recently, using the novel ligand [4,5-^{3}H-Leu^9]NKA (16) and highly selective NK-2 antagonists in receptor ligand binding assays, we provided additional evidence that receptors in hamster urinary bladder belong to the NK-2B subtype (16). Thus, it is of particular importance to characterize the pharmacology of, and perhaps classify, the hNK-2R.

In the present report we demonstrate that hNK-2R expressed in BV.Sf-21 possess high affinity and selectivity towards NK-2 ligands, are G protein coupled, and display pharmacological selectivity that is compatible with a NK-2A receptor subtype.

Materials and Methods

Baculovirus/insect cell expression. The molecular cloning of a functional hNK-2R has been described recently (11). The plasmid vector pMAMneoBlue containing the hNK-2R cDNA was restriction digested with *Eco*O109I and *Bam*HI, and the cDNA fragment was purified. The oligonucleotide 5′-d(GATCCACCTATAAATATGGG) was annealed to the oligonucleotide 5′-d(GTCCCCATATTTATAGG TG), which had been 5′ phosphorylated using polynucleotide kinase. This linker/adaptor was ligated into the *Eco*O109 site and the resulting fragment was cloned into the *Bam*HI site of pAcYM1 (17) and transformed into the *Escherichia coli* strain DH5α. The underlined sequence in the oligonucleotide corresponds to the 10-base sequence normally found in front of the initiation codon of the polyhedrin gene, and the bold sequence corresponds to the ATG initiation codon of the hNK-2R.

Recombinant plasmids with the insert in the correct orientation were isolated and plasmid DNA was purified by CsCl density gradients. DNA sequencing across the *Bam*HI sites confirmed that the expected sequences were present. Recombinant baculovirus (AcNPV) expressing hNK-2R under the control of the polyhedrin promoter was prepared using the co-transfection procedure of Summers and Smith (18). Briefly, *Spodoptera frugiperda* IPLB-Sf-21-AE cells grown in TC100 medium containing 10% fetal bovine serum were co-transfected by the calcium phosphate procedure with wild-type AcNPV DNA (1 μg) and recombinant pAcYM1-hNK-2R DNA (2 μg). Culture supernatants containing both recombinant and wild-type viruses were collected after 4 days of incubation at 27°. Plaques of recombinant virus on BV.Sf-21 were identified by microscopic inspection for the absence of polyhedra. hNK-2R-recombinant AcNPV were purified free of wild-type virus through two to four rounds of plaque purification. Confirmation of the virus obtained as hNK-2R-recombinant baculovirus was by Northern blot analysis of RNA prepared from BV.Sf-21, using a ^{32}P-labeled hNK-2R cDNA probe. For hNK-2R expression, Sf-21 cells were infected with recombinant virus at a multiplicity of infection of approximately 10 and were harvested after 48 hr of incubation at 27°.

To prepare large amounts of hNK-2R for pharmacological studies, the *S. frugiperda* cell line Sf-21 was grown in TC100 medium supplemented with 10% fetal bovine serum and 0.2% Pluronic F68, in a 20-liter stirred tank bioreactor (Chemap). At a cell density of 2.7×10^6 viable cells/ml the culture was infected with hNK-2R-recombinant baculovirus at a multiplicity of infection of 1.0. Growth at 27° was continued for an additional 54 hr and cells were recovered by centrifugation. The clones that demonstrated the highest binding of ^{125}I-NKA were scaled up to 6 liters, harvested, frozen in liquid nitrogen, and kept at −70° until used.

Membrane preparation. BV.Sf-21 membranes from cells infected with recombinant or wild-type viruses were prepared as described (16). Briefly, cells were homogenized at 4° (Brinkman PT-20 Polytron, setting 6, 15-sec bursts for 2 min on ice) in a buffer consisting of 50 mM Tris·HCl, pH 7.4, 5 mM KCl, 120 mM NaCl, and 10 mM EDTA and containing several protease inhibitors (100 mg/ml leupeptin, 20 μg/ml phenylmethylsufonyl fluoride, and 100 μg/ml soybean trypsin inhibitor). The homogenate was filtered through gauze and centrifuged at $1200 \times g$ for 10 min at 4° to remove cell debris. The supernatant was filtered again and centrifuged at $48{,}000 \times g$ for 30 min at 4°. The pellet was resuspended with a glass-Teflon motorized homogenizer in 30 volumes of ice-cold 50 mM Tris·HCl buffer, pH 7.4, containing 300 mM KCl and 10 mM EDTA and was incubated for 60 min at 4° with stirring. The suspension was centrifuged at $48{,}000 \times g$ for 30 min at 4° and then resuspended and centrifuged again.

The final membrane pellet was resuspended to a concentration of 2.23–3.71 mg of protein/ml in 50 mM Tris·HCl buffer, pH 7.4. Samples were stored at −70° until ready for use. No deterioration of receptor binding activity (as evident by consistent K_d and B_{max} values) was observed for periods of up to 2–4 months.

[^{3}H]NKA binding assays. Incubations were carried out in buffer consisting of 20 mM HEPES, pH 7.4, with 0.02% bovine serum albumin, 0.1 mM thiorphan, 0.3 mM dithiothreitol, 30 mM KCl, 0.3 mM $CaCl_2$, and [^{3}H]NKA (1.0–1.5 nM). Under these conditions, specific binding was consistently >85%. In competition experiments, incubation 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 (final concentration, 0.15 mg of protein/ml). 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 (using a Brandel MB-48R cell harvester equipped with Whatman GF/B filters that had been presoaked in 0.1% polyethylenimine).

In saturation kinetics experiments, incubation conditions were identical to those for the competition experiments except that the concentration of [^{3}H]NKA varied from 40 pM to 8.5 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 binding constants (K_d and K_i), receptor density (B_{max}), and kinetic rate constants (K_{obs}, k_{-1}, and k_1) and statistical analysis were carried out as published previously (16), using GraphPad InPlot software. 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 (19), based on the computation methods for ligand binding developed by Feldman (20). The differences between various binding models were tested with ANOVA and changes were considered significant for $p < 0.05$.

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

Materials. The synthesis and chemical and pharmacological validation of [4,5-^{3}H-Leu^9]NKA (specific activity, 137 Ci/mmol) have been described (16). 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

and were >95% pure by high performance liquid chromatographic analysis. Thiorphan, (±)-SR48,968, and (±)-CP96,345 were supplied by the Medicinal Chemistry Department, ICI Pharmaceuticals Group. Gpp(NH)p and GTPγS were purchased from Boehringer Mannheim. Sf-21 cells and pAcYM1 were obtained from R. D. Possee, National Environment Research Council Institute of Virology (Oxford, UK). All nonpeptide hormones and all other chemicals were purchased from Sigma Chemical Co. NKA was dissolved in assay buffer. All drugs and chemicals were dissolved in high performance liquid chromatography-grade dimethylsulfoxide or methanol. Final solvent concentrations in the assay did not exceed 0.5% (v/v).

Results

Fig. 1A illustrates that binding of [^{3}H]NKA to hNK-2R in BV.Sf-21 was highly specific (88 ± 5%, three experiments) and protein dependent (107 ± 19 fmol/mg of protein, three experiments). Fig. 1B also illustrates that, in contrast to recombinant cells, wild-type control cells demonstrated much lower ($p < 0.02$) and insignificant binding levels (14 ± 8%, 7 ± 10 fmol/mg of protein).

Fig. 2 illustrates that at low concentrations (0.1–3 mM) divalent cations greatly enhanced [^{3}H]NKA binding and that this effect was more prominent in HEPES buffer than in Tris·HCl buffer. Ca^{2+} was more potent (EC_{50} values of 31 ± 4 μM and 139 ± 17 μM, respectively; $p < 0.005$) and more efficacious than Mg^{2+} (6.4-fold versus 4.2-fold), regardless of the buffer used. Fig. 2 further illustrates that, with the exception of Tris·HCl with Mg^{2+}, all concentrations of either cation of >30 μM (with either buffer) demonstrated significantly ($p < 0.02$) higher binding than did the corresponding EDTA controls. The combination of Mg^{2+} plus Tris·HCl was significantly ($p < 0.05$) stimulatory only at 1–10 mM. Interestingly, at higher concentrations (>3 mM), both cations significantly inhibited ligand binding.

Under optimal conditions (HEPES buffer with 0.3 mM Ca^{2+}), Fig. 3 illustrates that binding of [^{3}H]NKA was potently inhibited by GTP analogs [IC_{50} values of 1 nM and 12 nM for GTPγS

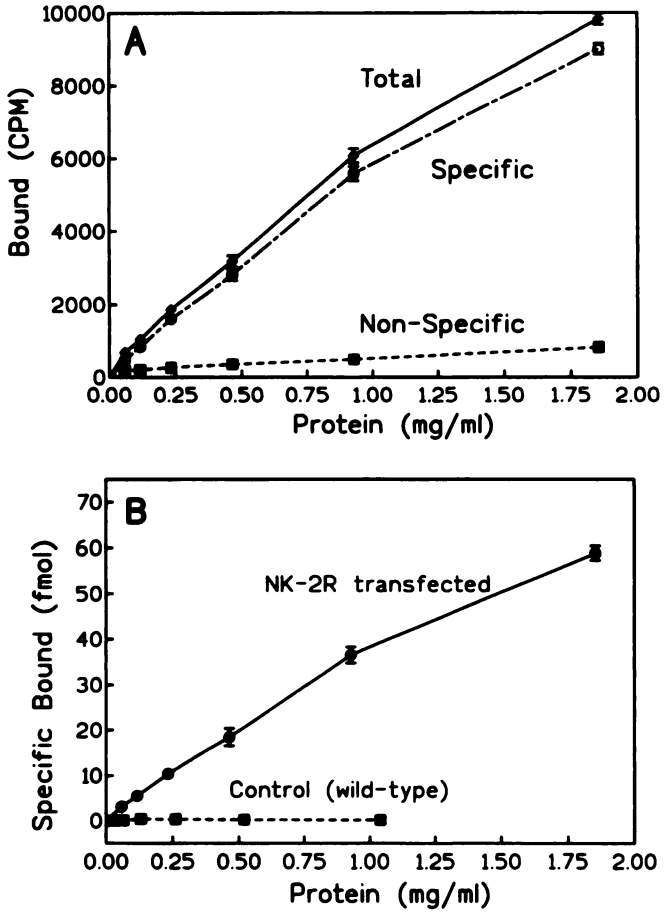

Fig. 1. Analysis of protein-dependent binding of [^{3}H]NKA. A, Binding to human NK-2 receptor-infected BV.Sf-21 membranes. Serial dilutions of membranes were incubated with 2 nM ligand. Protein content was determined with the Bio-Rad reagent. Nonspecific binding was determined with 1 μM NKA. B, Comparison of specific binding levels in receptor-transfected and wild-type controls. Data (mean ± standard deviation) are expressed as specific binding, in cpm (A) or fmol (B), from a typical experiment determined in triplicate. Standard error bars are not shown when they are smaller than the symbols.

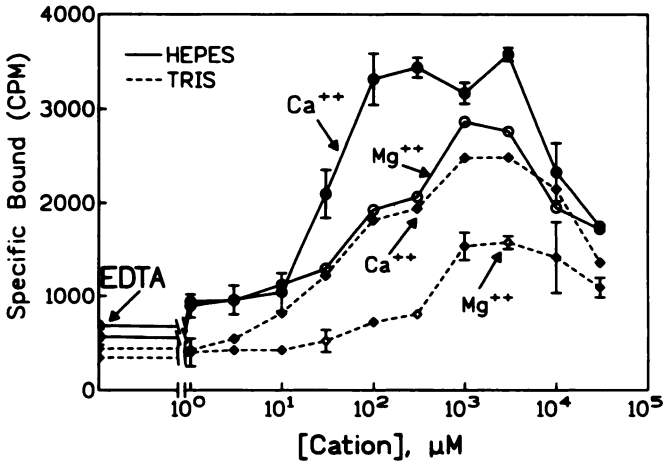

Fig. 2. Stimulation of [^{3}H]NKA binding to BV.Sf-21 membranes by divalent cations. Membranes were divided and incubated with [^{3}H]NKA (1.28 nM) in Tris·HCl or HEPES, in the presence of increasing concentrations of the indicated cation. A, Specific binding (cpm) was plotted for EDTA (1 mM)-treated controls and cation-treated membranes. Data are from a typical experiment determined in triplicate. Standard error bars are not shown when they are smaller than the symbols.

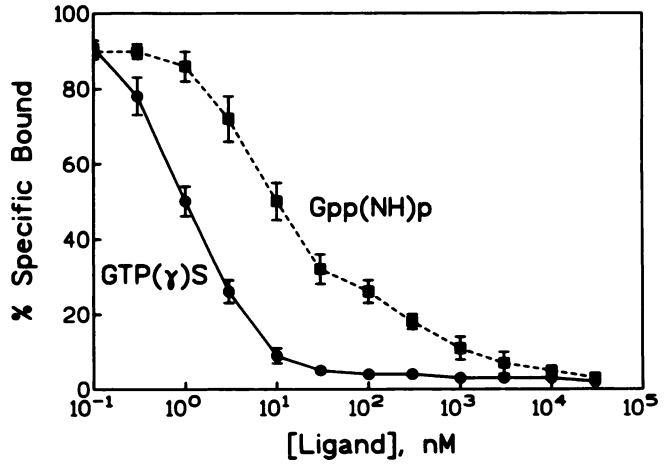

Fig. 3. Inhibition of [^{3}H]NKA binding to BV.Sf-21 membranes by GTP analogs. Membranes containing hNK-2R were incubated with [^{3}H]NKA (1.3 nM) in Ca^{2+} (0.3 mM)-containing HEPES buffer (20 mM, pH 7.4) for 30 min at 25°, in the presence of increasing concentrations of GTP(γ)S or Gpp(NH)p. Specific binding (percentage) was calculated relative to controls in the absence of GTP analogs. Data are mean ± standard deviation from two experiments, with duplicate determinations.

and Gpp(NH)p, respectively]. These data, combined with the stimulation by divalent cations, suggest that the cloned hNK-2R are coupled to G proteins in BV.Sf-21 and are prone to agonist-dependent transition of high and low affinity states (see below).

Kinetic analysis of [^{3}H]NKA binding (Fig. 4A) indicated that binding was rapid, was concentration dependent, and reached equilibrium proportionally to the ligand concentration. Fig. 4B demonstrates a significant ($p < 0.05$) linear correlation ($r = 0.8723$) of K_{obs} with ligand concentration. The kinetic rate constants calculated from the slope (k_1) and y-axis (k_{-1}) data were $k_1 = 0.085$ nM$^{-1}\cdot$min^{-1}, $k_{-1} = 0.124$ min^{-1}, and kinetic K_d (k_{-1}/k_1) = 1.47 nM. The calculated dissociation half-life ($t_{1/2}$) from these experiments was 5.9 min.

To further explore the kinetics of NKA binding, we conducted dissociation experiments with agonist and antagonists. Fig. 5 illustrates that binding was reversible and that agonist and antagonists could completely and rapidly dissociate bound [^{3}H]NKA. Dissociation of bound [^{3}H]NKA induced by antagonists (SR48,968 and MEN10,376) followed a monoexponential decay model, with very similar $t_{1/2}$ values (Table 1). In contrast, the agonist (NKA)-induced dissociation was biexponential and could be resolved into a fast component and a slow component

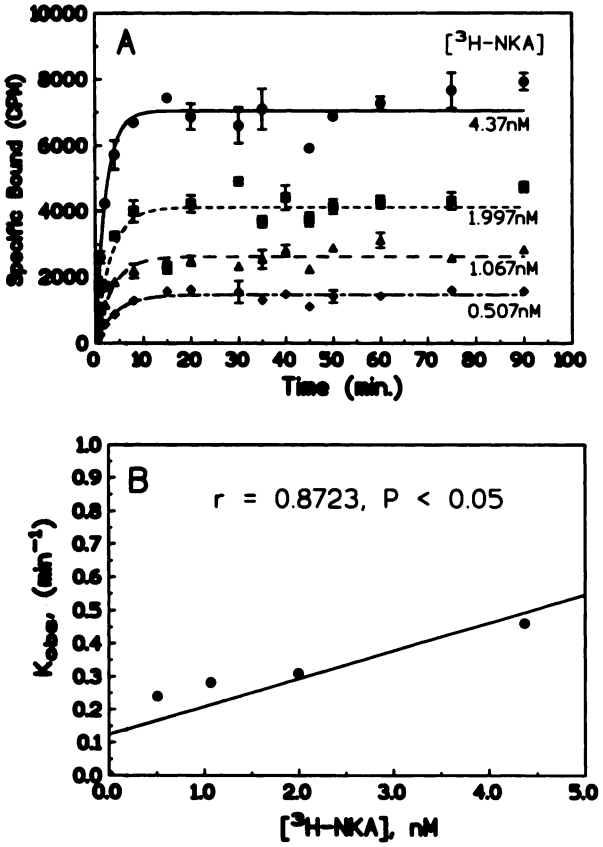

Fig. 4. Kinetics of [^{3}H]NKA association to BV.Sf-21 membranes. Membranes were incubated with the indicated concentrations of [^{3}H]NKA for 90 min at 25°. Nonspecific binding was defined with 1 μM NKA. A, Data were fit with a rate equation, $B_t = B_{eq}\cdot[1 - e^{(-Kobs\cdot t)}]$. B, K_{obs} determined from the kinetic data was plotted as a function of ligand concentration and fit to the equation $K_{obs} = k_1\cdot[L] - k_{-1}$, to obtain the association (k_1 = 0.085 nM$^{-1}\cdot$min^{-1}) and dissociation (k_{-1} = 0.124 min^{-1}) rate constants to yield kinetic K_d (k_{-1}/k_1) = 1.47 nM. Data are mean ± standard error of triplicate determinations from a typical experiment. Regression analysis demonstrated a significant linear correlation ($p < 0.05$, $r = 0.8723$) between ligand concentrations and K_{obs} values.

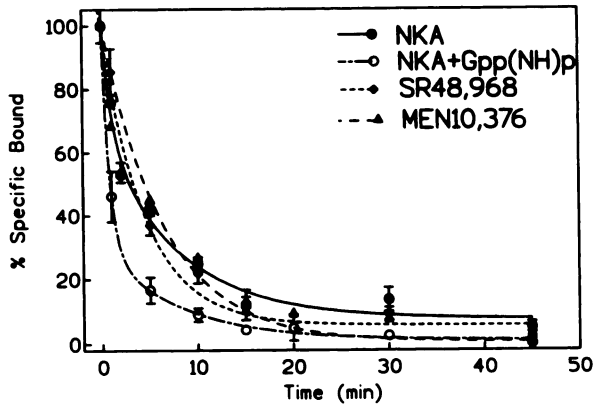

Fig. 5. Kinetics of dissociation of [^{3}H]NKA from BV.Sf-21 membranes induced by NKA and antagonists. Membranes were equilibrated for 30 min with 1.94 nM [^{3}H]NKA, before initiation of dissociation with 3 μM concentrations of the indicated compound. Data were collected for another 120 min and were fit with a biexponential rate equation, $B_t = B_{eq1}\cdot e^{(-K_{-1}\cdot t)} + B_{eq2}\cdot e^{(K_{-2}\cdot t)}$. NKA- and NKA- plus Gpp(NH)p-induced dissociations fit significantly better ($p < 0.05$, ANOVA) to a biexponetial model, whereas antagonist-induced dissociations fit a monoexponential dissociation. Data are mean ± standard error from two experiments, with triplicate determinations.

TABLE 1

Kinetic analysis of ligand binding to cloned hNK-2R in BV.Sf-21

Results are mean ± standard error from a typical experiment determined in tripicate.

Compound	Fast component		Slow component	
	$t_{1/2}$	Sites	$t_{1/2}$	Sites
	min	%	*min*	%
NKA	0.72 ± 0.13	41	5.32 ± 1.14	59
NKA + Gpp(NH)p	0.55 ± 0.07	70	5.62 ± 1.54	30
SR48,968	Undetected		3.11 ± 0.58	100
MEN10,376	Undetected		4.20 ± 0.82	100

(Table 1), suggesting the existence of two affinity states. The majority of the dissociation induced by NKA alone (59%) (Table 1) was from the slow component. However, addition of a GTP analog in combination with NKA caused a shift of the majority of the receptors (70%) to the fast-dissociating component, without changing the intrinsic rate constants (Table 1). The slower dissociation rate of NKA (5.3 min) is nearly identical to that calculated from association kinetics (5.9 min) and is also similar to the antagonist-induced dissociation rates (Table 1).

A saturation analysis of the equilibrium [^{3}H]NKA binding illustrates (Fig. 6) binding to a single population of high affinity and saturable receptor sites (K_d = 2.15 ± 0.15 nM, B_{max} = 777 ± 25 fmol/mg of protein). A computer-based statistical analysis of these data (with GraphPad InPlot or Lundon AccuFit Two-Site) did not yield a significantly better fit for a two-site versus a one-site binding model (ANOVA, $p > 0.05$), in spite of the fact that the concentration range used for [^{3}H]NKA (40 pM to 5.86 nM) in this experiment would permit detection of very high affinity sites. The mean data from several receptor batches were K_d = 2.28 ± 0.26 nM and B_{max} = 820 ± 167 fmol/mg of protein (mean ± standard error, four experiments).

In none of the four saturation experiments, conducted with a wide range of [^{3}H]NKA concentrations (40 pM to 8.7 nM), was there a significantly better fit to a two-site model, although the kinetic experiments did indicate the existence of high and low affinity states for agonist binding. However, due to limita-

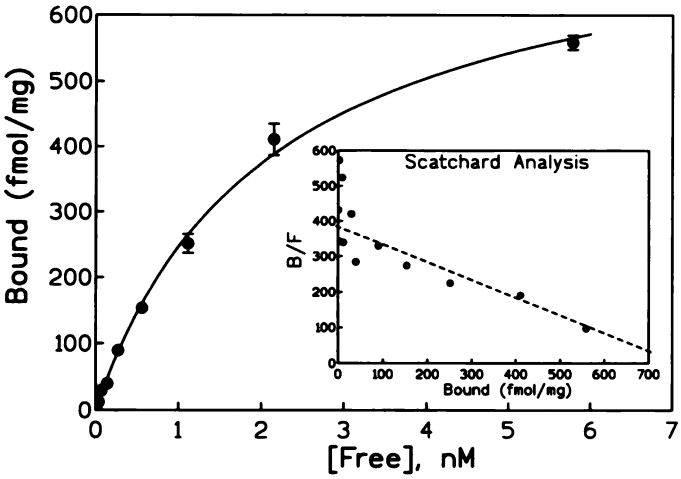

Fig. 6. Saturation analysis of [^{3}H]NKA binding to BV.Sf-21 membranes. Membranes were incubated with [^{3}H]NKA (40 pM to 5.86 nM) under standard conditions. Data were analyzed as discussed in Materials and Methods and were linearized according to the method of Scatchard (*inset*). There was no significantly better fit to a two-site model than to a one-site model, with either Lundon AccuFit or GraphPad InPlot software ($p > 0.05$, ANOVA). Data are mean of triplicate determinations from a typical experiment.

tions of ligand usage above 10 nM, it is possible that a significantly lower affinity state (e.g., $10 \times K_d$) may not be detected. To better evaluate both high and low affinity sites, we conducted equilibrium binding experiments with varying amounts of NKA (10 pM to 1000 nM) combined with [^{3}H]NKA and used two-site analysis to compute the saturation binding parameters.

In the majority of these experiments (four of six total), we were able to detect high and low affinity binding for NKA, as follows: $K_{d1} = 1.78 \pm 0.39$ nM, $B_{max1} = 458 \pm 98$ fmol/mg of protein (mean ± standard error, six experiments); $K_{d2} = 266 \pm 91$ nM, $B_{max2} = 147 \pm 57$ fmol/mg of protein (mean ± standard error, four experiments). Thus, these "ligand dilution" experiments yielded a K_d of 1.78 ± 0.39 nM, which was no different ($p > 0.05$) from the K_d of 2.28 ± 0.26 nM that was consistently obtained in saturation experiments with [^{3}H]NKA alone. Both affinity constants also agree well with the k_{-1}/k_1 value of 1.47 nM obtained independently in kinetic experiments. The lower affinity sites for NKA binding ($-\log K_d = 6.58$) represent only 24% of the total binding sites, which agrees with the affinity values for this site ($pK_i = 6.78$, 16% of the sites) obtained in competition experiments (Table 2).

The pharmacology of the NK-2 receptors in these BV.Sf-21 membranes was explored with a series of selective NK-2 agonists and antagonists. Fig. 7 illustrates that selective NK-2 (but not NK-1 or NK-3) agonists potently inhibited [^{3}H]NKA binding with the following order of potency: NK-2 ligands ≫ SP ≫ Senktide. Fig. 7 and Table 2 also illustrate that the binding of NKA and the selective NK-2 agonists [Nle^{10}]NKA(4–10) and [β-Ala^8]NKA(4–10) could be resolved into two populations of high and low affinity sites (Table 2). However, inhibition curves for SP (or Senktide) were compatible with binding to a single, low affinity, receptor population (Table 2). The pK_i values for NKA (8.92 and 6.78) are in excellent agreement with those obtained in the saturation experiments ($-\log K_d = 8.64$ and 6.58).

Moreover, Table 2 demonstrates that NKA and [Nle^{10}]NKA(4–10) bound predominantly (84% and 69%, respectively)

TABLE 2

Pharmacological analysis of cloned hNK-2R expressed in BV.Sf-21

Data are mean ± standard error of the number of experiments given in parentheses, in two batches of BV.Sf-21, determined in duplicate.

Competitor	Population 1		Population 2	
	pK_i	Sites	pK_i	Sites
		%		%
NKA	8.92 ± 0.05	84 ± 5	6.78 ± 0.37	16 ± 5 (4)
[Nle^{10}]NKA(4-10)	8.61 ± 0.36	69 ± 19	6.90 ± 0.32	31 ± 19 (3)
[β-Ala^8]NKA(4-10)	8.31 ± 0.55	15 ± 11	6.67 ± 0.21	85 ± 11 (3)
SR48,968	9.01 ± 0.10 (3)			
MEN10,376	7.97 ± 0.11 (3)			
L659,877	7.54 ± 0.12 (3)			
R396	6.59 ± 0.02 (2)			
SP	6.59 (2)			
(±)-CP96,345	NA* (2)			
Senktide	NA (2)			

* NA, not active up to 10 μM (<25% inhibition).

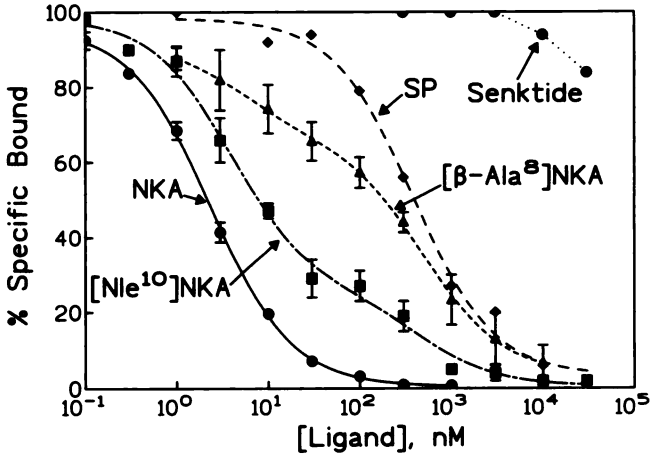

Fig. 7. Inhibition of [^{3}H]NKA binding to BV.Sf-21 membranes by tachykinins and selective NK-2 agonists. Membranes were incubated with 1.27 nM ligand in the presence of increasing concentrations of agonists. There was a significantly better fit for the NK-2 agonists (but not SP) to a two-site binding model than to a one-site model ($p < 0.05$, ANOVA). Nonspecific binding was determined with 1 μM NKA. Data are expressed as percentage of control and are from two or three experiments determined in duplicate.

to the high affinity state, whereas [β-Ala^8)NKA(4–10) bound preferentially to the low affinity state (85%), of the NK-2 receptors. The "intrinsic" affinities for NKA were somewhat higher (2–4 fold) than those of the other NK-2 agonists at the high affinity site, but all of them displayed equal affinity at the second site (Table 2), which was identical to that of the NK-1 ligand SP. However, the observed (Fig. 7) overall rank order of potency, NKA > [Nle^{10}]NKA(4–10) > [β-Ala^8]NKA(4–10), may be explained by the different distribution of binding to the high and low affinity states induced by selective NK-2 agonists (Table 2). These observations (for the selective agonists) with hNK-2R are in contrast to our observations with hamster urinary bladder membranes (a NK-2B subtype) (16), where these agonists are equipotent.

Fig. 8 illustrates that selective NK-2 antagonists inhibited binding and that the newly described nonpeptide antagonist SR48,968 (15) was the most potent antagonist ($pK_i = 9.01$), exceeding the potency of NKA. In contrast to the agonist data, antagonist binding conformed to a receptor model with a single

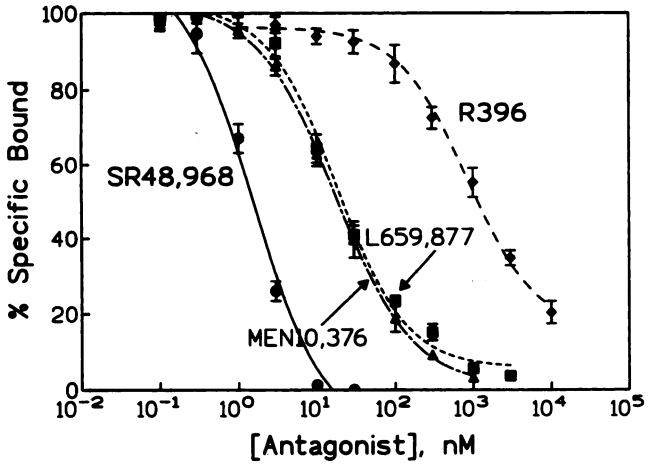

Fig. 8. Inhibition of [^{3}H]NKA binding to BV.Sf-21 membranes by selective NK-2 antagonists. Membranes were incubated with 2 nM ligand in the presence of increasing concentrations of antagonists. Antagonist binding data were fit best to a single-site binding model. Nonspecific binding was determined with 1 μM NKA. Data are expressed as percentage of control and are from two or three experiments determined in duplicate.

affinity state, in agreement with kinetic data that demonstrated complete dissociation from a single population with slower off-rates (Table 1). The observed order of potency (Table 2) was SR48,968 ≫ MEN10,376 ≥ L659,877 > R396, which is substantially different from our observation in hamster urinary bladder or trachea (SR48,968 > L659,877 > R396 ≫ MEN10,376) (16, 22, 23). Indeed, Table 3 illustrates that the most noticeable discrepancy in the selectivity of the antagonists for the different NK-2 receptor subtypes occurs with MEN10,376 and R396. The latter antagonist is much more potent than MEN10,376 in hamster tissues but is much less potent with the human or rabbit receptors.

Discussion

Infection of Sf-21 cells with a baculovirus expression vector harboring the cDNA of the hNK-2R resulted in detectable receptor expression. Northern analysis showed that hNK-2R mRNA was detectable, compared with the wild-type virus-infected control (data not shown). NKA ligand binding confirmed that functional receptors were expressed and that the level of receptors increased to a maximum value at 50–70 hr after infection. The receptor concentrations achieved (approximately 1 pmol/mg of protein) are equal to those found in native sources for NKA receptors (e.g., hamster urinary bladder) (16). We have scaled up the hNK-2R/Sf-21 culture, with harvesting at 54 hr after infection.

The cloned hNK-2R expressed in BV.Sf-21 bound [^{3}H]NKA rapidly, reversibly, and with high specificity. The analysis of NKA-induced dissociation indicated that [^{3}H]NKA dissociates from slow and fast kinetic components, which presumably represent high and low affinity states, respectively, induced by agonist binding. Gpp(NH)p in combination with NKA induced faster dissociation, an effect mediated by shifting the receptors to the lower affinity state. In contrast, antagonists induced dissociation only from the slower site, with rates similar to that observed with NKA at that site. The observation that the peptide MEN10,376 and nonpeptide SR48,968 induce complete and indistinguishable dissociation may suggest that they both compete for the same NKA binding site, albeit with different affinities.

These experiments, the enhancement by divalent cations, and the inhibition of [^{3}H]NKA binding by GTP analogs strongly suggest that the receptors are coupled to G proteins in these cells, as expected from the deduced structure, which contains seven putative membrane-spanning helices. Thus, it appears that the cloned hNK-2R can undergo an agonist-induced transition from high to low affinity state that is mediated by G proteins. Our data on the cloned hNK-2R are in agreement with those of others who demonstrated that cloned bovine or rat NK-2 receptors transfected in different host cells (i.e., murine B82 fibroblasts, Chinese hamster ovary cells, or monkey kidney COS cells) are highly sensitive to divalent cations (which stimulate) and GTP analogs (which inhibit) (24–27). These properties are also similar to those displayed by the native NK-2B receptors in hamster urinary bladder (16), except for the notably greater sensitivity of cloned hNK-2R to GTP analogs (nanomolar) versus the much weaker inhibition (micromolar) observed in hamster (16, 24).

Saturation analysis with [^{3}H]NKA alone demonstrated high affinity binding to a single class of saturable receptor sites. The binding affinity derived from equilibrium experiments (K_d = 2.28 ± 0.26 nM) agrees well with that observed in kinetic experiments (k_{-1}/k_1 = 1.47 nM) and the higher affinity site observed in competition experiments (K_d = 1.78 ± 0.39 nM). These results are similar to those obtained with native (hamster) (16, 24) or cloned (rat and bovine) (24–27) receptors, where the majority of the receptors bind NKA with affinitiy of 2–3 nM.

The saturation experiments did suggest a mostly homogeneous population of high affinity receptors. However, due to limitations of the concentration range of the ligand used in these experiments (<8 nM) and the observation of biexponential dissociation kinetics for NKA, we cannot completely exclude the existence of a small number of low affinity receptors (K_d > 20 nM). Indeed, in competition experiments that covered

TABLE 3
Comparative pharmacological properties of selective NK-2 peptide antagonists with NK-2 receptor subtypes

Receptor	Type	Method of evaluation	Affinity constant	Rank order (pK_i or pA_2)	Ref.
RPA[a]	NK-2A	SMC	pA_2	MEN10,376 (8.11) > L659,877 (6.78) > R396 (5.41)	22
Cloned hNK-2R	NK-2A	[^{3}H]NKA	pK_i	MEN10,376 (7.97) > L659,877 (7.54) > R396 (6.59)	This work
HT	NK-2B	SMC	pA_2	L659,877 (8.24) > R396 (7.46) > MEN10,376 (5.84)	22, 23
HUBM	NK-2B	[^{3}H]NKA	pK_i	L659,877 (8.13) > R396 (7.88) > MEN10,376 (5.84)	16

[a] RPA, rabbit pulmonary artery without endothelium; HT, hamster trachea; HUBM, hamster urinary bladder membranes; SMC, smooth muscle contraction.

a wider concentration range, we did observe a minor population (16–24%) of receptors in a low affinity state.

It is interesting to note that NK-2 receptors, either in their native tissues or cloned and expressed in host cells and regardless of species, possess 10–20 times lower affinity for NKA (K_d = 2–3 nM) than do the NK-1 receptors (native or cloned) for SP (K_d = 0.1–0.17 nM). This is probably due to a much slower rate of dissociation of SP from NK-1 receptors, compared with the rate of NKA dissociation from NK-2 receptors (16, 24, 26–28).

The cloned hNK-2R possesses high affinity for NKA over SP or Senktide (100–1000-fold) and preferential binding of NK-2-selective ligands over NK-1- or NK-3-selective agonists. These properties are in agreement with published data for the cloned rat and bovine receptors (24–26). The overall preference for NK-2 > NK-1 > NK-3 ligands is similar to the observed selectivity in native hamster receptors (16), suggesting that the natural tachykinins do not distinguish between the NK-2 receptor subtypes.

Although the selective NK-2 agonists [Nle^{10}]NKA(4–10) and [β-Ala^8]NKA(4–10) were not characterized in other studies of cloned receptors, they are widely used in functional and ligand binding assays of native NK-2 receptors and were used by us to characterize the cloned hNK-2R. We observed that both agonists potently displaced [^{3}H]NKA binding and that [Nle^{10}]NKA(4–10) predominantly bound to the high affinity state, in contrast to [β-Ala^8]NKA(4–10), which bound mainly to the lower affinity state, resulting in greater potency. These agonists, however, are equipotent at the hamster urinary bladder receptors (16), suggesting that they may discriminate between some NK-2 receptor subtypes. This hypothesis remains to be proven upon further testing of these agonists with other cloned receptors.

The competition studies with antagonists demonstrated that SR48,968, a novel and potent nonpeptide antagonist of NK-2 receptors, was the most potent ligand for hNK-2R, similar to its high affinity in other species (15, 16, 21). Moreover, the potency observed in our binding experiments (pK_i = 9.01) agrees well with its potency in functional studies in human bronchi (pA_2 = 9.4) (15). Interestingly, the potency of SR48,968 is only 3–7-fold higher than that observed in hamster bladder in binding (pK_i = 8.6) (16) or functional (pA_2 = 8.11) (21) assays, suggesting that this antagonist does not discriminate well between the NK-2 receptor subtypes. These results have been corroborated in a recent report on the binding of [^{3}H]SR48,968 to NK-2 receptors in rat, hamster, and guinea pig tissues (29), demonstrating only a small preference (7-fold) for NK-2A receptors in guinea pig over NK-2B receptors in hamster. Moreover, the low affinity for the cloned hNK-2R observed with R396 in this study (pK_i = 6.59 ± 0.02) is in excellent agreement with the low affinity of R396 (pK_B = 6.12 ± 0.32) observed in functional receptor assays in human bronchus (30).

This observation is in contrast to the inverse relation between the two receptors observed with the two peptide NK-2 antagonists, MEN10,376 and R396. Table 3 illustrates that, whereas with hNK-2R MEN10,376 is 24-fold more potent than R396, the opposite is true in hamster urinary bladder, where MEN10,376 is 173-fold less potent (16). This dramatic difference cannot be attributed to preferred metabolism of one antagonist over the other, because all these studies included protease inhibitors that prevented peptide metabolism. A similar trend with even larger discrimination was reported in hamster trachea versus rabbit pulmonary artery for these antagonists (Table 3), except that L659,877 and R396 were more potent (6- and 16-fold, respectively) in this study than in the functional receptor assay in rabbit pulmonary artery (Table 3). However this difference may be attributed to different methodologies (i.e., binding versus smooth muscle contractions), because there is a better correlation for these antagonists in binding assays, using either [^{3}H]NKA (here and in Ref. 16) or [^{3}H]SR48,968 (29). Indeed, large differences (10–40-fold) in absolute potency for either MEN10,376 or R396 are not uncommon in functional receptor assays even for the same class of receptor (31). Thus, pK_b values ranging from 6.93 to 8.02 have been reported for MEN10,376 in tissues containing NK-2A receptors, and up to 10-fold differences in pK_B values (5.07–6.12) were observed for R396 in similar studies (30).

The order of potency for selective NK-2 antagonists with the hNK-2R is similar to that in guinea pig and rabbit tissues, i.e., SR48,968 ≫ MEN10,376 ≥ L659,877 > R396 (30, 31), which is different from that obtained in hamster tissues, i.e., SR48,968 > L659,877 > R396 ≫ MEN10,376 (Table 3), supporting the hypothesis that these are indeed subtypes of NK-2 receptors (16, 22, 23, 30).

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