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Human Substance P Receptor (NK-1): Organization of the Gene, Chromosome Localization, and Functional Expression of cDNA Clones[†,‡]

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ABSTRACT: The gene for the human substance P receptor (NK-1) was cloned using cDNA probes made by the polymerase chain reaction from primers based on the rat sequence. The gene spans 45–60 kb and is contained in five exons, with introns interrupting at sites homologous to those in the NK-2 receptor gene. Analysis of restriction digests of genomic DNA from mouse/human cell hybrids indicates the NK-1 receptor is a single-copy gene located on human chromosome 2. Polymerase chain reaction using primers based on the 5′ and 3′ ends of the coding sequence was used to generate full-length cDNAs from human lung and from IM9 lymphoblast cells. When transfected into COS-7 cells, the NK-1 receptor binds ^{125}I-BHSP with a K_d of 0.35 ± 0.07 nM and mediates substance P induced phosphatidylinositol metabolism. The receptor is selective for substance P; the relative affinity for neurokinin A and neurokinin B is 100- and 500-fold lower, respectively. Human IM9 lymphoblast cells express relatively high levels of the NK-1 receptor, and Northern blot analysis indicates modulation of mRNA levels by glucocorticoids and growth factors, suggesting that this cell line may be useful as a model for studying the control of NK-1 receptor gene expression.

Over the past several years, it has become increasingly evident that the class of neuropeptides known as tachykinins possess a remarkable array of biological roles in the central and peripheral nervous system as well as in the immune system (Lundberg et al., 1983; Payan et al., 1983; Saria et al., 1983; Coleridge & Coleridge, 1984; Lee et al., 1986; Maggio, 1988). The three mammalian tachykinins, substance P, neurokinin A (substance K), and neurokinin B (neuromedin K), appear to mediate such diverse processes as transmission of sensory information, smooth muscle contraction, nociception, inflammation, sexual behavior, and possibly wound healing and nerve regeneration (Pernow, 1983; Mantyh et al., 1988, 1989; Skerrett, 1990). The tachykinin receptors have been classified with respect to their preferred ligands in the following order: NK-1, substance P; NK-2, neurokinin A (substance K); NK-3, neurokinin B (neuromedin K). However, because the tachykinins share a common C-terminal amino acid sequence, -Phe-X-Gly-Leu-Met-NH_2, which is essential for biological activity, there is a certain amount of cross reactivity among the receptors and their ligands (Buck et al., 1984; Burcher et al., 1986; Martling et al., 1987). This cross reactivity often confounds studies of the role of the ligand–receptor pairs involved in particular physiological or pathophysiological responses, and at present only pharmacologic methods are available to distinguish between involvement of one or more of the tachykinins.

The molecular characterization of the tachykinin receptors provides a new avenue to study the functions of these molecules. In initial work by Masu et al. (1987), the bovine NK-2 receptor was cloned by expression in *Xenopus* oocytes. The information provided from that cDNA was used to clone

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[‡] The nucleic acid sequence for the human NK-1 receptor gene reported in this paper has been submitted to GenBank under Accession Number M76675.

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cDNA for the rat NK-2 receptor by Sasai and Nakanishi (1989) and the human NK-2 receptor gene by our laboratory (Gerard et al., 1990). Hybridization under conditions of low stringency with rat NK-2 receptor cDNA yielded both the NK-1 and NK-3 receptors for that species (Yokota et al., 1989; Hershey & Krause, 1990; Shigemoto et al., 1990).

In the present paper we report cloning of the gene, isolation of expressible cDNAs from human lung and lymphocyte RNA samples, and chromosome assignment for the human NK-1 receptor.

Materials and Methods

Preparation of RNA. Human lung poly(A^+) RNA was obtained as described previously from an individual with cystic fibrosis (Gerard et al., 1990). RNA was extracted from IM9 cells grown in RPMI 1640 containing 10% fetal calf serum, with guanidinium thiocyanate containing 2-mercaptoethanol, and purified by centrifugation through cesium chloride as described (Glisin et al., 1974). Poly(A^+) RNA was then purified by passing the material over oligo(dT)–Sepharose (Aviv & Leder, 1972) and transcribed into cDNA by the method of Gubler and Hoffman (1983).

Polymerase Chain Reactions. Polymerase chain reaction (PCR)[1] was initially carried out using primers based on the cDNA sequence reported for the rat NK-1 receptor (Yokota et al., 1989). A sense primer from nucleotides 85–105, 5′-ACCTGGCAAATCGTTCTTTGG-3′, and an antisense primer from nucleotides 538–558, 5′-TGCATGATCGAGTGGCCGGAG-3′, or a sense primer from nucleotides 422–439, 5′-GGCTGTCAGCCACAGCCA-3′, and an antisense primer from nucleotides 988–1005, 5′-GATTATGAGGGGCTGGAA-3′, all with *Eco*R1 restriction sites three nucleotides from their 5′ ends, were synthesized using standard cyanoethyl phosphoramidite chemistry (Applied Biosystems Model 318A DNA synthesizer) and used with cDNA from human lung or IM9 cells and recombinant Taq DNA polymerase (Perkin-Elmer, Cetus) through 25 cycles consisting of 1 min at 95 °C, 2 min at 37 °C, and 3 min at 72 °C, with a final extension period of 7 min at 72 °C. Ten percent of the resulting reaction was subjected to a second round of 25 cycles of amplification under the same conditions. The PCR product was purified following agarose gel electrophoresis using GeneClean (Bio 101, LaJolla, CA), digested with *Eco*R1, repurified following a second electrophoresis, and ligated to pBluescript SK^- (Stratagene). Full-length cDNA for the human NK-1 receptor was prepared from human lung or IM9 cell cDNA using primers determined from the gene sequence, corresponding to nucleotides −18 to −1 and 1207–1224 with appended *Bam*H1 restriction sites at their 5′ ends. PCR was carried out as described for partial length cDNAs, and the products were purified, digested with *Bam*H1, and ligated to *Bam*H1-digested vector, pBluescript, SK^+, or the mammalian expression vectors, pCDNA (Invitrogen) or pMAM-neo (Clonetech).

Genomic DNA Library Screening. A human placental genomic DNA library (generous gift of Dr. S. Orkin, Children's Hospital and Harvard Medical School, Boston, MA) was screened with ^{32}P-labeled partial cDNAs corresponding to nucleotides 85–558 or 422–1005 from the human NK-1 receptor generated by PCR as previously described (Gerard et al., 1990).

[1] Abbreviations: PCR, polymerase chain reaction; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TAE, Tris–acetate–EDTA; BHSP, Bolton Hunter labeled substance P; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline: HBSS, Hank's balanced salt solution.

Sequence Analysis. The genomic clones were digested with *Hin*dIII or *Pst*1, electrophoresed on 1% agarose TAE (0.04 M Tris–acetate, pH 7.6, 1 mM EDTA) gels, blotted to nylon membranes (Genescreen Plus, Du Pont), and probed with ^{32}P-labeled cDNA corresponding to nucleotides 85–558 or 422–1005, or with synthetic oligonucleotides specific for each of the exons. Genomic fragments hybridizing with these cDNAs were isolated by agarose gel electrophoresis, purified with GeneClean, and ligated to pBluescript. Clones were expanded in *Escherichia coli* XL-1 Blue (Stratagene) and purified by alkaline lysis and centrifugation through cesium chloride as described previously (Birnboim & Doly, 1982). These subcloned fragments were subjected to double-stranded sequencing with DNA polymerase and ^{35}S-dATP (Sequenase, USBiochemicals, Cleveland, OH) (Chen & Seeburg, 1985). All of the exons, as well as the 5′ and 3′ flanking regions, were sequenced on both strands; intron–exon junctions were assigned by homology with the human NK-2 receptor gene sequence and the rat NK-1 receptor cDNA sequence. Further confirmation of the exon sequences was provided by sequence analysis of the human cDNAs prepared by PCR.

Chromosome Localization. Chromosome localization for the substance P receptor gene was accomplished using DNA from 40 cell hybrids involving 18 unrelated human cell lines and 4 mouse cell lines (Shows et al., 1978, 1982, 1984). The hybrids were characterized by karyotypic analysis and by mapped enzyme markers (Shows et al., 1978, 1982; Shows, 1983). The human substance P receptor cDNA 85–558 was hybridized to Southern blots containing *Eco*R1-digested DNA from the human–mouse hybrids as described above. Scoring was determined by the presence or absence of human bands in the hybrids on the blots.

Cell Cultures and Transfections. IM-9 cells (ATCC CCL 159) were grown in RPMI 1640 containing 10% fetal calf serum, penicillin, and streptomycin at 37 °C in an atmosphere of 5% CO_2. For studies of NK-1 receptor regulation, cells were diluted to 2×10^5/mL and grown for 24–48 h in the presence of 100 μM dexamethasone, 1 mM dibutyryl-cAMP, or 1 μM retinoic acid.

COS-7 cells (ATCC CRL 1651) were maintained in DMEM (high glucose) supplemented with 6 mM glutamine, nonessential amino acids, penicillin, streptomycin, and 10% fetal calf serum. Transfections for transient expression were carried out as described (Gerard & Gerard, 1991); cells were trypsinized and subcultured at 750000 per 10-cm dish 18–24 h prior to transfection. The NK-1 receptor cDNA in pCDNA, 2 μg/dish, was incubated with the cells for 2.5 h in DMEM containing 10% NuSerum (Collaborative Research), 400 μg/mL DEAE-dextran (Sigma), and 100 μM chloroquine (Sigma), at 37 °C in an atmosphere of 5% CO_2. Cells were shocked with 10% DMSO in PBS for 5–10 min at room temperature and replaced in DMEM containing 10% fetal calf serum for 72 h at 37 °C in 5% CO_2.

Transfections for stable expression in COS-7 cells were conducted using 20 μg/dish of linearized NK-1 receptor cDNA in pMAM-neo with 10 μg/mL lipofectin (Gibco BRL) in 5 mL of serum-free DMEM (Felgner et al., 1987). After 12–18 h, media were restored to 10% fetal calf serum and transfected cells were selected for neomycin resistance in the presence of 500 μg/mL geneticin (Gibco BRL).

Binding. COS cells transfected with the NK-1 receptor cDNA were washed twice with binding buffer composed of 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 3 mM $MnCl_2$, and 0.02% BSA and incubated in 2 mL/dish of the same buffer containing the protease inhibitors bacitracin (40 μg/mL),

leupeptin, chymostatin, pepstatin (each at 4 μg/mL), and phosphoramidon (5 μM), with 0.1 nM ^{125}I-BHSP (2000 Ci/mmol) (New England Nuclear) and increasing concentrations of unlabeled tachykinin peptides for 45–60 min at room temperature (Coats & Gerard, 1989). Cells were washed three times with binding buffer and solubilized with 3 mL of 1 N NaOH, and cell-associated ligand was determined by γ counting. Nonspecific binding was assessed by incubating the cells in the presence of 10 μM unlabeled substance P.

NK-1 receptor binding studies with IM9 cells were carried out with cells diluted to 2×10^5/mL 18–24 h before use. Cells were washed with binding buffer (above), resuspended at 1×10^7/mL in the same buffer containing protease inhibitors (above), and incubated for 60 min at room temperature with 0.1 nM ^{125}I-BHSP and increasing concentrations of unlabeled substance P. Free ligand was removed by filtering 0.5-mL aliquots through glass fiber filters (GF/F; Whatman) and washing three times with the same buffer. Nonspecific binding was assessed by incubating the cells with 10 μM unlabeled substance P as described for COS cell binding above.

Northern Blot Analysis. Ten-microgram poly(A$^+$) RNA samples were electrophoresed on 1% agarose formaldehyde gels as described (Lehrach et al., 1977), and the RNA was blotted to nylon membranes (Genescreen Plus, Du Pont) and hybridized with ^{32}P-labeled substance P receptor cDNA for 16 h at 42 °C, washed as described above for DNA blots, and exposed to X-ray film. The relative amounts of RNA were assessed by densitometry and normalized to actin.

Inositol Phosphate Metabolism. NK-1R COS-7 cells which display stable expression of the human NK-1 receptor were studied for agonist-induced metabolism of phosphatidylinositol as previously described (Guicheney et al., 1991). Cells were incubated with 2 μCi/mL [^{3}H]inositol (120 Ci/mmol; Amersham) in medium 199 containing 0.5% fetal calf serum for 18–24 h at 37 °C. Cells were washed with HBSS, preincubated for 10 min at 37 °C in HBSS containing 20 mM LiCl, and stimulated following addition of the same protease inhibitors as used for the binding experiments with 0.1 μM substance P for 20 min at 37 °C. Reactions were terminated by the addition of an equal volume of 4.5% perchloric acid containing 10 μg/mL phytic acid at 4 °C. The mixtures were neutralized by addition of 2 M potassium carbonate, and inositol phosphates were separated by chromatography on Dowex 1X8 (formate form) as described by Berridge et al. (1983).

Results

Preparation of Partial cDNA Probes for the Human Substance P Receptor and Isolation of Genomic Clones. Oligonucleotide primers were designed on the basis of the sequence reported for the rat NK-1 receptor cDNA (Yokota et al., 1989; Hershey & Krause, 1990) from regions of the molecule not homologous to the human neurokinin A (NK-2) receptor (Gerard et al., 1990). One such pair included nucleotides 85–105 (sense) and 538–558 (antisense). When these primers were used in polymerase chain reaction with cDNA prepared from human lung tissue, they generated a 473 bp cDNA fragment. Sequence analysis confirmed the identity of the cDNA as deriving from the human substance P receptor on the basis of homology with the rat molecule.

Initial screening of the human genomic DNA library was conducted using the 473 bp cDNA encompassing nucleotides 85–558 and yielded seven positive clones among approximately 1×10^6 bacteriophage plaques. Southern analysis following digestion with *Hin*dIII revealed two DNA fragments hybridizing with the probe, one of 1.3 kb, contained in four of the seven clones, and one of 1.8 kb, contained in the other three. No clones were isolated which contained more than one positive band. Sequence analyses revealed that the 1.3-kb *Hin*dIII fragment encodes the amino-terminal coding region and 224 nucleotides of 5′ flanking sequence (exon 1) and the 1.8-kb *Hin*dIII fragment encodes exon 2. The coding sequence and 5′ and 3′ flanking sequences are shown in Figure 1. An additional 0.8 kb of 5′ flanking sequence for exon 1 was identified by hybridization of the 1.3-kb *Hin*dIII fragment with an overlapping *Pst* fragment containing exon 1. This fragment extends the structure to −630 bp relative to the initiating ATG (Figure 1) and contains a TATAA box at nucleotide −608 and a CREB/Ca^{2+} response element consensus sequence (Montminy et al., 1990) at nucleotide −627. Primer extension analysis using IM9 mRNA revealed several potential cap sites (data not shown). The most frequent transcripts map within a CGC triplet located 22–24 bp downstream from the putative TATAA box.

In order to identify genomic clones encoding the remaining exons, another partial cDNA, encompassing nucleotides 422–1005, was made by PCR and used to rescreen the library, yielding four additional clones. *Hin*dIII digestion and hybridization with oligonucleotide probes specific for exons 3–5 revealed that one of these clones, clone 1-B, contained all three exons (but not exons 1 or 2). Two of the clones contained only exon 3, and one clone contained both exons 3 and 4. The coding sequences for exons 3–5 are also shown in Figure 1 with their intron–exon junctional sequences.

Chromosome Localization. Preliminary data indicated that *Eco*R1, *Pst*1, and *Hin*dIII digestion of genomic DNA from mouse and human gave different patterns of hybridization with the lung NK-1 receptor cDNA probe (not shown). Southern blots of human–mouse hybrid cell DNAs digested with *Eco*R1 were probed with the human NK-1 receptor cDNA 85–558 and analyzed by concordance/discordance ratios (Table I). These data indicate that the receptor is encoded on human chromosome 2.

Molecular Cloning of Human NK-1 Receptor cDNA Using PCR. On the basis of the sequences determined from exons 1 and 5 of the genomic clones, oligonucleotides corresponding to the 5′ and 3′ ends of the cDNA were used to amplify a product of approximately 1.2 kb from either human lung cDNA or IM9 cell cDNA (not shown). Sequence analyses yielded the predicted DNA sequence based on the genomic clones and confirmed the intron–exon splice junctions. The cDNA sequence obtained from IM9 cells was identical to that obtained from lung. The deduced protein sequence for the human NK-1 receptor is presented in Figure 2 and compared with the rat NK-1 receptor and the human NK-2 receptor.

Expression of the Human NK-1 Receptor in COS-7 Cells. Transfection of the NK-1 receptor cDNA into COS-7 cells conferred upon them the expression of high-affinity binding sites for substance P (Figure 3). Nontransfected cells display no detectable substance P binding. Scatchard analysis of ^{125}I-BHSP binding by displacement indicates a K_d of 0.35 ± 0.07 nM and expression of $\sim 1 \times 10^6$ sites per transfected cell, on the basis of control transfections using β-galactosidase cDNA. The receptor is selective for substance P compared with other members of the tachykinin family, requiring 100-fold higher concentrations of neurokinin A and 500-fold higher concentrations of neurokinin B to effect similar displacement of ^{125}I-BHSP (Figure 3). Under the conditions used, the kinetics of binding of ^{125}I-BHSP to NK-1-transfected COS-7 cells is similar to that observed using tissue membrane preparations (Buck et al., 1984; Coats & Gerard, 1989); uptake

Table I: Segregation of NK-1 Receptor with Human Chromosomes in *Eco*R1-Digested Human–Mouse Cell Hybrid DNA[a]

	concordant no. of hybrids		discordant no. of hybrids		
chromosome	(+/+)	(−/−)	(+/−)	(−/+)	% discordancy
1	5	15	7	3	33
2	13	19	0	0	0
3	9	8	4	11	47
4	9	13	5	6	33
5	9	12	5	7	36
6	9	16	5	3	24
7	9	7	4	11	48
8	9	10	5	9	42
9	4	16	10	2	37
10	11	7	3	12	45
11	9	9	5	9	44
12	10	8	4	11	45
13	6	13	8	6	42
14	12	5	2	14	48
15	10	12	3	7	31
16	5	12	9	7	48
17	12	7	1	12	41
18	10	10	4	9	39
19	7	14	7	5	36
20	10	9	4	10	42
21	11	8	3	11	42
22	5	13	8	5	42
X	6	4	5	11	62

[a] The human NK-1 receptor mapped to chromosome 2 by somatic cell hybrids as described under Materials and Methods. Scoring was determined by the presence or absence of human bands in the hybrids on the blots. Concordant hybrids have either retained or lost the human bands together with a specific human chromosome. Discordant hybrids have either retained the human bands, but not a specific chromosome, or the reverse. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordancy is the basis for chromosome assignment.

Table II: Stimulation of Phosphatidylinositol Metabolism in NK-1R COS-7 Cells by Substance P[a]

substance P concn (nM)	^{3}H cpm in inositol phosphates (% above base line)
0	0
100	52 ± 3
1:10 serum	329 ± 7

[a] NK-1R COS-7 cells were preincubated with [^{3}H]inositol and stimulated with substance P or a 1:10 dilution of fetal calf serum as described under Materials and Methods. The percent increase in inositol phosphates in response to substance P is the mean (±SEM) of duplicate determinations for two separate experiments.

of ligand is relatively slow, reaching equilibrium only after 30–45 min, and binding can be displaced by addition of excess unlabeled ligand at equilibrium (data not shown).

In order to assess signal transduction via the transfected receptor, a stable cell line NK-1R COS-7 was developed. Stimulation of NK-1R COS-7 cells with substance P results in increased metabolism of phosphatidylinositol relative to unstimulated cells (Table II). Stimulation with 0.1 μM substance P results in 52 ± 3% elevation of inositol phosphates compared with control cells. These data indicate productive coupling of the expressed receptor with the COS cell GTP-binding proteins.

Regulation of Expression of the NK-1 Receptor in IM9 Cells. Preliminary experiments suggested that IM9 cells are a relatively rich source of NK-1 receptor mRNA (data not shown). In order to ascertain the usefulness of these cells for studying the regulation of NK-1 receptor gene expression, cells were cultured with or without dexamethasone (100 μM), retinoic acid (1 μM), or dibutyryl-cAMP (1 mM). Cells were harvested at 24 and 48 h. RNA was extracted at the 24-h time point, and receptor binding measurements were made at 48 h. As shown in Figure 4 and confirmed by densitometric analysis, a representative blot reveals relatively small (<2-fold) changes in mRNA levels after 24 h, relative to the actin control. These changes in mRNA levels were correlated with expression of functional receptor as assessed by binding activity (Table III). No significant difference was observed in the number of sites per cell for cells cultured with cAMP or retinoic acid. In contrast, dexamethasone treatment led to a reduction in receptor specific content of ~7-fold.

Table III: Regulation of Expression of the NK-1 Receptor in Human IM9 Lymphoblasts

treatment[a]	K_d (nM)	B_{max} (fmol/10^6 cells)
none	0.60	4.35
1 μM retinoic acid	0.59	3.74
1 mM dibutyryl-cAMP	0.47	3.46
100 μM dexamethasone	0.62	0.63

[a] IM9 cells were treated for 48 h with the transcriptional modulators indicated. Scatchard analyses were performed from displacement of binding curves in triplicate to determine the K_d and B_{max}.

Discussion

The original report of Masu and co-workers described the molecular cloning of the bovine NK-2 receptor (Masu et al., 1987) and provided the key probe used subsequently to clone the rat NK-1 (Sasai & Nakanishi, 1989; Hershey & Krause, 1990), NK-2 (Yokota et al., 1989), and NK-3 receptors (Shigemoto et al., 1990). Our interest in the expression of the NK-1 and NK-2 receptors led us to clone and characterize the human genes for these molecules, which simultaneously provides cDNA clones via the polymerase chain reaction. We have previously reported the molecular characterization of the human NK-2 receptor gene and cDNA (Gerard et al., 1990) and now extend this study to the NK-1 receptor. Our strategy relied on the high degree of sequence identity between species for particular regions of the NK-1 receptor to provide PCR primers useful with human RNA samples. On the basis of previous pharmacological studies (Payan et al., 1983; Stimler-Gerard, 1987), we expected and confirmed the presence of NK-1 receptor RNA in lung tissues and IM9 lymphoblasts by PCR. The partial cDNAs were then used to isolate a series of overlapping genomic clones spanning some 45–60 kb, which were characterized by sequence analyses (Figures 1 and 2) and contained the entire coding sequence.

Comparison of the human NK-1 and NK-2 receptor gene organization is remarkable in that both are found to consist of five exons, and the relative placement of the introns is identical for the two receptors. In contrast to the NK-2 receptor gene, which was isolated in a single 20-kb genomic fragment, the human NK-1 receptor extends some 45–60 kb and required three ~20-kb clones to encompass all five exons. The rat NK-1 receptor gene is organized in the same way and spans a length of DNA similar to the human NK-1 receptor gene (Hershey et al., 1991). The assignment to chromosome 2 for the NK-1 receptor also contrasts with the NK-2 receptor, which was mapped to chromosome 10 (Gerard et al., 1990).

Comparison of the deduced protein sequences for the human and rat NK-1 receptors indicates that the NK-1 receptors from these species are ~92% identical (Figure 2). In comparison, the human NK-1 and NK-2 receptors are only 47% identical. The rat NK-1 receptor sequence is identical in length to the human; 21 amino acid changes occur spread over the molecule, with the highest frequency in the N-terminal and C-terminal sequences. Perhaps the most interesting are the charge inversions at positions 97 and 186, where Val/Glu replacements occur in the second and third extracellular loops. Within the

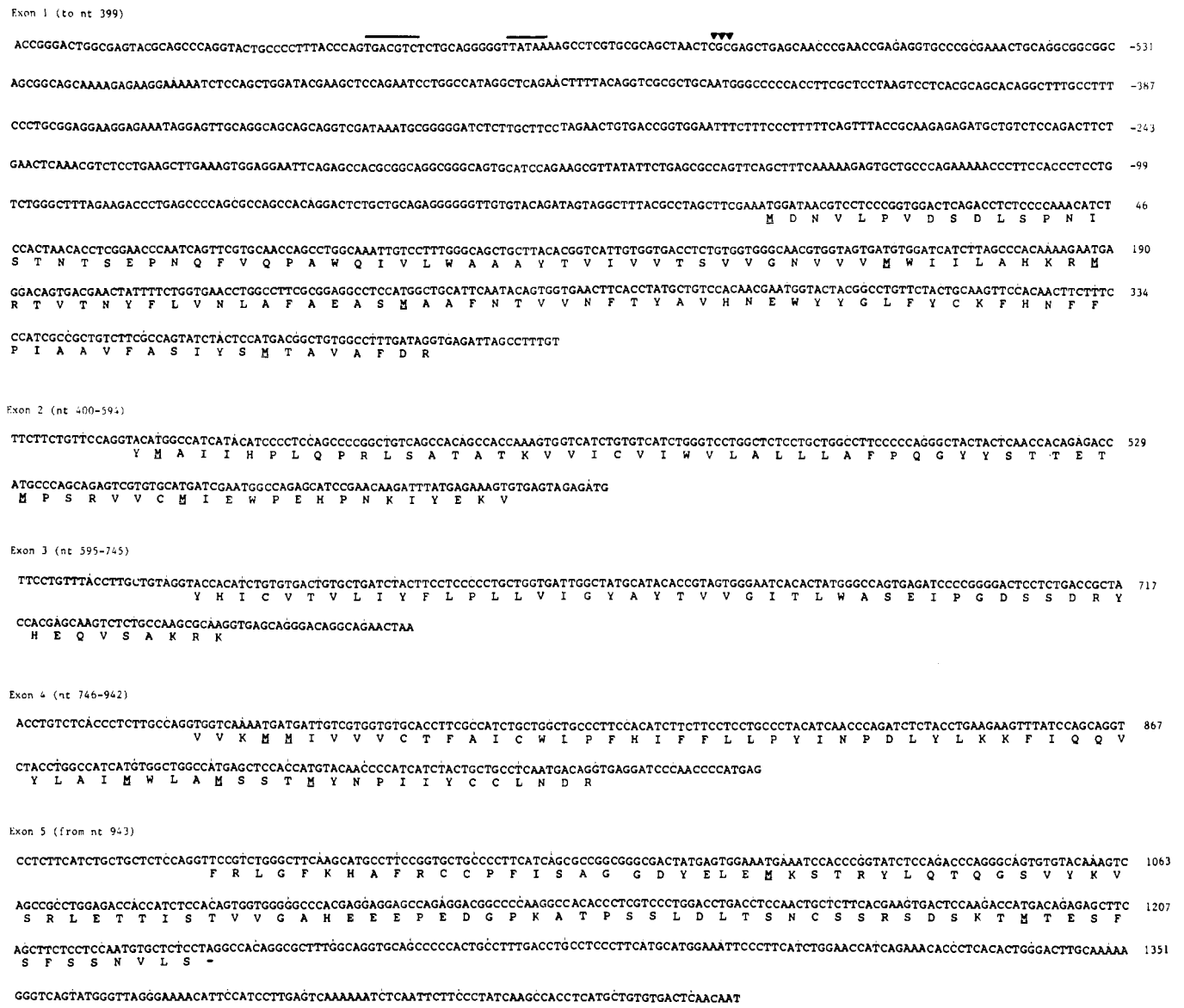

FIGURE 1: Exon and junctional sequences for the human NK-1 receptor gene. Numbering of the nucleotide sequence is based on the initiating ATG as 1, and subsequent exons are numbered as transcribed. Splice junctions follow the typical pattern (GT donor, AG acceptor). A calcium/cAMP response element (CREB) is located 11 bp upstream from the putative TATAA box, as indicated by the bars. The transcription start site appears to be within the CGC triplet indicated in the figure (▼).

transmembrane segments a cysteine in M2 for the rat is replaced by serine in human, while a new cysteine appears in M4 of the human molecule, which is a phenylalanine in rat. In the case of rhodopsin and the adrenergic and muscarinic receptors, these transmembrane segments constitute the putative ligand binding site (Findlay & Eliopoulos, 1990). These changes could account for subtle pharmacologic species differences in responsiveness toward agonists and antagonists.

The full-length NK-1 receptor cDNA clones amplified by PCR from IM9 lymphoblasts and from human lung tissues were expressed in transient and stable transfections in COS-7 cells and were found to bind substance P with high affinity (Figure 3). Previous reports on the expression of the rat NK-1 receptor have not explored signal transduction in transfected eukaryotic cells. Tachykinin-induced phosphatidylinositol metabolism is reported for isolated tissues and cultured cells (Mantyh et al., 1984; Hughes et al., 1988); therefore, we measured this response in COS-7 cells stably transfected with the NK-1 receptor and observed an increase in [^{3}H]inositol phosphates in these cells following stimulation with substance P (Table III). This is the first direct evidence relating to the mechanism of signal transduction for this receptor.

Analysis of the 5′ flanking region reveals a CREB/Ca^{2+} response element consensus sequence at 627 bp upstream from the initiating methionine and almost adjacent to the TATAA box. The CREB element allows genes to respond to elevated levels of calcium or cAMP with enhanced gene transcription (Montminy et al., 1990). When the IM9 cell was stimulated with dibutyryl-cAMP, no increase in NK-1 receptor mRNA relative to actin was observed (Figure 4). Conceivably, the high message level present in IM9 cells reflects maximal expression, or this cell line may lack CREB.

Ihara and Nakanishi (1990) reported that expression of the rat NK-1 receptor gene was virtually silenced in pancreatic acinar AR42J cells following incubation with dexamethasone for as little as 4 h. IM9 cells respond in a much less dramatic fashion, with less than 50% decrease in the NK-1 receptor mRNA level relative to actin after 24-h incubation (Figure 4). The decreased message content, however, was reflected in an ~7-fold drop in NK-1 receptor binding sites measured after 48 h (Table III). A slight enhancement (~2-fold) in message level was observed with retinoic acid, which was not

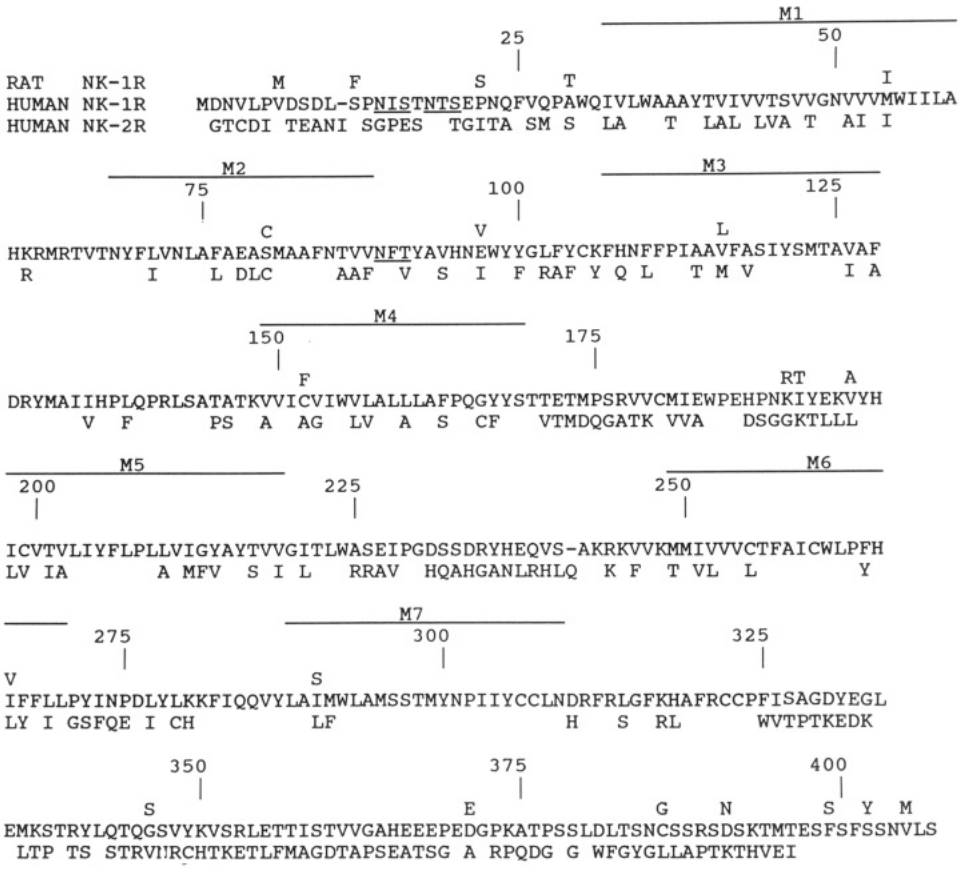

FIGURE 2: Deduced amino acid sequence of the human substance P (NK-1) receptor. cDNA clones from lung and IM9 lymphoblast confirmed the nucleotide sequence determined for the exons. Three potential N-linked oligosaccharide binding sites at positions 14, 18, and 89 are underlined. The human NK-2 (substance K) receptor and rat substance P receptor are presented for comparison.

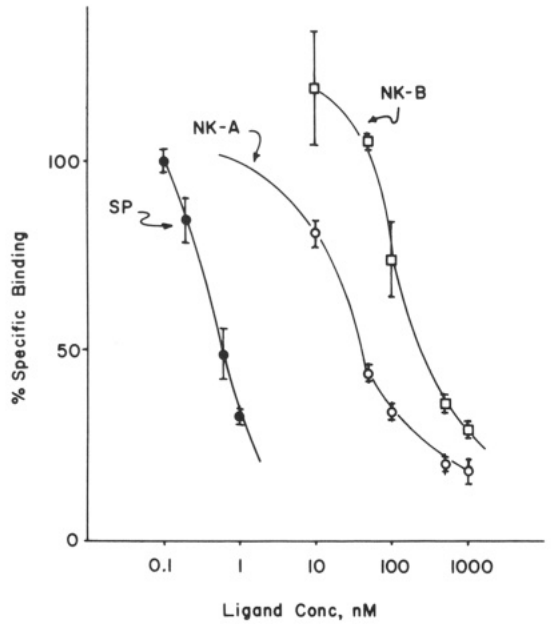

FIGURE 3: Receptor binding of substance P by transfected cDNA clones. COS-7 cells were transfected with cDNA clones isolated from lung or IM9 lymphoblast under conditions of transient expression. Displacement of specifically bound ^{125}I-BHSP was measured with unlabeled substance P (SP), substance K (NK-A), and neurokinin B (NK-B). Scatchard analysis reveals an estimated K_d at 0.35 ± 0.07 nM, and neurokinin A and neurokinin B display 100- and 500-fold lower K_d.

reflected by a similar change in the number of binding sites per cell. Whether these data reflect the transformed nature of the IM9 lymphoblast and are not representitive of other cell types and tissues requires further investigation. Strong endogenous promoter activity is observed when CAT constructs containing the 0.8-kb 5′ flanking sequence are transfected into COS-7 cells (L. A. Garraway, unpublished observations), indicating the potential for transcriptional activation.

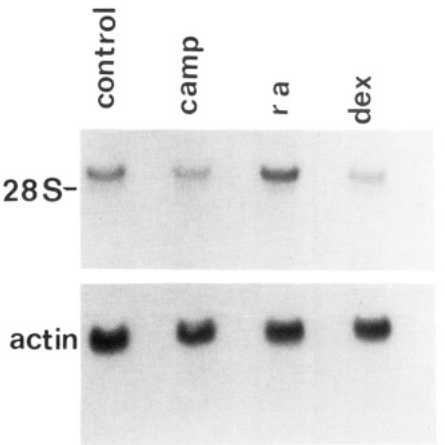

FIGURE 4: Northern analysis of the lymphoid substance P receptor. Ten micrograms of poly(A$^+$) RNA was analyzed from IM9 lymphoblasts, untreated or treated with 1 mM dibutyryl-cAMP (camp), 1 μM retinoic acid (ra), or 100 mM dexamethasone (dex). Blots were hybridized with radiolabeled substance P or actin. Substance P receptor message (~5.0 kb) is depicted following 4-h exposure at −70 °C using an intensifying screen.

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