Cloning and Expression of a Rat Neuromedin K Receptor cDNA*

(Received for publication, August 21, 1989)

Ryuichi Shigenoto, Yoshifumi Yokota, Kunihiro Tsuchida, and Shigetada Nakanishi

From the Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto 606, Japan

Functional cDNA clones for rat neuromedin K receptor were isolated from a rat brain cDNA library by cross-hybridization with the bovine substance K receptor cDNA. Injection of the mRNA synthesized in vitro from the cloned cDNA into Xenopus oocytes elicited electrophysiological responses to tachykinins, with the most potent sensitivity being to neuromedin K. Ligand-binding displacement in membranes of mammalian COS cells transfected with the cDNA indicated the rank order of affinity of the receptor to tachykinins: neuromedin K > substance K > substance P. The hybridization analysis showed that the neuromedin K receptor mRNA is expressed in both the brain and the peripheral tissues at different levels. The rat neuromedin K receptor consists of 452 amino acid residues and belongs to the family of G protein-coupled receptors, which are thought to have seven transmembrane domains. The sequence comparison of the rat neuromedin K, substance P, and substance K receptors revealed that these receptors are highly conserved in the seven transmembrane domains and the cytoplasmic sides of the receptors. They also show some structural characteristics, including the common presence of histidine residues in transmembrane segments V and VI and the difference in the numbers and distributions of serine and threonine residues as possible phosphorylation sites in the cytoplasmic regions. This paper thus presents the first comprehensive analysis of the molecular nature of the multiple peptide receptors that exhibit similar but pharmacologically distinguishable activities.

The mammalian tachykinin system represents a typical example of biologically active peptides that exhibit a high degree of functional diversity within the same group of peptides (1, 2). It consists of three distinct peptides: substance P, substance K, and neuromedin K. The biosyntheses of these three peptides are regulated by various cellular mechanisms including differential expression of the duplicated genes and alternative RNA splicing (3–7). The three peptides possess a common spectrum of biological activities including a sensory transmission in the nervous system and contraction/relaxation of peripheral smooth muscles (2, 8). However, they show markedly different biological potencies, depending on the pharmacological preparations tested. These pharmacological studies as well as ligand-binding experiments suggest that there are three different types of tachykinin receptors, each specific for the respective peptide (2, 8). Thus, the regulation at the level of not only peptide production but also peptide-receptor interaction plays an important role in expressing the functional diversity of the mammalian tachykinin system.

In a previous study (9), we isolated and determined the nucleotide sequence of a cDNA clone for the bovine substance K receptor (SKR) by combining molecular cloning with an electrophysiological assay in Xenopus oocytes. In the subsequent study (10), we reported the amino acid sequence of rat substance P receptor (SPR) deduced from the cloned cDNA and discussed the sequence comparison between rat SPR and rat SKR. The results indicated that SPR and SKR belong to the family of receptors coupled to G proteins. In order to elucidate the structures and functions of the three mammalian tachykinin receptors, we have isolated a cDNA clone for rat neuromedin K receptor (NKR) in this investigation. We report here the complete amino acid sequence of rat NKR deduced from the cloned cDNA and the electrophysiological and ligand-binding characterization of this receptor expressed in Xenopus oocytes and in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: cDNA synthesis kit from Pharmacia LKB Biotechnology Inc.; 

The abbreviations used are: SKR, substance K receptor; NKR, neuromedin K receptor; SPR, substance P receptor.

* This work was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan; the Institute of Physical and Chemical Research; and the Science and Technology Agency of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05189.
whereas the other differed from both the SPR and the SKR cDNA clones in the restriction pattern. One representative clone (prTKR3) of the latter group was then electrophysiologically tested for the expression of the tachykinin receptor in the oocyte system; the mRNA was synthesized in vitro from the prTKR3 clone and injected into oocytes in order to examine an electrophysiological response to the application of tachykinin peptides (9). The cloned receptor was found to respond specifically with tachykinin peptides and most potently to neuromedin K. The cDNA insert of prTKR3 was subjected to sequence determination of both strands by the chain termination method (11).

**NKR cDNA Expression in COS Cells and Binding Assay of NKR**

The EcoRI cDNA fragment of prTKR3 was subcloned into the Xho I site of the expression vector CDM8 (12). Transfection of the resultant NKR cDNA-CDM8 in COS cells was conducted according to the procedures described previously for the expression of SPR (10). DNA-transfected cells were harvested and homogenized with a Dounce homogenizer at 4°C in a solution containing 0.25 M sucrose, 25 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, and 1 mM EDTA. The homogenate was centrifuged at 500 x g for 10 min, and the pellet was suspended in the same buffer, homogenized, and centrifuged. The two supernatants were combined and centrifuged at 100,000 x g for 1 h. The crude cell membranes thus isolated were suspended in a solution containing 55 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, and 1 mM EDTA at a concentration of approximately 1 mg of protein/ml, and stimulated with ligand binding of NKR. The cell membranes (200 µg/ml) were incubated with various concentrations (saturation experiments) or 0.20 mM (displacement experiments) of [³H]-labeled Bolton-Hunter edeoidin in 0.1-0.2 ml of 50 mM Tris-Cl, pH 7.4, 1 mM MnCl₂, 50 µg/ml chymostatin, 40 µg/ml bacitracin, 4 µg/ml leupeptin, and 200 µg/ml bovine serum albumin at 22°C for 90 min. The reaction was terminated by the addition of 2.5 ml of an ice-cold solution containing 55 mM Tris-Cl, pH 7.4, and 1 mM MnCl₂, and the mixture was rapidly filtered over a Whatman GF/B filter; the filter was presoaked overnight in 0.5% bovine serum albumin at 4°C. The incubation tube and filter were washed four times with 2.5 ml of the above reaction termination solution. Radioactivity on the filter was determined in a γ-counter. Each experiment was carried out at least three times in duplicate. The nonspecific binding was defined as the binding activity in the presence of 1 µM edeoidin and was subtracted from the total binding activity for determination of the specific binding. The specific binding activity amounted to 92-98% of the total binding activity. The theoretical curves for displacement of [³H]-labeled Bolton-Hunter edeoidin binding by tachykinin peptides were drawn by nonlinear least squares analysis as described (10).

**RNA Blot Hybridization Analysis**

Total RNA and poly(A)⁺ RNA were isolated from various tissues of male rats as described previously (10). The yields of poly(A)⁺ RNA/total RNA shown in Fig. 7 were 6.1% (brain), 4.9% (stomach), 6.9% (duodenum), and 7.2% (large intestine). RNA blot hybridization analysis of poly(A)⁺ RNA each was carried out by using a nylon membrane as described previously (10). The 1371-base pair EcoT141I fragment was excised from clone prTKR3 and used as a probe. The size-markers used were rat ribosomal RNAs.

**RESULTS AND DISCUSSION**

The Nucleotide and Amino Acid Sequences for NKR—Fig. 1 shows the 2159-nucleotide sequence determined for the longest cDNA insert of the four functional NKR cDNA clones and the amino acid sequence of NKR deduced from the cloned cDNA. The size of the cDNA insert presented in Fig. 1 was smaller than that of the NKR mRNA estimated from RNA blot hybridization analysis (approximately 4.7 kilonucleotides; see Fig. 7). This hybridization analysis gave rise to only a single hybridization band. Furthermore, the four functional cDNA clones all contained a common protein-coding region but terminated with different positions at the 3' ends. Thus, the small size of the cDNA insert probably resulted from priming of adenine-rich sequences of the 3'-untranslated region of the mRNA.

The amino acid sequence of NKR was assigned from the longest open reading frame of the cDNA. The nucleotide sequence surrounding the initiation codon agrees reasonably well with the consensus sequence (13). The NKR polypeptide consists of 452 amino acid residues with a relative molecular weight of 61,104. The hydrophaticity profile and the sequence homology analyses indicated that NKR possesses seven hydrophobic segments and shares a significant sequence similarity with the members of G protein-coupled receptors (reviewed in Ref. 14). In addition, similar to other G protein-coupled receptors, there are potential N-glycosylation sites at the amino-terminal region and many serine and threonine residues as possible phosphorylation sites at the carboxyl-terminal region. Therefore, it is conceivable that NKR, like other G protein-coupled receptors, has a structure consisting of seven membrane-spanning domains with an extracellular amino terminus and a cytoplasmic carboxyl terminus.

**Comparison of the Amino Acid Sequences of the Three Rat Tachykinin Receptors**—Fig. 2 shows the comparison of the amino acid sequences of NKR, SPR, and SKR of the rat species. Although NKR is longer than two other tachykinin receptors at the amino-terminal region and the three receptors diverge in the sequences of both amino-terminal and carboxyl-terminal regions, the core sequences covering the seven putative transmembrane regions are strikingly homologous among the three receptors. The percentages of the homology in the core segments of the three receptors (assigned as the sequence corresponding to residues 63-394 of NKR) are as follows: 66.3% between NKR and SPR, 54.9% between NKR and SKR, and 53.7% between SPR and SKR. Of the 157 amino acid residues that are identical in the core sequences of the three tachykinin receptors, certain amino acids are also conserved in the sequences of all of the adrenergic and muscarinic receptors (14, 15), and these conserved amino acid species are cysteine (3/6), proline (4/11), glycine (2/4), phenylalanine (4/12), tryptophan (4/9), tyrosine (3/13), asparagine (3/8), and serine (2/6) (the numbers in parentheses show the respective amino acids conserved throughout the tachykinin, adrenergic, and muscarinic receptors, relative to those conserved in the three tachykinin receptors). Among the three cysteine residues that are conserved throughout all three families of receptors, it has been suggested by the analysis of β₂-adrenergic receptor that a disulfide bond is formed by 2 of the conserved cysteine residues that are situated in the first and second extracellular loops (18). The 3rd cysteine residue, which immediately follows transmembrane segment VII, is evidenced to be palmitoylated, and this results in the anchoring of the receptor to the plasma membrane (17). It has also been suggested that the proline and glycine residues in the membrane-spanning domains induce bends in the transmembrane helix, thus facilitating the interlock of adjacent helices (14). The 3 amino acids with aromatic side chains are all conserved in the three families of receptors, and these amino acids can be characterized as possessing the property to interact with the adjacent aromatic or charged amino acids. Thus, the amino acids discussed here should play a crucial role in the formation of a fundamental structure of the G protein-coupled receptors.

Fig. 3 illustrates the amino acids conserved in the three tachykinin receptors in the light of a transmembrane model of the tachykinin receptor. It is clear from this figure that the amino acids are conserved in not only the transmembrane helices but also their extending cytoplasmic portions close to the transmembrane helices. The tachykinin receptors are thought to express their functions by activating a phosphatidylinositol-calcium second message system (8, 18), and the observed conservation on the cytoplasmic side, particularly on the short homologous sequences of the third cytoplasmic loop near transmembrane segments V and VI (10), may be important in the coupling of the receptors to common cyto-
**Structure and Expression of Cloned Neumedin K Receptor**

ATCTCTGTGTTCTCTCTGCCCAGAGAGACGGCAGCCACCGTTGACGAGTCTGCTTCAACCCCAATCAAGCTTCCAGGTGGCTACAG

1 20 40

**FIG. 1.** The eDNA sequence for rat NKR and its deduced amino acid sequence. The amino acid sequence deduced for NKR is shown above the nucleotide sequence. Positions of the putative transmembrane segments I-VII of NKR are indicated above the amino acid sequence; the termini of each segment are tentatively assigned on the basis of a hydropathy profile and comparison with SPR and SKR. Triangles, potential N-glycosylation sites in the amino-terminal region.

**FIG. 2.** Alignment of the amino acid sequences of rat NKR (upper), SPR (middle), and SKR (lower). The enclosed amino acids represent residues that are identical in two or all of the sequences. Hyphens show deletions of the amino acid residues when compared with the other two sequences. Dots show the amino acids that are conserved in the sequences of the tachykinin, adrenergic, and muscarinic receptors. Triangles, potential N-glycosylation sites.

plasmic effecters. Interestingly, SPR and NKR are highly conserved not only throughout the third cytoplasmic loops but also in portions of the carboxyl-terminal regions (Fig. 2). In contrast, most of these regions diverge between SKR and SPR/NKR. Furthermore, the number of serine and threonine residues in the third cytoplasmic loops and in the carboxyl-terminal cytoplasmic regions differs among the three tachykinin receptors. SPR, NKR, and SKR possess 5, 2, and 1 residues of these amino acids in the third cytoplasmic loops, respectively, and 26, 28, and 14 residues in the carboxyl-terminal regions, respectively. G protein-coupled receptors exhibit a reduction in their responsiveness after repeated

Downloaded from http://www.jbc.org/ by guest on November 13, 2017
Application of agonists (reviewed in Refs. 14 and 19). A number of studies of the adrenergic and muscarinic receptors and rhodopsin have shown that the desensitization of these receptors correlates with the phosphorylation of serine and threonine residues at the carboxyl termini and/or the third cytoplasmic loops of these proteins (14, 19). Furthermore, mutational analysis of the β-adrenergic receptor has indicated that the phosphorylation of serine and threonine residues in the carboxyl tail is responsible for evoking rapid desensitization of this receptor (20). All three tachykinin receptors show desensitization in response to repeated application of agonists, but the desensitization behavior is different among the three receptors (10, 18) (see also Fig. 4). SPR is strongly desensitized to agonists, whereas desensitization is evoked moderately and weakly for NKR and SKR, respectively. Thus, it is possible that the sequence divergence and/or the different distribution of threonine and serine residues in the third cytoplasmic loops and the carboxyl-terminal regions could participate in evoking differing desensitization behavior of the three tachykinin receptors.

Fig. 3 also discloses an extreme sequence conservation of transmembrane segment VII among the three tachykinin receptors. Analogous to adrenergic receptors (21, 22), this membrane domain may play a crucial role in interacting with tachykinins, particularly with the carboxyl-terminal sequence common to the three tachykinin peptides (8). The seven hydrophobic transmembrane domains of G protein-coupled receptors generally contain several aspartic acids and glutamic acids (14). Except for Asp/Glu, which is present commonly in transmembrane segment II of the G protein-coupled receptors (14), no other aspartic acids or glutamic acids are present in the tachykinin receptors. In the structures of the tachykinin receptors, 1 histidine residue each is present at transmembrane segments V and VI, and these histidine residues, which are characteristic of the tachykinin receptors, may also play an important role in governing the interaction with receptor-specific ligands.

Electrophysiological Characterization of NKR—To determine the specificity of the tachykinin receptor encoded by a single cloned cDNA, we investigated electrophysiological responses to three mammalian tachykinin peptides in the oocyte expression system. As shown in Fig. 4, the application of 10⁻⁷ M neuromedin K induced a potent electrophysiological response in an oocyte injected with the mRNA derived from the cloned cDNA. The observed response was reduced by the second application of neuromedin K after a 10-min interval, but this desensitization was relieved by a 20-min washing prior to repeated application of neuromedin K. In the same oocyte, 10⁻⁶ M substance K and 10⁻⁶ M substance P applied at 20-25-min intervals induced a moderate and a minimal electrophysiological response, respectively. Thus, the rank order of potencies determined electrophysiologically for the three tachykinins is neuromedin K > substance K > substance P, and this order is consistent with that reported by ligand-binding experiments of rat brain NKR (9).

Ligand Binding of NKR—In order to determine accurately the affinities of NKR for tachykinin peptides, we examined the ligand-binding properties of the receptor expressed transiently in monkey kidney COS cells after transfection of the cloned cDNA. The NKR cDNA was inserted into the eukaryotic expression vector CDM8, and this plasmid was introduced into COS cells. The specific expression of the NKR mRNA in the transfected cells was verified by RNA blot hybridization analysis, whereas the NKR mRNA was not detectable in untransfected cells (data not shown). Since edeoidin, an octopod tachykinin, is known to be an agonist that binds to NKR with a high affinity (8), we measured binding of ¹²⁵I-Bolton-Hunter edeoidin to membranes prepared from NKR cDNA-transfected cells. As shown in Fig. 5, binding of ¹²⁵I-Bolton-Hunter edeoidin was saturable with a dissociation constant (Kᵦ) of 0.64 ± 0.17 nM. This value agrees well with that of a previously published report on binding of ¹²⁵I-Bolton-Hunter edeoidin to rat brain cortex membranes (23). The average density of binding sites in three experiments was 265 fmol/mg of protein of NKR cDNA-transfected cell membranes. No such sites were detected on membranes prepared from untransfected cells or cells transfected with the vector DNA alone.

Fig. 6 shows the result of displacement of ¹²⁵I-Bolton-Hunter edeoidin binding to membranes derived from DNA-transfected cells by three tachykinin peptides. The result demonstrated that ¹²⁵I-Bolton-Hunter edeoidin binding was most effectively inhibited by neuromedin K and less effec-
Experimental Procedures. Results are shown from one of three independent experiments. Inset, Scatchard plot of [125I]-Bolton-Hunter ededoisin binding; in this experiment, \( B_{\text{max}} \) value was 314 fmol/mg of protein, but \( B_{\text{max}} \) values in the three experiments were dependent on the efficiency of transfection.

FIG. 5. Saturation isotherms of specific binding of [125I]-Bolton-Hunter ededoisin to membranes prepared from NKR cDNA-transfected cells. Experimental details are described under “Experimental Procedures.” Results are shown from one of three independent experiments. Inset, Scatchard plot of [125I]-Bolton-Hunter ededoisin binding; in this experiment, \( B_{\text{max}} \) value was 314 fmol/mg of protein, but \( B_{\text{max}} \) values in the three experiments were dependent on the efficiency of transfection.

FIG. 6. Displacement of [125I]-Bolton-Hunter ededoisin binding by tachykinin peptides. Experimental details are described under “Experimental Procedures.” Each point represents the mean of three separate experiments. The unlabeled tachykinin peptides added to the binding assay are as follows: 0, neuromedin K; 1, substance K; 2, substance P.

Hybridization Analysis of NKR mRNA—The properties of the brain NKR have been well characterized by ligand-binding studies (8, 23, 24). Although pharmacological experiments with a selective agonist for NKR have suggested the presence of NKR in peripheral tissues such as the gastrointestinal tract (25), the NKR in these peripheral tissues has not yet been well characterized or even reported to be undetectable by ligand-binding studies. We therefore examined expression of the NKR mRNA in the rat brain and the gastrointestinal tract by blot hybridization analysis. As shown in Fig. 7, poly(A)+ RNAs isolated from the brain, stomach, duodenum, and large intestine gave rise to a single hybridization band with an estimated mRNA size of approximately 4.2 kilonucleotides. The mRNA was found in high amounts in the brain and in a lesser amount (about 1–10% of the relative amount of the brain NKR mRNA) in the gastrointestinal tract. The result thus explicitly demonstrates that the NKR mRNA is expressed in both the brain and the peripheral tissues but in different amounts.

In summary, this investigation presents the complete amino acid sequence of rat NKR and discusses the sequence characteristics of the tachykinin receptors on the basis of the sequence comparison of the three rat tachykinin receptors. The three tachykinin receptors belong to the family of G protein-coupled receptors and show sequence similarities and divergences that are segmented from one another. The three receptors underlie the expression of common but clearly distinguishable biological activities of the three mammalian tachykinin peptides. The characterization of the three receptors presented in this study as well as in our previous studies (9, 10) explicitly demonstrates the differing affinity rank orders of these receptors: for SPR, substance P > substance K > neuromedin K; for SKR, substance K > neuromedin K > substance P; and for NKR, neuromedin K > substance K > substance P. The desensitization effect observed by repeated administration of agonists is also different in the three receptors, and the desensitization effect is manifested in the order of SPR, NKR, and SKR. The sequence similarities and divergences discussed in this paper may thus contribute to the emergence of similar but distinct properties of the three receptors.

Hybridization Analysis of NKR mRNA—The properties of the brain NKR have been well characterized by ligand-binding studies (8, 23, 24). Although pharmacological experiments with a selective agonist for NKR have suggested the presence of NKR in peripheral tissues such as the gastrointestinal tract (25), the NKR in these peripheral tissues has not yet been well characterized or even reported to be undetectable by ligand-binding studies. We therefore examined expression of the NKR mRNA in the rat brain and the gastrointestinal tract by blot hybridization analysis. As shown in Fig. 7, poly(A)+ RNAs isolated from the brain, stomach, duodenum, and large intestine gave rise to a single hybridization band with an estimated mRNA size of approximately 4.2 kilonucleotides. The mRNA was found in high amounts in the brain and in a lesser amount (about 1–10% of the relative amount of the brain NKR mRNA) in the gastrointestinal tract. The result thus explicitly demonstrates that the NKR mRNA is expressed in both the brain and the peripheral tissues but in different amounts.

In summary, this investigation presents the complete amino acid sequence of rat NKR and discusses the sequence characteristics of the tachykinin receptors on the basis of the sequence comparison of the three rat tachykinin receptors. The three tachykinin receptors belong to the family of G protein-coupled receptors and show sequence similarities and divergences that are segmented from one another. The three receptors underlie the expression of common but clearly distinguishable biological activities of the three mammalian tachykinin peptides. The characterization of the three receptors presented in this study as well as in our previous studies (9, 10) explicitly demonstrates the differing affinity rank orders of these receptors: for SPR, substance P > substance K > neuromedin K; for SKR, substance K > neuromedin K > substance P; and for NKR, neuromedin K > substance K > substance P. The desensitization effect observed by repeated administration of agonists is also different in the three receptors, and the desensitization effect is manifested in the order of SPR, NKR, and SKR. The sequence similarities and divergences discussed in this paper may thus contribute to the emergence of similar but distinct properties of the three receptors.

REFERENCES
Cloning and expression of a rat neuromedin K receptor cDNA.
R Shigemoto, Y Yokota, K Tsuchida and S Nakanishi


Access the most updated version of this article at http://www.jbc.org/content/265/2/623

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/2/623.full.html#ref-list-1