The EMBO Journal vol.11 no.10 pp.3585-3591, 1992

Delineation of structural domains involved in the subtype specificity of tachykinin receptors through chimeric formation of substance P/substance K receptors

Yoshifumi Yokota[1], Chihiro Akazawa, Hiroaki Ohkubo[2] and Shigetada Nakanishi

Institute for Immunology, Kyoto University Faculty of Medicine, Yoshida, Sakyo-ku, Kyoto 606 and [2]Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto 862, Japan

[1]Present address: Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto 862, Japan

Communicated by A.D.Strosberg

The mammalian tachykinin receptors belong to the family of G protein-coupled receptors and consist of the substance P, substance K and neuromedin K receptors (SPR, SKR and NKR). We constructed 14 chimeric receptors in which seven transmembrane segments were sequentially exchanged between the rat SPR and SKR and examined the subtype specificity of the chimeric receptors by radioligand binding and inositol phosphate measurements after transfection into COS cells. All chimeric receptors showed maximum responses in agonist-induced inositol phosphate stimulation. Detailed analysis of five receptors with agonist selectivity similar to SPR indicated that the selectivity is mainly determined by the region extending from transmembrane segment II to the second extracellular loop together with a minor contribution of the extracellular N-terminal portion. This conclusion was more directly confirmed by an additional chimeric formation in which the introduction of the above middle portion of SPR into the corresponding region of SKR conferred a high affinity binding to substance P. The tachykinin receptors can thus be divided into two functional domains: the region covering transmembrane segments V-VII and responsible for fundamental recognition of the common tachykinin sequence; and its preceding portion involved in evoking subtype specificity by interacting with the divergent sequences of the peptides.

Key words: chimeric formation/G protein-coupled receptor/ligand binding domain/signal transduction/tachykinin receptor

Introduction

The mammalian tachykinin system is a typical example of biologically active peptides that exhibit a high degree of functional diversity through interaction with the selective receptors (Nakanishi *et al.*, 1990; Nakanishi, 1991). It consists of three distinct peptides, substance P, substance K and neuromedin K, and possesses three different receptors (Nakanishi *et al.*, 1990; Nakanishi, 1991). We and another group reported the molecular cloning of cDNAs for the substance P receptor (SPR) (Yokota *et al.*, 1989; Hershey and Krause, 1990), substance K receptor (SKR) (Masu *et al.*, 1987; Sasai and Nakanishi, 1989) and neuromedin K receptor (NKR) (Shigemoto *et al.*, 1990). The three receptors belong to the family of G protein-coupled receptors with seven transmembrane segments. The overall identity of the amino acid sequences of these receptors is ~50%, indicating that they form a subgroup within the superfamily of G-coupled receptors (Nakanishi, 1991). All three receptors are coupled to the stimulatory cascades of both phosphatidylinositol (PI) hydrolysis and cyclic AMP formation (Nakajima *et al.*, 1992). However, they differ in affinities for the three tachykinins, efficacies of agonist-evoked desensitization and expression sites of their mRNAs (Tsuchida *et al.*, 1990; Shigemoto *et al.*, 1990; Ingi *et al.*, 1991). The different physiological responses of the three tachykinins thus occur as a result of the selectivities, different properties and distributions of the three receptors.

Our previous studies using functional expression of the tachykinin receptors in COS cells indicated that they show a common feature of peptide receptors in that they cross-recognize peptides of the same peptide family (Ingi *et al.*, 1991). Furthermore, the binding characteristics of various tachykinin peptides and their related peptide fragments revealed that the tachykinin receptors interact with several key amino acids of the respective tachykinin peptide through recognition of the fundamental core sequence of this peptide family (Ingi *et al.*, 1991). The structural basis for the subtype specificity of the tachykinin receptors, however, remains to be elucidated. The construction of chimeric receptor proteins has proven to be a very useful approach in identifying structural domains which determine the subtype specificity of the G protein-coupled receptors for small molecules such as catecholamines and acetylcholine (Frielle *et al.*, 1988; Kobilka *et al.*, 1988; Kubo *et al.*, 1988; Marullo *et al.*, 1990; Wess *et al.*, 1991). It would be interesting to examine structural determinants for the ligand binding specificity of the tachykinin receptors that interact with peptide molecules larger than catecholamines and acetylcholine. In this investigation, we constructed a series of chimeric receptors of SPR-SKR and assessed their agonist selectivity by measuring radioligand binding and inositol phosphate formation in cDNA-transfected COS cells. Here we report that high affinity binding of the tachykinin receptors is determined by the middle portion around transmembrane segments (TMs) II-IV and also partly by the extracellular sequence of these receptors.

Results

Construction and expression of chimeric receptors

We constructed chimeric receptors for SPR-SKR by exchanging cDNA restriction fragments at the corresponding restriction sites situated in the four extracellular and three cytoplasmic regions of the two rat receptors. These sites allowed the exchange of unmodified transmembrane domains together with their flanking regions. We sequentially replaced

transmembrane segments according to a membrane spanning model of the tachykinin receptors and constructed 14 chimeric receptors which consisted of two series of reciprocal receptors, termed the KP and PK series, as illustrated in Figure 1. In the KP series, the amino acid sequences of SPR were substituted for the homologous regions of SKR in the direction from the N-terminus to the C-terminus. Chimeras in the PK series were mirror images of those in the KP series in that the amino acid sequences of SKR were replaced with the corresponding regions of SPR in the same direction. We transiently expressed the chimeric receptor cDNAs in COS-7 cells and its variant COS-m6 cells by DNA transfection. The COS-m6 cell line was used for the PI hydrolysis experiments because inositol phosphate formation was relatively low under ligand-untreated control culture conditions (Wong *et al.*, 1990). The expression of each chimeric receptor protein was examined by immunostaining with polyclonal antibodies against SPR and SKR. The expression levels of the chimeric receptors were estimated to be similar to those of the parental SPR and SKR (data not shown).

Measurements of PI hydrolysis

Our previous studies indicated that both SPR and SKR are coupled to the activation of PI hydrolysis through a G protein as a major signalling pathway (Nakajima *et al.*, 1992). To assess the subtype specificity of the chimeric receptors, we examined responses of PI hydrolysis to the application of substance P or substance K in COS-m6 cells expressing individual chimeric receptors. We first determined dose–response curves of the parental SPR and SKR in inducing inositol phosphate formation after 30 min incubations of cDNA-transfected cells with substance P and substance K (Figures 2A and 3A). The effective concentrations of substance K and substance P in inducing a half-maximal response (EC_{50}) of SKR were $\sim 3 \times 10^{-9}$ M and 3×10^{-7} M, respectively. These values were in good agreement with the previous data obtained from CHO cells stably expressing SKR (Nakajima *et al.*, 1992). The EC_{50} values of substance P and substance K for SPR were $\sim 3 \times 10^{-10}$ M and 1×10^{-8} M, respectively, and both values were slightly lower than those of the SPR expressed in CHO cells (Nakajama *et al.*, 1992). This probably reflects a coupling efficiency difference of SPR with a G protein between COS-m6 and CHO cells.

We determined dose–response curves of the KP and PK series of chimeric receptors by measuring inositol phosphate formation after application of substance P and substance K. The results presented in Figures 2B–H and 3B–H show that all chimeric receptors were capable of responding to both peptides and evoking full activation of inositol phosphate formation at a concentration of at least 1×10^{-6} M of the peptide. The results thus indicated that they are functionally expressed and are coupled to the PI hydrolysis signalling pathway in COS-m6 cells. However, the different chimeric receptors showed distinct potencies and selectivities in response to substance P and substance K, and they were classified into two groups termed group H (high affinity) and group L (low affinity). The former showed a potency and selectivity comparable with those of the native receptor in inducing the stimulation of inositol phosphate formation, whereas the latter, though maintaining a fundamental receptor activity, lost a high affinity or selectivity, or both. This difference in potency between the two groups of the chimeric receptors could be more clearly appreciated at a concentration of 10^{-8} M of the agonist added. At this concentration, the receptors of group H showed a marked increase in inositol phosphate formation in response to the addition of the selective agonist, whereas those of group L exhibited no appreciable stimulation of PI hydrolysis. According to this approximation, several features characteristic of the different chimeric receptors can be pointed out as summarized in Table I.

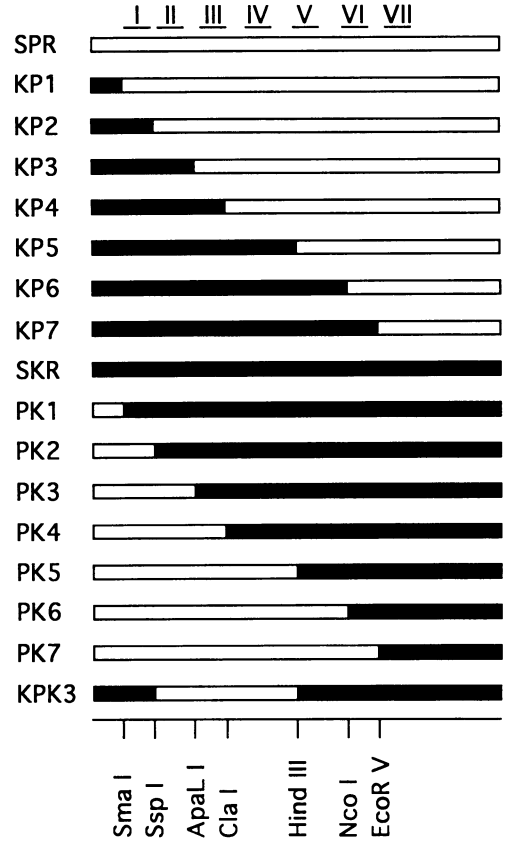

Fig. 1. Schematic structures of chimeric receptors constructed from rat SPR and SKR. Empty and solid boxes represent the amino acid sequences derived from the SPR and SKR, respectively. The KP series of the chimeric receptors was constructed by exchanging the SPR and SKR sequences at the restriction sites corresponding to the following amino acid positions of the SPR sequence: KP1 (28–29); KP2 (56–58); KP3 (94–95); KP4 (129–131); KP5 (194–196); KP6 (249–251) and KP7 (276–278). Chimeras in the PK series were mirror images of those of the KP series. The KPK3 receptor contained the SPR sequence from amino acid residue 58 to residue 195. Note that the chimeric formations result in no insertion or deletion of amino acid residues in the chimeric receptors constructed. The locations of the seven transmembrane segments of the tachykinin receptors and seven restriction sites used for the chimeric formation are indicated above and below the structures of the chimeric receptor, respectively.

(i) In the KP series, KP1 and KP2 were categorized as group H (Figure 2B and C). These receptors were made by replacing the extracellular N-terminal sequence, or together with its extending TM I of SPR, with the corresponding region of SKR. Both receptors retained a subtype specificity characteristic of SPR in responding to substance P and substance K, although the response curve for substance P slightly shifted to the right in both receptors when compared with that of the native SPR.

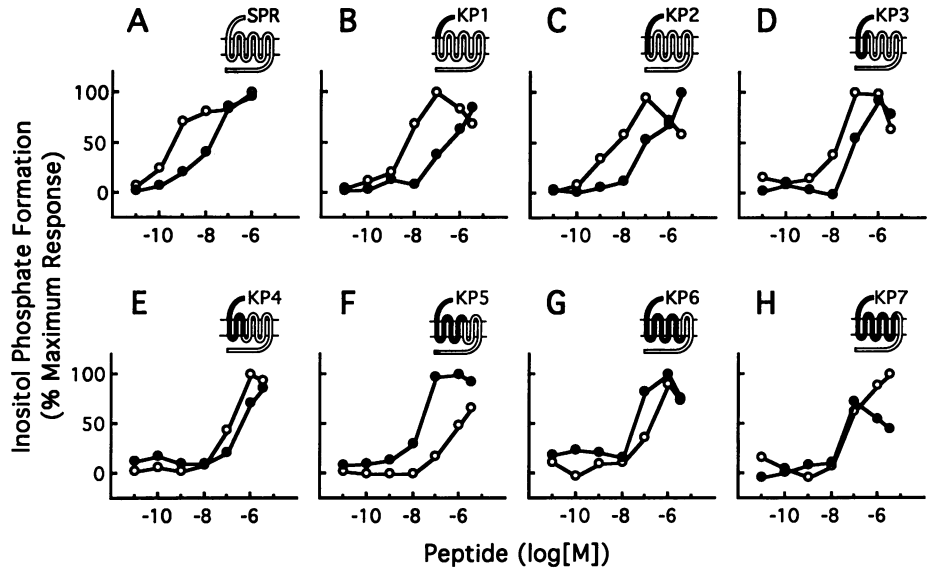

Fig. 2. Dose–response curves of SPR and the KP series of chimeric receptors for inositol phosphate formation. COS-m6 cells expressing SPR (**A**), KP1 (**B**), KP2 (**C**), KP3 (**D**), KP4 (**E**), KP5 (**F**), KP6 (**G**) and KP7 (**H**) were incubated with the indicated concentrations of substance P (○) and substance K (●) for 30 min, and the amounts of total inositol phosphates were determined. Inositol phosphate formation is expressed as a percentage of the maximal response for the respective receptor. Each curve shown is representative of three or four experiments. The maximal response of SPR was a 2.8-fold increase over basal levels of agonist-untreated cells, while those of the chimeric receptors ranged from 2.1- to 3.4-fold over basal levels. In insets, the sequence derived from SPR is indicated by an open line, while that derived from SKR is displayed by a solid line.

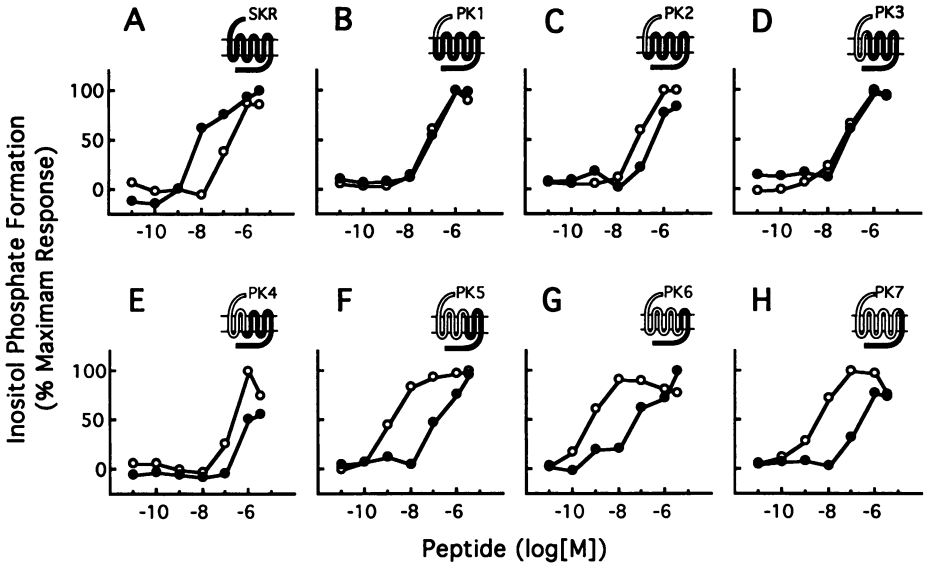

Fig. 3. Dose–response curves of SKR and the PK series of chimeric receptors for inositol phosphate formation. Total inositol phosphate formations in COS-m6 cells expressing SKR (**A**), PK1 (**B**), PK2 (**C**), PK3 (**D**), PK4 (**E**), PK5 (**F**), PK6 (**G**) and PK7 (**H**) were examined after incubation with substance P (○) or substance K (●). Each curve shown is representative of three to six experiments and is drawn as a percentage of the maximal stimulation. The maximal response of SKR was 2.1-fold over basal levels, while those of the chimeric receptors ranged within 2.4- to 3.4-fold. For other explanations, see Figure 2.

(ii) In the PK series, PK5, PK6 and PK7 showed a ligand binding selectivity identical to that of the native SPR (Figure 3F–H), indicating that the C-terminal region extending from TM V to the cytoplasmic tail is not important in determining the SPR subtype specificity.

(iii) The sequential replacements of TM II and TM III in the KP series (KP3 and KP4) resulted in the loss of the agonist potency to stimulate inositol phosphate formation in response to substance P (Figure 2D and E). Thus, taken together with the above results, this finding suggested that the region from TM II to the second extracellular loop of SPR contributes to determining the subtype specificity of this receptor.

(iv) Although the reciprocal chimeric receptors (PK1–PK4 and KP6–KP7) were examined to delineate the domains involved in the subtype specificity of SKR, none of them showed a potency or selectivity comparable with that of SKR (Figures 2F–H and 3D, E). We thus failed to assign a structural determinant for the SKR selectivity within the receptor molecule. Notably, however, a significant selectivity for substance K was reproducibly observed in the KP5 receptor (Figure 2F), suggesting that similar to

SPR, the sequence preceding TM V may contribute to determining a subtype specificity of SKR.

Radioligand binding assays

To assess the subtype specificity of the chimeric receptors more quantitatively, we examined the ability of each receptor to bind to a radiolabeled ligand in membrane fractions after introduction and expression of the chimeric receptor cDNAs in COS-7 cells. Among the 14 chimeric receptors, five receptors classified as group H (KP1, KP2, PK5, PK6 and PK7) showed a high affinity binding to [^{125}I]Bolton–Hunter(BH)-substance P, but not to [^{125}I]substance K, at a concentration of 0.1 nM of the respective radioligand (Figure 4). The remaining nine receptors classified as group L, however, revealed no significant binding to either [^{125}I]BH-substance P or [^{125}I]substance K even by the addition of high concentrations of membrane fractions to a binding assay mixture (data not shown).

Table I. Classification and IC_{50} values of native and various chimeric receptors

Receptor	Group	IC_{50} (nM)	
		Substance P	Substance K
KP1	H	0.37 ± 0.07* (n = 4)	4.7 ± 1.3** (n = 4)
KP2	H	0.36 ± 0.05* (n = 5)	7.8 ± 1.1** (n = 5)
KP3	L	–	–
KP4	L	–	–
KP5	L	–	–
KP6	L	–	–
KP7	L	–	–
PK1	L	–	–
PK2	L	–	–
PK3	L	–	–
PK4	L	–	–
PK5	H	0.16 ± 0.02 (n = 3)	21 ± 2 (n = 3)
PK6	H	0.12 ± 0.01 (n = 3)	20 ± 1 (n = 3)
PK7	H	0.14 ± 0.03 (n = 3)	20 ± 6 (n = 3)
KPK3	H	0.60 ± 0.14* (n = 3)	7.7 ± 2.0* (n = 3)
SPR	H	0.19 ± 0.02 (n = 3)	23 ± 6 (n = 3)
SKR	H	110 ± 30 (n = 3)	0.33 ± 0.05 (n = 3)

Each receptor was classified into group H or group L according to the potency and selectivity in the response of agonist-induced PI hydrolysis; see also text for explanation. Data of PI hydrolysis were obtained from at least three separate experiments. IC_{50} values are given as the means ± SEM of data obtained from the indicated number (n) of separate displacement experiments of [^{125}I]BH-substance P binding. In the third column (substance P), *$P < 0.05$ compared with IC_{50} of SPR in binding to substance P. In the fourth column (substance K), *$P < 0.05$ and **$P < 0.01$ compared with IC_{50} in binding to substance K.
– not measurable.

We determined displacement curves of substance P and substance K in the binding of [^{125}I]BH-substance P to the five chimeric receptors of group H. The results of displacement experiments of the native SPR and the five chimeric receptors are presented in Figure 4 and are summarized in Table I. The displacement experiments indicated that the affinities of binding to both substance P and substance K in the PK5, PK6 and PK7 receptors were virtually identical to those of the native SPR. The data thus supported our conclusion that TM V, TM VI and TM VII, together with their connecting loops and the C-terminal tail, are not essential for determining subtype specificity of SPR. Furthermore, the analysis of KP1 and KP2 revealed the role of the N-terminal sequence of the receptor in the selective binding to a tachykinin peptide. The IC_{50} values of substance P for KP1 and KP2 were determined to be 0.37 ± 0.07 nM and 0.36 ± 0.05 nM, respectively (IC_{50}: the concentration of the peptide required to inhibit the specific binding by 50%). The affinity for substance P was thus reduced in these chimeric receptors when compared with that of the native SPR (0.19 ± 0.02 nM). Notably, both receptors showed significant increases in the affinity for substance K. The IC_{50} values of substance K for KP1 and KP2 were 4.7 ± 1.3 nM and 7.8 ± 1.1 nM, respectively, and these values were much lower than that for the native SPR (23 ± 6 nM). This observation thus strongly suggested

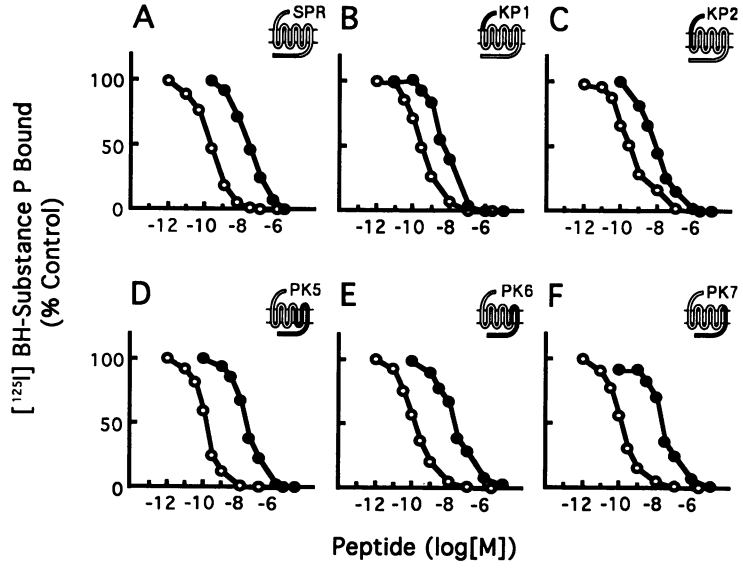

Fig. 4. Displacements of [^{125}I]BH-substance P binding to SPR and five chimeric receptors by substance P and substance K. Competition of binding of [^{125}I]BH-substance P at a final concentration of 0.1 nM was assessed by addition of various concentrations of unlabeled substance P (○) and substance K (●) to the binding assays of SPR (**A**) and the chimeric receptors of KP1 (**B**), KP2 (**C**), PK5 (**D**), PK6 (**E**) and PK7 (**F**). Each curve shown is representative of three to five experiments done in triplicate.

that the extracellular N-terminal domain partly contributes to determining agonist subtype specificity of the tachykinin receptor.

Chimera KPK3

To explore more directly the role of the region covering TM II to the second cytoplasmic loop in determining subtype specificity of SPR, we constructed a new chimeric receptor KPK3 and examined a profile of the subtype specificity of this receptor by measuring inositol phosphate formation and radioligand binding displacement. In this receptor, the sequence from the first cytoplasmic loop to the second extracellular loop of SPR was introduced into the SKR structure by exchanging the corresponding sequence of SKR. As shown in Figure 5A, analysis of inositol phosphate formation indicated that the KPK3 receptor retained not only a relatively high affinity in interacting with substance P but also a subtype specificity characteristic of SPR with the rank order of substance P > substance K. This binding feature of the KPK3 receptor was more explicitly revealed by radioligand displacement experiments (Figure 5B). The IC_{50} values of substance P and substance K were determined to be 0.60 ± 0.14 nM and 7.7 ± 2.0 nM, respectively. Thus, similar to the KP1 and KP2 receptors, the affinity of the KPK3 receptor for substance P was slightly lower than that of SPR but considerably higher than that of SKR. Furthermore, the affinity for substance K increased as compared with the native SPR, consistent with the above finding that the N-terminal portion of the receptor is partly involved in determining the agonist selectivity. Thus, the analysis of the KPK3 receptor indicated more directly that the subtype specificity of the tachykinin receptor is mainly determined by the region extending from TM II to the second extracellular loop and also partly by the extracellular N-terminal domain of this receptor.

Discussion

In this investigation, we attempted to delineate the structural domains involved in the ligand binding specificity of the tachykinin receptors by construction and expression of a series of chimeric receptors between SPR and SKR. The agonist selectivity of the resultant receptors was assessed by radioligand binding and inositol phosphate measurements. Replacement of the C-terminal region up to the TM V of SPR with the corresponding region of SKR showed a subtype specificity of SPR in both inositol phosphate and radioligand binding assays. Furthermore, when the similar region of SPR (from the middle portion of the second extracellular loop to the carboxyl tail) was exchanged with the NKR in separate experiments, this chimeric receptor also showed a high affinity binding to substance P (U.Gether, T.E.Johansen, R.M.Snider, J.A.Lowe, S.Nakanishi and T.W.Schwartz, manuscript in preparation). These findings indicate that the region extending from TM V to the C-terminal tail is not a major determinant involved in conferring a high affinity binding of the receptor, and suggest that its preceding region is essential for governing the ligand binding selectivity of the tachykinin receptor. In fact, the importance of the middle portion of the tachykinin receptor in determining subtype specificity was evidenced by analysis of the KPK3 chimeric receptor in which the region around TM II–TM VI of SPR was replaced in the SKR structure. Furthermore, the exchange of the extracellular N-terminal domain also affected the affinity of the tachykinin receptor. Thus, taken together, it can be concluded that the ligand binding specificity of the tachykinin receptor is mainly determined by the region around TM II–TM IV and also partly by the extracellular N-terminal domain of this receptor.

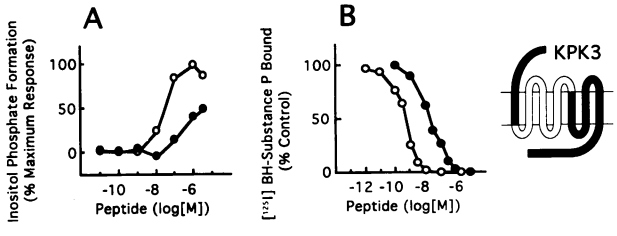

Fig. 5. Characterization of the property of the KPK3 chimeric receptor. Dose–response curves of total inositol phosphate formation (**A**) and displacement of [^{125}I]BH-substance P binding (**B**) of the KPK3 chimeric receptor were determined. The results shown in (A) and (B) are representatives from three and four experiments, respectively. For other explanations, see Figures 2 and 4.

Although all chimeric receptors showed functional expression in the analysis of inositol phosphate formation, none of these receptors exhibited a distinct subtype specificity characteristic of SKR with a high affinity for substance K. The possibility that the loss of this subtype specificity is due to insufficient synthesis or inappropriate membrane incorporation of these proteins can be excluded from the following observations. (i) Immunostaining analysis using antibodies against SPR and SKR showed appreciable amounts of expression of chimeric receptors in membranes of cDNA-transfected cells. (ii) Maximal responses in inositol phosphate formation were observed in all chimeric receptors by the application of a tachykinin peptide. (iii) Dose–response curves for substance K did not vary significantly among the chimeric receptors, indicating that they retained the fundamental ability to interact with the core sequence of the tachykinin peptide. We suspect that the observed loss of the SKR selectivity may reflect some molecular incompatibility to maintain a high affinity binding to substance K in the chimeric receptor formation. This was unexpected for some chimeric receptors, particularly for the KP7 receptor in which the exchanged TM VII and the third extracellular sequence are highly conserved between SPR and SKR; amino acids are identical at 28 of 33 positions in these regions of the two receptors. Similar chimeric incompatibilities were reported for some of the chimeric receptors constructed between the adrenergic α_2 and β_2 receptors (Kobilka *et al.*, 1988) and between the thyrotropin and lutropin–choriogonadotropin receptors (Nagayama *et al.*, 1991). Furthermore, it was found that the potency of SKR to interact with substance K was lower than that of SPR for substance P in PI hydrolysis. Thus we suggest that a subtle molecular incompatibility may produce a marked effect on the high-affinity binding ability of SKR. In spite of this limitation, a significant selectivity for substance K was observed in the KP5 receptor in which the C-terminal region up to TM V of SKR was replaced with the corresponding region of SPR. It is thus conceivable that the structural basis for governing the subtype specificity is similar between SPR and SKR.

It has previously been demonstrated that several transmembrane domains are important in determining the selectivity of agonist and antagonist binding in the adrenergic

and muscarinic receptors by formation of a ligand binding pocket (Frielle *et al.*, 1988; Kobilka *et al.*, 1988; Kubo *et al.*, 1988; Marullo *et al.*, 1990; Wess *et al.*, 1991). For example, TM VI and TM VII of β-adrenergic receptors have been shown to play a crucial role in conferring antagonist binding specificity, whereas TM IV is important for agonist binding selectivity (Frielle *et al.*, 1988), although other domains are also involved (Strader *et al.*, 1987, 1989; Frielle *et al.*, 1988; Marullo *et al.*, 1990). In contrast, in glycohormone receptors such as the lutropin–choriogonadotropin receptor and the thyrotropin receptor, the large extracellular domains have been shown to be responsible for binding these glycoprotein hormones and for conferring a high affinity binding (Xie *et al.*, 1990; Nagayama *et al.*, 1991). Thus, the mode of ligand binding in the tachykinin receptors represents an intermediate form between the adrenergic/muscarinic receptors and the glycohormone receptors, and this notion is conceivable, taking into consideration that tachykinin peptides are about 10 times larger than acetylcholine and catecholamines.

Our previous binding studies of various tachykinin peptides and their related peptide fragments indicated that the minimum sequence which manifests a fundamental binding is contained in the C-terminal part of the tachykinin peptides, and that a high-affinity and selective binding originates from the heterogeneity of the N-terminal sequences of the peptide molecules (Ingi *et al.*, 1991). This structure–activity relationship of the tachykinin peptides coincides well with the concept of the 'message–address' sequence of the peptide (Schwyzer, 1977; Portoghese, 1989). A message sequence is an amino acid sequence common within the same group of peptides and is believed to be essential in both binding and signal transduction. An address sequence, on the other hand, confers high affinity and selectivity of the peptides without participating in signal transduction. The common C-terminal -Phe-X-Gly-Leu-Met-NH_2 sequence of the tachykinin peptides corresponds to a message sequence, while the divergent N-terminal sequence represents an address sequence. In relation to the 'message–address' concept, the functional domains of the tachykinin receptors defined in this investigation are postulated as schematically illustrated in Figure 6. Because the exchange of the C-terminal portion between SPR and SKR resulted in no significant change in the affinity of SPR, we assume that this portion contributes to the interaction with the message sequence of the tachykinin peptides. Its preceding N-terminal portion including the extracellular N-terminal domain, on the other hand, is predicted to be involved in recognition of the address sequence of the tachykinin peptides, thus providing the subtype specificity characteristic of each tachykinin receptor. This structural map may be much too simplified, but this model could be useful for investigating the structural basis of the peptide–receptor interaction not only for understanding the functions of the tachykinin receptors but also for developing new agonists and antagonists as human therapeutic agents.

Materials and methods

Materials

Inositol-free Dulbecco's modified Eagle's medium was obtained from Dainippon Seiyaku. *In vitro* mutagenesis kit was purchased from Bio-Rad. A COS-m6 cell line (a subline of COS-7 cells) was kindly provided by Dr E.M.Ross (University of Texas Southwestern Medical Center).

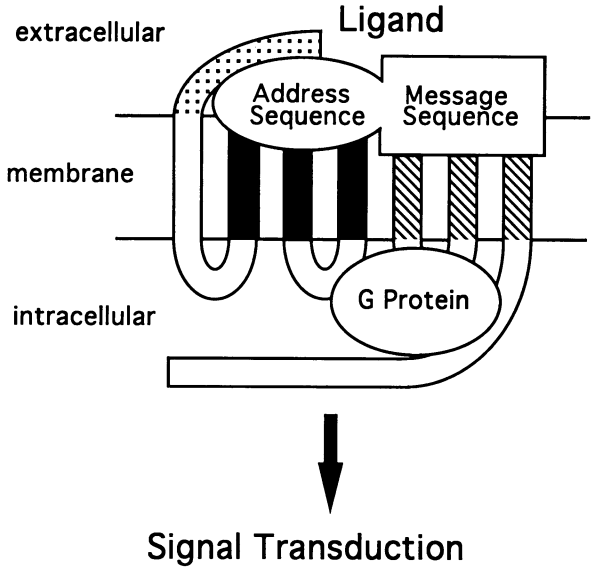

Fig. 6. A model of the peptide binding sites of the tachykinin receptor. For an explanation of this model, see text. Hatched areas represent the binding site for a message sequence, while solid and dotted areas illustrate major and minor binding sites for an address sequence, respectively.

Other reagents were obtained as described (Ingi *et al.*, 1991; Nakajima *et al.*, 1992).

Construction of chimeric receptor cDNAs

Seven restriction sites shown in Figure 1 (*Sma*I, *Ssp*I, *Apa*LI, *Cla*I, *Hin*dIII, *Nco*I and *Eco*RV) at equivalent positions in the cDNAs for rat SPR (Yokota *et al.*, 1989) and for rat SKR (Sasai and Nakanishi, 1989) were used to construct chimeric receptor cDNAs. These sites were chosen because they are unique in both cDNA sequences and the chimeric formations cause no insertion or deletion in the amino acid sequences of SPR and SKR. The *Nco*I site pre-existed in the coding region of the SKR cDNA, while the others were introduced by site-directed mutagenesis (Kunkel, 1985; Kakizuka *et al.*, 1990) using the *in vitro* mutagenesis kit after subcloning the cDNAs into *Hin*dIII–*Pst*I sites of pTZ19U (Mead *et al.*, 1986). The mutagenized SPR and SKR cDNAs containing the above restriction site were digested with a mixture of *Hin*dIII, *Pst*I and one more appropriate restriction enzyme; the former two were used to cut at the 5′ and 3′ ends of the cDNAs, while the third enzyme was included to digest the cDNAs at the mutagenized site. Fragments were ligated into the *Hin*dIII–*Pst*I sites of the eukaryotic expression vector CDM8 (Seed, 1987) to generate chimeric tachykinin receptor cDNAs. The authenticity of the SPR and SKR cDNAs used for chimeric formation and the various chimeric receptor cDNAs constructed was confirmed by sequence determination in combination with restriction enzyme analysis. Chimera KPK3 was generated by recombination of the KP2 and PK5 cDNAs at the *Stu*I site of the SPR sequence.

Expression of chimeric receptors

Transfection of each chimeric receptor cDNA into mammalian cells was conducted by the calcium phosphate method (Graham and van der Eb, 1973). COS-m6 (Wong *et al.*, 1990) and COS-7 cells were used as host cells for measurements of PI hydrolysis and ligand binding displacement of the chimeric receptors, respectively. Cell culture was performed as described (Yokota *et al.*, 1989) except that COS-m6 cells were cultured in 10% CO_2 during their maintenance and then shifted to 5% CO_2 during transfection and [^{3}H]inositol incorporation. The expression of each chimeric receptor protein was confirmed by immunostaining with polyclonal antibodies against SPR and SKR, which were kindly provided by Dr R.Shigemoto (Kyoto University).

Measurements of PI hydrolysis

The formation of total inositol phosphate (inositol mono-, bis- and tris-phosphates) was measured as described previously (Nakajima *et al.*, 1992). Briefly, COS-m6 cells were trypsinized 20–24 h after DNA transfection, seeded in 12-well plates at a density of $2.5–5.0 \times 10^4$ cells per well and cultured for 24 h. The cells were labeled with myo-[2-^{3}H]inositol (1 mCi/ml) in inositol-free Dulbecco's modified Eagle's

medium containing 10% dialyzed fetal calf serum and antibiotics for 20–24 h. They were washed twice with phosphate-buffered saline solution (PBS) and incubated with the same solution for 30 min at 37°C to reduce basal levels of inositol phosphate formation and then with PBS containing 10 mM LiCl (PBS-Li) for 30 min at 37°C. The cells were challenged with various concentrations of an agonist dissolved in PBS-Li by incubation for 30 min at 37°C. Extraction and separation of ^{3}H-labeled inositol phosphates were carried out as described (Nakajima *et al.*, 1992). The radioactivity of ^{3}H-labeled products was determined by a liquid scintillation spectrometer. All experiments were carried out at least three times in triplicate. The value of standard deviation was $<15\%$ in each triplicated sample. COS-m6 cells transfected with the vector DNA alone showed no stimulation of inositol phosphate formation after application of substance P and substance K within the concentration ranges of 1×10^{-12} to 3×10^{-6} M.

Radioligand binding assays

Cell culture and membrane preparation of receptor-expressing cells were performed according to the procedures described previously (Shigemoto *et al.*, 1990). Displacement binding experiments were carried out by using [^{125}I]BH-substance P and [^{125}I]substance K as described (Ingi *et al.*, 1991); cell membranes (1–20 μg/ml) were incubated with 0.1 nM [^{125}I]BH-substance P or 0.1 nM [^{125}I]substance K in the presence and absence of various concentrations of substance P and substance K, respectively. Each experiment was carried out at least three times in triplicate. The non-specific binding was defined as the binding activity in the presence of 2.5 μM of the corresponding unlabeled tachykinin and was subtracted from the total binding activity for determination of the specific binding. The non-specific binding activity amounted to $<5\%$ of the total binding activity. The IC_{50} values in the displacement analysis were calculated by non-linear least-squares analysis.

Acknowledgements

This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan, the Uehara Memorial Foundation and the CIBA-Geigy Foundation.

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Received on May 29, 1992