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STUDY ON THE INTRACELLULAR SIGNAL TRANSDUCTION INDUCED BY NEUROKININ RECEPTORS

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ABBREVIATIONS

Abbreviations used are those recommended by IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN): *Eur. J. Biochem.*, **138**: 9, (1984).

Ala: L-alanine
β-Ala: β-alanine
Arg: L-arginine
Asn: L-asparagine
Asp: L-aspartic acid
Cys: L-cysteine
Gln: L-glutamine
Glu: L-glutamic acid
pGlu: L-pyroglutamic acid
Gly: glycine
His: L-histidine
Ile: L-isoleucine
Leu: L-leucine
Lys: L-lysine
MePhe: N-methyl-L-phenylalanine
Met: L-methionine
Phe: L-phenylalanine
Pro: L-proline
Ser: L-serine
Thr: L-threonine
Trp: L-tryptophan
D-Trp: D-tryptophan
Tyr: L-tyrosine
Val: L-valine

NKA: neurokinin A
NKB: neurokinin B
NPγ: neuropeptide γ
NPK: neuropeptide K
SP: substance P

NK_1: neurokinin-1
NK_2: neurokinin-2
NK_3: neurokinin-3

CP-96,345: (2S, 3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine

MDL 28,564: [Leu$^9\psi(CH_2NH)$Leu^{10}]neurokinin A(4-10)

MEN 10,376: [Tyr^5,D-Trp^{6,8,9},Lys^{10}]neurokinin A(4-10)

senktide: succinyl-[Asp^6, MePhe^8]substance P

SR 142801: (S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)-propyl)-4-phenylpiperidin-4-yl)-N-methylacetamide

SR 48968: (S)-N-methyl-N-[4-acetylamino-4-phenylpiperidino-2-(3,4-dichlorophenyl)butyl]benzamide

Boc: t-butyloxycarbonyl

BOP: benzotriazolyl N-oxytrisdimethylamino phosphonium

DCC: N,N'-dicyclohexylcarbodiimide

DCM: dichloromethane

Fmoc: 9-fluorenylmethyloxycarbonyl

HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate

HF: hydrogen fluoride

HOBt: 1-hydroxybenzotriazole

MBHA resin: 4-methylbenzhydrylamine resin

NMP: N-methyl-2-pyrrolidone

RP-HPLC: reverse-phase high performance liquid chromatography

SAL resin: super acid labile resin, 4-(2',4'-dimethoxyphenyl- fluorenyl-aminomethyl)-phenoxy resin

TFA: trifluoroacetic acid

A.A.: arachidonic acid

AC: adenylate cyclase

$[Ca^{2+}]_i$: concentration of intracellular free Ca^{2+}

CHO cells: Chinese hamster ovary cells

cyclic AMP: cyclic adenosine 3',5'-monophosphate

DG: diacylglycerol

ET: endothelin

G protein: GTP-binding regulatory protein

GRP-10: gastrin-releasing peptide related decapeptide

Ins(1,4,5)P_3: inositol 1,4,5-trisphosphate

Ins(1,3,4,5)P_4: inositol 1,3,4,5-tetrakisphosphate

IP_3: inositol trisphosphate

PGE_2: prostaglandin E_2

PI: phosphatidylinositol

PIP_2: phosphatidylinositol 4,5 bis-phosphate

PKC: protein kinase C

PLC: phospholipase C

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethylsulfoxide

EGTA: ethyleneglycol bis(2-aminoethylether) tetraacetic acid

FCS: fetal calf serum

fura-2/AM: fura-2 acetoxymethyl ester

IBMX: 3-isobutyl-1-methylxanthine

SDS: sodium dodecyl sulfate

SSC: sodium citrate-sodium chloride

U73122: 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione

EC_{50}: median effective concentration

IC_{50}: median inhibitory concentration

CHAPTER I

GENERAL INTRODUCTION

Tachykinin is a family of peptides which share the common C-terminal sequence, Phe-Xaa-Gly-Leu-Met-NH_2 (Xaa; aromatic or aliphatic amino acid) as shown in TABLE I-1. Substance P was the first characterized peptide of this family. In 1931, von Euler and Guddam (1931) isolated a substance from horse brain and intestine, which had the ability to induce contraction of rabbit intestine and to cause hypotension by intravenous administration into rabbits. These effects were not inhibited by atropine, an antagonist for acetylcholine receptors, indicating that this active compound was not identical to acetylcholine (von Euler and Guddam, 1931). Because this crude compound was obtained as powder, it was named substance P (SP; "P" means powder). However, its primary sequence was not determined until the early 1970s. In 1970, SP was purified from horse hypothalamus by Chang and Leeman (1970), and its primary structure was determined in 1971 (Chang et al., 1971).

Since the discovery of SP, non-mammalian tachykinins such as physalaemin (Erspamer et al., 1964), eledoisin (Erspamer and Anastasi, 1962) and kassinin (Anastasi et al., 1977) have also been characterized (TABLE I-1). However, these peptides have been shown to induce higher contractile responses in several smooth muscle preparations from mammals than does SP. These findings led researchers to predict the presence of other mammalian tachykinins. In 1983, two novel peptides, neurokinin A (NKA) and neurokinin B (NKB), whose primary structures are similar to that of SP, were discovered from porcine spinal cord using a contractile assay of guinea-pig ileum by Kimura et al. (1983). Moreover, neuropeptide K (NPK) (Tatemoto et al., 1985) and neuropeptide γ (NPγ) (Kage et al., 1988) which both possess a NKA sequence at the C-terminus have been isolated from porcine brain and rabbit intestine, respectively. At present, these mammalian tachykinins are called neurokinins (Munekata, 1991).

Neurokinins are distributed throughout the central nervous system as well as in the peripheral tissues and have a wide variety of biological activities, as

shown in TABLE I-2. Relatively high amounts of SP are distributed in thin nerve fibers such as those of the C-group which are associated with pain transmission (Hokfelt et al., 1975). SP also has a depolarization effect on spinal cord preparations from new-born rats (Konishi and Otsuka, 1974). These findings indicate that SP is a neurotransmitter involved in the transmission of primary sensory neurons. In addition, neurokinins released from the nerve endings induce various functions. For example, neurokinins cause vasodilatation acting on the endothelium cells, possibly through the generation of nitric oxide (e.g., D'Orleans-Juste et al., 1985), and induce the contraction of gastrointestinal and vascular smooth muscle (Munekata, 1991; Maggi et al., 1993; Buck, 1994). Furthermore, neurokinins are involved in various inflammatory responses such as plasma protein extravasation and recruitment or stimulation of inflammatory cells (Munekata, 1991; Maggi et al., 1993; Buck, 1994; Bozic et al., 1996).

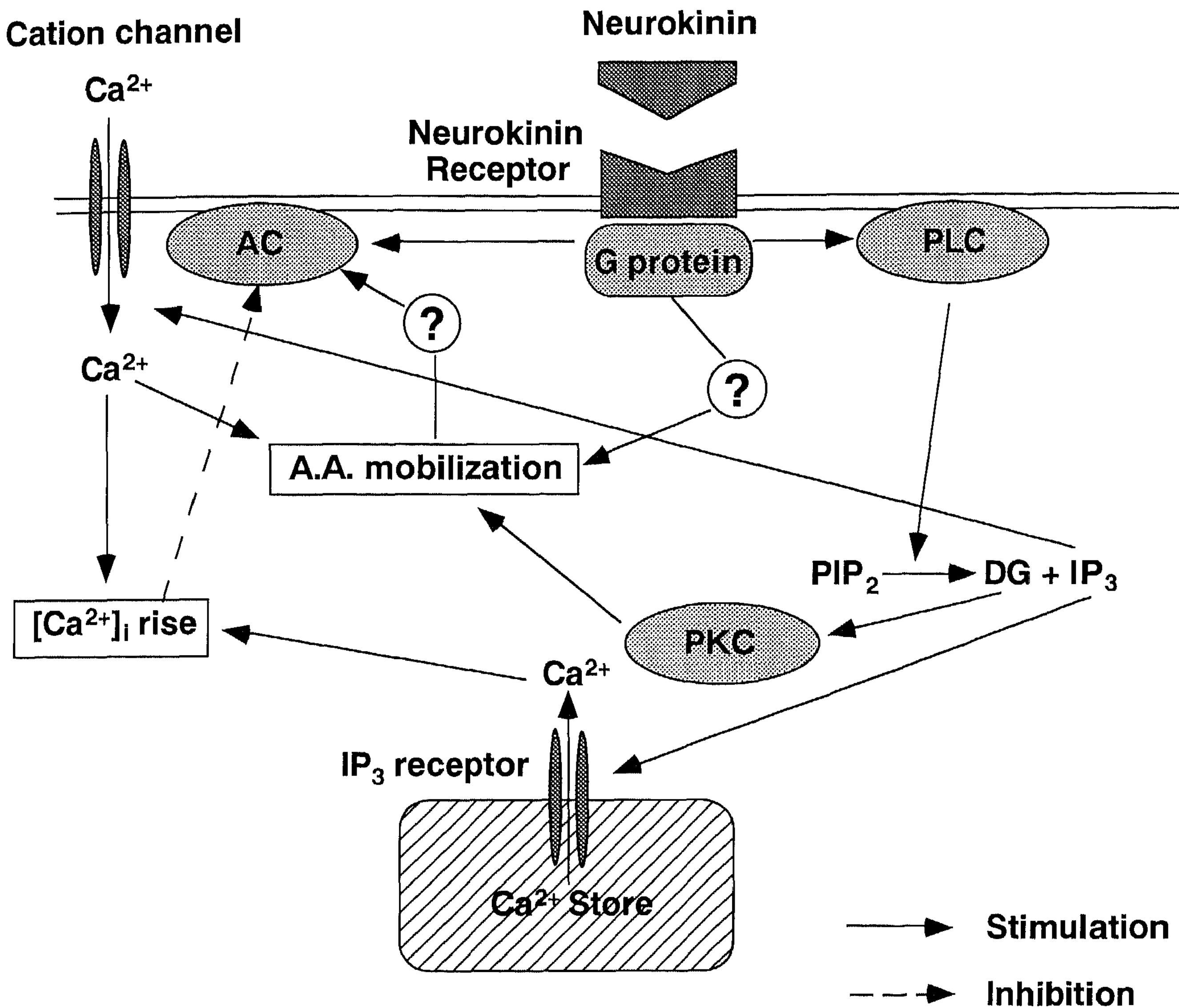
Most of these biological functions are thought to be mediated by three pharmacologically distinct cell surface receptors classified as neurokinin-1, 2 and 3 receptors (NK_1, NK_2 and NK_3 receptors, respectively) (Nakanishi, 1991; Buck, 1994). The preferred endogenous ligand appears to be SP for NK_1 receptors, NKA for NK_2 receptors and NKB for NK_3 receptors. The tissue distribution of each receptor type in the central nervous system as well as in the peripheral tissues, is listed in TABLE I-3. Recently, the heterogeneity of each neurokinin receptor type has been suggested (Maggi et al., 1993). For instance, NK_2 receptors have been classified into two subtypes, NK_{2A} and NK_{2B} receptors, and although there is no distinction made by native neurokinin peptides or by selective agonists with the exception of MDL 28,564 [[$Leu^9\psi(CH_2NH)Leu^{10}$]NKA(4-10)], the subtypes can be recognized by their very different affinity for NK_2 receptor antagonists (Maggi et al., 1990; Patacchini et al., 1991). Functional cDNAs for neurokinin receptors corresponding to NK_1, NK_2 and NK_3 receptors have also been cloned (Sasai

and Nakanishi, 1989; Yokota et al., 1989; Hershey and Krause, 1990; Shigemoto et al., 1990). These cloned receptors display a different rank order of affinity for each of the neurokinins: for the NK_1 receptors, SP>NKA>NKB; for the NK_2 receptors, NKA>NKB>SP; for the NK_3 receptors, NKB>NKA>SP. These rank orders are also identical to those of pharmacological potency for the neurokinins as shown in TABLE I-3.

These cloned neurokinin receptors belong to the family of GTP-binding regulatory protein (G protein)-coupled receptors possessing seven putative membrane-spanning domains (Nakanishi, 1991; Buck, 1994). Individual intracellular signal transduction induced by neurokinin receptors has been investigated using cell systems, in which each neurokinin receptor cDNA was transfected as shown in TABLE I-4 and Fig. I-1 (Henderson et al., 1990; DeBernardi et al., 1991; Eistetter et al., 1991; Nakajima et al., 1992; Takeda et al., 1992; Subramanian et al., 1994). These systems have overcome the problems associated with mixed neurokinin receptor populations expressed in tissues and cells endogenously. These studies indicate that stimulating neurokinin receptors activates phospholipase C (PLC) to cause phosphatidylinositol (PI) hydrolysis. However, various cells have different signaling modes, except that for PI hydrolysis. For instance, the activation of rat NK_1, NK_2 and NK_3 receptors causes cyclic AMP generation by the direct stimulation of adenylate cyclase in Chinese hamster ovary (CHO) cells (Nakajima et al., 1992). In contrast, the activation of bovine NK_2 receptors expressed in CHO cells indirectly stimulates adenylate cyclase, which is probably due, at least in part, to the autocrine stimulation by endogenously generated eicosanoids, particularly prostaglandin E_2 (PGE_2) (Eistetter et al., 1991). Furthermore, the activation of bovine NK_2 receptors inhibits isoproterenol-stimulated adenylate cyclase activity through the elevation of the intracellular free Ca^{2+} level in C6-2B rat glioma cells (DeBernardi et al., 1991). These findings suggest that not only the property of each receptor, but

also that of each cell type in which neurokinin receptors are artificially expressed, determines the intracellular signal transduction involved, and that these signals may not be reflected in physiological phenomena. Therefore, cell systems that express endogenous neurokinin receptors, should be studied to examine the intracellular signal transduction that induces the functions of neurokinins under physiological conditions.

In the present study, the individual intracellular signal transduction involved in endogenously expressed NK_1, NK_2 and NK_3 receptors was investigated to elucidate the signaling pathway responsible for the functions of neurokinins. The cell systems expressing endogenous NK_2 and/or NK_3 receptors have been little studied. Therefore, such cell systems were firstly screened, and it was found that NKA and NKB stimulate a rise in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) in the murine neuroblastoma C1300 cell line. In CHAPTER II, the types of neurokinin receptors expressed in the C1300 cell line are characterized. As a result, C1300 cells were demonstrated to express both NK_2 and NK_3 receptors, and these receptors were independently activated using selective agonist and antagonist. In CHAPTER III, the intracellular signal transduction induced by each of the endogenous NK_2 and NK_3 receptors in this cell system is discussed. In addition, it has been reported that SP induces PI hydrolysis and stimulates the secretion of amylase in the AR42J rat pancreatic acinar cell line (Womack et al., 1985; Christophe, 1994). Therefore, in CHAPTER IV, I discuss the type(s) of neurokinin receptors present in the AR42J cell line, having found only NK_1 receptors, as well as the NK_1 receptor-mediated signal transduction.



Abbreviations

A.A.; arachidonic acid

AC; adenylate cyclase

DG; diacylglycerol

IP_3; inostiol 1,4,5-trisphosphate

PIP_2; phosphatidylinositol 4,5 bis-phosphate

PKC; protein kinase C

Fig. I-1. Intracellular signal transduction induced by artificially expressed neurokinin receptors

TABLE I-1. Primary structures of tachykinins.

Tachykinin	Primary structure
Mammalian	
Substance P (SP)	Arg - Pro - Lys - Pro - Gln - Gln - **Phe** - Phe - **Gly - Leu - Met - NH_2**
Neurokinin A (NKA)	His - Lys - Thr - Asp - Ser - **Phe** - Val - **Gly - Leu - Met - NH_2**
Neurokinin B (NKB)	Asp - Met - His - Asp - Phe - **Phe** - Val - **Gly - Leu - Met - NH_2**
Neuropeptide K (NPK)	Lys - Arg - His - Lys - Thr - Asp - Ser - **Phe** - Val - **Gly - Leu - Met - NH_2** His - Ser - Ile - Gln - Gly - His - Gly - Tyr - Leu - Ala - Lys - Leu - Leu Asp - Ala - Asp - Ser - Ser - Ile - Glu - Lys - Gln - Val - Ala
Neuropeptide γ (NKγ)	His - Lys - Thr - Asp - Ser - **Phe** - Val - **Gly - Leu - Met - NH_2** Arg - Lys - His - Ser - Ile - Gln - Gly - His - Gly - Ala - Asp
Avian	
[Arg³]substance P	Arg - Pro - Arg - Pro - Gln - Gln - **Phe** - Phe - **Gly - Leu - Met - NH_2**
Amphibian	
Physalaemin	pGlu - Ala - Asp - Pro - Asn - Lys - **Phe** - Tyr - **Gly - Leu - Met - NH_2**
Phyllomedusin	pGlu - Asn - Pro - Asn - Arg - **Phe** - Ile - **Gly - Leu - Met - NH_2**
Uperolein	pGlu - Pro - Asn - Pro - Asn - Ala - **Phe** - Tyr - **Gly - Leu - Met - NH_2**
Kassinin	Asp - Val - Pro - Lys - Ser - Asp - Gln - **Phe** - Val - **Gly - Leu - Met - NH_2**
Pisces	
Scyliorhinin I	Ala - Lys - Phe - Asp - Lys - **Phe** - Tyr - **Gly - Leu - Met - NH_2**
Molluscan	
Eledoisin	pGlu - Pro - Ser - Lys - Asp - Ala - **Phe** - Ile - **Gly - Leu - Met - NH_2**

TABLE I-2. Biological activities of neurokinins.

---

(1) Neuronal stimulation
Neurotransmitter and/or neuromodulator on primary sensory and sympathetic neurons

(2) Endothelium-dependent vasodilatation

(3) Smooth muscle contraction
Contractions of gastrointestinal and vascular smooth muscles

(4) Inflammatory responses
Plasma protein extravasation
Mast cell degranulation
Recruitment or stimulation of inflammatory cells
- Stimulation of chemotaxis to neutrophils
- Enhancement of lymphocyte proliferation
- Stimulation of alveolar macrophages and monocytes

(5) Stimulation of secretion
Salivary secretion
Secretion of digestive enzymes from pancreatic and parotid acini

(6) Cell proliferation
Growth stimulation of T-lymphocytes, skin fibroblasts, arterial smooth muscle cells and keratinocytes

---

TABLE I-3. Neurokinin receptors and their distribution

Type of Receptor	NK_1	NK_2	NK_3
Rank Order of Agonist Potency	SP>NPγ>NKA =NPK>NKB	NPK=NPγ>NKA NKB>SP	NKB>NKA>SP
Distribution (mRNA)[a]			
CNS	Hypothalamus		Cortex
	Striatum		Hypothalamus
	Olfactory bulb		Olfactory bulb
	Spinal cord		Cerebellum
Peripheral Tissues	Small intestine	Stomach	Eye
	Large intestine	Large intestine	
	Parotid gland	Adrenal gland	
	Submandibular and sublingual glands	Urinary bladder	
	Urinary bladder		

a Distribution of neurokinin receptor mRNAs was assessed by blot-hybridization analysis in rat (Tsuchida et al., 1990).

TABLE I-4. Intracellular signaling pathways induced by artificially expressed neurokinin receptors

Cell Type	Transfected Receptor Type	PI Hydrolysis	$[Ca^{2+}]_i$ Incresae	cAMP Cascade	Arachidonic Acid Release	Reference
CHO Cells	NK_1, NK_2, NK_3 (Rat)	Stim.	Stim.	Stim.[a]		Nakajima et al. (1992)
CHO Cells	NK_2 (Bovine)	Stim.	Stim.	Stim.[b]	Stim.	Eistetter et al. (1991)
C6-2B Rat Glioma Cells	NK_2 (Bovine)	Stim.	Stim.	Inhib.[c]		DeBernardi et al. (1991)

"Stim." and "Inhib." mean the stimulation and the inhibition, respectively.

[a] This effect was shown to be dependent on the direct coupling to adenylate cyclase.

[b] This effect was a secondary response, and might be mediated by endogenously generated eicosanoids, particularly PGE_2.

[c] This effect was dependent on the elevation of $[Ca^{2+}]_i$.

CHAPTER II

IDENTIFICATION OF NEUROKININ RECEPTOR TYPES IN A MURINE NEUROBLASTOMA C1300 CELL LINE

II-1. INTRODUCTION

NK_1 receptors are reported to be widely distributed in both the nervous system and peripheral tissues (Munekata, 1991; Maggi et al., 1993). In contrast, blot-hybridization and RNase-protection analyses show that the expression of NK_2 receptor mRNA is restricted to the peripheral tissues, being abundant in the urinary bladder, large intestine, stomach and adrenal gland, and that NK_3 receptor is predominantly expressed in the central nervous system, particularly in the cortex, hypothalamus and cerebellum (Tsuchida et al., 1990). These findings suggested that NK_2 and NK_3 receptors may be involved in neurokinin activity in peripheral tissues and the central nervous system, respectively. Furthermore, pharmacological experiments indicate that NK_2 receptors are expressed in the tracheal smooth muscle of many species, the activation of which causes bronchoconstriction, possibly contributing to bronchial hyperreactivity (Frossard and Advenier, 1991). In the case of NK_3 receptors, neurokinins induce the contraction of smooth muscle via NK_3 receptors in rat portal vein (Mastrangelo et al., 1986). In addition, it has been demonstrated that guinea-pig ileum expresses both NK_1 and NK_3 receptors, and that the activation of NK_3 receptors causes smooth muscle contraction dependent on the release of acetylcholine from nerve endings (Guard and Watson, 1987; Laufer et al., 1988), whereas NK_1 receptors directly act on the smooth muscle cells (Burcher et al., 1986; Tousignant et al., 1991).

However, it is not clear which intracellular signaling pathway is responsible for the functions elicited by neurokinins, since little is known about the cell systems that have endogenous NK_2 and NK_3 receptors. In the case of NK_2 receptors, although Nagaki et al. (1994) reported that NKA stimulates an increase in $[Ca^{2+}]_i$, probably through the activation of NK_2 receptors in the acinar cells of the feline tracheal submucosal gland, this is the

only description, and a system with endogenous NK_3 receptors has not been found.

In an attempt to solve such problems, an established cell line expressing endogenous NK_2 and/or NK_3 receptors was screened and as a result, it was found that murine neuroblastoma C1300 cells responded to neurokinins, and that the responses to NKA and NKB were much greater than that of SP. In this chapter, I have identified the NK_2 and NK_3 receptors in the C1300 cell line using blot-hybridization analysis and pharmacological techniques.

II-2. MATERIALS AND METHODS

II-2-1. Materials

Materials were purchased as follows: RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM), from Gibco (Grand Island, New York, USA); murine neuroblastoma C1300 cells, from RIKEN Cell Bank (Tsukuba, Japan); fetal calf serum (FCS), from Boehringer Mannheim (Tokyo, Japan); [α-^{32}P]dCTP (- 3000 Ci/mmol), from Amersham International plc (Bucks, UK); fura-2/acetoxymethyl ester (fura-2/AM), from Dojin (Kumamoto, Japan). SR 48968 [(S)-N-methyl-N-[4-acetylamino-4-phenylpiperidino-2-(3,4-dichlorophenyl)butyl]benzamide] and SR 142801 [(S)-(N)-(1-(3-(1-benzoyl-3-(3, 4-dichlorophenyl) piperidin-3-yl) propyl) -4-phenylpiperidin-4-yl) - N -methylacetamide] were provided by Dr. X. Emonds-Alt (Sanofi Recherche, France). CP-96,345 [(2S, 3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine] was provided by Dr. A. Nagahisa (Pfizer Inc., Japan). The cDNAs for the rat neurokinin receptors, prTKR1-1 (Sasai et al., 1989), prTKR2 (Yokota et al., 1989) and prTKR3 (Shigemoto et al., 1990), were from Dr. S. Nakanishi (Kyoto University, Japan). AR42J rat pancreatic acinar cell line was a gift from Dr. T. Ohtaki (Takeda Chemical Industries, Japan). All other chemicals were of reagent grade.

II-2-2. Synthesis of peptides

All peptides were synthesized using the solid phase methodology developed by Merrifield (Merrifield, 1963; Munekata et al., 1987) in conventional glass reaction vessels. SP, NKA, NKB, [βAla^{8}]NKA(4-10) and [Tyr^{5},D-Trp^{6,8,9},Lys^{10}]NKA(4-10) (MEN 10,376) were synthesized by Boc strategy (TABLE II-1), and succinyl-[Asp^{6},MePhe^{8}] SP (senktide) by Fmoc strategy (TABLE II-2).

II-2-2-a. Boc strategy

Amino acids used for the synthesis were protected with an N$^{\alpha}$-tert-butyloxycarbonyl (Boc) group. The side-chain protecting groups employed were as follows: 2-chlorobenzyloxycarbonyl group for Lys, 2,4-dinitrophenyl group for His, benzyl group for Ser and Thr, cyclohexyl group for Asp and 2,6-dichlorobenzyl group for Tyr. MBHA (4-methylbenzhydrylamine) resin-HCl was used to synthesize C-terminal amidating peptides. The resin was swelled in dichloromethane (DCM) for 15 min, and neutralized by triethylamine. After washing with DCM and methanol, the C-terminal amino acid residues were coupled to the resin with N,N'-dicyclohexylcarbodiimide (DCC) in N,N-dimethylformamide. Coupling reactions in chain elongation were carried out by DCC/HOBt or the symmetrical anhydride method. Every coupling reaction was checked by Kaiser's ninhydrin test (Kaiser et al., 1970). When the ninhydrin test was positive, the reactions were repeated until the ninhydrin test was negative. After acylation, the Boc group was removed by treatment with 50% trifluoroacetic acid (TFA) in DCM containing 1% 1,2-ethanedithiol for 30 min at room temperature. The resin was washed with DCM and n-propanol, and neutralized with 10% triethylamine in DCM. After the completion of chain elongation, the peptide resin was treated with anhydrous hydrogen fluoride (HF) at 0°C for 1 h in the presence of p-cresol,

p-thiocresol and dimethyl sulfide to cleave the peptide from the solid support and remove the protecting groups of each amino acid residue. HF and scavengers were removed in vacuo and residual crude product was washed with ether, extracted with aqueous acetic acid, and applied onto a Dowex 1 × 2 (acetate form) column to remove F^-.

II-2-2-b. Fmoc strategy

Amino acids used for the synthesis were protected with a Nα-9-fluorenylmethyloxycarbonyl (Fmoc) group. The side-chain protecting groups used were 2,2,5,7,8-pentamethylchroman-6-sulfonyl group for Arg, Boc group for Lys and His, *tert*-butyl ester group for Asp and Glu, triphenylmethyl group for Gln, *tert*-butyl group for Ser and Thr. SAL resin was used to synthesize C-terminal amidating peptides. Coupling reactions in chain elongation were carried out by the BOP/HOBt or HBTU/HOBt method. Kaiser's ninhydrin test (Kaiser er al., 1970) was performed as described above. After the coupling reaction of each amino acid, the Fmoc group was deprotected by treatment with 20% piperidine in N-methyl-2-pyrrolidone (NMP) for 30 min at room temperature. In the case of senktide, the N-terminus of the peptide was succinylated with succinic anhydride in DCM for 1h. To cleave peptides from the solid support, the resin was treated with TFA in the presence of trimethylsilyl bromide, thioanisole, m-cresol and 1,2-ethanedithiol. Crude peptides were extracted with aqueous acetic acid and the solution was washed with ether.

II-2-2-c. Purification and homogeneity of synthetic peptides

Crude peptides were purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) using Nucleosil 7C18 column (6.7 × 250 mm). The peptide was applied into the column under the gradient elution of acetonitrile-H_2O containing 0.1% TFA. Purity of peptides was

confirmed by analytical RP-HPLC using Nucleosil 5C18 column (4.6 × 150 mm), and all peptides proved to possess a purity of > 95%. The synthetic peptides were treated with 6 N HCl at 110°C for 22 h, and resulting hydrolysates were analyzed with a Hitachi L8500 amino acid analyzer to assess the amino acid composition of the peptides. The molecular weight of peptides was checked by fast atom bombardment mass spectrometry.

II-2-3. Cell culture

Murine neuroblastoma C1300 cells were grown in 75-cm^2 plastic tissue culture flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% (v/v) FCS and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified atomosphere of 5% CO_2/95% air. The cells were passaged every 4-5 days. The cells (passage 6-35) were used after reaching confluence. AR42J rat pancreatic acinar cells were also cultured in DMEM supplemented with 14% (v/v) FCS and antibiotics under the same conditions as described above.

II-2-4. Poly(A)+ RNA preparation and RNA blot hybridization

Total RNA was extracted from cells and tissues as described by Chomczynski and Sacchi (1987), and eluted through oligo(dT)-cellulose to select poly(A)+ RNA. The yield of poly(A)+ RNA was 2.5-7.5%. The cDNA probes used for analysis of the NK_1, NK_2 and NK_3 receptor mRNAs were as follows: for the NK_1 receptor mRNA, the 1176-bp *Stu*I-*Bst*EII fragment derived from clone prTKR2; for the NK_2 receptor mRNA, the 1497-bp *Pst*I fragment derived from clone prTKR1-1; for the NK_3 receptor mRNA, the 1370-bp *Eco*T14I fragment derived from clone prTKR3. The probes were labeled using a random primer DNA labeling kit (Amersham International plc, Bucks, UK) with [α-^{32}P]dCTP. Poly(A)+ RNA (2 μg each) was denatured with glyoxal, fractionated on a 1.2% agarose gel, and blotted onto a GeneScreen

Plus membrane (Du-Pont New England Nuclear, USA). RNA hybridization proceeded as described (Hatsuzawa et al., 1990). The blot was hybridized with each probe in 1 M NaCl, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS) and 0.1 mg/ml salmon sperm DNA at 60°C for 12 h. The blot was then washed sequentially in 2 × sodium citrate-sodium chloride (SSC) (room temperature, 10 min), 2 × SSC, 1% SDS (60°C, 1 h) and 0.1 × SSC (room temperature, 20 min). The rinsed blot was exposed on an Imaging Plate BAS-III (Fuji Photo Film Co. Ltd., Tokyo, Japan) and visualized using a bio-imaging analyzer BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

II-2-5. The measurement of $[Ca^{2+}]_i$

Confluent C1300 cells were detached by pipetting, then washed twice with HEPES-buffered solution (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM HEPES, 11 mM glucose, 0.2% bovine serum albumin, pH 7.4). The $[Ca^{2+}]_i$ was measured using fura-2, a sensitive luminescent Ca^{2+} chelator (Takuwa et al., 1989; Takuwa et al., 1991). The cells were incubated with 4 μM fura-2/AM for 1 hr at 20°C. The fura-2-loaded cells were washed twice, resuspended in fresh HEPES-buffered solution without fura-2/AM, and stored at 20°C until use. About 10^6 cells/ml were examined for each measurement. The fluorescence of fura-2-loaded cells at 500 nm excited at 340 and 350 nm was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy Inc. Tokyo, Japan). The cell suspension in the cuvette was constantly stirred while measuring the fluorescence. The cells were stimulated by the agonist at 30°C instead of 37°C, because intracellular fura-2 mostly leaked and the exact calculation of $[Ca^{2+}]_i$ level was difficult at 37°C as described elsewhere (Takuwa et al., 1991). The responses at 30°C were a little lower than those at 37°C, but the concentration-dependence of the peptides at 30°C was same as that at 37°C. The $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985). Several peptides were

dissolved in dimethylsulfoxide (DMSO) because they were poorly soluble in distilled water. The same amount of DMSO was added to a comparative cell suspension, and the final concentration in the buffer did not exceed 1.0%, which had no effect upon either the basal $[Ca^{2+}]_i$ or the peptide-induced increase in $[Ca^{2+}]_i$. In experiments to assess the inhibitory effects of antagonists, the cells were stimulated by agonists after exposure to each antagonist for 2 min.

II-2-6. Statistics

Each value in the text, tables or figures is expressed as a mean ± SEM. To determine statistical significance, the values were compared using Student's *t* test. The differences were considered significant if *p* values were less than 0.05.

II-3. RESULTS

II-3-1. The rise in $[Ca^{2+}]_i$ induced by neurokinins

Figure II-1. shows typical tracings representing the effects of SP, NKA and NKB on $[Ca^{2+}]_i$ in C1300 cells. Both NKA and NKB at 10 μM induced an immediate but transient increase in $[Ca^{2+}]_i$. SP at 10 μM also induced a Ca^{2+} transient, although its response was much smaller than those of NKA and NKB. The $[Ca^{2+}]_i$ reached maximal concentration within about 20 s after the addition of NKA and NKB. The effects of NKA and NKB on $[Ca^{2+}]_i$ were concentration-dependent [median effective concentration (EC_{50}) values: NKA 87 ± 13 nM, NKB 97 ± 15 nM; n=3]. The maximal increase in $[Ca^{2+}]_i$ induced by NKA and NKB was about 200 nM. Figure II-2 shows the concentration-dependent increase in $[Ca^{2+}]_i$ induced by neurokinins and related peptides. Although 10 μM [βAla^8]-NKA(4-10), a selective agonist for NK_2 receptors (Rovero et al., 1989), slightly induced the increase in $[Ca^{2+}]_i$, the response was

much less than those of NKA and NKB. In addition, senktide, which is a selective agonist for NK_3 receptors (Wormser et al., 1986), concentration-dependently evoked the rise in $[Ca^{2+}]_i$ (EC_{50} value: 84 ± 27 nM; n=4), but the maximal response was about 10-fold less than that of NKA and NKB.

II-3-2. Expression of neurokinin receptor mRNAs

Since the existence of neurokinin receptors in C1300 cells was suggested, an RNA blot analysis using each cDNA probe of NK_1, NK_2 and NK_3 receptors was performed to investigate which type of neurokinin receptors were endogenously expressed. The autoradiograph of the RNA hybridization of NK_1, NK_2 and NK_3 receptor mRNAs from mouse C1300 cells as well as from mouse brain, mouse intestine and rat AR42J cells is shown in Fig. II-3. The blot hybridization of poly(A)+ RNA from C1300 cells by a cDNA probe for NK_2 receptors revealed three mRNA bands of about 2.3, 3.1 and 5.3 kb. Similarly, three bands of NK_2 receptor mRNA were detected in poly(A)+ RNA from the mouse intestine, and these were about 2.7, 3.1 and 5.3 kb. NK_2 receptor mRNA was not detected in mouse brain or AR42J cells. RNA hybridization using a cDNA probe for NK_3 receptors showed that the poly(A)+ RNAs isolated from the C1300 cells as well as those from the mouse brain and intestine, but not those from AR42J cells, yielded a single band with an estimated size of 4.2 kb. NK_1 receptor mRNA was not detectable in poly(A)+ RNA from C1300 cells, whereas it was evident in mouse brain, mouse intestine and AR42J cells. These results revealed that C1300 cells expressed mRNAs of NK_2 and NK_3 receptors, but not that of NK_1 receptors.

II-3-3. Functional expression of NK_2 and NK_3 receptors

The inhibitory effects of selective antagonists for neurokinin receptors (Fig. II-4) on the NKA- and NKB-induced Ca^{2+} mobilization were examined to determine which type of neurokinin receptors are functionally expressed in

this cell line. CP-96,345, a selective antagonist for NK_1 (Snider et al., 1991), affected neither the NKA- nor the NKB-induced Ca^{2+} mobilization at a concentration of 10 μM (Fig. II-5).

NKA- and NKB-stimulated Ca^{2+} mobilization was inhibited by 10 nM and 500 nM of SR 48968, a selective antagonist for NK_2 (Fig. II-4 and II-6., Emonds-Alt et al., 1992), although such concentrations of SR 48968 did not affect the basal $[Ca^{2+}]_i$ or the Ca^{2+} mobilization evoked by 1.0 mM carbachol (data not shown). In addition, the inhibitory effect of another NK_2 receptor antagonist, MEN 10,376 (Fig. II-4., Maggi et al., 1993), was also examined. As shown in Fig. II-7 and TABLE II-3, this antagonist (0.3 and 1.0 μM) not only produced a concentration-dependent rightward shift of the curves for both NKA and NKB, but also attenuated their maximal responses (when stimulated by 10 μM of NKA and NKB), although 10 μM of it had no effect on the basal $[Ca^{2+}]_i$ or Ca^{2+} mobilization evoked by 1.0 mM carbachol (data not shown).

Since 10 μM SR 142801, a selective antagonist for NK_3 receptors (Fig. II-4., Emonds-Alt et al., 1995), slightly increased the $[Ca^{2+}]_i$, and partially inhibited the response to 1.0 mM carbachol (reduced to about 70% of the level in its absence), its inhibitory effects were examined at less than 3.3 μM. Such concentrations had no effect on basal $[Ca^{2+}]_i$ or the response to carbachol. The compound at 0.3 μM produced a slightly rightward shift of the curve for NKB (EC_{50} values: 110 and 480 nM in the absence and presence of SR 142801, respectively), whereas it did not affect the curve for NKA (Fig. II-8). Although 1.0 and 3.3 μM SR 142801 inhibited the increase in the $[Ca^{2+}]_i$ evoked by both NKA and NKB, the response to NKB was more sensitive than that to NKA (Fig. II-8).

To investigate whether the $[Ca^{2+}]_i$ increase evoked by NKA and NKB was via only NK_2 receptors or both NK_2 and NK_3 receptors, the concentration-dependency of the inhibitory effects of SR 48968 on these responses was

examined. The response to NKA at 0.33 μM was completely inhibited by concentrations above 0.3 μM SR 48968. In the presence of 0.3 μM SR 48968, the response to 0.33 μM NKB was also attenuated to about 8% of that in its absence. However, even if the concentration was increased up to 10 μM, the response was not inhibited completely (TABLE II-4). Next, whether or not NK_3 receptors were responsible for the resistance to SR 48968 was investigated. As shown in Fig. II-9, NKB concentration-dependently stimulated the increase in $[Ca^{2+}]_i$ in the presence of 1.0 μM SR 48968 (EC_{50} value: 7.1 ± 1.2 nM, n=3). Furthermore, although the $[Ca^{2+}]_i$ increase evoked by 0.33 μM of NKB in the absence and presence of 1.0 μM SR 48968 was inhibited by SR 142801, the response in the presence of SR 48968 was more sensitive to SR 142801 than that in its absence. The values of median inhibitory concentration (IC_{50}) in the absence and presence of SR 48968 were 1.2 ± 0.1 μM and 24.8 ± 2.3 nM, respectively ($P < 0.01$, n=3) (Fig. II-10). As described above, it was shown that senktide stimulated the increase in $[Ca^{2+}]_i$, but the maximal response was about 10-fold less than that induced by NKA or NKB. Thus, the inhibitory effects of SR 142801 and SR 48968 on the senktide-induced $[Ca^{2+}]_i$ increase were assessed. In the presence of 0.1 μM SR 142801, the response induced by 0.33 μM senktide was reduced to 15 ± 1% of that in its absence (n=3, data not shown). In contrast, the concentration-response curve of Ca^{2+} mobilization evoked by senktide was not affected by 1.0 μM SR 48968 (EC_{50} values; absence of SR 48968, 84 ± 27 nM; presence of it, 68 ± 28 nM; n=4; Fig. II-11). These findings suggested that functional NK_2 and NK_3 receptors were endogenously expressed in C1300 cells, and that most of the $[Ca^{2+}]_i$ increase evoked by NKA and NKB was dependent on the activation of NK_2 receptors, however the portion unaffected by SR 48968 was caused through NK_3 receptors.

It was found that NK_2 and NK_3 receptors in C1300 cells could be independently activated using selective agonist and antagonist as shown in Fig.

II-12. To induce only NK_2 receptor-mediated response, the stimulation by NKA was carried out under the condition that the response through NK_3 receptors was selectively inhibited by SR 142801, since a selective NK_2 receptor agonist which could activate NK_2 receptors in C1300 cells has not been found. Indeed, the cells were stimulated by 3.3 μM NKA in the presence of 1.0 μM SR 142801, which completely inhibited the $[Ca^{2+}]_i$ increase evoked by 3.3 μM NKB in the presence of 10 μM SR 48968 (NK_3 receptor-mediated response, data not shown). To observe the response mediated by NK_3 receptors, the cells were stimulated by 1.0 μM senktide.

II-4. DISCUSSION

This study demonstrated that NKA and NKB stimulated the increase in the $[Ca^{2+}]_i$ in the C1300 cell line (Fig. II-1 and 2), and that this response was inhibited by NK_2 receptor antagonists such as SR 48968 and MEN 10,376 (Fig. II-6 and 7). However, [βAla^8]NKA(4-10), a selective agonist for NK_2 receptors, slightly stimulated the $[Ca^{2+}]_i$ increase (Fig. II-2), even though this peptide induces a contraction of smooth muscle with a similar efficacy to that by NKA in the endothelium-denuded rabbit pulmonary artery and hamster trachea both of which express NK_2 receptors (Maggi et al., 1990). Moreover, a higher concentration of NKA is required to activate the NK_2 receptors expressed in C1300 cells than to activate known NK_2 receptors (Maggi et al., 1990; Sundelin et al., 1992; Maggi et al., 1993). In addition, MEN 10,376 not only produced a concentration-dependent rightward shift of the curves for both NKA and NKB, but also attenuated their maximal responses, suggesting that the compound did not inhibit these responses competitively. These findings suggested that C1300 cells expressed functional NK_2 receptors, which differed from the known NK_2 receptors. The reason for this may be ascribed to the different isoforms of NK_2 receptors, as these cells expressed three forms of

NK_2 receptor mRNA, one of which was distinct from that of mouse intestine (C1300 cells, 2.3 kb; mouse intestine, 2.7 kb, Fig. II-3). Tsuchida et al. (1990) indicated that there are two large forms of rat NK_2 receptor mRNA expressed in the peripheral tissues, and that two additional small forms of the mRNA are expressed specifically in the adrenal gland and eye. It was also shown that the multiple forms of rat NK_2 receptor mRNA differ in the lengths of the 5' mRNA portions, and that two small forms of the mRNA, if translated, encode a truncated NK_2 receptor, lacking the first two transmembrane domains. In addition, production of multiple isoforms by alternative splicing has been reported in members of the G protein-coupled receptor family such as NK_1 receptors (Fong et al., 1992), neuropeptide Y-Y1 receptors (Nakamura et al., 1995), metabotropic glutamate receptors (Tanabe et al., 1992) and prostaglandin-E EP_3 subtype receptors (Namba et al., 1993; Sugimoto et al., 1993). All these isoforms differ only in the third cytoplasmic loop or carboxyl-terminal tail. In the case of human NK_1 receptors, the existence of two isoforms was demonstrated, and they differ only in the length of the carboxyl-terminal tail (Fong et al., 1992). Affinity between the long form of human NK_1 receptors and SP was consistent with that of native NK_1 receptors in mammalian tissues, whereas the short form had at least a 10-fold lower affinity than that of the long form. Although SP elicited chloride current via the long and short forms of human NK_1 receptors expressed in *Xenopus* oocytes, the response through the short form was much lower than that evoked by the activation of the long form. These findings suggested that the mechanisms for the generation of neurokinin receptor mRNAs were different among tissues, and that multiple isoforms of neurokinin receptors may be produced by mechanisms such as alternative splicing. Therefore, the multiple isoforms of NK_2 receptors may also explain the differences between NK_2 receptors present in C1300 cells and known NK_2 receptors. Cloning of the NK_2 receptors expressed in C1300 cells should be performed to elucidate this.

In the present study, the existence of functional NK_3 receptors in C1300 cells was suggested by the following findings: 1) about 8% of the response to NKB could not be inhibited by SR 48968, although the $[Ca^{2+}]_i$ increase induced by NKA was completely abolished by this compound (TABLE II-4); 2) NKB elicited the $[Ca^{2+}]_i$ increase in a concentration-dependent manner even in the presence of 1.0 μM SR 48968, which completely abolished the response to NKA (Fig. II-9); 3) the $[Ca^{2+}]_i$ increase resistant to SR 48968 induced by NKB was completely inhibited by SR 142801 (Fig. II-10); 4) the senktide-evoked $[Ca^{2+}]_i$ increase was inhibited by SR 142801, but not by SR 48968 (Fig. II-11).

SR 142801 is a competitive and highly potent non-peptide antagonist of the NK_3 receptor, but its inhibitory activity is highly species dependent. This compound has a high affinity for NK_3 receptors in the human, gerbil and guinea-pig, but a much lower affinity in the rat. Furthermore, this compound at concentrations above micromolar interacts with not only NK_3 receptors, but also NK_1 and NK_2 receptors (Emonds-Alt et al., 1995). In the murine C1300 cell line, SR 142801 at nanomolar concentrations inhibited the response to NKB resistant to SR 48968, and at concentrations above micromolar, blocked those to NKA and NKB. These results suggested that SR 142801 at nanomolar concentrations specifically inhibited the response through NK_3 receptors, and blocked those through both NK_3 and NK_2 receptors in this cell line at micromolar concentrations.

It was demonstrated that cells of an established cell line, C1300, endogenously express functional NK_2 and NK_3 receptors. In addition, the response through endogenous NK_2 or NK_3 receptors could be independently observed using this cell system (Fig. II-12). These findings show that the use of the C1300 cell line will contribute to the understanding of the intracellular signaling pathway elicited by endogenous NK_2 and NK_3 receptors as well as to the pharmacological investigation of neurokinins.

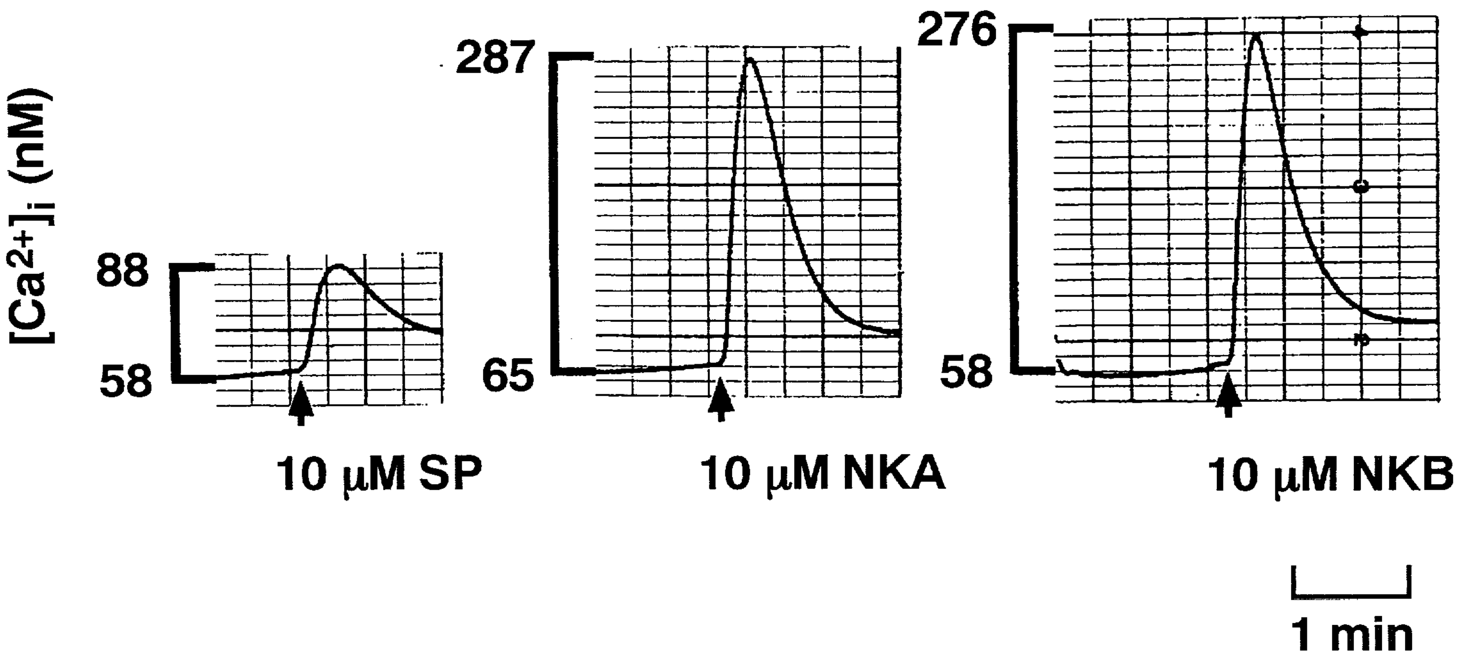


Fig. II-1. Time course of neurokinin induced-$[Ca^{2+}]_i$ increase in fura-2-loaded C1300 cells. Arrowheads indicate the addition of each stimulant. The fluorimetric recordings of representative experiments are shown.

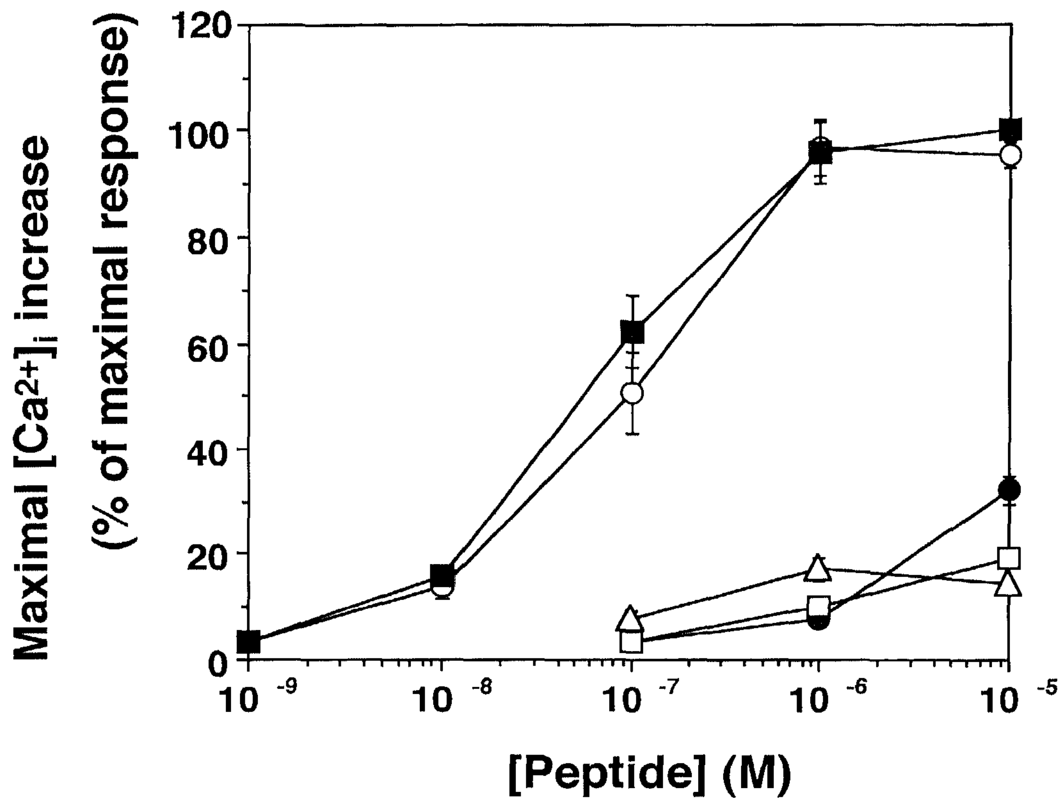


Fig. II-2. Concentration-response curves of the $[Ca^{2+}]_i$ increase in C1300 cells induced by neurokinin and related peptides. Cells were stimulated by the indicated concentrations of SP (closed circles), NKA (closed squares), NKB (open circles), [βAla⁸]NKA(4-10) (open squares) and senktide (open triangles). The increase in $[Ca^{2+}]_i$ at each concentration is plotted as a percentage relative to the maximal response induced by NKA at 10 μM. Data are mean ± SEM (bars) values of three separate experiments.

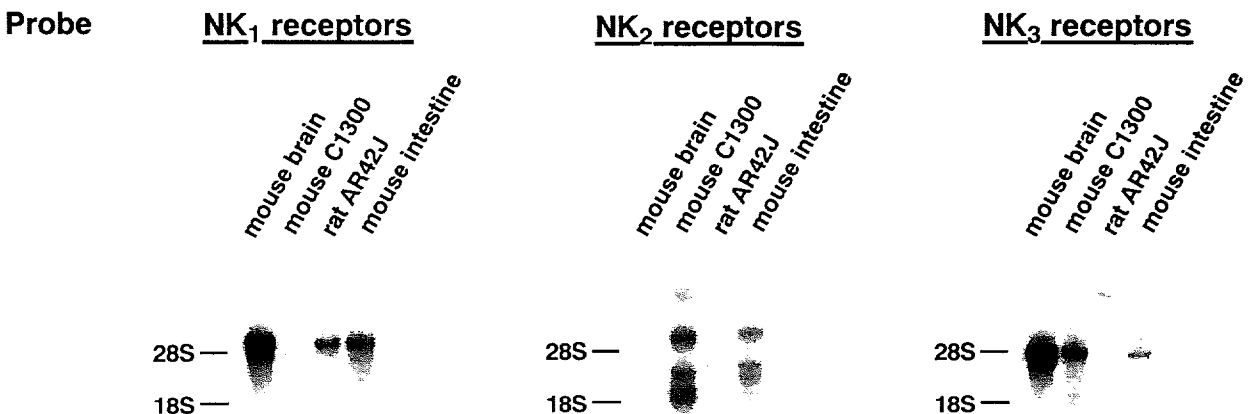


Fig. II-3. RNA blots of poly $(A)^+$ RNA from C1300 cells, AR42J cells, mouse intestine, and mouse brain using each cDNA probe for NK_1, NK_2, and NK_3 receptors.

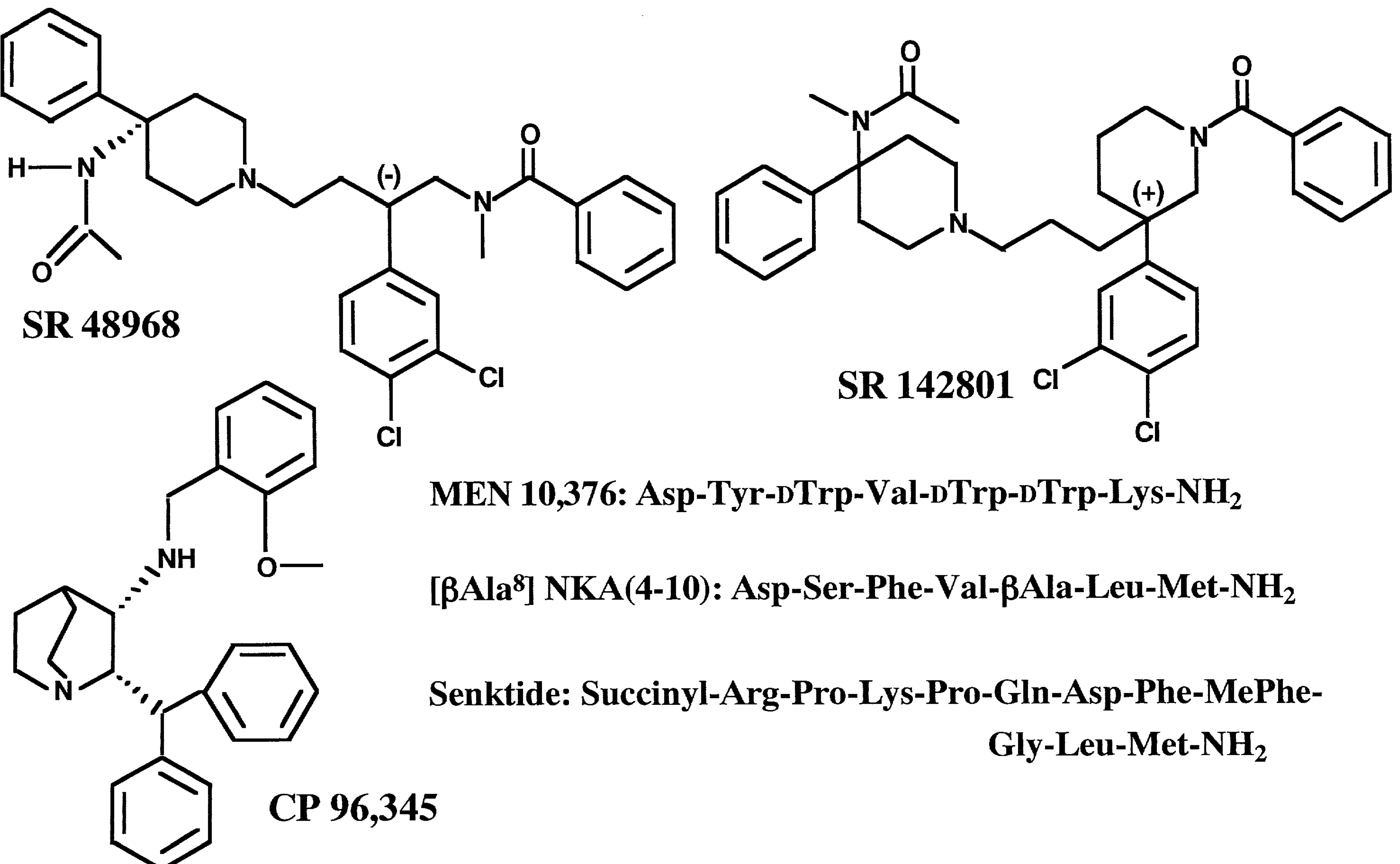


Fig. II-4. Structures of the selective agonists and antagonists used in this study.

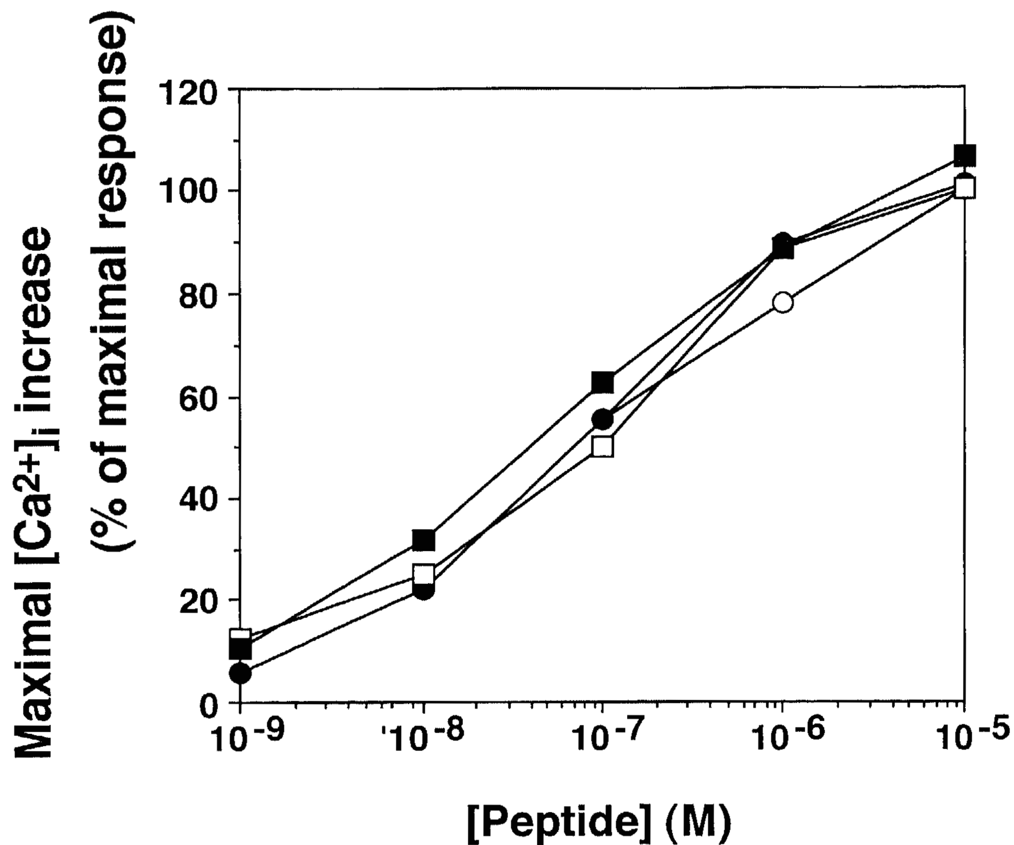


Fig. II-5. Inhibitory effects of CP-96,345, an NK_1 receptor antagonist. Cells were stimulated by the indicated concentrations of NKA (circles) and NKB (squares) in the absence (open symbols) and presence (closed symbols) of 10 μM CP-96,345.

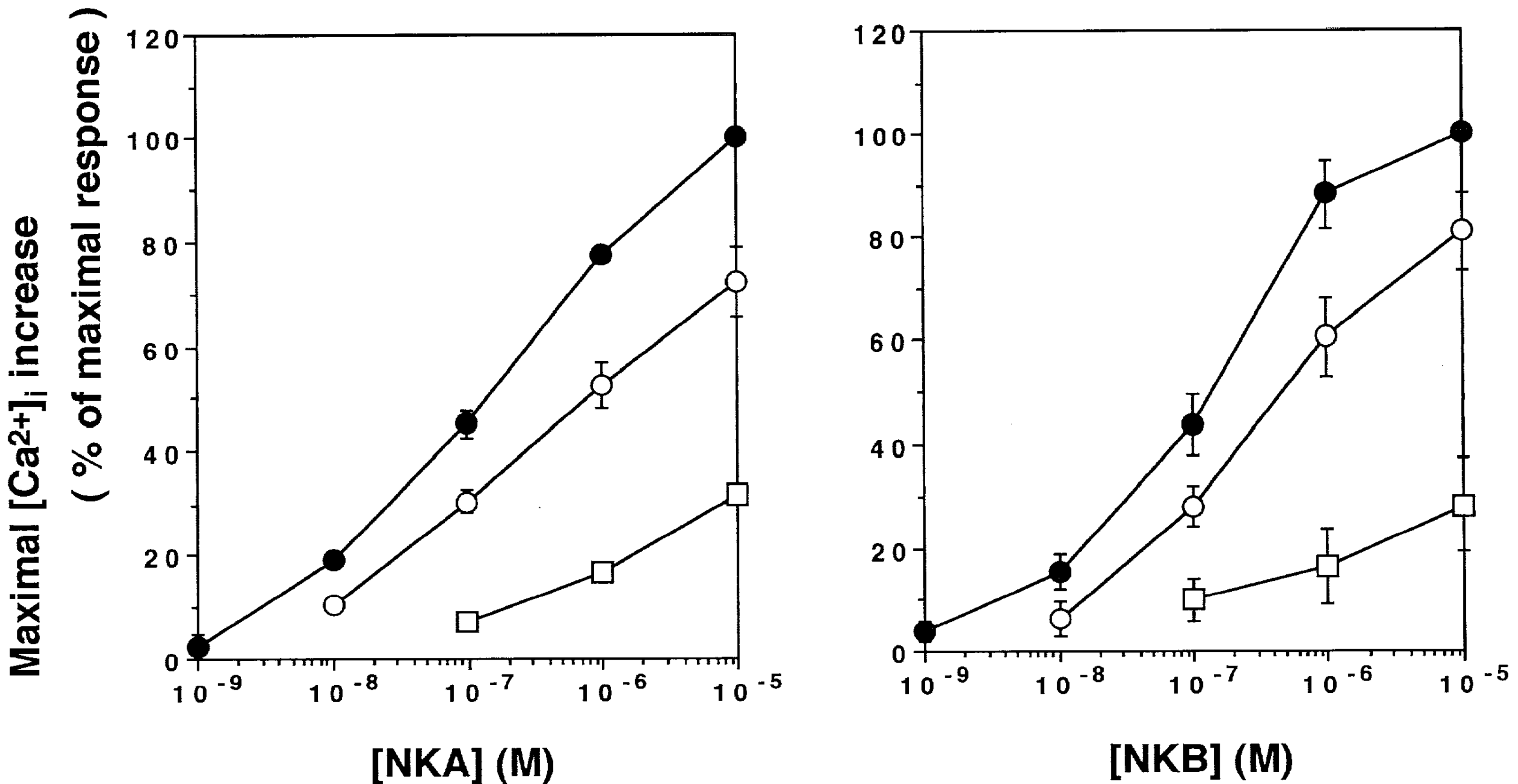


Fig. II-6. Inhibitory effects of SR 48968, an NK_2 receptor antagonist. Cells were stimulated by the indicated concentrations of NKA (left) and NKB (right) in the absence (closed circles) and presence of 10 nM (open circles) or 500 nM (open squares) SR 48968. The increase in $[Ca^{2+}]_i$ at each concentration is plotted as a percentage relative to the maximal response induced by NKA (left) or NKB (right) at 10 μM. Data are mean ± SEM (bars) values of three separate experiments.

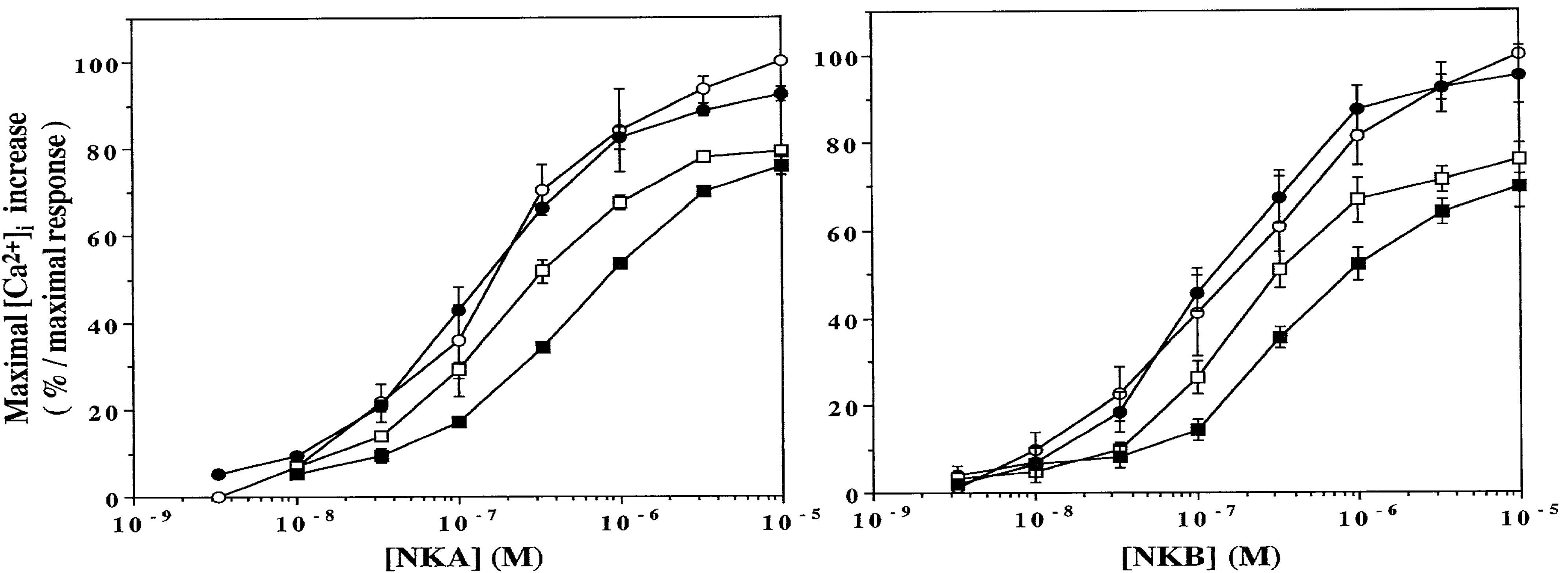


Fig. II-7. Inhibitory effects of MEN 10,376, an NK_2 receptor antagonist. Cells were stimulated with the indicated concentrations of NKA (left) and NKB (right) in the absence (open circles) and presence of 0.1 (closed circles), 0.3 (open squares), or 1.0 μM (closed squares) MEN 10,376. The increase in $[Ca^{2+}]_i$ at each concentration of agonist is plotted as a percentage relative to the maximal response induced by NKA or NKB at 10 μM. Data are mean ± SEM (bars) values of three separate experiments.

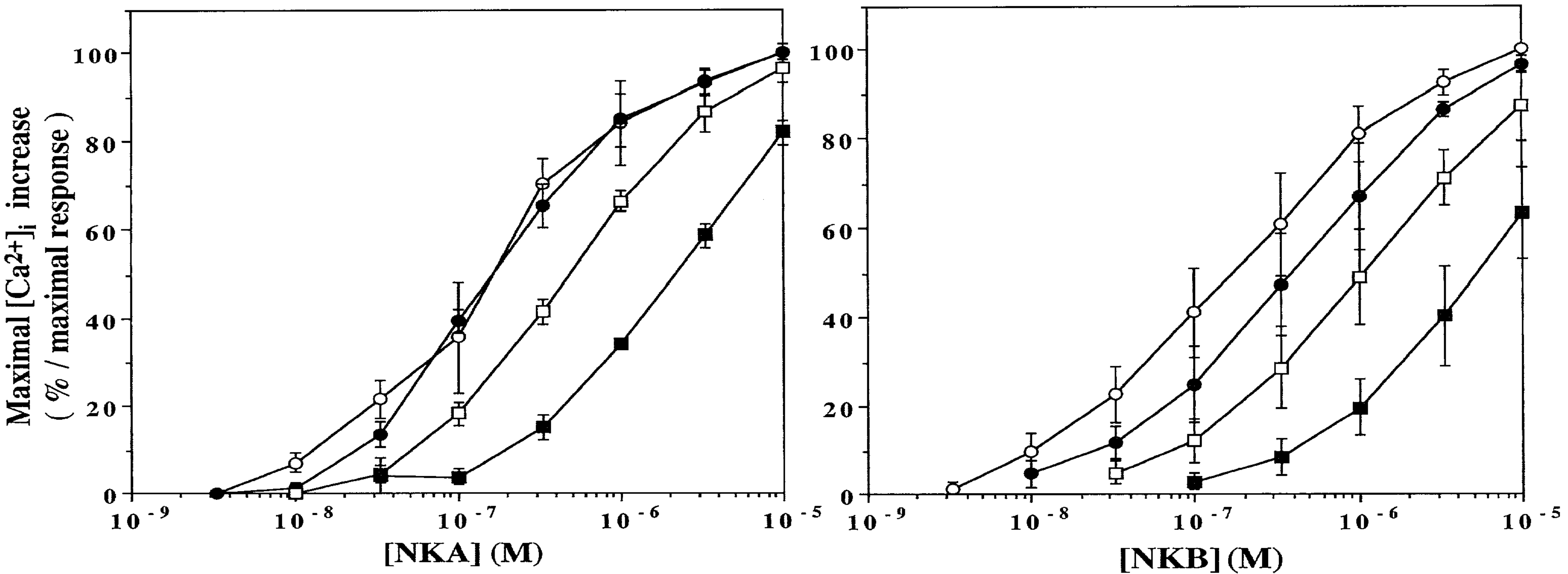


Fig. II-8. Inhibitory effects of SR 142801, an NK_3 receptor antagonist. Cells were stimulated with the indicated concentrations of NKA (left) and NKB (right) in the absence (open circles) and presence of 0.3 (closed circles), 1.0 (open squares), or 3.3 μM (closed squares) SR 142801. The increase in $[Ca^{2+}]_i$ at each concentration of agonist is plotted as a percentage relative to the maximal response induced by NKA or NKB at 10 μM. Data are mean ± SEM (bars) values of three separate experiments.

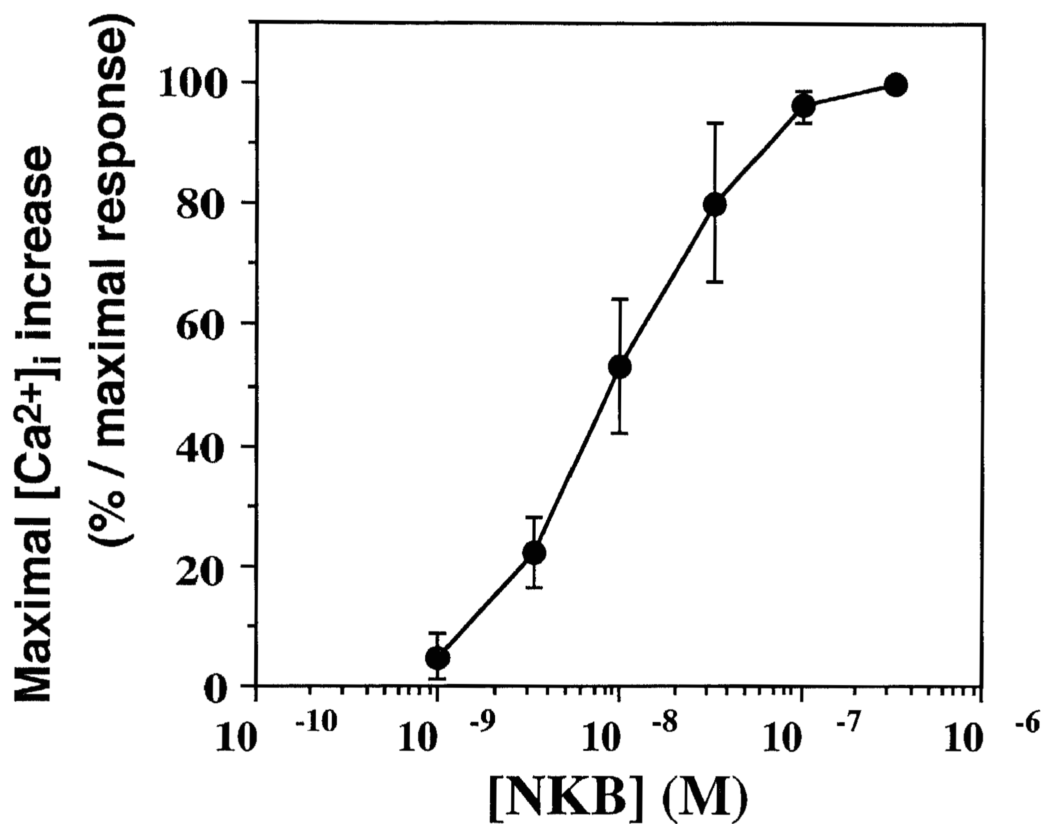


Fig. II-9. Concentration-dependence of the $[Ca^{2+}]_i$ increase evoked by NKB in the presence of SR 48968. Cells were stimulated with the indicated concentrations of NKB in the presence of 1.0 μM SR 48968. The increase in $[Ca^{2+}]_i$ at each concentration is plotted as a percentage relative to the maximal response induced by NKB at 0.3 μM. Data are mean ± SEM (bars) values of four separate experiments.

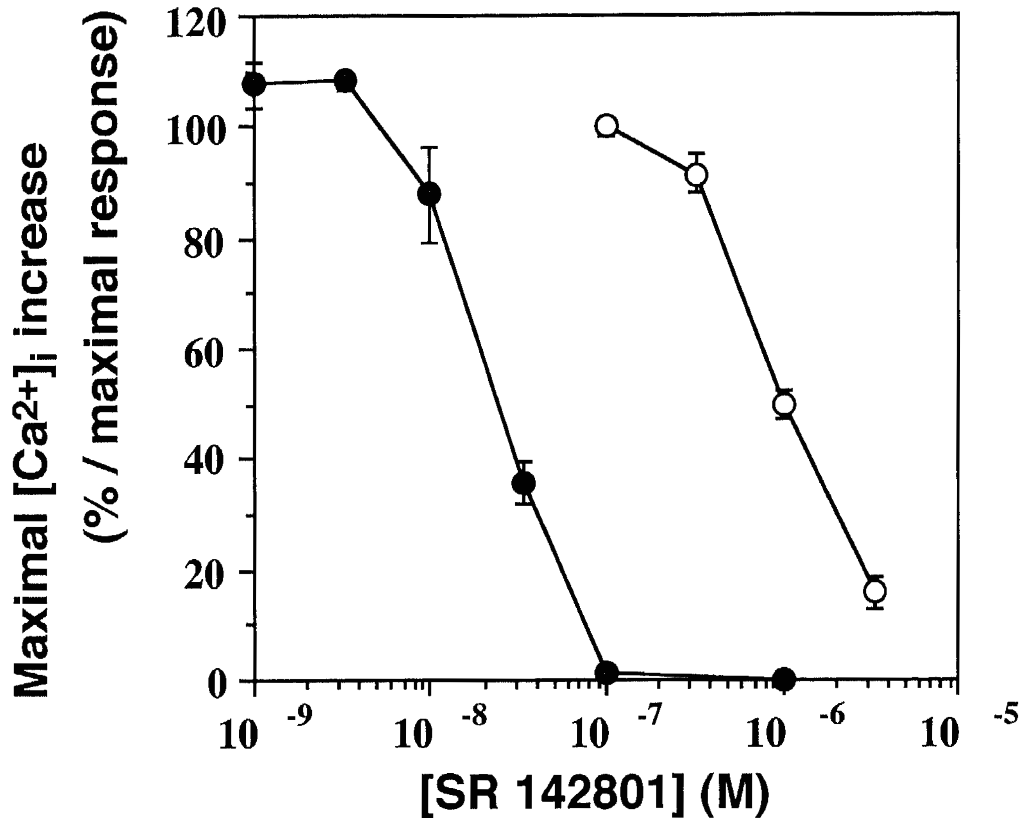


Fig. II-10. Concentration-dependent inhibition of the NKB-induced $[Ca^{2+}]_i$ increase by SR 142801 in the presence or absence of SR 48968. Cells were stimulated with 0.33 μM NKB in the absence (open circles) or presence (closed circles) of 1.0 μM SR 48968 in the presence of SR 142801 at the indicated concentrations. The increase in $[Ca^{2+}]_i$ at each concentration is plotted as a percentage relative to the response in the absence of SR 142801. Data are mean ± SEM (bars) values of four separate experiments.

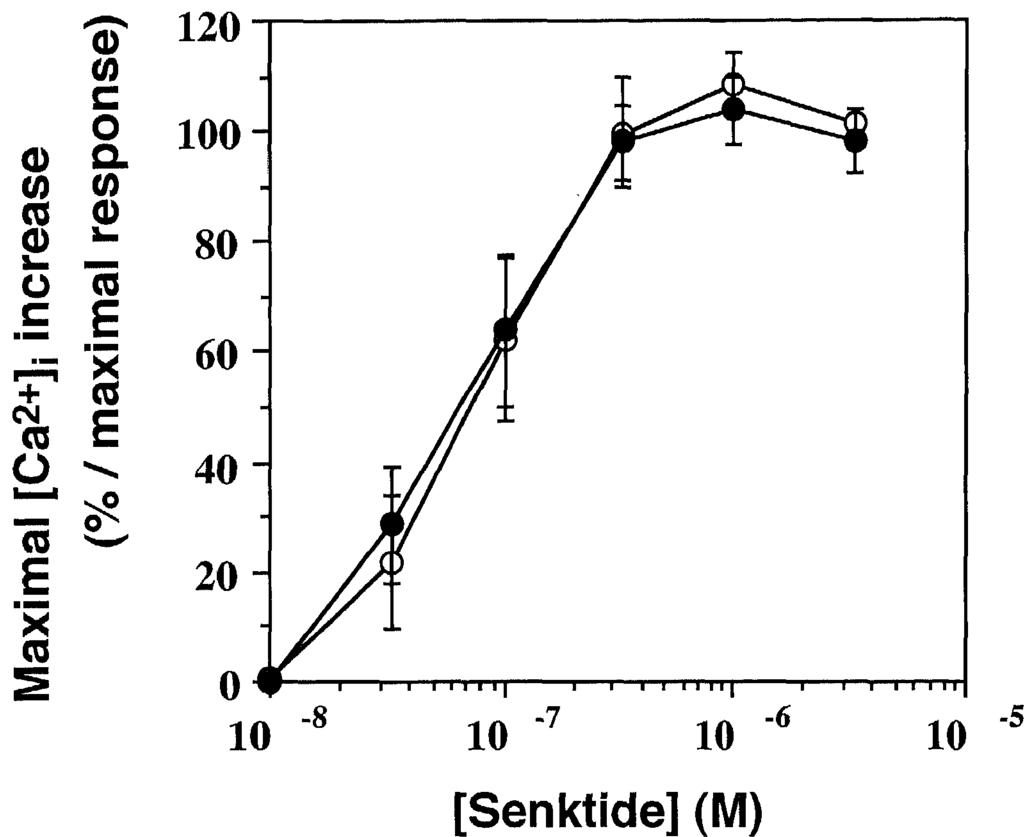
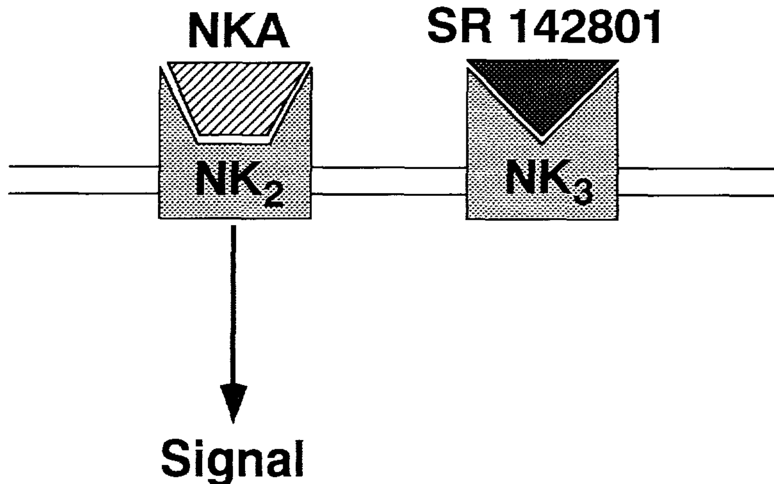
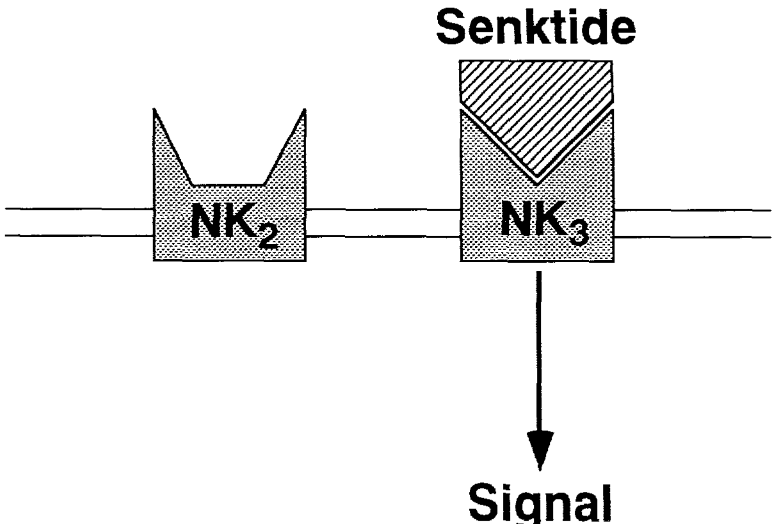


Fig. II-11. Inhibitory effect of SR 48968 on the $[Ca^{2+}]_i$ increase evoked by senktide, a selective agonist for NK_3 receptors. Cells were stimulated with the indicated concentrations of senktide in the absence (open circles) or presence (closed circles) of 1.0 μM SR 48968. The increase in $[Ca^{2+}]_i$ at each concentration is plotted as a percentage relative to the response induced by 3.3 μM senktide in the absence of SR 48968. Data are mean ± SEM (bars) values of four separate experiments.





SR 142801; NK$_3$ receptor antagonist
Senktide; NK$_3$ receptor agonist

Fig. II-12. Independent activation of NK$_2$ and NK$_3$ receptors endogenously expressed in the C1300 cell line.

TABLE II-1. Boc solid-phase peptide synthesis.

STEP		*min*
(1) CH_2Cl_2	wash ×2	2
(2) 50% TFA in CH_2Cl_2 deprotection of Boc-group		30
(3) n-PrOH	wash ×2	2
(4) 10% Et_3N in CH_2Cl_2 neutralization		2
(5) n-PrOH	wash ×2	2
(6) CH_2Cl_2	wash ×2	2
(7) Boc-AA-OH, DCC, and HOBt coupling		60<
(8) MeOH	wash ×2	2
(9) CH_2Cl_2	wash ×2	2
(10) MeOH	wash ×2	2

TABLE II-2. Fmoc solid-phase peptide synthesis.

STEP		*min*
(1) CH_2Cl_2	wash ×2	2
(2) 20% piperidine in NMP deprotection of Fmoc-group		30
(3) MeOH	wash ×2	2
(4) CH_2Cl_2	wash ×2	2
(5) Fmoc-AA-OH, BOP, and HOBt coupling in NMP		60<
(6) MeOH	wash ×2	2
(7) CH_2Cl_2	wash ×2	2
(8) MeOH	wash ×2	2

TABLE II-3. The inhibitory effect of MEN 10,376 on the $[Ca^{2+}]_i$ increase induced by NKA and NKB

MEN 10,376	NKA		NKB	
(μM)	EC_{50} (nM)	Max. (%)[a]	EC_{50} (nM)	Max. (%)[a]
0	103±18	100	111±25	100
0.1	118±10	94±2	120±8	97±7
0.3	183±26	83±1	181±27	77±3
1.0	492±48	84±4	366±70	74±6

Data are mean ± SEM values of three separate experiments.

[a] Maximal responses in the presence of MEN 10,376 are expressed as a percentage relative to the value of the maximal response induced by 10 μM NKA or NKB in its absence.

TABLE II-4. The inhibitory effect of SR 48968 on the $[Ca^{2+}]_i$ increase induced by NKA and NKB.

SR48968 (μM)	NKA (%)	NKB (%)
0	100	100
0.01	73.2±7.2	76.1±4.2
0.03	25.7±5.1	29.2±4.2
0.1	5.9±2.1	9.9±0.9
0.3	0.2±0.2	7.7±1.0**
1.0	0	7.9±1.0**
10.0	0	7.3±1.2**

Cells were stimulated by 0.33 μM of NKA and NKB in the absence or presence of SR 48968 at the indicated concentrations. The maximal increase in the $[Ca^{2+}]_i$ is expressed as a percentage relative to the response in the absence of SR 48968. Data are mean ± SEM values of four separate experiments. The $[Ca^{2+}]_i$ increase induced by NKB was compared with that by NKA in the presence of each concentration of SR 48968, and the differences were considered significant when *P*-values of the Student's *t*-test were below 0.05 (**: $P<0.01$).

CHAPTER III

INDIVIDUAL INTRACELLULAR SIGNAL TRANSDUCTION INDUCED BY NK_2 AND NK_3 RECEPTORS EXPRESSED IN A C1300 CELL LINE

III-1. INTRODUCTION

Signaling pathways mediated by endogenous neurokinin receptors have been studied using various tissue preparations. For example, NK_3 receptors endogenously expressed in guniea-pig ileum were reported to stimulate PI hydrolysis (Guard et al., 1988). However, NK_1 receptors were also expressed in guinea-pig ileum, and furthermore the activation of NK_3 receptors induced the release of acetylcholine and SP (Guard and Watson, 1987). Thus, the results of studies using tissue preparations are complicated, since such preparations contain various types of cells such as smooth muscle cells and neurons. These findings indicated that studies using systems, which consist of a single type of cell, represent a better approach for investigating signaling pathways.

At present, cell lines expressing endogenous NK_2 and/or NK_3 receptors have yet to be established, although several systems with endogenous NK_1 receptors have been reported. Therefore, the intracellular signal transduction involved in endogenous NK_2 and NK_3 receptors has been little investigated. As described in CHAPTER II, it has been demonstrated that murine neuroblastoma C1300 cells expressed endogenous NK_2 and NK_3 receptors, and furthermore that the NK_2 and NK_3 receptors could be independently activated using selective agonist and antagonist. These results show that the C1300 cell line is a useful system with which to examine signaling pathways of endogenous NK_2 and NK_3 receptors. In this chapter, the signaling pathway induced by each of the endogenous NK_2 and NK_3 receptors was investigated using this cell system.

III-2. MATERIALS AND METHODS

III-2-1. Materials

The materials used in CHAPTER II were obtained as described in CHAPTER II-2-1. All other materials were purchased as follows: myo-[2-^{3}H] inositol (10 - 20 Ci/mmol), from Amersham International plc (Bucks, UK); U73122 [1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione], from Funakoshi (Tokyo, Japan); ω-conotoxin GVIA, from Peptide Institute, Inc. (Osaka, Japan); nifedipine, ionomycin, 3-isobutyl-1-methylxanthine (IBMX) and forskolin from Wako Pure Chemical Industries (Osaka, Japan); Medium 199, from Nissui Pharmaceutical (Tokyo, Japan). All other chemicals were of reagent grade.

III-2-2. Synthesis of peptides

All peptides used in this study except for ω-conotoxin were synthesized using solid phase chemistry in conventional glass reaction vessels as described in CHAPTER II. Senktide and secretin were synthesized by Fmoc strategy, and NKA by Boc strategy.

III-2-3. Cell culture

Murine neuroblastoma C1300 cells were cultured under the same conditions as described in CHAPTER II.

III-2-4. The measurement of $[Ca^{2+}]_i$

The measurement of $[Ca^{2+}]_i$ was performed as described in CHAPTER II. To omit extracellular free Ca^{2+} from the buffer, 4 mM EGTA (final concentration) was added before the stimulation. In experiments to examine the effects of nickel, nifedipine, ω-conotoxin and U73122, these drugs were added 2 min before the stimulation.

III-2-5. The measurement of PI hydrolysis

Formation of $[^3H]$inositol phosphates was measured by a modification of the method of Berridge et al. (1983). Dispersed cells were prelabeled for 24 h with 3 μCi/ml of myo-[2-^{3}H] inositol in Medium 199 containing 1% FCS under an atmosphere of 5% CO_2/95% air at 37°C. Then, the cells were washed twice with HEPES-buffered solution containing 10 mM LiCl, and suspended in the same solution at about 3.0 × 10^6 cells/ml. Aliquots of the cell suspension (360 μl) were preincubated for 20 min in the presence or absence of 3.3 μM U73122. The cells were then stimulated by the addition of 40 μl stimulant, incubated for 10 s at 37°C, and the reaction quickly terminated by the addition of 80 μl of ice-cold 20% perchloric acid. After incubation for 20 min on ice, the precipitate was removed by centrifugation at 2,000 × g for 10 min at 4°C. The supernatant (430 μl) was neutralized with 1.5 M KOH containing 60 mM HEPES, kept on ice for 24 h, then the precipitated $KClO_4$ was removed by centrifugation at 2,000 × g for 10 min at 4°C. Water-soluble radiolabeled inositol phosphates were separated by anion exchange chromatography. The supernatant was applied to a column containing 1 ml of Dowex 1 × 8 resin (100 - 200 mesh, formate form; Muromachi Kagaku, Tokyo, Japan). Free inositol and glycerophosphoinositol were eluted with 10 ml of water and 5 mM disodium tetraborate/60 mM sodium formate, respectively. Inositol monophosphate, inositol bisphosphates and inositol trisphosphates (IP_3) were then sequentially eluted with 6 ml of 0.1 M formic acid/0.2 M ammonium formate, 0.1 M formic acid/0.4 M ammonium formate and 0.1 M formic acid/1.0 M ammonium formate, respectively. The IP_3 eluate was mixed with aqueous counting scintillant, and $[^3H]IP_3$ was quantified by scintillation counting.

III-2-6. Cyclic AMP assay

The detached cells were washed twice with HEPES-buffered solution, and resuspended in the same buffer. After incubation for 15 min at 37°C, the

cells (cell density: about 5.0 × 10^5 cells/190 μl) were resuspended in HEPES-buffered solution containing 1.0 mM IBMX, which inhibits the degradation of cyclic AMP, and incubated at 37°C for 20 min. Then the cells were stimulated by addition of 10 μl stimulant. After incubation at 37°C for 10 min, the reaction was terminated by addition of 50 μl of 25% (w/v) ice-cold trichloroacetic acid into each tube. To extract the intracellular cyclic AMP completely, each tube was frozen at -70°C for 2 min, and immediately thawed at 37°C; this process was carried out three times. The extracts were centrifuged at 8,000 rpm for 10 min, and the supernatant was washed three times with 3 volumes of diethylether saturated with water to remove the trichloroacetic acid. Thereafter the supernatant was incubated at 65°C for 15 min to remove residual diethylether, and then the contents of cyclic AMP was measured by radioimmunoassay using a cyclic AMP kit (YAMASA, Tokyo, Japan).

III-2-7. Statistics

Statistical analysis was carried out as described in CHAPTER II.

III-3. RESULTS

III-3-1. Involvement of internal and external Ca^{2+} in NK_2 and NK_3 receptor-mediated Ca^{2+} mobilization

Firstly, it was examined whether internal and/or extracellular Ca^{2+} was responsible for the $[Ca^{2+}]_i$ increases involved in NK_2 and NK_3 receptors. To activate the NK_2 or NK_3 receptors expressed in the C1300 cells independently, these cells were stimulated by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) or 1.0 μM senktide, respectively, as described in CHAPTER II. As shown in Fig. III-1, the $[Ca^{2+}]_i$ increases dependent on the activation of NK_2 and NK_3 receptors occurred even in the absence of extracellular Ca^{2+}, but the

maximal increases were reduced to 57 ± 2 and 43 ± 4% of the levels seen in the presence of extracellular Ca^{2+}, respectively. In addition, when NK_2 and NK_3 receptors were activated in the presence of 1.0 mM nickel, an inorganic Ca^{2+} influx blocker (Rink et al., 1990), the maximal increases in $[Ca^{2+}]_i$ were attenuated to 84 ± 3 and 77 ± 6% of the levels in the absence of nickel, respectively (Fig. III-1). These findings indicated that the increases in $[Ca^{2+}]_i$ through the activation of both NK_2 and NK_3 receptors are due to the combined mobilization of internal Ca^{2+} and the entry of extracellular Ca^{2+}.

III-3-2. The influx of extracellular Ca^{2+}

To elucidate whether the Ca^{2+} influx mediated by NK_2 and NK_3 receptors depends on voltage-dependent Ca^{2+} channels, the effects of nifedipine and ω-conotoxin, blockers of L- and N-type voltage-dependent Ca^{2+} channels (Reuter, 1983; Olivera et al., 1984; Rink, 1990), respectively, were examined (Fig. III-2). The increases in $[Ca^{2+}]_i$ induced by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) and 1.0 μM senktide, were unaffected by 0.1 μM nifedipine and 1.0 μM ω-conotoxin. In addition, the presence of extracellular K^+ at 60 mM did not stimulate Ca^{2+} mobilization (data not shown). These findings indicated that the influx of extracellular Ca^{2+} by the activation of NK_2 and NK_3 receptors was independent of voltage-dependent Ca^{2+} channels.

III-3-3. NK_2 and NK_3 receptor-mediated PI hydrolysis and the inhibitory effect of U73122 on these responses

To test whether the activation of NK_2 and NK_3 receptors induces PLC-dependent PI hydrolysis, the formation of IP_3 was measured as shown in Fig. III-3 and TABLE III-1. In these experiments, the cells were stimulated by NKA or senktide for 10 s, because IP_3 levels were found to maximize about 10 s after the addition of stimulant (data not shown). In the presence of 1.0 μM

SR 142801, 3.3 μM NKA stimulated the formation of IP_3 (119.3 ± 4.9% of basal, n=6), and this response was abolished by 3.3 μM U73122, a putative PLC inhibitor (Bleasdale et al., 1990), (when stimulated in the presence of U73122, 102.5 ± 2.1% of basal, n=6). Senktide at 1.0 μM also enhanced the formation of IP_3, which was inhibited by 3.3 μM U73122 (absence of U73122; 108.1 ± 2.0% of basal, presence of it; 102.9 ± 3.7% of basal, n=6). These results indicated that stimulation of NK_2 and NK_3 receptors in C1300 cells induced PLC activation.

III-3-4. The inhibitory effect of U73122 on NK_2 and NK_3 receptor-mediated Ca^{2+} mobilization

To determine whether the activation of PLC is essential for NK_2 and NK_3 receptor-mediated Ca^{2+} mobilization, the inhibitory effect of U73122 was examined. U73122 inhibited the increase in $[Ca^{2+}]_i$ induced by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) and 1.0 μM senktide, in a concentration dependent manner (IC_{50} values: NKA in the presence of SR 142801, 0.55 ± 0.05 μM; n=3, senktide, 0.47 ± 0.03 μM; n=3) (Fig. III-4). However, the increase in $[Ca^{2+}]_i$ induced by 1.0 μM ionomycin, a calcium ionophore, was not affected by this compound up to 3.3 μM (Fig. III-4). These results suggested the involvement of PLC for Ca^{2+} mobilization via NK_2 and NK_3 receptors.

III-3-5. Effect of the activation of NK_2 and NK_3 receptors on the cyclic AMP cascade

To investigate whether NK_2 and NK_3 receptors expressed in C1300 cells couple to adenylate cyclase, the effects of NKA (in the presence of SR 142801) and senktide on the level of cyclic AMP in C1300 cells were examined. The accumulation of cyclic AMP was not induced by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) or 1.0 μM senktide, whereas secretin, which is known

to activate the adenylate cyclase via G protein-coupled receptors (Roth et al., 1984), and forskolin, which appears to directly activate the adenylate cyclase, induce about 4- and 18-fold increases in the level of cyclic AMP, respectively (TABLE III-2). In addition, it was also examined whether the accumulation of cyclic AMP induced by secretin and forskolin was affected by the activation of NK_2 and NK_3 receptors. 3.3 μM NKA (in the presence of 1.0 μM SR 142801) partially inhibited the responses to secretin and forskolin (both at 10 μM) (about 17% and 10% inhibition, respectively), whereas 1.0 μM senktide did not (TABLE III-3). These findings suggested that the NK_2 receptors in C1300 cells might negatively regulate the cyclic AMP cascade.

III-4. DISCUSSION

When the cells were stimulated by NKA (in the presence of SR 142801) and senktide, immediate but transient increases in $[Ca^{2+}]_i$ occurred. These responses were partially attenuated in the absence of extracellular Ca^{2+} or in the presence of nickel (Fig. III-1), indicating that the transient increase in $[Ca^{2+}]_i$ involved in NK_2 and NK_3 receptors was dependent on both the mobilization of internal Ca^{2+} and the entry of extracellular Ca^{2+}. However, this finding is not in agreement with other results concerning $[Ca^{2+}]_i$ increase by the activation of NK_2 receptors as shown in TABLE III-4. For instance, the activation of NK_2 receptors artificially expressed in B82 fibroblasts caused a rapid and transient increase in $[Ca^{2+}]_i$ dependent on only mobilization of internal Ca^{2+} (Henderson et al., 1991). In the acinar cells of the feline tracheal submucosal gland (Nagaki et al., 1994) as well as CHO cells transfected with bovine and human NK_2 receptor cDNAs (Eistetter et al., 1991; Subramanian et al., 1994), NKA elicited a $[Ca^{2+}]_i$ increase consisting of both transient and sustained phases, which depended on, both the Ca^{2+} influx and the Ca^{2+} released from intracellular Ca^{2+} store(s) (transient phase) and a Ca^{2+} influx

only (sustained phase). These and present findings suggested that the property of each cell type would determine the mechanisms by which the activation of NK_2 receptors increases the $[Ca^{2+}]_i$.

In C1300 cells, PI hydrolysis was stimulated by the activation of NK_2 and NK_3 receptors, but these responses were inhibited by U73122 (Fig. III-3 and TABLE III-1). This inhibitor also blocked NK_2 and NK_3 receptor-mediated $[Ca^{2+}]_i$ increases, indicating the involvement of PLC in these responses (Fig. III-4). In various cell types, inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which is produced by the activation of PLC, interacts with $Ins(1,4,5)P_3$ receptors on Ca^{2+}-storing intracellular organelles, and induces the release of Ca^{2+} (Fisher et al., 1987). These and the present findings suggested that the NK_2 and NK_3 receptor-mediated mobilization of internal Ca^{2+} was caused by $Ins(1,4,5)P_3$, which was generated by PLC activation.

To characterize the nature of the Ca^{2+} influx by NK_2 and NK_3 receptors, the effects of nifedipine and ω-conotoxin were examined. Neither of these compounds affected NK_2 and NK_3 receptor-mediated Ca^{2+} mobilization (Fig. III-2). The depolarization by 60 mM extracellular K^+ did not affect the $[Ca^{2+}]_i$. These findings indicated that the influx of extracellular Ca^{2+} by NK_2 and NK_3 receptors is independent of L- or N-type voltage-dependent Ca^{2+} channels, suggesting that these responses occur through voltage-independent channels. In addition, since U73122 almost completely abolished Ca^{2+} mobilization by NK_2 and NK_3 receptors (Fig. III-4), the influx of extracellular Ca^{2+} may also be dependent on the activation of PLC. The formation of $Ins(1,4,5)P_3$ and/or inositol 1,3,4,5-tetrakisphospate ($Ins(1,3,4,5)P_4$) induced by the activation of PLC leads to a Ca^{2+} influx in several cell systems (Irvine and Moor, 1986; Kuno and Gardner, 1987; Pittet et al., 1989; Irvine, 1990; Mochizuki-Oda et al., 1994). Moreover, capacitative Ca^{2+} entry is also one of the mechanisms involved in Ca^{2+} entry through the activation of PLC, which itself is activated by the depletion of intracellular

Ca^{2+} stores (Putney and Bird, 1993). Therefore, the mechanisms of Ca^{2+} influx elicited by the activation of NK_2 and NK_3 receptors in C1300 cells remain to be clarified.

The activation of NK_2 receptors slightly reduced the accumulation of cyclic AMP induced by secretin and forskolin, although that did not induce the increase in the level of cyclic AMP. The involvement of NK_2 receptors in the cyclic AMP cascade have been reported by several groups (DeBernardi et al., 1991; Eistetter et al., 1991; Nakajima et al., 1992). For example, when NK_2 receptors were artificially expressed in CHO cells, NKA caused cyclic AMP generation by the direct or indirect stimulation of adenylate cyclase (Eistetter et al., 1991; Nakajima et al., 1992). In contrast, the activation of NK_2 receptors, which were artificially expressed in C6-2B rat glioma cells, inhibited isoproterenol-stimulated adenylate cyclase activity through the elevation of $[Ca^{2+}]_i$ (DeBernardi et al., 1991). Taken together, these findings suggested that the property of cells, in which NK_2 receptors were expressed, would determine the NK_2 receptor-mediated signaling pathways involved in the cyclic AMP cascade. In addition, it was suggested that the activation of NK_2 receptors endogenously expressed in the C1300 cell line might negatively regulate the adenylate cyclase, although further analysis is required to confirm this. On the other hand, the activation of NK_3 receptors affected neither the accumulation of cyclic AMP induced by secretin or forskolin nor the basal level, indicating that NK_3 receptors expressed in C1300 cells did not couple to adenylate cyclase. However, the effect of NK_3 receptors on the cyclic AMP cascade may be too little to be detectable, because the NK_3 receptor-mediated $[Ca^{2+}]_i$ increase was about 10-fold less than that mediated by NK_2 receptors.

In conclusion, this study indicated that C1300 cells endogenously express functional NK_2 and NK_3 receptors, the activation of which induced both the formation of IP_3 and the $[Ca^{2+}]_i$ increase. It was suggested that the $[Ca^{2+}]_i$ increase mediated by NK_2 and NK_3 receptors is dependent on both the

mobilization of internal Ca^{2+} and the entry of extracellular Ca^{2+} through voltage-independent channels. The mobilization of internal Ca^{2+} and the entry of extracellular Ca^{2+} may both result from PLC activation. In addition, this study also suggested the possibility that NK_2 receptors expressed in the C1300 cell line negatively regulate the cyclic AMP cascade.

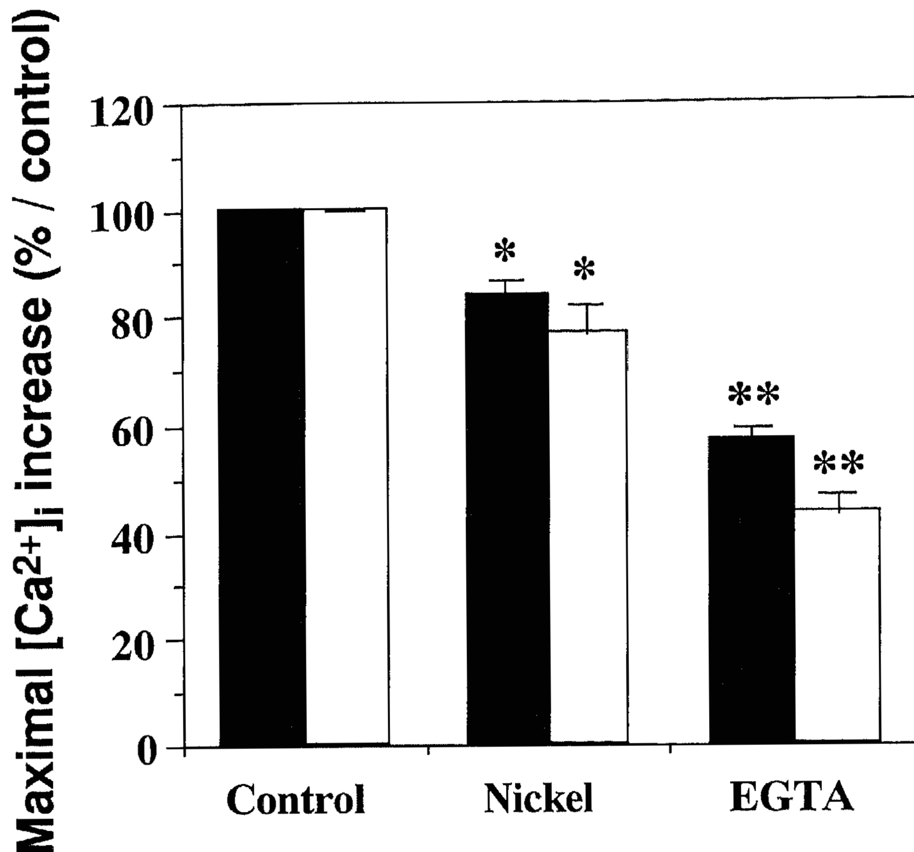


Fig. III-1. Involvement of extracellular Ca^{2+} in the $[Ca^{2+}]_i$ increase through the activation of NK_2 and NK_3 receptors. Cells were stimulated with 3.3 μM NKA (in the presence of 1.0 μM SR 142801) (closed bars) and 1.0 μM senktide (open bars), and these stimulations were done in the presence of 1.28 mM extracellular Ca^{2+} (Control), in the presence of 1.0 mM nickel and 1.28 mM extracellular Ca^{2+} (Nickel), and in the absence of extracellular Ca^{2+} (EGTA). The increase in $[Ca^{2+}]_i$ is plotted as a percentage relative to the response in the presence of extracellular Ca^{2+}. Data are mean ± SEM (bars) values (n=3). The values of $[Ca^{2+}]_i$ stimulated by NKA (in the presence of 1.0 μM SR 142801) and senktide in the absence of extracellular Ca^{2+} or in the presence of nickel were compared with those in the presence of extracellular Ca^{2+} using Student's *t* test, and differences with *p* values of <0.05 were considered significant: $*p < 0.05$, $**p < 0.01$.

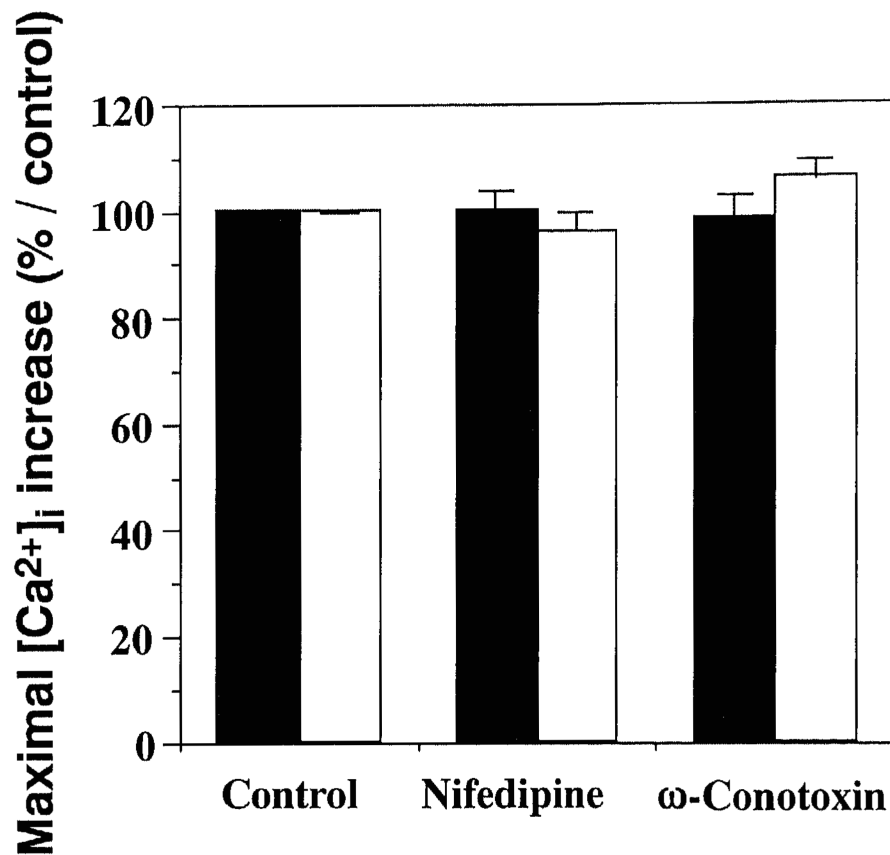


Fig. III-2. Effects of nifedipine and ω-conotoxin on NK_2 and NK_3 receptor-mediated $[Ca^{2+}]_i$ increase. Cells were stimulated by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) (closed bars) and 1.0 μM senktide (open bars), after incubation with 0.1 μM nifedipine or 1.0 μM ω-conotoxin for 2 min. The increase in $[Ca^{2+}]_i$ is plotted as a percentage relative to the control response. Data are mean ± SEM values of three separate experiments.

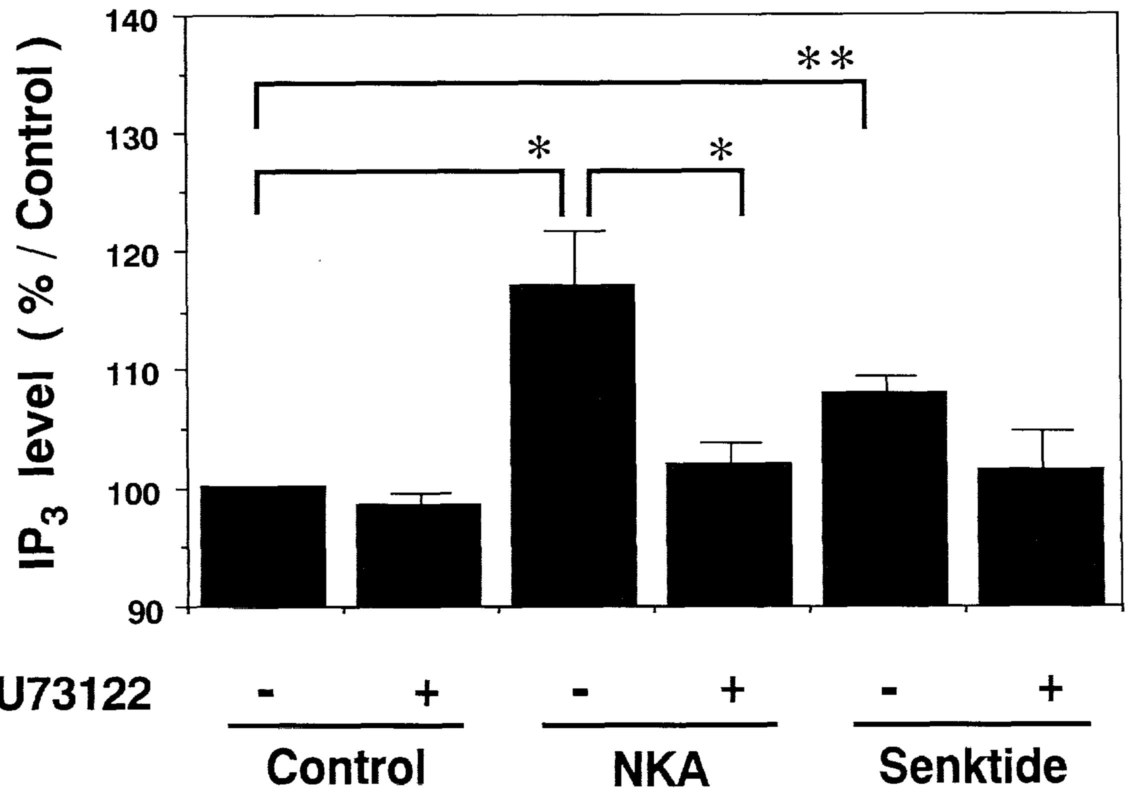


Fig. III-3. Formation of IP_3 mediated by the activation of NK_2 and NK_3 receptors. In the presence or absence of 3.3 μM U73122, cells were stimulated by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) and 1.0 μM senktide. The formation of IP_3 was expressed as a percentage relative to the basal level. Data are mean ± SEM values of six separate experiments. The differences between the values were considered significant when *p* values by Student's *t* test were <0.05: $^*p < 0.05$, $^{**}p < 0.01$.

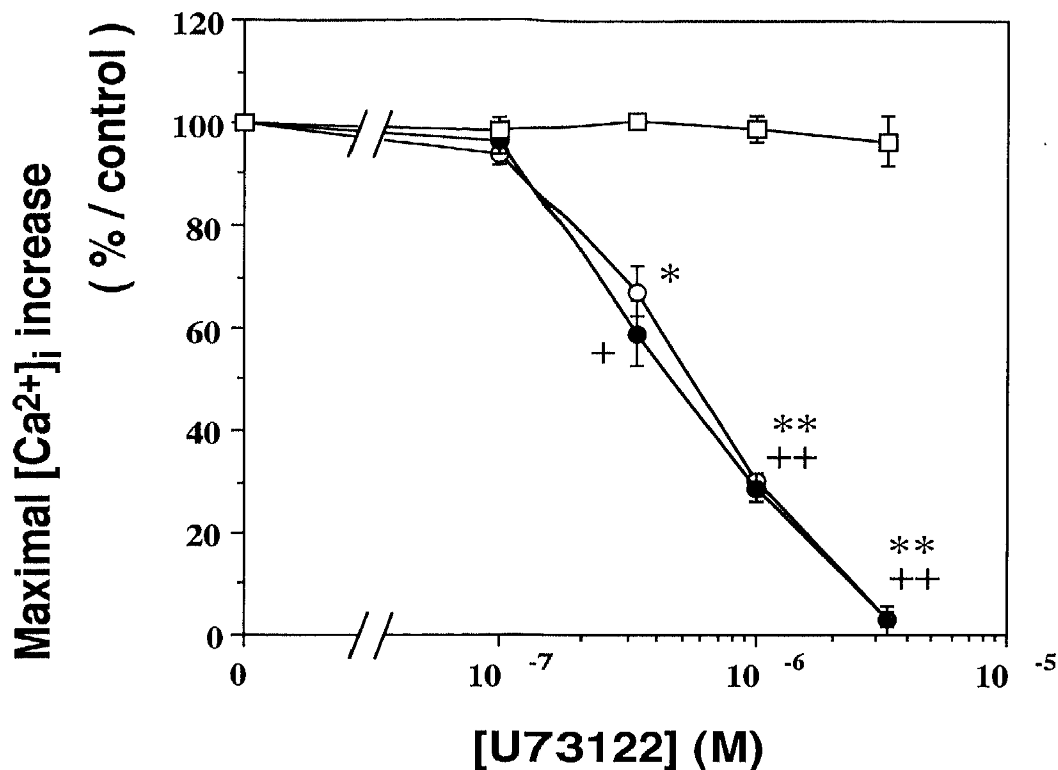


Fig. III-4. Concentration-dependent inhibition of NK_2 and NK_3 receptor-mediated $[Ca^{2+}]_i$ increase by U73122. After incubation with the indicated concentrations of U73122, cells were stimulated by 3.3 μM NKA (in the presence of 1.0 μM SR 142801) (open circles), 1.0 μM senktide (closed circles), and 1.0 μM ionomycin (open squares). The increase in $[Ca^{2+}]_i$ at each concentration is plotted as a percentage relative to the response to each stimulant in the absence of U73122. Data are mean ± SEM (bars) values of three separate experiments. The differences from the values in the absence of U73122 were considered significant when p values by Student's t test were <0.05: $^{*}p < 0.05$, $^{**}p < 0.01$ for NKA in the presence of SR 142801; $^{+}p < 0.05$, $^{++}p < 0.01$ for senktide.

TABLE III-1. Formation of IP_3 evoked by the activation of NK_2 and NK_3 receptors.

	U73122 (3.3 μM)	
Stimulant.	-	+
Control	100	98.0 ± 1.5
NKA (3.3 μM)[a]	119.3 ± 4.9*	102.5 ± 2.1^{+}
Senktide (1.0 μM)	108.1 ± 2.0**	102.9 ± 3.7

[a] Cells were stimulated by 3.3 μM NKA in the presence of 1.0 μM SR 142801.

Data are mean ± SEM values of six independent experiments.

The differences between the values were considered significant when p values by Student's t test were <0.05. Asterisks show the significance between the NKA- or senktide-stimulated IP_3 formation and the control level (* $p < 0.05$, ** $p < 0.01$). $^{+}$ shows the significance between the response in the absence of U73122 and that in its presence ($^{+}$ $p < 0.01$).

TABLE III-2. Effects of NKA, senktide, secretin and forskolin on the level of cyclic AMP in C1300 cells.

Stimulant	Cyclic AMP level (fold/control)
Control	1
NKA (3.3 μM) *a*	0.91 ± 0.06
Senktide (1.0 μM)	0.99 ± 0.06
Secretin (10.0 μM)	3.92 ± 0.09***
Forskolin (10.0 μM)	17.56 ± 1.37***

a The cells were stimulated by NKA in the presence of 1.0 μM SR 142801.
Data are mean ± SEM values of three separate experiments.
The level of cyclic AMP induced by each stimulant was compared with that of control, and the differences were considered significant when p values by Student's t test were < 0.05: *** $p < 0.001$

TABLE III-3. Effects of NKA and senktide on the secretin- and forskolin-induced cyclic AMP formation.

	Cyclic AMP level (fold/control)		
Stimulant	-	+ NKA [a]	+ Senktide
Control	1.00	0.91 ± 0.06	0.99 ± 0.06
Secretin	3.92 ± 0.09	3.27 ± 0.09**	3.873 ± 0.10
Forskolin	17.56 ± 1.37	15.91 ± 0.70	17.56 ± 1.00

[a] The cells were stimulated by NKA in the presence of 1.0 μM SR 142801.

Data are mean ± SEM values of three separate experiments.

Secretin- and forskolin-induced cyclic AMP formation in the presence of NKA or senktide was compared with that in their absence, and the differences considered significant when p values by Student's t test were < 0.05: ** $p < 0.01$

TABLE III-4. Diverse properties of Ca^{2+} signaling evoked by NK_2 receptors.

Cell Type	NK_2 Receptor	Calcium Signaling[a]	
		Transient Phase	Sustained Phase
CHO Cells[b]	transfected (human and bovine)	+ (Intra. and Extra.)	+ (Extra.)
B82 fibroblasts[c]	transfected (bovine)	+ (Intra.)	-
Acini of tracheal submucosal gland[d]	endogenous (feline)	+ (Intra. and Extra.)	+ (Extra.)
C1300 Cells[e]	endogenous (murine)	+ (Intra. and Extra.)	-

[a] "Intra." and "Extra." mean "the release from intracellular Ca^{2+} stores" and "the influx of extracellular Ca^{2+}", respectively, and it is indicated which of the two each phase was dependent on.
[b] Eistetter et al. (1991) and Subramanian et al. (1994).
[c] Henderson et al. (1991).
[d] Nagaki et al. (1994).
[e] Indicated in this study.

CHAPTER IV

IDENTIFICATION OF NK_1 RECEPTORS IN AN AR42J RAT PANCREATIC ACINAR CELL LINE AND INTRACELLULAR SIGNAL TRANSDUCTION INDUCED BY ITS RECEPTORS

IV-1. INTRODUCTION

NK_1 receptors are reported to be endogenously expressed in cell systems such as rat parotid gland (Hanley et al., 1980; Merritt and Rink, 1987; Guillemain et al., 1992), guinea-pig pancreatic acini (Sjödin et al., 1980), astrocytes (Torrens et al., 1986; Marriot et al., 1991), and neonatal rat spinal neurons (Parsons et al., 1995). In addition, several established cell lines, such as AR42J rat pancreatic acinar cells (Womack et al., 1985; Horstman et al., 1988), U 373 MG human astrocytoma cells (Lee et al., 1989; Pradier et al., 1993), and IM-9 human lymphoblast cells (Payan et al., 1984), also express endogenous NK_1 receptors. Results of studies of the neurokinin-related signalings in these cell systems indicated that the endogenous NK_1 receptors are predominantly coupled to PLC. However, several exceptions have been observed. For instance, although SP activated PLC in the iris sphincters of rabbit, bovine and pig, it stimulated the accumulation of cyclic AMP in those of dog, cat and human (Tachado et al., 1991). In canine thyroid slices, SP caused a prompt but transient rise in the level of cyclic AMP and also increased the release of thyroid hormones (Yamashita et al., 1983). In addition, the activation of NK_1 receptors, which are artificially expressed in CHO cells, not only stimulated the PI hydrolysis, but also activated the cyclic AMP cascade (Eistetter et al., 1991; Nakajima et al., 1992). These findings suggested that type of cell, in which NK_1 receptors were expressed, might determine the intracellular signaling systems involved. Therefore, studies on the signaling pathways evoked by endogenous NK_1 receptors must not only contribute to the elucidation of the mechanisms of NK_1 receptor-mediated signaling, but also answer why the signaling modes of NK_1 receptors differ among the cell types.

In this chapter, I investigated which signaling pathways were involved in the NK_1 receptors expressed in AR42J rat pancreatic acinar cells, in which SP

was reported to stimulate amylase release (Womack et al., 1985; Horstman et al., 1988).

IV-2. MATERIALS AND METHODS

IV-2-1. Materials

Materials used in this chapter were obtained as described in CHAPTER II-2-1. or III-2-1.

IV-2-2. Synthesis of peptides

All peptides used in this study were synthesized using solid phase chemistry in conventional glass reaction vessels as described in CHAPTER II. Secretin and gastrin-releasing peptide related decapeptide (GRP-10) was synthesized by Fmoc strategy, and SP, NKA and NKB by Boc strategy.

IV-2-3. Cell culture

AR42J rat pancreatic acinar cells were cultured in DMEM supplemented with 14% (v/v) FCS and antibiotics under the same conditions as described in CHAPTER II.

IV-2-4. Poly(A)+ RNA preparation and RNA blot hybridization

Poly(A)+ RNA preparation and RNA blot hybridization were carried out as described in CHAPTER II.

IV-2-5. The measurement of amylase release

Confluent AR42J cells were detached by brief exposure to 0.02% ethylenediaminetetraacetic acid/0.25% trypsin and immediately neutralized with an equal volume of DMEM medium containing 14% (v/v) FCS. After being washed twice with the same medium, the cells were seeded in 24-well

plates at a density of 1.5×10^5 cells/well and cultured for 72 h. Then, the cells were washed twice with the Hank's solution (138 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 0.3 mM Na_2HPO_4, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.4 mM $MgSO_4$, 10 mM HEPES, 22 mM glucose, 0.4% bovine serum albumin, pH 7.2), and incubated with the same solution for 15 min at 37°C. The supernatant was then immediately removed, and 300 μl of Hank's solution containing the peptides added to initiate the stimulation, followed by incubation for the indicated time. Next the supernatant was collected, and was centrifuged at 7,000 rpm for 1 min at 4°C to remove the residual cells. In addition, the cells plated in the well were treated with Hank's solution containing 1.0% Triton X-100 to extract and estimate the total amylase in the cells. The contents of amylase in the supernatant or the extract from the cells were measured by the kit for amylase measurement, Neo·Amylase Test "DAIICHI" (Daiichi Chemical, Tokyo, Japan).

IV-2-6. The measurement of $[Ca^{2+}]_i$

Basically, the measurement of $[Ca^{2+}]_i$ was performed as described in CHAPTER II, except that the cells were stimulated at 37°C.

IV-2-7. The measurement of PI hydrolysis

Formation of ^3H-inositol phosphates was measured by a minor modification of the method as described in CHAPTER III. Briefly, confluent AR42J cells were detached as described in IV-2-5, and the cells were seeded in 24-well plates at a density of 1.5×10^5 cells/well and cultured for 48 h. The cells were then labeled with 3 μCi/ml of myo-[2-^3H] inositol in Medium 199 containing 1% FCS under an atmosphere of 5% CO_2/95% air at 37°C for 24 h. The cells were washed twice with HEPES-buffered solution containing 10 mM LiCl, and incubated with the same solution for 15 min at 37°C. There after the supernatant was removed, and 500 μl of HEPES-buffered solution containing

the peptides was added to initiate the stimulation. After incubation for the indicated time, the reaction was terminated by addition of 100 μl of ice-cold 20% (w/v) perchloric acid, followed by further incubation on ice for 20 min to completely extract the inositol phosphates. The extracts were then centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was neutralized with 1.5 M KOH containing 60 mM HEPES. Next, the water-soluble radiolabeled inositol phosphates were separated by anion exchange chromatography as described in CHAPTER III, and $[^3H]IP_3$ was quantified by scintillation counting.

IV-2-8. Cyclic AMP assay

The stimulation by agonists and the measurement of the contents of cyclic AMP were performed as described in CHAPTER III.

IV-2-9. Statistics

Statistical analysis was carried out as described in CHAPTER II.

IV-3. RESULTS

IV-3-1. Amylase release induced by SP and GRP-10

It has already been reported that SP stimulates the release of amylase in AR42J cells (Womack et al., 1985). Therefore, the effect of SP on the amylase release was examined to determine whether neurokinin receptors were expressed in AR42J cells, as well as to confirm the results reported by Womack et al. As shown in Fig. IV-1, the amylase was spontaneously released even without the stimulation. Both 1.0 μM SP and 10 nM GRP-10, whose receptors are known to be expressed in this cell line (Christophe, 1994), enhanced the basal amylase release, and their effects continued for at least 45 min after the stimulation (Fig. IV-1). In addition, the effects of SP and GRP-

10 were concentration-dependent (EC_{50}: SP 0.53 nM, GRP-10 10 pM, Fig. IV-2). These findings suggested the presence of neurokinin receptors in the AR42J cell line.

IV-3-2. Identification of NK_1 receptors in the AR42J cell line

To study which types of neurokinin receptors are involved in the amylase release evoked by SP, a blot-hybridization analysis of poly(A)+ RNA using each of the cDNA probes for NK_1, NK_2 and NK_3 receptors was performed (Fig. IV-3). NK_1 receptor mRNA was expressed in this cell line, whereas NK_2 and NK_3 receptor mRNAs were not, suggesting that only NK_1 receptors are expressed in AR42J cells. Therefore, the effects of neurokinins and neurokinin receptor agonists on both the $[Ca^{2+}]_i$ and the amylase release were examined to elucidate whether these receptors are functional. As shown in Fig. IV-4, SP, NKA, NKB and SP-OMe (an NK_1 receptor agonist) induced the $[Ca^{2+}]_i$ increase in a concentration-dependent manner (EC_{50} values: SP 4.8 ± 0.8 nM, NKA 86 ± 37 nM, NKB 81 ± 16 nM, SP-OMe 35 ± 10 nM; n=5), the order of potency being SP>SP-OMe>NKA=NKB, which is in agreement with the pharmacological characterization of NK_1 receptors (Maggi et al., 1993; Buck, 1994). In contrast, [βAla⁸]NKA(4-10) and senktide, which are selective agonists for NK_2 and NK_3 receptors, respectively, did not stimulate the $[Ca^{2+}]_i$ increase. In addition, SP, NKA and NKB also induced the amylase release in a concentration-dependent manner, the order of potency being SP>NKA=NKB (EC_{50} values: SP 1.5 nM, NKA 44 nM, NKB 26 nM; n=5-6) (Fig. IV-5). These results indicated that AR42J cells endogenously expressed functional NK_1 receptors, but not NK_2 and NK_3 receptors.

IV-3-3. PI hydrolysis and Ca^{2+} mobilization induced by the activation of NK_1 receptors

It was investigated whether NK_1 receptors present in AR42J cells stimulate PI hydrolysis to induce the formation of IP_3 and the rise in $[Ca^{2+}]_i$. Both 1.0 μM SP and 10 nM GRP-10 stimulated the formation of IP_3 (Fig. IV-6). The accumulation of IP_3 induced by SP reached peak level within 5-10 s, but had decreased to basal level within 180 s. In contrast, GRP-10-induced IP_3 formation also reached peak level within 5-10 s, but was sustained. These findings suggested that SP and GRP-10 stimulated the PI hydrolysis in AR42J cells, and furthermore that the NK_1 receptors expressed in AR42J cells coupled to PLC.

SP and GRP-10 also caused a rise in $[Ca^{2+}]_i$ in a concentration-dependent manner (EC_{50}: SP 0.7 nM, GRP-10 21 pM, Fig. IV-7). Even when the cells were stimulated by 0.1 μM SP or 1.0 nM GRP-10 in the absence of extracellular Ca^{2+}, a rise in $[Ca^{2+}]_i$ still occurred, but maximal responses were reduced to about 29 and 22% of the levels observed in its presence, respectively (Fig. IV-7 and 8). Furthermore, the inhibitory effects of U73122, a putative PLC inhibitor, on the $[Ca^{2+}]_i$ increases induced by SP and GRP-10 were examined to determine whether these responses were due to the PLC activation. In the presence of 10 μM U73122, the maximal $[Ca^{2+}]_i$ increases evoked by 0.1 μM SP and 10 nM GRP-10 in both the presence and absence of extracellular Ca^{2+} were reduced to about 5% of the levels in its absence (Fig. IV-9). These results suggested that NK_1 receptor-mediated $[Ca^{2+}]_i$ increase was dependent on the Ca^{2+} release from intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+}, both of which might be mediated by the activation of PLC.

IV-3-4. Effect of the activation of NK_1 receptors on the cyclic AMP cascade

To study whether the NK_1 receptors were coupled to the cyclic AMP cascade, the effects of SP, secretin and forskolin on the level of cyclic AMP in AR42J cells were examined. Secretin and forskolin appear to activate the

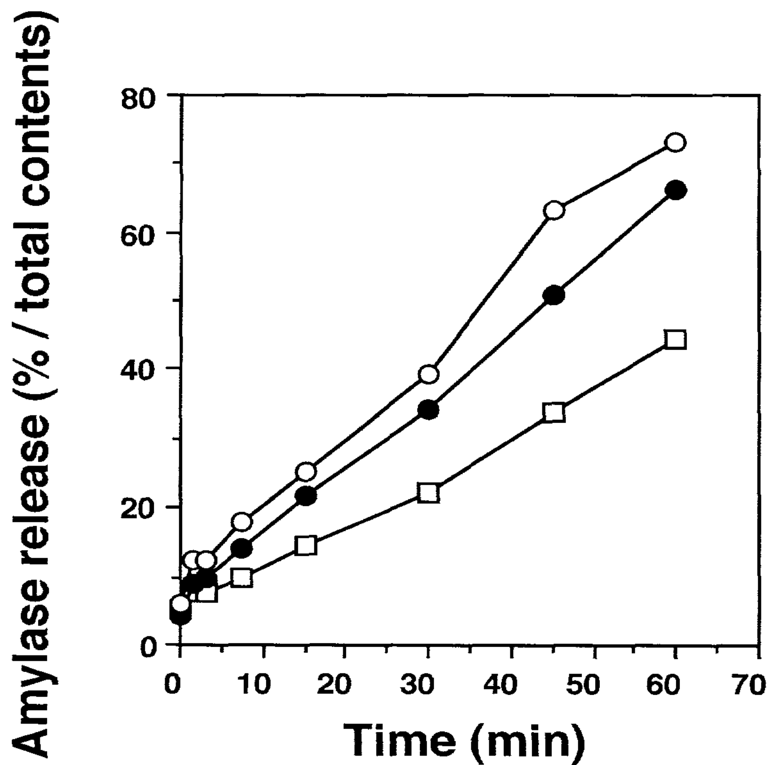
adenylate cyclase by indirect (via the G protein-coupled receptors) and direct processes, respectively. As shown in TABLE IV-1, 1.0 μM SP did not affect the level of cyclic AMP, although 1.0 μM secretin and 10 μM forskolin induced about a 3- and 6-fold increase, respectively. In addition, the effect of SP on the secretin- and forskolin-induced cyclic AMP generation was also studied to determine whether NK_1 receptors negatively regulate adenylate cyclase (TABLE IV-2). The formation of cyclic AMP evoked by 1.0 μM secretin and 10 μM forskolin was unaffected by 1.0 μM SP. These findings indicated that the NK_1 receptors expressed in AR42J cells did not couple to the cyclic AMP cascade.

IV-4. DISCUSSION

In the present study, a $[Ca^{2+}]_i$ rise mediated by the activation of NK_1 receptors was observed even in the absence of extracellular Ca^{2+} (Fig. IV-7 and 8). Horstman et al. (1988) also indicated that SP evoked the Ca^{2+} mobilization in the presence and absence of extracellular Ca^{2+} in the AR42J cell line. In addition, the NK_1 receptor-stimulated $[Ca^{2+}]_i$ increase was markedly inhibited by U73122 (Fig. IV-9). It has been shown that $Ins(1,4,5)P_3$, which is generated from phosphatidylinositol 4,5-bisphosphate by the activation of PLC, activates the $Ins(1,4,5)P_3$ receptors on internal Ca^{2+} stores to induce the release of Ca^{2+} in a variety of cell types (Fisher and Agranoff, 1987). Furthermore, it has been reported that $Ins(1,4,5)P_3$ induced the Ca^{2+} release from internal Ca^{2+} stores in permeabilized AR42J cells (Bird et al., 1991). Taken together with the result that the NK_1 receptors activated the PLC to generate IP_3, it is suggested that the $[Ca^{2+}]_i$ rise induced by NK_1 receptors was, at least in part, dependent on the Ca^{2+} release from the internal Ca^{2+} stores, which might be mediated by $Ins(1,4,5)P_3$.

On the other hand, the maximal $[Ca^{2+}]_i$ rise mediated by NK_1 receptors in the absence of extracellular Ca^{2+} was much smaller than that in the presence of extracellular Ca^{2+} (Fig. IV-7 and 8). Therefore, the NK_1 receptor-mediated $[Ca^{2+}]_i$ rise might be partially due to the Ca^{2+} influx from extracellular Ca^{2+}. Indeed, Gallacher et al. (1990) reported that, although SP induced the $[Ca^{2+}]_i$ rise in the presence of extracellular Ca^{2+}, this response was abolished in the absence of extracellular Ca^{2+}, which suggested that it occurred via the Ca^{2+} influx. However, this finding is not in agreement with the present result indicating that SP induced the $[Ca^{2+}]_i$ increase even in the absence of extracellular Ca^{2+}. Therefore, further investigation is required to elucidate the mechanisms of the influx from extracellular Ca^{2+} mediated by NK_1 receptors in AR42J cells.

In conclusion, the present study indicated that the AR42J cells expressed endogenous NK_1 receptors, but not NK_2 or NK_3 receptors. It was also suggested that NK_1 receptors activated the PLC to generate IP_3 which, in turn, caused the Ca^{2+} release from the intracellular Ca^{2+} stores to induce the $[Ca^{2+}]_i$ rise, and that these receptors did not couple to the cyclic AMP cascade. In addition, this study showed that the AR42J cell line is a useful tool with which to investigate the pharmacological functions and the signaling pathways of endogenous NK_1 receptors.



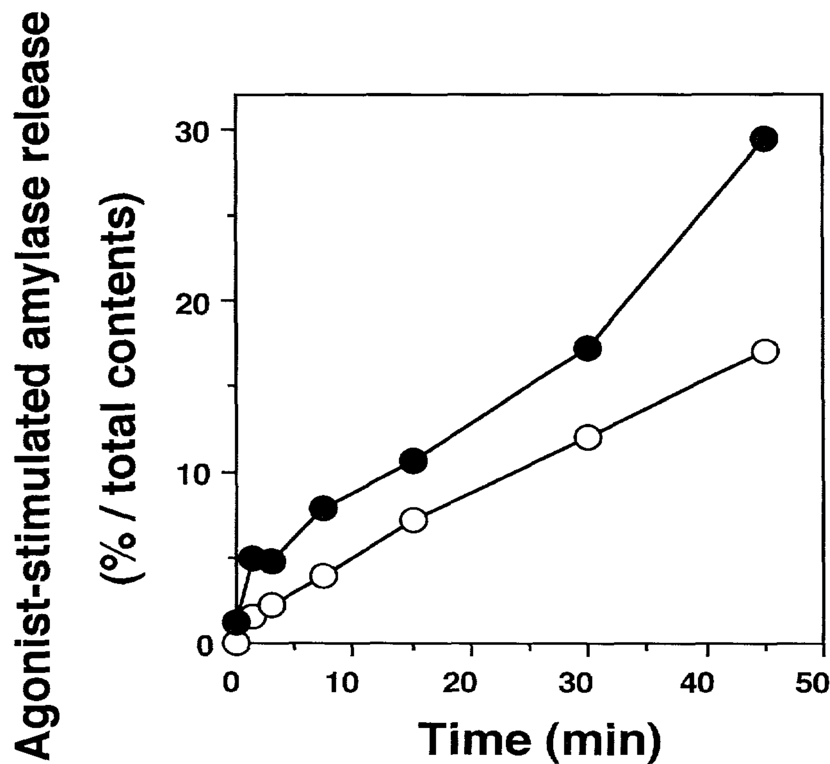


Fig. IV-1. Time course of amylase release induced by SP and GRP-10. AR42J cells were stimulated by 1.0 μM SP, 10 nM GRP-10 or vehicle for the indicated time. (Left panel) Amylase release is expressed as the value of the basal plus agonist-stimulated release. Symbols indicate as follows: closed circles; SP, open circles; GRP-10, open squares; vehicle. (Right panel) Amylase release is expressed as the value of agonist-stimulated release. Symbols indicate as follows: open circles; SP, closed circles; GRP-10.

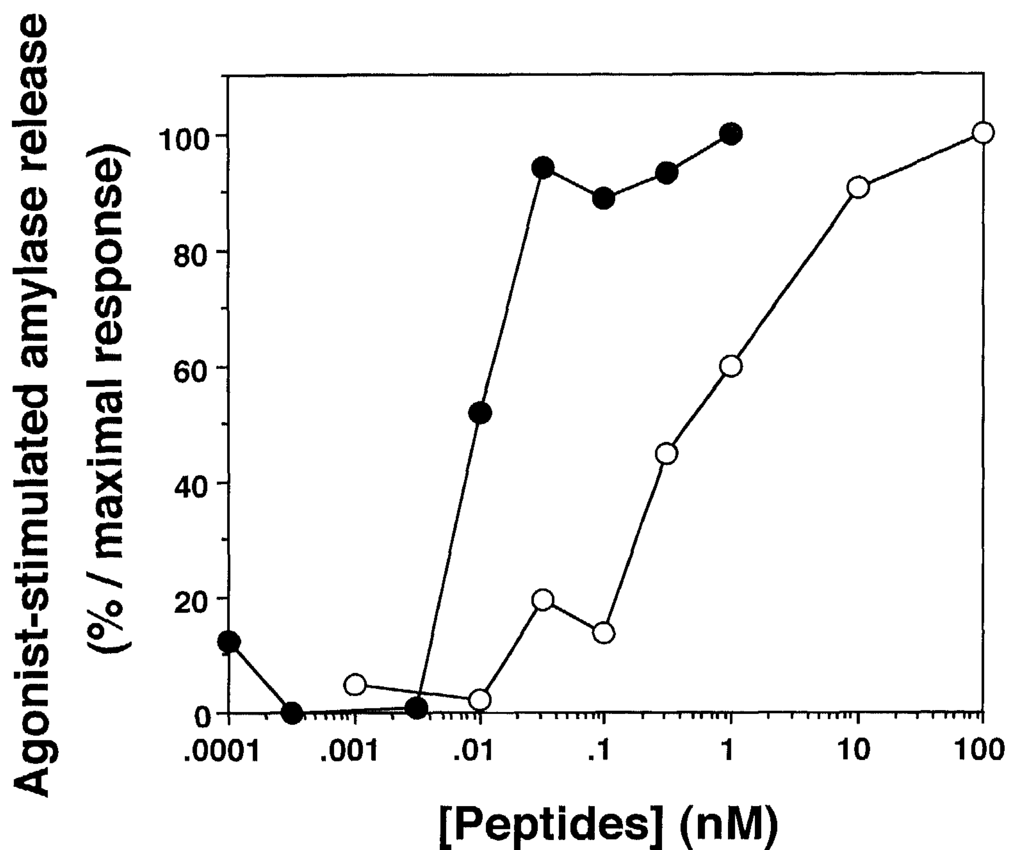


Fig. IV-2. Concentration-response curves of SP and GRP-10 for amylase release. AR42J cells were stimulated by the indicated concentration of SP (open circles) and GRP-10 (closed circles) for 30 min. The values are expressed as a percentage relative to the maximal response induced by each peptide.

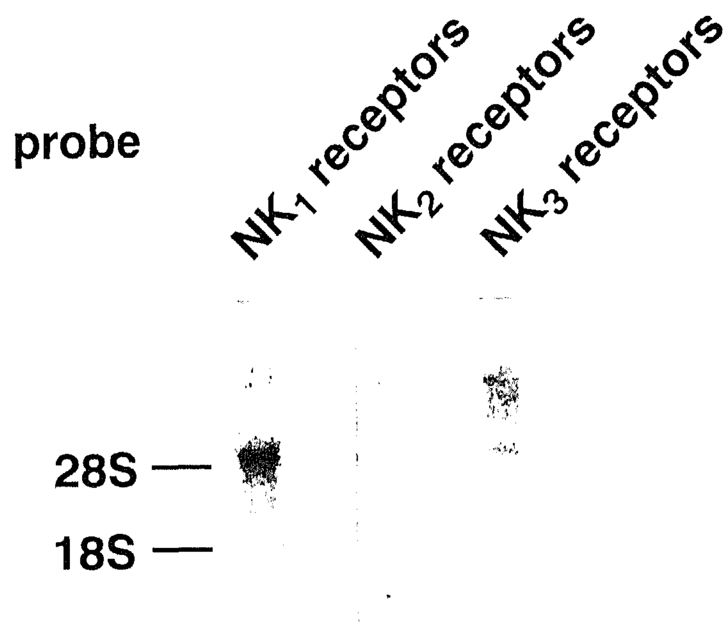


Fig. IV-3. RNA blots of poly $(A)^+$ RNA from AR42J cells using each of the cDNA probes for NK_1, NK_2, and NK_3 receptors.

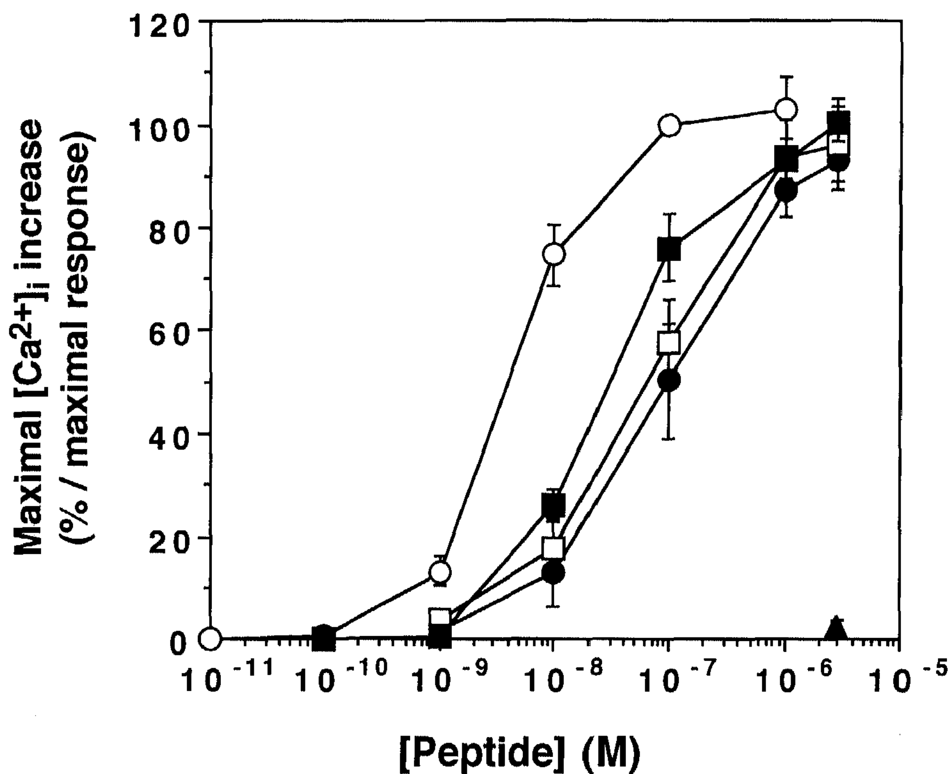


Fig. IV-4. Concentration-response curves of the $[Ca^{2+}]_i$ increase evoked by neurokinin and related peptides in AR42J cells. Cells were stimulated with the indicated concentrations of SP (open circles), NKA (closed circles), NKB (open squares), SP-OMe (closed squares), [βAla[8]]NKA(4-10) (open triangles) and senktide (closed triangles). The $[Ca^{2+}]$i increases are expressed as a percentage relative to the maximal response induced by 0.1 μM SP. Data are mean ± SEM (bars) values of five independent experiments.

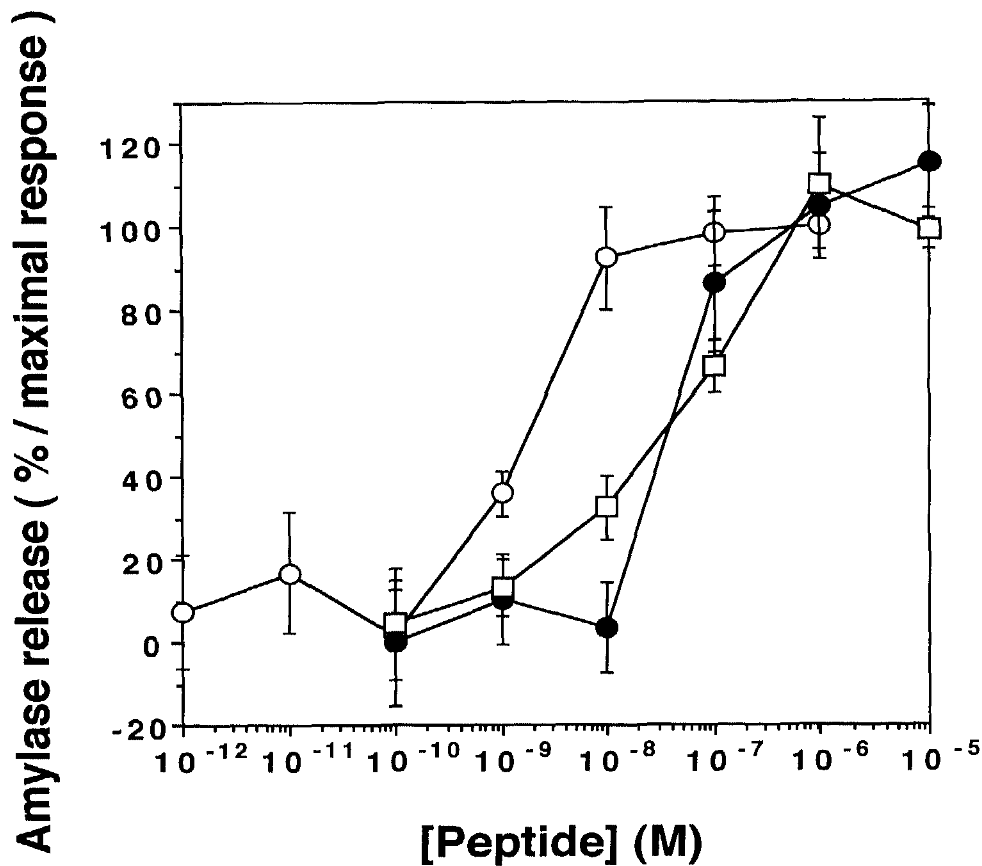


Fig. IV-5. Concentration-response curves of the amylase release evoked by SP, NKA and NKB in AR42J cells. Cells were stimulated with the indicated concentrations of SP (open circles), NKA (closed circles) and NKB (open squares). Amylase release is expressed as a percentage relative to the maximal response induced by 1.0 μM SP. Data are mean ± SEM (bars) values of five or six independent experiments.

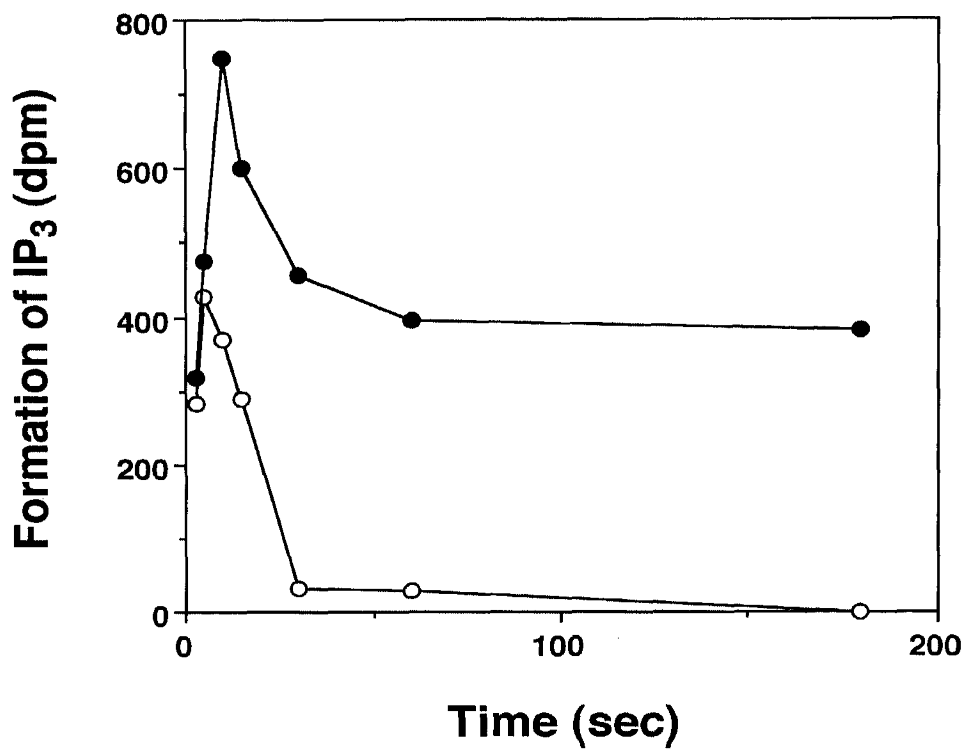


Fig. IV-6. Time couse of IP_3 formation induced by SP and GRP-10. AR42J cells were exposed to 1.0 μM SP (open circles) and 10 nM GRP-10 (closed circles). Formation of IP_3 was expressed as the values of the agonist-stimulated formation.

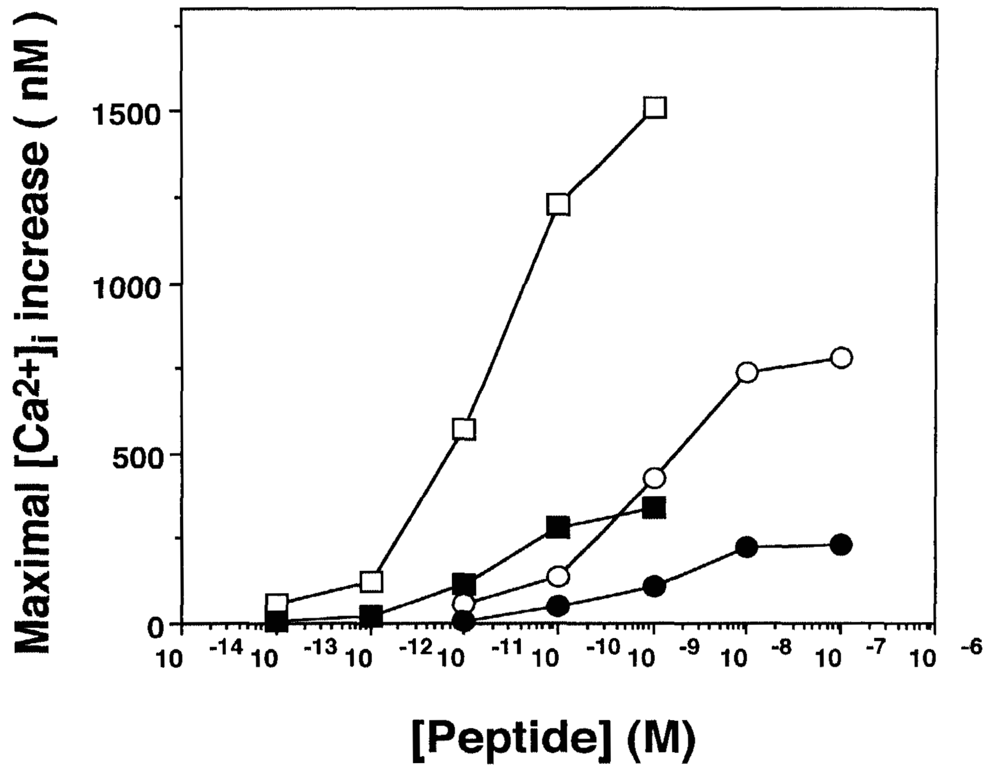


Fig. IV-7. Concentration-response curves of the $[Ca^{2+}]_i$ increase evoked by SP and GRP-10 in the presence or absence of extracellular Ca^{2+}. AR42J cells were stimulated by the indicated concentrations of SP (circles) and GRP-10 (squares) in the presence (open symbols) or absence (closed symbols) of extracellular Ca^{2+}.

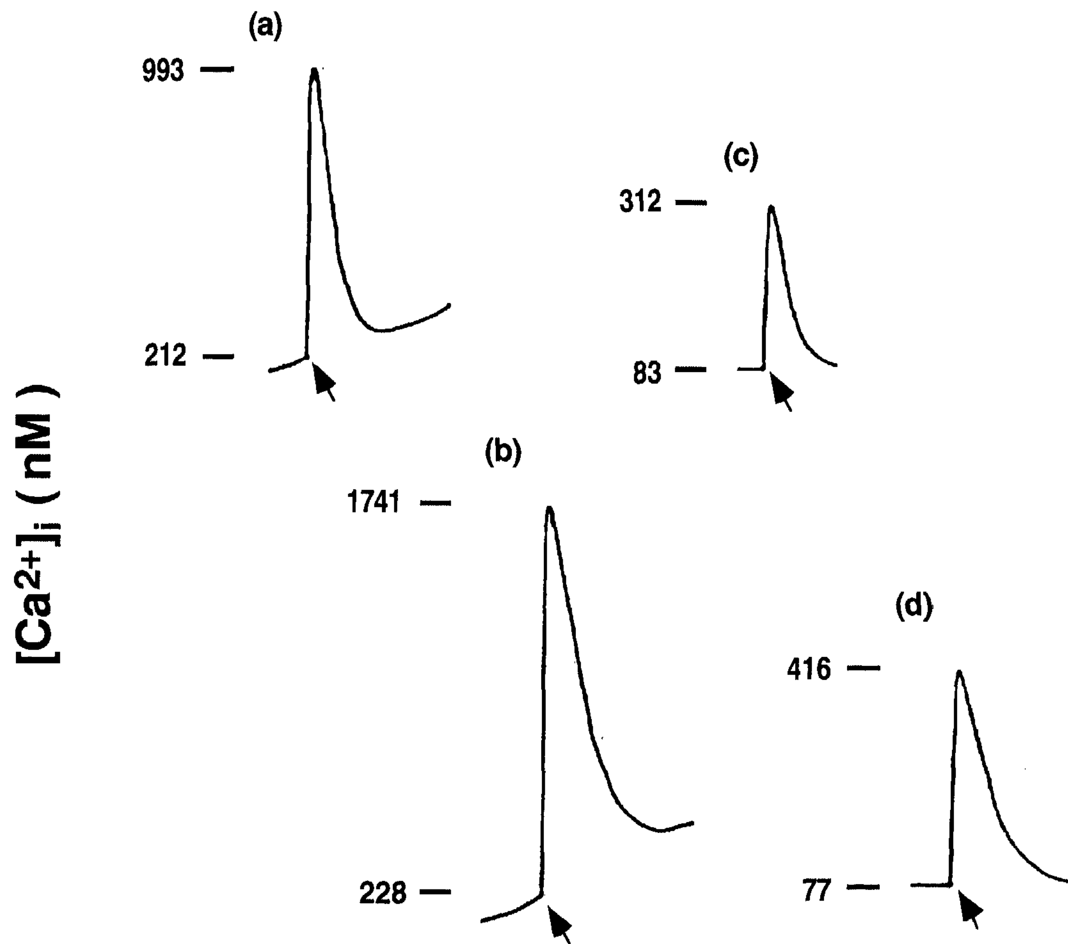


Fig. IV-8. Effects of SP and GRP-10 on $[Ca^{2+}]_i$ in the presence or absence of extracellular Ca^{2+}. AR42J cells were stimulated by 0.1 μM SP (a, c) and 1.0 nM GRP-10 (b, d) in the presence (a, b) or absence (c, d) of extracellular Ca^{2+}. Arrowheads indicate the addition of SP or GRP-10.

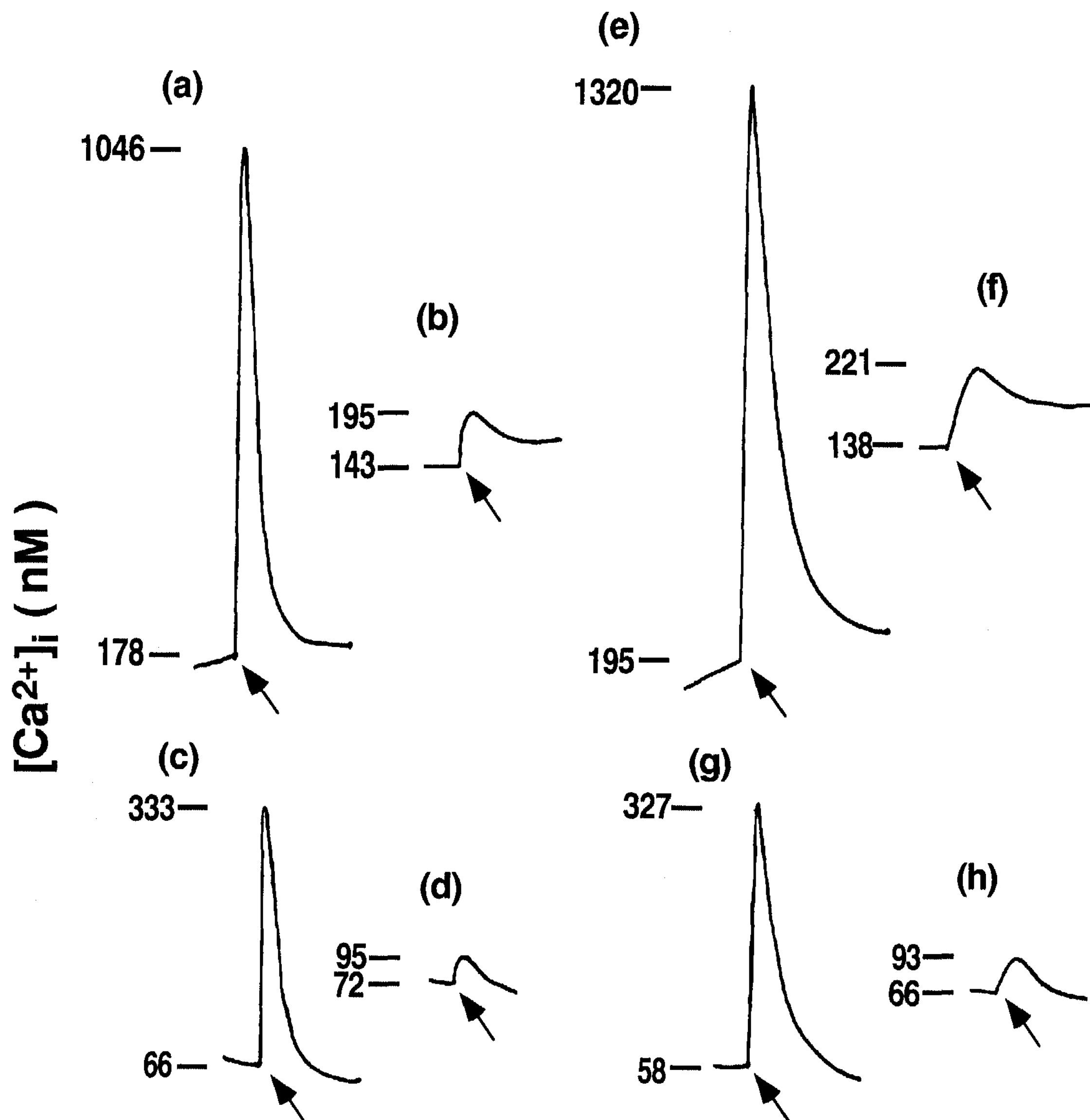


Fig. IV-9. Inhibitory effect of U73122, a putative phospholipase C inhibitor, on the $[Ca^{2+}]_i$ increase induced by SP and GRP-10. AR42J cells were stimulated by 0.1 μM SP (a, b, c, d) and 1.0 nM GRP-10 (e, f, g, h) in the presence (b, d, f, h) and absence (a, c, e, g) of 10 μM U73122 and in the presence (a, b, e, f) and absence (c, d, g, h) of extracellular Ca^{2+}. Arrowheads indicate the addition of SP and GRP-10.

TABLE IV-1. Effects of substance P, secretin and forskolin on the level of cAMP in AR42J cells.

Stimulant	Cyclic AMP level (pmol/10^5 cells)
Control	3.0 ± 0.3
Substance P (1 μM)	3.2 ± 0.4
Secretin (1 μM)	8.9 ± 0.9*
Forskolin (10 μM)	15.0 ± 1.3***

AR42J cells were stimulated by 1.0 μM substance P, 1.0 μM secretin and 10 μM forskolin for 10 min.
Data are mean ± SEM values of three separate experiments.
The levels of cyclic AMP stimulated by substance P, secretin, and forskolin were compared with that of control, and the differences considered significant when p values by Student's t test were <0.05: * $P > 0.05$, *** $P > 0.001$.

TABLE IV-2. Effect of substance P on secretin- and forskolin-induced cAMP formation.

	cAMP level (pmol/10^5 cells)	
	Substance P (1 μM)	
Stimulant	-	+
Control	3.0 ± 0.2	3.2 ± 0.2
Secretin (1 μM)	9.5 ± 1.4	9.6 ± 1.4
Forskolin (10 μM)	16.5 ± 1.8	17.7 ± 1.3

AR42J cells were stimulated by 1.0 μM secretin and 10 μM forskolin in the absence or presence of 1.0 μM substance P for 10 min.

Data are mean ± SEM values of three separate experiments.

CHAPTER V

GENERAL DISCUSSION

The present study shows that all neurokinin receptors have the ability to stimulate PI hydrolysis. The stimulation of PI hydrolysis was shown to regulate a variety of cell functions such as exocytosis, contraction of smooth muscles and cell proliferation (Dennis et al., 1991; Rhee et al., 1991). Therefore, the biological functions of neurokinins may be also elicited through this signaling pathway. On the other hand, many other studies indicated that neurokinin receptors could stimulate or inhibit the adenylate cyclase in particular cell systems (Eistetter et al., 1991; DeBernardi et al., 1991; Nakajima et al., 1992). These findings suggested that the functions induced by neurokinins could occur through the cyclic AMP cascade. To elucidate the mechanisms of the biological functions of neurokinins, the intracellular signal transduction pathways responsible for each function should be investigated.

Why do the signaling modes of neurokinin receptors differ among cell types? G protein-coupled receptors such as neurokinin receptors appear to transduce the extracellular signal to several types of G proteins which, in turn, activate effectors such as PLC and adenylate cyclase (Gilman, 1987). The activation of each effector is mediated by a specific type of G protein (Gilman, 1987). For instance, the G_q type of G protein is involved in the activation of PLC, while the G_s and G_i types are concerned with the activation and inhibition of adenylate cyclase, respectively. Therefore, neurokinin receptor-mediated signaling pathways may be determined by the affinity between these receptors and each type of G protein, that is to say, the neurokinin receptors may interact with type G_q to activate PLC predominantly, since neurokinin receptors have high affinity with G_q. However, because the affinity between the neurokinin receptors and type G_s is lower than that between these receptors and G_q, the neurokinin receptors may be able to transduce the signal to G_s and to activate the adenylate cyclase only in particular cell types expressing high amounts of G_s type G protein, or overexpressing the neurokinin receptors. Indeed, although neurokinin receptors artificially

expressed in CHO cells activate the adenylate cyclase to generate cyclic AMP, the expression level of the receptors in this system was much higher than that in the cell systems expressing endogenous receptors (e.g. NK_1 receptors; CHO cells, about 2.75×10^5 receptors/cell; AR42J cells, about 1.0×10^4 receptors/cell) (Nakajima et al., 1992; Womack et al., 1985). Furthermore, although endothelin-1 (ET-1) did not mediate inhibitory action on forskolin-stimulated cyclic AMP accumulation in COS-7 cells transfected with each type of endothelin receptors, it could when cotransfected with G_i type G protein (Takigawa et al., 1995). These findings support the hypothesis that the signaling pathways involved in not only neurokinin receptors, but also other G protein-coupled receptors may be determined by the affinity between each type of G protein and these receptors, that is, the amounts of receptors and each type of G proteins in a cell as well as the distribution of them control the coupling efficiency between them. It is necessary to estimate the affinity between receptors and G proteins directly to elucidate this hypothesis.

CHAPTER VI

CONCLUSION

In the present study, the intracellular signal transduction involved in each type of endogenous NK_1, NK_2 and NK_3 receptor was investigated to elucidate the signaling pathways essential to the biological functions of neurokinins. Because cell systems expressing endogenous NK_2 and/or NK_3 receptors have yet to be established, cell lines were firstly screened as described in CHAPTER II. It was demonstrated that the murine neuroblastoma C1300 cell line expressed the NK_2 and NK_3 receptors endogenously, and that this NK_2 receptor subtype differed from known receptors. In addition, this study revealed that the NK_2 and NK_3 receptors in this cell line could be independently activated using selective agonist and antagonist, which made it possible to investigate the individual signal transductions evoked by these endogenous receptors. As shown in Fig. II-12, only NK_2, and not NK_3 receptors, were activated by NKA in the presence of an NK_3 receptor antagonist, SR 142801. In contrast, activation of NK_3 receptors was only induced by the NK_3 receptor agonist, senktide.

In CHAPTER III, a study of the individual intracellular signaling pathways involved in endogenous NK_2 and NK_3 receptors using the C1300 cell line is presented. As shown in Fig. VI-1, this study indicated that the activation of NK_2 and NK_3 receptors activated the PLC to generate IP_3. It was also revealed that their activation caused increases in $[Ca^{2+}]_i$, which were dependent on both the Ca^{2+} release from the intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+} via voltage-independent channels. Furthermore, it was suggested that both the mobilization from internal Ca^{2+} and the Ca^{2+} influx result from the activation of PLC. In addition, this study also suggested that the activation of NK_2 receptors might inhibit the increased activity of adenylate cyclase evoked by stimulants such as secretin and forskolin.

In CHAPTER IV, a study showing that AR42J rat pancreatic acinar cells expressed only NK_1, and not NK_2 or NK_3 receptors is described. Further, the signaling pathways induced by NK_1 receptors is discussed (Fig. VI-2). It was

demonstrated that the NK_1 receptors expressed in AR42J cells increased the activity of PLC to generate IP_3, but did not activate or inhibit that of adenylate cyclase. In addition, this study also showed that the activation of NK_1 receptors elicited the rise in $[Ca^{2+}]_i$, which was, at least in part, dependent on the Ca^{2+} mobilization from the intracellular Ca^{2+} stores.

Finally, this study shows that C1300 cells and AR42J cells are very useful to investigate the signaling pathways induced by neurokinin receptors as well as to examine pharmacological functions of neurokinins.

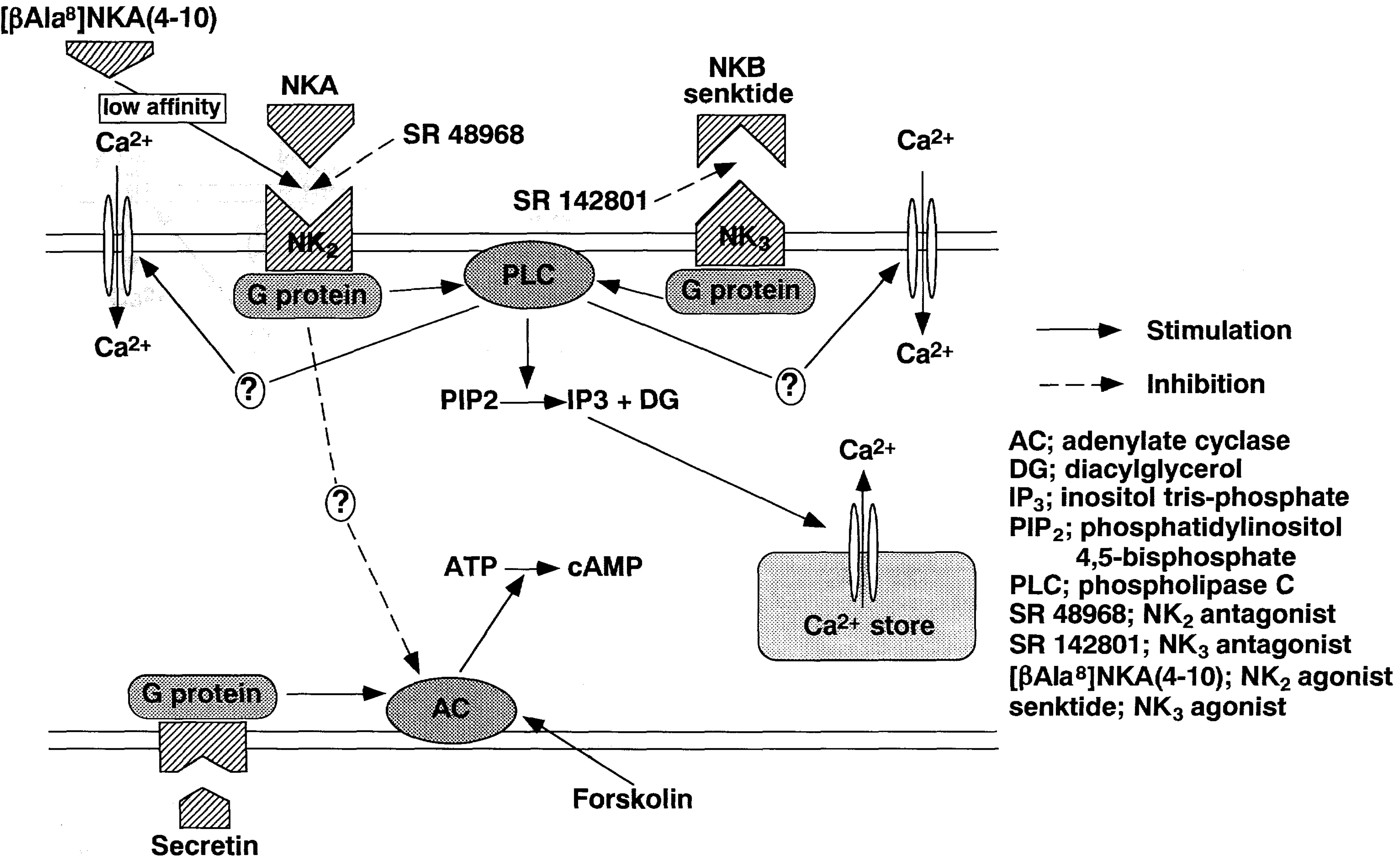


Fig. VI-1. Neurokinin receptors expressed in C1300 cells and individual intracellular signal transduction induced by them.

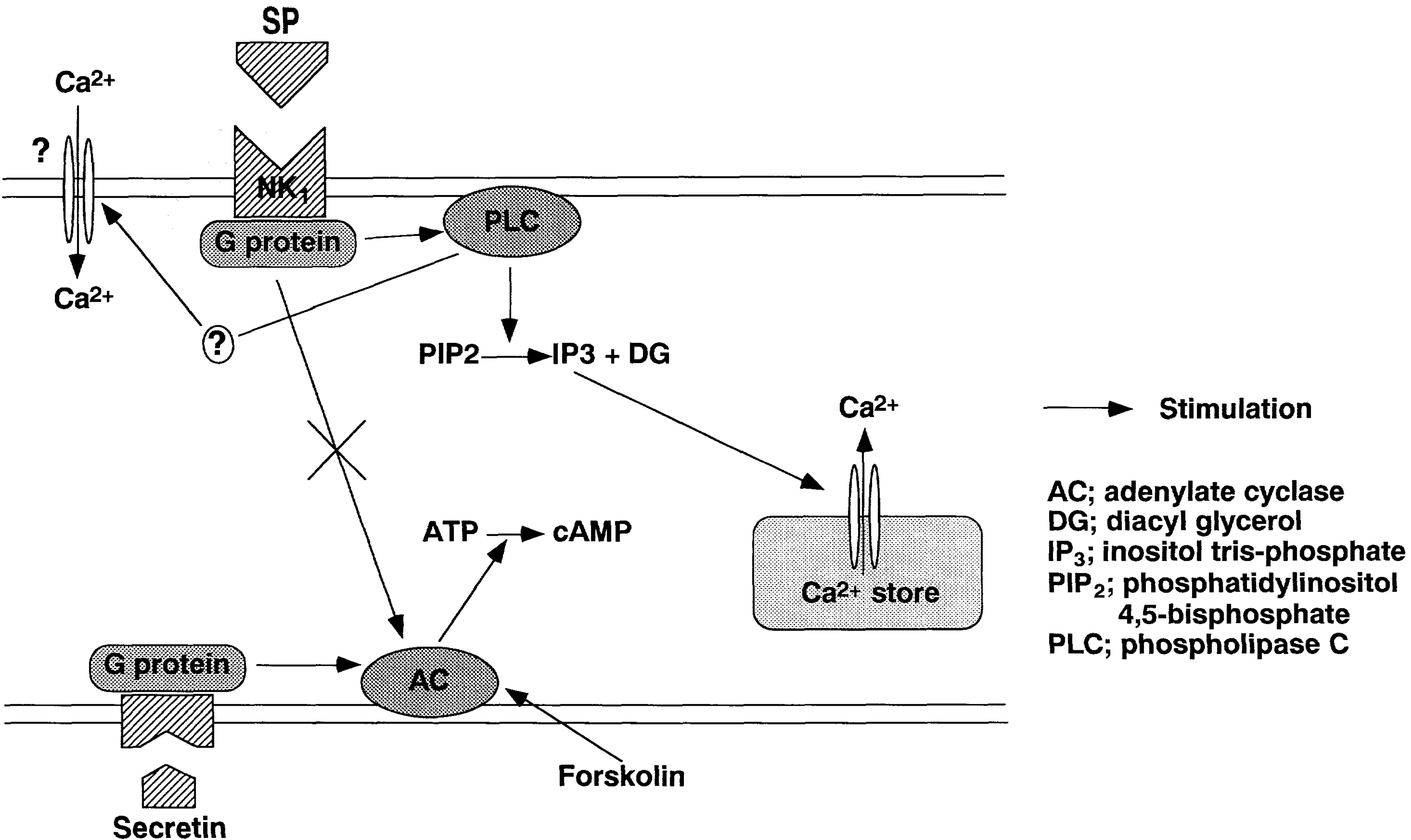


Fig. VI-2. NK_1 receptors expressed in AR42J cells and the intracellular signal transduction induced by its receptors.

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