Optimum Cross-linking Spacer Length of Dimeric Neurokinin B Analogs for Interaction with NK-1 Tachykinin Receptors

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A series of dimeric analogs of neurokinin B (NKB) COOH-terminal heptapeptide were synthesized in order to find an optimum length of cross-linking spacer for bivalent interaction with tachykinin receptors. Dimerization was carried out at the NH_2-terminus of heptapeptide with succinyl bis[$(Gly)_n$–OH], in which the number of glycine varies from 0 to 4. Dimers D–$(Gly)_n$–$NKB_{4—10}$, namely succinyl bis[$(Gly)_n$–Asp–Ser–Phe–Val–Gly–Leu–Met–NH_2] (n=0—4), were almost inactive in the assay using rat vas deferens, indicating that they have no interaction with NK-2 receptor subtype. In contrast, these dimers were very active in guinea pig ileum (GPI) containing all of NK-1, 2, and 3 receptors. Relative activity was highest when the cross-linking spacer with oligoglycine of n=2, and decreased sharply for dimers with shorter and longer chain lengths (n=0 and 1; 3 and 4). In order to specify receptor subtype (NK-1 or NK-3) in GPI to which dimers bind, dimers were examined under the conditions that NK-1 receptors are desensitized by substance P methyl ester. All dimers exhibited drastically diminished contractile activity, indicating that dimers exclusively interact with NK-1. This was further confirmed by blocking the response of NK-3 receptors with atropine, which had no effect on the contractile activity of dimers. The results suggested that dimers interact bivalently with NK-1 receptors by bridging adjacent two binding sites.

Mammalian tachykinins include substance P (SP),[1] neurokinin A (NKA) and neurokinin B (NKB). These tachykinins function as neuropeptides through their own endogenous receptors named NK-1, -2, and -3, respectively.[2,3] Although their affinities for each receptor differ considerably,[4] they can cross-interact with all of these subtypes when they were administered exogenously. In order to clarify the functions of each subtype in such a multiple receptor system, highly specific and selective ligands are required.

The studies on the structure-activity relationships of bioactive peptides have shown that ligand dimerization often creates a novel analog having high receptor specificity and selectivity.[5,6] In our recent studies on dimerization of SP, NKA, and NKB, it was found that dimers of their COOH-terminal heptapeptide, minimum fragments to elicit a full activity, interact very strongly with receptors in the isolated smooth muscle preparations of guinea pig ileum (GPI) and rat vas deferens (RVD).[7] Furthermore, we found that dimerization changes the receptor selection of monomers, shifting the selectivity to NK-1 subtype.[8] This shift in receptor selectivity was particularly prominent for dimerization of $NKB_{4—10}$, a ligand specific for NK-3 subtype. $NKB_{4—10}$ became specific for NK-1 by its dimerization. These results suggested that NK-1 receptor has a structure to which dimers can bind preferentially.

In the present study, we have designed and synthesized a series of dimeric analogs of $NKB_{4—10}$ with varying cross-linking spacer lengths. If the dimeric ligand interact bivalently with receptors, there might be an optimal length of the cross-linking spacers.[9—13] In order to find such an optimal spacer length, elongation of spacers for $NKB_{4—10}$ was carried out by utilizing oligoglycines bridged with succinic acid. Figure 1 shows the dimeric analogs of $NKB_{4—10}$, namely D–$(Gly)_n$–$NKB_{4—10}$, cross-linked by succinyl bis[$(Gly)_n$–OH] (n= 0, 1, 2, 3, and 4). Dimers prepared were evaluated for their contractile activities in GPI and RVD as a function of cross-linking spacer lengths.

Results and Discussion

Synthesis. Synthetic schemes of peptide fragments are shown in Fig. 2. The α-amino groups were protected with t-butoxycarbonyl (Boc) group, and the COOH-terminal carboxyl groups with benzyl or methyl ester. The β-carboxyl group of Asp residue was protected with benzyl group. Methionine sulfoxide, Met(O), was used to avoid an inconstant oxidation of the methylthio group of methionine during the synthesis. Couplings were carried out by the mixed anhydride (MA) method or carbodiimide-additive method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt). For deprotection, Boc group was cleaved with HCl/dioxane or trifluoroacetic acid (TFA), and benzyl (Bzl) and methyl (Me) esters were chopped off by catalytic hydrogenation and saponification, respectively. Fragment condensation was carried out by the EDC-HOBt method. Homogeneity was confirmed by thin-layer chromatography (TLC), high-performance (HP)-TLC, and elemental analysis.

Synthetic schemes for cross-linking spacer molecules are shown in Fig. 3. Spacers with n=1 and 2 were prepared as follows; (i) succinylation of H–Gly–OBzl or H–$(Gly)_2$–OBzl with succinic anhydride, and (ii) coupling of the resulting Succinyl–Gly–OBzl or Succinyl–$(Gly)_2$–OBzl with another equimolar amount of H–Gly–OBzl,

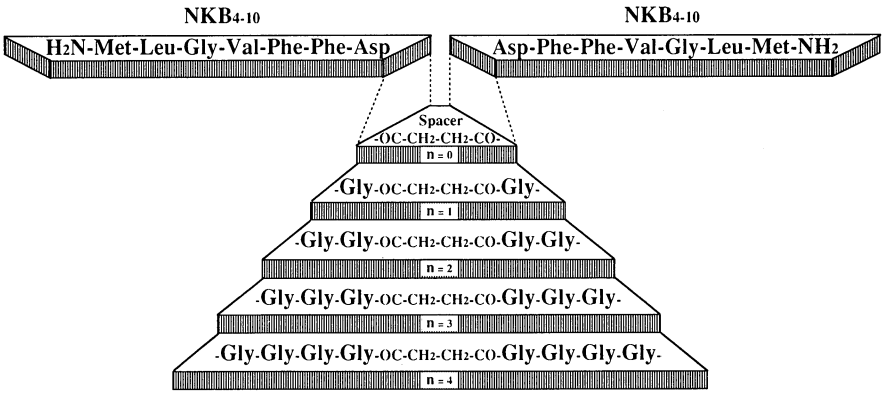

Fig. 1. Structure of the dimeric analogs of $NKB_{4—10}$ with spacers of succinyl oligoglycines.

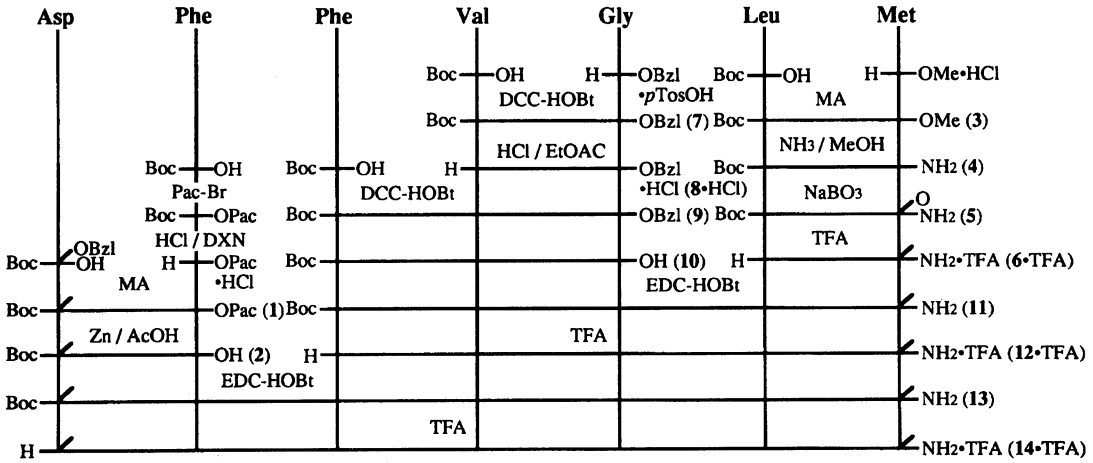

Fig. 2. Synthetic scheme of monomeric $NKB_{4—10}$.

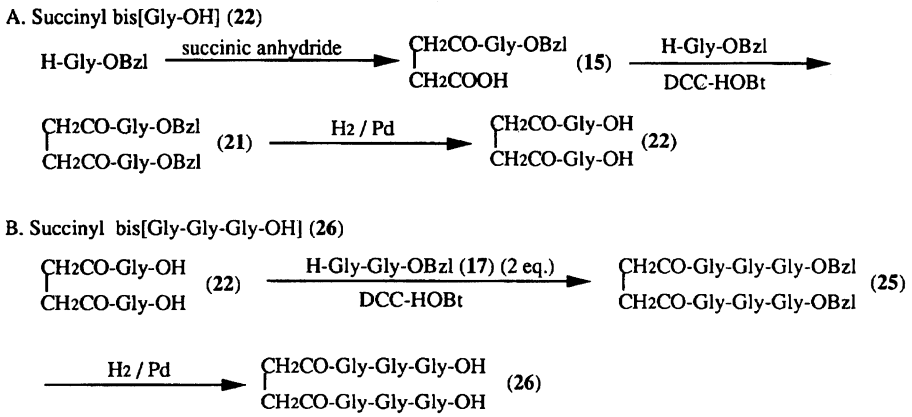

Fig. 3. Synthetic scheme of cross-linking spacers of succinyl oligoglycines, succinyl bis[Gly–OH] (A) and succinyl bis[$(Gly)_3$–OH] (B). Succinyl bis[$(Gly)_2$–OH] was prepared as in scheme A, while succinyl bis[$(Gly)_4$–OH] was as in scheme B.

or H–$(Gly)_2$–OBzl by the EDC-HOBt method.

Synthesis of spacers with $n=3$ and 4 was carried out by coupling of succinyl bis[Gly–OH] with 2-fold molar excess of H–$(Gly)_2$–OBzl or H–$(Gly)_3$–OBzl by the EDC-HOBt method. A serious difficulty came about in preparation of succinyl bis[$(Gly)_4$–OH]. The major problem was that hydrogenation or saponification of succinyl bis[$(Gly)_4$–OBzl] could not be monitored by TLC because of immobility of both benzyl ester and free acids in any solvent systems.[14] Since the product mixture by dimerization reaction appeared to be easily separable by gel filtration, the contaminated material was used directly for coupling of $NKB_{4—10}$ without further purification. After coupling of peptide, four

different kinds of peptides were expected; i.e., succinyl bis[$(Gly)_4$-OBzl], [$(Gly)_4$-OBzl]-succinyl-$(Gly)_4$-protected $NKB_{4—10}$, [$(Gly)_4$-OH]-succinyl-$(Gly)_4$-protected $NKB_{4—10}$, and the desired succinyl bis[$(Gly)_4$-protected $NKB_{4—10}$]. Separation of succinyl bis-[$(Gly)_4$-protected $NKB_{4—10}$] from others was achieved by gel filtration using Sephadex LH-20 in DMF.

Figure 4 shows the synthetic scheme of dimer analogs of $NKB_{4—10}$. Dimerization was carried out by coupling of each spacer molecule ($n=1$, 2, and 3) with two equimolar amounts of monomer $NKB_{4—10}$ by the EDC-HOBt method. Protecting groups were removed by the method using low and high concentrations of hydrogen fluoride (HF).[15] This method also reduced Met(O) to Met simultaneously. Dimers were purified by gel filtration with Sephadex LH-20 in DMF.

Biological Activity. Biological activities of a series of dimeric $NKB_{4—10}$ analogs synthesized were evaluated by estimating their ability to contract GPI and RVD. GPI contains all the receptor subtypes of NK-1, -2, and -3, while RVD contains exclusively NK-2.[16] Relative intensities of contractions were calculated against the maximal contraction obtained with carbachol (1.0×10^{-5} M, $1\,M=1\ mol\,dm^{-3}$) in the GPI assay and with noradrenaline (1.0×10^{-5} M) in the RVD assay. The dose-response curves were constructed with 4—8 dose levels using the relative potencies expressed as a percentage. Figure 5 shows the curves of $NKB_{4—10}$ dimers in GPI and RVD, and Table 1 the potencies expressed by EC_{50} values calculated from Fig. 5. Monomer $NKB_{4—10}$ exhibited a considerably strong activity in GPI (68% of native NKB). Its potency in GPI was almost 40-fold stronger than that in RVD, indicating that monomer $NKB_{4—10}$ is highly selective for receptors in GPI. When this $NKB_{4—10}$ was dimerized by spacers with various lengths, all the resulting dimers were found to be very active in GPI, but not in RVD. They were virtually inactive in RVD (Fig. 5B and Table 1). It is thus evident that dimers can not interact with NK-2.

The potencies of dimers in GPI varied, depending upon the length of spacers. The contraction activity in GPI was maximized by a dimer with the spacer of $n=2$ (Fig. 6). D-$(Gly)_2$-$NKB_{4—10}$ was 2.5—3.9-fold more potent than D-$(Gly)_0$-$NKB_{4—10}$, D-$(Gly)_1$-$NKB_{4—10}$, D-$(Gly)_3$-$NKB_{4—10}$, and D-$(Gly)_4$-$NKB_{4—10}$. It was also 2.5-fold more potent than even native NKB. These results suggest that receptors in GPI have a structure with which dimers of $NKB_{4—10}$, especially D-$(Gly)_2$-$NKB_{4—10}$, can interact preferentially.

Since dimers do not interact with NK-2, they would interact with NK-1 and/or NK-3 in GPI. NK-1 subtype is known to desensitize efficiently with SP methyl ester (SP-OMe), a highly selective ligand for NK-1.[17] Thus, if GPI was pre-treated with SP-OMe, the contraction that was induced by peptide injected afterwards would represent the activity mediated through only NK-3. In this study, SP-OMe (1.0×10^{-7} M) was injected twice before administration of dimers for complete desensitization. When contractile responses were compared in the absence and presence of SP-OMe, all dimers showed a drastically diminished activity in GPI (Fig. 7). This result suggested that biological response induced by D-$(Gly)_n$-$NKB_{4—10}$ in GPI is due predominantly to their interaction with NK-1. In contrast, $NKB_{4—10}$ was almost equally active under the conditions with and without SP-OMe, indicating that this monomer is interacting exclusively with NK-3. Native NKB showed only slightly decreased activity in desensitized GPI, suggesting that NKB interacts mainly with NK-3 as reported by others.[18]

The predominant interaction of D-$(Gly)_n$-$NKB_{4—10}$ with NK-1 was further confirmed by preinjection of atropine, a specific blocker of NK-3 response. When atropine (50—100 μM) was added beforehand, NKB analogs (1.0×10^{-9} M) of $NKB_{4—10}$ and senktide, a NK-3 selective peptide ligand,[19] showed no contraction. This indicates that these monomers interact exclusively with NK-3 in GPI. However, dimers showed almost unchanged contraction with and without atropine. Dimers elicited only slightly diminished (0—20%) contractions of GPI after pre-incubation with atropine, indicating that the receptor signal of dimers was mainly mediated through NK-1.

The strengths of interactions between ligands and receptors can be estimated indirectly through by examining the extents in washing-out of ligands from the assay system. Contraction profiles of $NKB_{4—10}$, D-$(Gly)_0$-$NKB_{4—10}$ and D-$(Gly)_2$-$NKB_{4—10}$ in such a washing examination in GPI are shown in Fig. 8. The wash was repeated just after reaching maximum contractions and by changing buffer twice every 15 s. Monomer $NKB_{4—10}$ was rapidly washed out from the assay system, namely from GPI tissue, as shown by no occurrence of contraction after several washings (Fig. 8A). By contrast, contractions caused by D-$(GIy)_0$-$NKB_{4—10}$ and D-$(Gly)_2$-$NKB_{4—10}$ required repeated washings to reach a basal level (Figs. 8B and 8C). These results suggest that in GPI dimers hardly dessociate from receptors in contrast to monomers. Similar difficulty in washing was also reported for dimers of a neurokinin A fragment.[7,8]

Since $NKB_{4—10}$ and its dimeric analogs contain the exactly same pharmacore of heptapeptide, the change in receptor selection by dimerization might be attributed to the conformational changes. Schwyzer[20] has postulated membrane-assisted molecular mechanisms for selection of tachykinin receptor subtypes by SP, NKA, and NKB. NK-1 selection by SP was ascribed to a strong hydrophobic membrane interaction of the C-terminal message segment as a perpendicularly oriented α-helical domain,[21] while NK-3 selection was characterized by electrostatic repulsion from the anionic charge layer

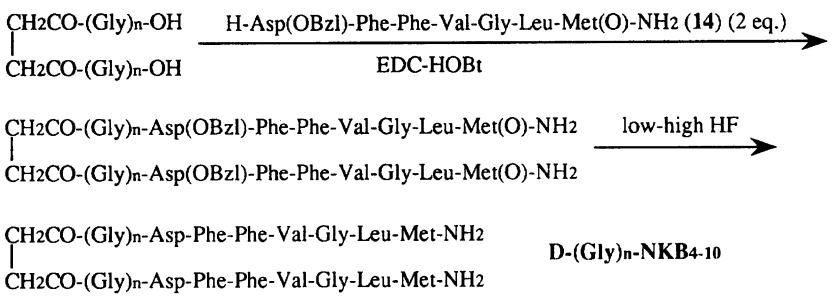

Fig. 4. Synthetic scheme of dimeric analogs of $NKB_{4—10}$. Dimerization of monomeric $NKB_{4—10}$ with spacers of succinyl oligoglycine (n=0—4).

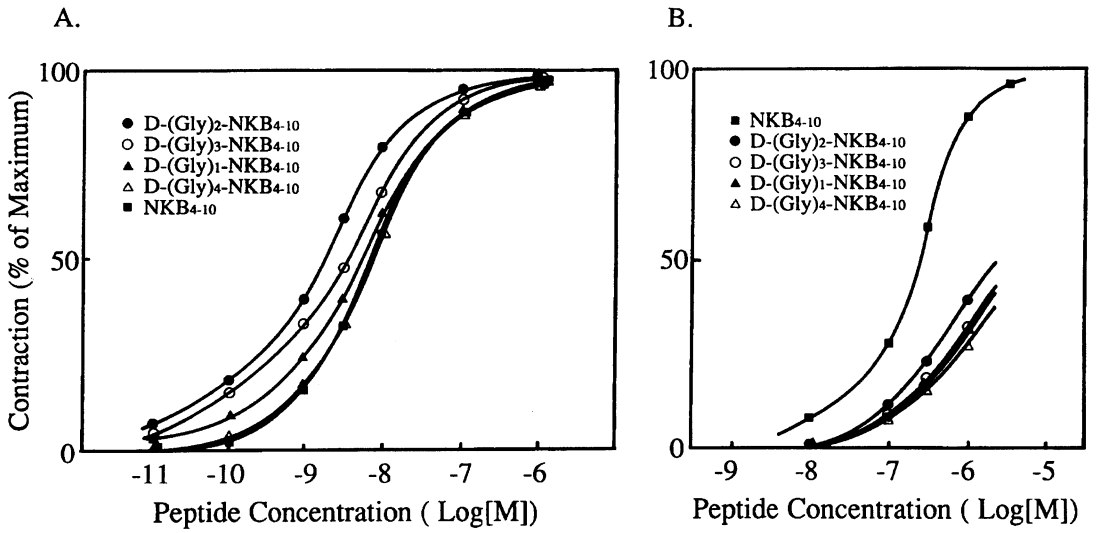

Fig. 5. Dose-response curves of monomeric and dimeric analogs of $NKB_{4—10}$ in the contraction assays in guinea pig ileum (A) and rat vas deferens (B).

Table 1. Biological Activities of Monomeric and Dimeric analogs of $NKB_{4—10}$

Peptides	GPI		RVD	
	EC_{50}(nM)	RP[a)]	EC_{50} (nM)	RP[a)]
SP	2.4	2.8	2950	0.093
NKA	13	0.51	178	1.5
NKB	4.5	1.5	2190	0.13
$NKB_{4—10}$	6.6	1.0	275	1.0
D–$(Gly)_0$–$NKB_{4—10}$	12	0.55	1350	0.20
D–$(Gly)_1$–$NKB_{4—10}$	5.9	1.1	>5000	<0.10
D–$(Gly)_2$–$NKB_{4—10}$	1.8	3.7	>3000	<0.10
D–$(Gly)_3$–$NKB_{4—10}$	4.5	1.5	>5000	<0.10
D–$(Gly)_4$–$NKB_{4—10}$	7.1	0.92	>5000	<0.10

a) RP, potencies relative to monomeric $NKB_{4—10}$ (=1.0).

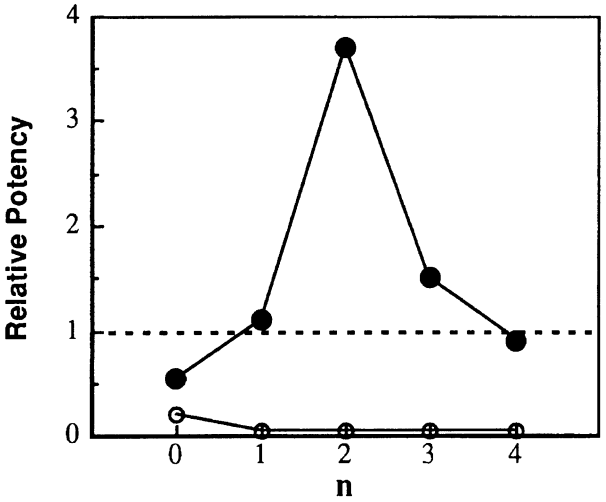

Fig. 6. Relative potency of dimers of $NKB_{4—10}$ in guinea pig ileum (●—●) and rat vas deferens (○—○) as a function of cross-linking spacer lengths. Potency of each analog was estimated by calculating the ratio of EC_{50} value against that of monomeric $NKB_{4—10}$. The numbers "n" on the abscissa denotes the number of glycine in D–$(Gly)_n$–$NKB_{4—10}$.

and by no requirement of such an α-helical structure. These assessments prompted us to measure the circular dichroism (CD) spectra of peptides and compare their secondary conformations. As reported by Rolka et al.,[22] SP adopted partially the α-helical conformations in hydrophobic environments. In Fig. 9, the CD spectrum of SP in 60% trifluoroethanol in 50 mM phosphate buffer is depicted to show such a helical struc-

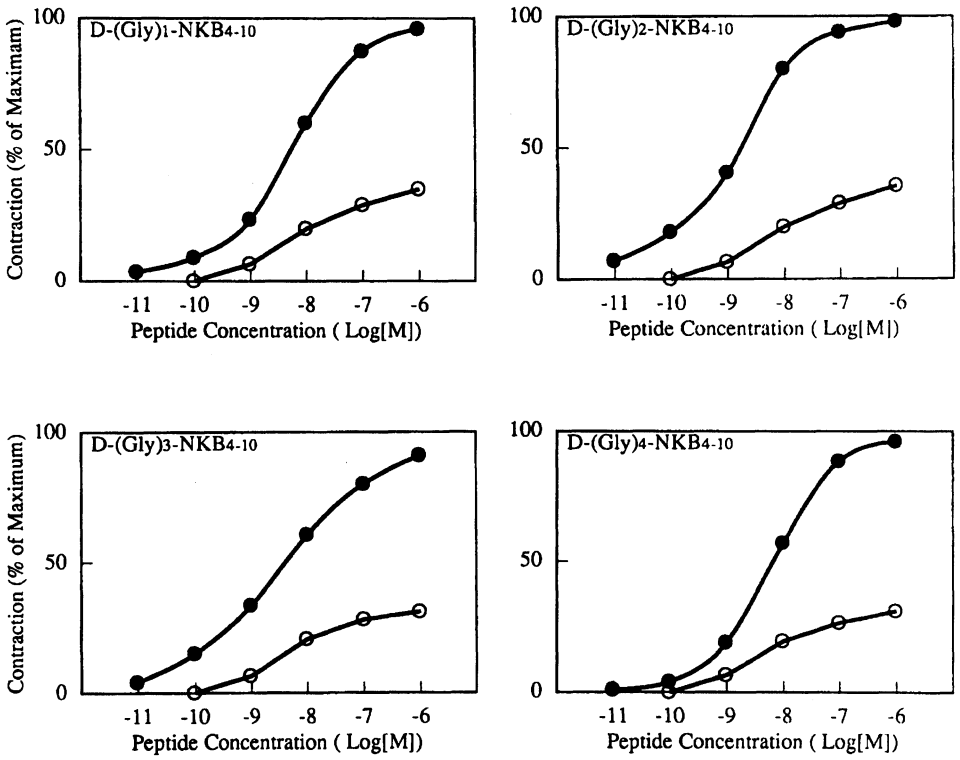

Fig. 7. Contractile activity of dimeric analogs of NKB_{4-10} in the presence (○—○) and absence (●—●) of substance P methyl ester. For desensitization substance P methyl ester (1×10^{-7} M) was incubated twice before injection of test solution of peptides. The standard contraction was obtained with carbachol (1.0×10^{-5} M) without substance P methyl ester.

ture. NKB exhibited almost the same CD profile as SP (data not shown). From the CD spectra of D-$(Gly)_2$-NKB_{4-10} measured under the same conditions of those for SP and NKB monomers, it was found that the content of α-helix fairly increased, for example, as shown by increased mean residue ellipticites at 208 and 222 nm (Fig. 9). Such a reinforcement of α-helical conformations is assumed to stabilize the interaction of dimers with NK-1 receptors. Since the predominant interaction of NKB_{4-10} monomer with NK-3 in GPI appears to be assured by the presence of Asp^4, it is supposed that this residue in dimers is mostly shielded not to gain access to NK-3 receptors, resulting in exclusive selection of NK-1 by dimers.

The fact that the contraction activity of NKB dimers was maximized with the spacer of succinyl bis[$(Gly)_2$-OH] ($n=2$) suggests that NK-1 receptor may have a structure with two adjacent ligand binding sites. Molecular cloning of NK-1 in rat has already been reported, and it was a single chain protein that can take a structure having seven transmembrane domains. There might be several possibilities that dimers bind bivalently to NK-1 receptor subtype. For instance, NK-1 may contain a subsite for ligand binding along with the major binding site. More likely, however, microaggregation of receptors may provide an opportunity for dimers to interact with receptors bivalently, dimers bridging two adjacent receptors. The assessment of these possibilities would require more detailed biochemical and molecular pharmacological studies.

Experimental

Analytical Methods. All melting points are uncorrected. TLC was carried out on silica gel G (Merck, Frankfurt) with the following solvent systems (by volume): R_f^1, $CHCl_3$-MeOH (5:1); R_f^2, $CHCl_3$-MeOH (9:1); R_f^3, $CHCl_3$-MeOH (20:1); R_f^4, $CHCl_3$-MeOH-concd aq ammonia (50:10:2); R_f^5, $CHCl_3$-MeOH-concd aq ammonia (95:5:1); R_f^6, $CHCl_3$-MeOH-AcOH (50:10:2); R_f^7, $CHCl_3$-MeOH-AcOH (95:5:1); R_f^8, *n*-BuOH-AcOH-H_2O (4:1:5, organic phase); R_f^9, *n*-BuOH-AcOH-pyridine-H_2O (4:1:1:2); R_f^{10}, 0.1% AcOH-*n*-BuOH-H_2O (11:5:3, organic phase); R_f^{11}, *n*-BuOH-AcOH-EtOAc-H_2O (1:1:1:1); R_f^{12}, *n*-PrOH-concd aq ammonia (6:4).[14] The R_f values for (HP)-TLC (Silica Gel G, Merck) are designated as R_f^n (HP) with the solvent system number (n) indicated above. Optical rotations were measured with a Union high sensitivity polarimeter PM-71. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolysis with 6 M HCl in a sealed tube at 110°C for 24 h.

High-performance liquid chromatography (HPLC) was carried out using a self-packed column of Hitachi 3063 ODS gel (4×150 mm). Peptides were eluted at a flow rate of 1.0 ml min^{-1} by a linear gradient from 10 to 60% of 0.1% TFA/CH_3CN over 60 min, and detected by monitoring UV absorption at 210 nm. Peak positions were shown as retention times (RT) in min.

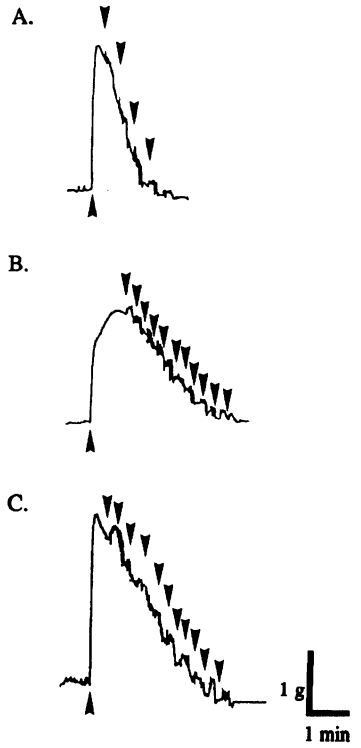

Fig. 8. Contractile profiles of monomeric and dimeric analogs of $NKB_{4—10}$ with successive washings. Upward arrowheads indicate the injection of peptide, while downward ones show the each washing. A, monomeric $NKB_{4—10}$; B, dimeric D-$(Gly)_0$-$NKB_{4—10}$ and; C, dimeric D-$(Gly)_2$-$NKB_{4—10}$.

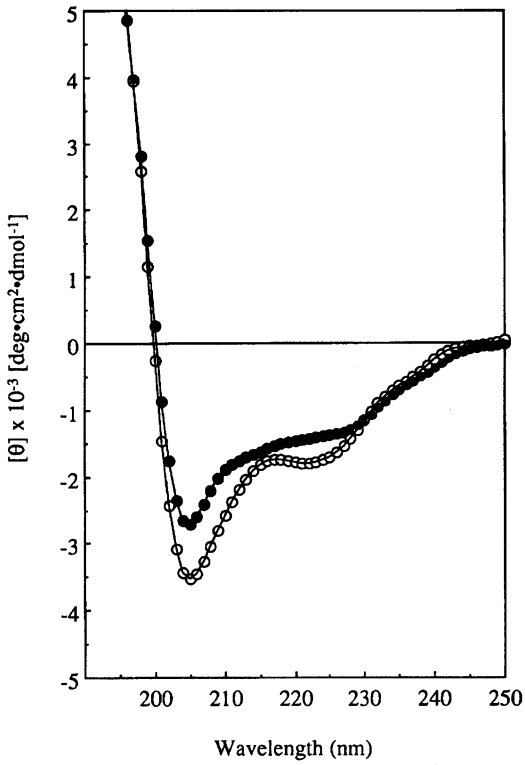

Fig. 9. CD spectra of substance P (●—●) and dimeric D-$(Gly)_2$-$NKB_{4—10}$ (○—○). Spectra were obtained in 60% trifluoroethanol in 50 μM phosphate buffer with 50 mM peptide concentration at 25°C. For calculation of mean residue ellipticity of D-$(Gly)_2$-$NKB_{4—10}$, the glycine residues of the cross-linking spacer were also involved due to the presence of the carbonyl groups in glycine.

Peptide Synthesis. DCC and HOBt were purchased from Peptide Institute (Osaka). EDC hydrochloride and TFA were purchased from Watanabe Chemical Industries (Hiroshima).

Synthesis of Neurokinin $B_{4—10}$. Boc-Asp(OBzl)-Phe-OPac (1): Isobutyl chloroformate (3.01 ml, 23 mmol) was added to a solution of Boc-Asp(OBzl)-OH (7.44 g, 23 mmol) and Et_3N (3.22 ml, 23 mmol) in tetrahydrofuran (THF) (90 ml) at −15°C. After 10 min, a chilled solution of H-Phe-OPac·HCl (7.29 g, 23 mmol) and Et_3N (3.22 ml, 23 mmol) in DMF (50 ml) was added to the reaction mixture. The reaction mixture was stirred for 2 h at 0°C and overnight at room temperature, and evaporated in vacuo. The residue dissolved in EtOAc was successively washed with 5% $KHSO_4$, 5% $NaHCO_3$, and H_2O, and dried over Na_2SO_4. After evaporation, the residue was solidified by the addition of ether-pet. ether: Yield, 9.98 g (74%); mp 96—97°C (reported value,[7] 96—97°C); $[\alpha]_D^{20}$ −25.8° (*c* 1.0, DMF) (reported value,[8] −25.6°); R_f^2 0.77.

Boc-Asp(OBzl)-Phe-OH (2): Zinc powder (32.6 g) was added to a solution of compound **1** (5.89 g, 10 mmol) in AcOH (10 ml), and the mixture was stirred for 2 h at room temperature. After removal of insoluble material, the residue evaporated was purified on a Sephadex LH-20 column (4.5×120 cm) eluted with DMF: Yield, 4.32 g (92%); mp 92—93°C (reported value,[8] 90—93°C): $[\alpha]_D^{20}$ −4.4° (*c* 1.0, DMF) (reported value,[8] −4.2°); R_f^5 0.42.

Boc-Leu-Met-OMe (3): This was prepared from Boc-Leu-OH (23.1 g, 100 mmol) and H-Met-OMe·HCl (20.0 g, 100 mmol) as described for **1**: Yield, 27.8 g (74%); mp 100—102°C; $[\alpha]_D^{20}$ −24.8° (*c* 1.0, DMF); R_f^2 0.70. Found: C, 54.07; H, 8.58; N, 7.42%. Calcd for $C_{17}H_{32}O_5N_2S$: C, 54.23; H, 8.57; N, 7.44%.

Boc-Leu-Met-NH_2 (4): Compound **3** (11.3 g, 30 mmol) was dissolved in MeOH (160 ml) saturated with NH_3 and the solution was left for 3 d at room temperature. After evaporation, the residue was crystallized by the addition of ether: Yield, 10.0 g (93%); mp 157—159°C; $[\alpha]_D^{20}$ −28.9° (*c* 0.5, DMF); R_f^2 0.54. Found: C, 53.14; H, 8.75; N, 11.65% Calcd for $C_{16}H_{31}O_4N_3S$: C, 53.16; H, 8.64; N,11.62%.

Boc-Leu-Met(O)-NH_2 (5): To a solution of compound **4** (6.93 g, 19 mmol) in MeOH (200 ml) was added $NaBO_3 \cdot 4H_2O$ (3.12 g, 20 mmol) in H_2O (50 ml).[23] The reaction mixture was stirred for 1.5 h at room temperature, and evaporated. The residue was purified on a silica-gel column (3.2×20 cm) eluted with $CHCl_3$-MeOH (9:1): Yield, 7.15 g (99%); mp 132—135°C (reported value,[8] 126—127°C); $[\alpha]_D^{20}$ −25.8° (*c* 1.0, MeOH) (reported value,[8] −25.8°); R_f^2 0.31.

H-Leu-Met(O)-NH_2·TFA (6·TFA): Compound **5** (6.42 g, 17 mmol) was dissolved in TFA (50 ml) at 0°C. After 30 min, the solution was evaporated and the residual oil was solidified by the addition of ether: Yield, 7.67 g (100%); R_f^9 0.46. This TFA-salt was used for the next reaction without further purification because of its hygroscopicity.

Boc–Val–Gly–OBzl (7): HOBt (6.49 g, 48 mmol) and DCC (9.08 g, 44 mmol) were added to a solution of Boc–Val–OH (8.69 g, 40 mmol), H–Gly–OBzl·TosOH (13.5 g, 40 mmol) and Et_3N (5.6 ml, 40 mmol) in CH_2Cl_2 (200 ml) at 0°C. The reaction mixture was stirred for 2 h at 0°C and stored overnight at room temperature. After removal of dicyclohexylurea, the solution was treated as described for **1**: Yield, 12.6 g (86%); mp 70—72°C (reported value,[8] 63—69°C); $[\alpha]_D^{20}$ −9.0° (*c* 1.0, DMF) (reported value,[8] −9.1°); R_f^2 0.67.

H–Val–Gly–OBzl·HCl (8·HCl). Compound **7** (12.4 g, 34 mmol) was dissolved in 2.4 M HCl in EtOAc (142 ml, 340 mmol) and the solution was left for 1 h at 0°C. The solution was evaporated to leave an oil: Yield, 10.9 g (100%); R_f^4 0.76.

Boc–Phe–Val–Gly–OBzl (9). This was prepared from Boc–Phe–OH (9.02 g, 34 mmol) and **8**·HCl (10.2 g, 34 mmol) by the DCC-HOBt method as described for **7**: Yield, 14.8 g (85%); mp 150—151°C (reported value,[8] 151—152°C); $[\alpha]_D^{20}$ −10.9°C (*c* 1.0, DMF) (reported value,[8] −10.7°); R_f^2 0.56.

Boc–Phe–Val–Gly–OH (10): Compound **9** (13.8 g, 27 mmol) was dissolved in MeOH (200 ml) and treated with hydrogen for 5 h in the presence of Pd-black. After removal of Pd-black, the filtrate was evaporated. The residual solid was recrystallized from MeOH-ether: Yield, 10.7 g (94%); mp 175—177°C (reported value,[8] 175—177°C); $[\alpha]_D^{20}$ −10.2° (*c* 1.0, DMF) (reported value,[8] −10.7°); R_f^6 0.60.

Boc–Phe–Val–Gly–Leu–Met(O)–NH_2 (11): To a solution of compound **10** (1.26 g, 3 mmol), compound **6**·TFA (1.17 g, 3 mmol) and Et_3N (0.42 ml, 3 mmol) in DMF (10 ml) were added HOBt (486 mg, 3.6 mmol) and EDC·HCl (632 mg, 3.3 mmol) at 0°C. The reaction mixture was stirred for 2 h at 0°C and overnight at room temperature. The residue obtained by evaporation was triturated with water to afford a precipitate. The solid collected was washed successively with 5% $NaHCO_3$, 5% $KHSO_4$, and H_2O, and dried: Yield, 1.38 g (89%); mp 226—227°C (reported value,[8] 226—228°C); $[\alpha]_D^{20}$ −4.8° (*c* 0.1, DMF) (reported value,[8] −4.8°); R_f^1 0.53.

H–Phe–Val–Gly–Leu–Met(O)–NH_2·TFA (12·TFA): This was prepared from compound **11** (1.36 g, 2 mmol) and TFA (10 ml) as described for **6**·TFA: Yield, 1.08 g (78%); mp 174—176°C (reported value,[8] 173—175°C); $[\alpha]_D^{20}$ +8.0° (*c* 0.1, DMF) (reported value,[8] +8.4°) ; R_f^1 0.16.

Boc–Asp(OBzl)–Phe–Phe–Val–Gly–Leu–Met(O)–NH_2 (13): This heptapeptide was prepared from Boc-dipeptide **2** (710 mg, 1.5 mmol) and pentapeptide amide **12**·TFA (1.04 g, 1.5 mmol) by the EDC-HOBt method as described for **11**. Recrystallization from hot DMF gave the desired pure peptide **13**: Yield, 1.38 g (88%); mp 209—210°C (reported value,[8] 207—210°C); $[\alpha]_D^{20}$ −7.8° (*c* 0.1, DMF) (reported value,[8] −8.0°); R_f^1 0.66.

H–Asp(OBzl)–Phe–Phe–Val–Gly–Leu–Met(O)–NH_2·TFA (14·TFA): This was prepared from compound **13** (1.24 g, 1.2 mmol) by treatment with TFA as described for **6**·TFA: Yield, 1.21 g (93%); mp 205—207°C (reported value,[8] 204—205°C); $[\alpha]_D^{20}$ −0.4° (*c* 0.1, DMF) (reported value,[8] −0.4°); R_f^9 0.86.

Synthesis of Succinyl Oligoglycine Cross-Linking Spacer. Succinyl–Gly–OBzl (15): To a solution of H–Gly–Bzl·*p*TosOH (3.37 g, 10 mmol) and Et_3N (1.4 ml, 10 mmol) in DMF (30 ml) was added succinic anhydride (1.10 g, 11 mmol) at 0°C. The solution was stirred for 2 h at 0°C and overnight at room temperature. The reaction mixture was evaporated and the residue was triturated with water to afford a precipitate. The solid collected was washed successively with 5% $NaHCO_3$, 5% $KHSO_4$ and H_2O, and dried: Yield, 2.11 g (80%); mp 100—101°C; R_f^1 0.47. Found: C, 58.97; H, 5.70; N, 5.27%. Calcd for $C_{13}H_{15}O_5N$: C, 58.86; H, 5.70; N, 5.28%.

Boc–Gly–Gly–OBzl (16): This was prepared from Boc–Gly–OH (7.01 g, 40 mmol) and H–Gly–OBzl·*p*TosOH (13.5 g, 40 mmol) by the DCC-HOBt method as described for **7**: Yield, 11.6 g (90%); mp 78—79°C; R_f^2 0.58. Found: C, 59.62; H, 6.88; N, 8.69%. Calcd for $C_{16}H_{22}O_5N_2$: C, 59.71; H, 6.94; N, 8.71%.

H–Gly–Gly–OBzl·HCl (17·HCl): This was prepared from Boc–Gly–Gly–OBzl (11.0 g, 34 mmol) and 4.2 M HCl in dioxane (130 ml, 540 mmol) as described for **8**·HCl: Yield, 8.60 g (97%); mp 155—157°C; R_f^2 0.11. Found: C, 50.90; H, 5.85; N, 10.75%. Calcd for $C_{11}H_{14}O_3N_2$·HCl: C, 51.07; H, 5.84; N, 10.83%.

Succinyl–Gly–Gly–OBzl (18): This was obtained from compound **17**·HCl (5.17 g, 20 mmol) and succinic anhydride (2.20 g, 22 mmol) as described for **15**: Yield, 4.64 g (72%); mp 148—150°C; R_f^1 0.10. Found: C, 55.89; H, 5.64; N, 8.68%. Calcd for $C_{15}H_{18}O_6N_2$: C, 55.90; H, 5.63; N, 8.69%.

Boc–Gly–Gly–Gly–OBzl (19): This was prepared from Boc–Gly–OH (350 mg, 2 mmol) and **17**·HCl (517 mg, 2 mmol) by the EDC-HOBt method as described for **11**: Yield, 601 mg (79%); mp 115—116°C; R_f^1 0.69. Found: C, 56.96; H, 6.62; N, 10.98%. Calcd for $C_{18}H_{25}O_6N_3$: C, 56.98; H, 6.64; N, 11.08%.

H–Gly–Gly–Gly–OBzl·TFA (20·TFA): This was prepared from compound **19** (379 mg, 1 mmol) and TFA (4 ml) as described for **6**·TFA: Yield, 392 mg (100%); mp 133—134°C; R_f^2 0.10. Found: C, 41.05; H, 4.62; N, 7.32%. Calcd for $C_{13}H_{17}O_4N_2$·TFA: C, 41.17; H, 4.52; N, 7.39%.

Succinyl Bis[Gly–OBzl] (21): This was prepared from **15** (5.31 g, 20 mmol) and H–Gly–OBzl·TosOH (6.75 g, 20 mmol) by the DCC-HOBt method as described for **7**: Yield, 5.93 g (72%); mp 143—144°C; R_f^2 0.50. Found: C, 63.94; H, 5.80; N, 6.79%. Calcd for $C_{22}H_{24}O_6N_2$: C, 64.07; H, 5.87; N, 6.79%.

Succinyl Bis[Gly–OH] (22): Compound **21** (5.36 g, 13 mmol) was hydrogenated in DMF (150 ml) as described for **10**: Yield, 2.99 g (99%); mp 200—201°C; R_f^{12} 0.48. Found: C, 41.19; H, 5.16; N, 11.92%. Calcd for $C_8H_{12}O_6N_2$: C, 41.38; H, 5.21; N, 12.06%.

Succinyl Bis[Gly–Gly–OBzl] (23). This was prepared from **18** (645 mg, 2 mmol) and **17**·HCl (517 mg, 2 mmol) by the EDC-HOBt method as described for **11**: Yield, 993 mg (94%); mp 189—192°C; R_f^1 0.60. Found: C, 59.27; H, 5.71; N, 10.64%. Calcd for $C_{26}H_{30}O_8N_4$: C, 59.31; H, 5.74; N, 10.64%.

Succinyl Bis[Gly–Gly–OH] (24): Compound **23** (158 mg, 0.3 mmol) was hydrogenated in DMF (10 ml) as described for **10**: Yield, 95.6 mg (92%); mp 229—230°C; R_f^{12} 0.48. Found: C, 41.46; H, 5.25; N, 16.01%. Calcd for $C_{12}H_{18}O_8N_4$: C, 41.62; H, 5.25; N, 16.18%.

Succinyl Bis[Gly–Gly–Gly–OBzl] (25): This was

prepared from **22** (69.7 mg, 0.3 mmol) and **17**·HCl (171 mg, 0.66 mmol) by the EDC-HOBt method as described for **11**: Yield, 156 mg (81%); mp 237—242°C (decomp). Found: C, 55.97; H, 5.66; N, 13.12%. Calcd for $C_{30}H_{36}O_{10}N_6$: C, 56.24; H, 5.66; N, 13.12%.

Succinyl Bis[Gly–Gly–Gly–OH] (26): Compound **25** (76.9 mg, 0.12 mmol) was hydrogenated in DMF (100 ml) as described for **10**: Yield, 46.0 mg (83%); mp 230—233°C (decomp); R_f[12] 0.52. Found: C, 42.04; H, 5.57; N, 18.41%. Calcd for $C_{16}H_{24}O_{10}N_6$: C, 41.74; H, 5.25; N, 18.25%.

Succinyl Bis[Gly–Gly–Gly–Gly–OBzl] (27): This was prepared from **22** (69.7 mg, 0.3 mmol) and **20**·TFA (261 mg, 0.6 mmol) by the EDC-HOBt method as described for **11**: Yield, 207 mg (81%); mp 239—245°C (decomp). Found: C, 57.01; H, 6.69; N, 13.18%. Calcd for $C_{40}H_{54}O_{12}N_8$: C, 57.27; H, 6.49; N, 13.36%.

Succinyl Bis[Gly–Gly–Gly–Gly–OH] (28): Compound **27** (83.9 mg, 0.1 mmol) was hydrogenated in DMF (100 ml) in the presence of Pd-black for 12 h. After filtration, the filtrate was evaporated in vacuo and the residue was triturated with water. The solid obtained was used for the next reaction without further purification, although it would be contaminated with **25** and/or monohydrogenated derivative.

Synthesis of Dimeric Neurokinin $B_{4—10}$ (D-$(Gly)_n$-$NKB_{4—10}$). Succinyl Bis[Gly–Asp(OBzl)–Phe–Phe–Val–Gly–Leu–Met(O)–NH_2] (29): This was prepared from **22** (21.1 mg, 91 μmol) and **14**·TFA (197 mg, 200 μmol) by the EDC-HOBt method as described for **11**: Yield, 120 mg (62%); mp 240—250°C (decomp); R_f[9] 0.76. Found: C, 57.43; H, 6.41; N, 11.78%. Calcd for $C_{88}H_{124}O_{22}N_{18}S_2$: C, 57.61; H, 6.45; N, 11.86%.

Succinyl Bis[Gly–Asp–Phe–Phe–Val–Gly–Leu–Met–NH_2] (D-$(Gly)_1$-$NKB_{4—10}$) (30): Compound **29** (50 mg, 24 μmol) was treated with anhydrous liquid HF (2.5 ml) in the presence of *p*-cresol (1.0 ml) and dimethyl sulfide (6.5 ml) for 2 h at 0°C. After evaporation of HF and dimethyl sulfide by aspiration, the residue was retreated with anhydrous HF (9.0 ml) in the presence of *p*-cresol (1.0 ml) for 1 h at 0°C. After removal of HF, the oily residue was solidified by the addition of EtOAc and 30% AcOH. The solid collected was purified by gel filtration using Sephadex LH-20 column (1.0×120 cm) eluted with DMF. Fractions containing the desired product were collected. After evaporation, the residue was crystallized by the addition of ether: Yield, 35.2 mg (81%); mp 250—253°C (decomp); RT 29.04; amino acid ratios in acid hydrolysate; Gly 2.02, Asp 1.04, Phe 2.00, Val 0.99, Leu 1.00, Met 0.87. Found: C, 55.49; H, 6.80; N, 12.81%. Calcd for $C_{88}H_{124}O_{22}N_{18}S_2$·$2H_2O$: C, 56.04; H, 6.84; N, 13.37%.

Succinyl Bis[Gly–Gly–Asp(OBzl)–Phe–Phe–Val–Gly–Leu–Met(O)–NH_2] (31): This was prepared from **30** (31.5 mg, 91 μmol) and **14**·TFA (197 mg, 200 μmol) by the EDC-HOBt method as described for **11**: Yield, 163 mg (80%); mp 228—230°C (decomp); R_f[10] 0.64. Found: C, 56.42; H, 6.31; N, 11.93%. Calcd for $C_{106}H_{142}O_{30}N_{20}S_2$: C, 56.82; H, 6.39; N, 12.50%.

Succinyl Bis[Gly–Gly–Asp–Phe–Phe–Val–Gly–Leu–Met–NH_2] (D-$(Gly)_2$-$NKB_{4—10}$) (32): This was prepared from protected compound **31** (46 mg, 23 μmol) by the HF-treatment as described for **30**: Yield, 25.6 mg (56%); mp 234—240°C (decomp); RT 29.16; R_f[11] 0.57. Amino acid ratios in acid hydrolysate; Gly 3.08, Asp 0.98, Phe 2.01, Val 1.01, Leu 1.00, Met 0.84. Found: C, 54.93; H, 6.62; N, 14.55%. Calcd for $C_{92}H_{130}O_{24}N_{20}S_2$: C, 55.25; H, 6.67; N, 14.26%.

Succinyl Bis[Gly–Gly–Gly–Asp(OBzl)–Phe–Phe–Val–Gly–Leu–Met(O)–NH_2] (33). This was prepared from **26** (21.8 mg, 46 μmol) and **14**·TFA (98.3 mg, 100 μmol) by the EDC-HOBt method as described for **11**: Yield, 36.6 mg (35%); mp 237—240°C (decomp); R_f[1] 0.58, R_f(HP)[9] 0.77. Found: C, 55.79; H, 6.83; N, 11.85%. Calcd for $C_{124}H_{160}O_{38}N_{22}S_2$: C, 56.61; H, 6.13; N, 11.71%.

Succinyl Bis[Gly–Gly–Gly–Asp–Phe–Phe–Val–Gly–Leu–Met–NH_2] (D-$(Gly)_3$-$NKB_{4—10}$) (34): This was prepared from protected compound **33** (25 mg, 9.5 μmol) by the HF-treatment as described for **30**: Yield, 11 mg (48%); mp 226—234°C (decomp); RT 29.83; R_f(HP)[11] 0.43. Amino acid ratios in acid hydrolysate; Gly 4.11, Asp 1.03, Phe 1.96, Val 1.04, Leu 1.00, Met 0.75. Found: C, 55.89; H, 6.51; N, 13.21%. Calcd for $C_{110}H_{148}O_{32}N_{22}S_2$: C, 56.11; H, 6.34; N, 13.09%.

Succinyl Bis[Gly–Gly–Gly–Gly–Asp(OBzl)–Phe–Phe–Val–Gly–Leu–Met(O)–NH_2] (35): This was prepared from **28** (10.0 mg, 29 μmol) and **14**·TFA (56.8 mg, 57.8 μmol) by the EDC-HOBt method as described for **11**. Purification was performed on a column (1.0×120 cm) of Sephadex LH-20 in DMF. The fractions (30—38 ml) were combined and evaporated. The solid residue was triturated with water to afford a powder: Yield, 13.0 mg (29%); mp 239—245°C (decomp); R_f(HP)[9] 0.75. Found: C, 56.38; H, 5.92; N, 14.04%. Calcd for $C_{142}H_{178}O_{46}N_{24}S_2$: C, 56.45; H, 5.94; N, 14.01%.

Succinyl Bis[Gly–Gly–Gly–Gly–Asp–Phe–Phe–Val–Gly–Leu–Met–NH_2] (D-$(Gly)_4$-$NKB_{4—10}$) (36): This was prepared from protected compound **35** (10.0 mg, 3.3 μmol) by the HF-treatment as described for **30**: Yield, 6.34 mg (70%); mp 234—242°C (decomp); RT 29.90; R_f(HP)[9] 0.38. Amino acid ratios in acid hydrolysate; Gly 5.20, Asp 1.04, Phe 1.98, Val 1.03, Leu 1.00, Met 0.68.

Biological Assays. Preparations of Peptide Solutions: The dimeric peptides were dissolved in a small volume of neat formic acid and the solution was then diluted with 0.1% bovine serum albumin. Concentration of formic acid used was kept below 1%, which gave no detectable effect on muscular tissues in the contraction experiments. In each measurement, 50 μl of peptide solution was injected directly into the organ bath.

GPI Assays: The GPI assay was carried out essentially as described previously.[7] From male guinea pigs (350—450 g) ileum was taken out rapidly to remove longitudinal strips. The strip (1.0—1.5 cm) was hung in a 5 ml organ bath containing Krebs-Ringer hydrogencarbonate buffer (composition in mM: NaCl, 127; KCl, 25; $CaCl_2$, 1.8; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; and glucose, 10; pH 7.4) gassed with the 95% O_2/5% CO_2 mixture at 37°C. After equilibration for 60 min, the strips were washed every 15 min with Krebs buffer before measurements. Contractions were recorded isotonically under the resting load of 1.0 g using an isometric force transducer (NEC San-Ei Instrument Co., Tokyo). Peptide solution was injected at 15 min intervals to the reaction bath. The results of each assay were expressed as percentages for maximal contraction obtained with carbachol (1.0×10^{-5} M). Dose-response curves

were constructed utilizing 4—8 dose levels, and potencies were assessed by calculating EC_{50}.

Desensitization in GPI: The GPI strip was first incubated with 10^{-7} M SP–OMe until the response had faded to a baseline level.[17] The tissue was then washed and immediately re-incubated with 10^{-7} M SP–OMe for 2 min. Peptides were then added and contractions were recorded, and the results are shown in Fig. 7.

Blockade of NK-3 by Atropine: The GPI strip was first incubated with 50—100 μM atropine for 30 min, and then peptide was injected. Contractions with and without atropine were compared to evaluate the effect of blockade of NK-3 receptors.

RVD Assays: Vas deferens of male Winster rats (200—250 g) were excised and hung in the organ bath. The contractile activity was measured as described for the GPI assay. Maximal contraction was obtained with noradrenaline (1.0×10^{-5} M). The results were shown in Fig. 5B.

Washing Examination: The wash-out examination was performed at all the concentrations used for construction of the dose-response curves in both GPI and RVD as described previously.[7,8] After observing maximum contractions, buffer in the organ bath was changed twice every 15 s by suction followed by pipetting until contraction reached to a basal level (Fig. 8).

CD Measurements. All spectra were measured on a JASCO J-720 spectropolarimeter equipped with a data analysis system J-700 (JASCO Inc., Tokyo). The CD data were analyzed as the mean residue ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) against the wavelength (nm) for each measurement. Quartz cell of 1 mm path length was used. Peptides were dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing 60% trifluoroethanol. All spectra were recorded in at 0.2 nm intervals over the wavelength range 195—250 nm at 25°C and analyzed at 1 nm intervals to depict Fig. 9.

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1) Abbreviations: Boc, *t*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; D–NKB, dimeric neurokinin B; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MA, mixed anhydride; NKA, neurokinin A; NKB, neurokinin B; RT, retention time; RVD, rat vas deferens; SP, substance P; SP–OMe, substance P methyl ester; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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