Full Paper

Bidirectional Regulation of Human Colonic Smooth Muscle Contractility by Tachykinin NK₂ Receptors

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Abstract. In this study, we attempted to clarify the mechanism of tachykinin-induced motor response in isolated smooth muscle preparations of the human colon. Fresh specimens of normal colon were obtained from patients suffering from colonic cancer. Using mucosa-free smooth muscle strips, smooth muscle tension with circular direction was monitored isometrically. Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) produced marked contraction. All of these contractions were inhibited by saredutant, a selective NK₂-R antagonist, but not by CP122721, a selective NK₁-R antagonist or talnetant, a selective NK₃-R antagonist. β Ala⁸-NKA(4-10) induced concentration-dependent contraction similar to NKA, but Sar⁹-Met¹¹-SP and Met-Phe⁷-NKB did not cause marked contraction. Colonic contraction induced by β Ala⁸-NKA(4-10) was completely blocked by saredutant, but not by atropine. Tetrodotoxin or N^G-nitro-L-arginine methyl ester pretreatment significantly enhanced β Ala⁸-NKA(4-10)-induced contraction. Immunohistochemical analysis showed that the NK₂-R was expressed on the smooth muscle layers and myenteric plexus where it was also co-expressed with neuronal nitric oxide synthase in the myenteric plexus. These results suggest that the NK₂-R is a major contributor to tachykinin-induced smooth muscle contraction in human colon and that the NK₂-R-mediated response consists of an excitatory component via direct action on the smooth muscle and an inhibitory component possibly via nitric oxide neurons.

Keywords: NK₁ receptor, NK₂ receptor, NK₃ receptor, human colon, smooth muscle

Introduction

It is well known that tachykinins such as substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) act as neurotransmitters (1). Three tachykinin receptors have been identified including neurokinin 1 receptor (NK₁-R), neurokinin 2 receptor (NK₂-R), and neurokinin 3 receptor (NK₃-R) (1). These are Gq-coupled receptors and their activation causes Ca²⁺ influx into the cells (2, 3). SP, NKA, and NKB preferentially bind to NK₁-R, NK₂-R, and NK₃-R, respectively; however their selectivity is limited and it has been reported that each tachykinin has the potential to act as a full agonist to each tachykinin

receptors (3). Since the late 20th century, a number of selective tachykinin receptor agonists and antagonists have been developed and are useful as pharmacological tools for investigating the role of tachykinins and their receptors (4 – 8).

Tachykinins and their receptors are abundantly expressed in gut tissues (9), and it is thought that tachykinins play an important role in gut motility. The role of tachykinin receptors in gut motility has been intensively investigated in guinea pigs (10 – 12), rats (13), mice (14), and dogs (15). It has been suggested that there are species differences for each tachykinin receptor (16, 17), human tissue is essential for studies relevant to humans. Although excellent reviews (18 – 20) have summarized the currently available information about tachykinin and their receptors, their role in gut contraction remains controversial. Therefore, more evidence is still needed.

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To better understand the involvement of tachykinins and their receptors in the human colon we performed the following experiments: evaluated the contribution of tachykinin receptor subtypes with selective receptor antagonists on tachykinin-induced smooth muscle contraction in isolated colonic strips; evaluated the effects of muscarinic receptor blockade, inhibition of nitric oxide synthesis, neuronal conductance on NK₂-R-mediated contraction; and conducted immunohistological studies to visualize the NK₂-R expression on the smooth muscle layers and the myenteric plexus in relation to neuronal nitric oxide synthase (nNOS).

Materials and Methods

Binding assay: tachykinin receptor binding assay

For the NK₁-R binding assay, cell membrane of IM9 cells, a human lymphoblastoma cell line, which expresses the NK₁ receptor (21), and [¹²⁵I]-Bolton-Hunter-labeled Lys³ Substance P (GE Healthcare Japan, Tokyo) were used as a source of NK₁-R and its radio-labeled ligand, respectively. Briefly, various concentrations of test compounds and radiolabeled ligand (130 pM) were incubated with IM9 membranes (12.5 µg) in a final volume of 0.2 ml buffer [50 mM Tris-HCl (pH 7.4) containing 0.5% DMSO, 2.3 mM MnCl₂, 0.02% BSA, 1.5 µg/mL chymostatin, 30 µg/mL bacitracin, and 30 µg/mL APMSF] at room temperature for 30 min. The reactions were terminated by rapid filtration through a GF/C filter plate (PerkinElmer Japan, Yokohama) presoaked with 0.3% polyethyleneimine, following by ten washes with washing buffer [50 mM Tris-HCl (pH 7.4) containing 0.02% BSA]. Filters were dried and soaked in 20 µl/well of Microscint 0 (PerkinElmer Japan) and receptor-bound radioactivity was then measured using TopCount liquid Scintillation counter (PerkinElmer Japan).

For NK₂-R binding assay, various concentrations of test compounds and [¹²⁵I]Neurokinin A (100 pM, GE Healthcare Japan) were incubated with the cell membrane of CHO cells expressing human NK₂-R (2 µg) in a final volume of 0.2 ml reaction buffer at room temperature for 30 min. For the NK₃-R binding assay, various concentrations of test compounds and [¹²⁵I]His, MePhe⁷-Neurokinin B (130 pM, PerkinElmer Japan) were incubated with the cell membrane of CHO cells expressing human NK₃-R (15 µg) in a final volume of 0.2 ml reaction buffer at room temperature for 30 min.

Preparation of smooth muscle preparation of human colon

All studies were conducted on normal smooth muscle strips of human descending or sigmoidal colon obtained from 43 patients (age, 28–87 years; female:male, 26:17)

who underwent abdominal surgery for colonic cancer. The responses to tachykinin were the same in these two preparations, so we combined the data from both of them to analyze the data. Isolated colon was immediately immersed in ice-cold Krebs solution of the following composition: 120.7 mM NaCl, 15.5 mM NaHCO₃, 5.9 mM KCl, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgCl₂, and 11.5 mM glucose. The specimens were transferred to the Takeda Pharmaceutical Company research center. After washing the tissue with Krebs solution, mucosa was removed using fine forceps and small pieces of smooth muscle with circular direction (approx. 5 mm × 1.5 cm) were prepared. These were placed in a thermostated (35°C) glass-made organ bath filled with 10 mL of Krebs solution bubbled with 95% O₂ / 5% CO₂ and arranged for isometric recording of circular muscle mechanical activity with 1 g of tension. The signal was delivered to an electric recorder (MLT-870; ADInstruments, Colorado Springs, CO, USA) via an amplifier (MLT-119, ADInstruments). All experiments were conducted according to the protocol approved by the ethical committees of Saiseikai-Nakatsu Hospital and Takeda Pharmaceutical Company.

Reagents

The following substances were used: Substance P (SP; Peptide Institute, Inc., Osaka); neurokinin A (NKA, Peptide Institute); neurokinin B (NKB, Peptide Institute); Sar⁹-Met¹¹-SP (American Peptide Company Inc., Sunnyvale, CA, USA), a selective NK₁-R agonist; βAla⁸-NKA(4-10) (Bachem, Bubendorf, Switzerland), a selective NK₂-R agonist; Met-Phe⁷-NKB (Bachem), a selective NK₃-R agonist. These were dissolved in dimethylsulfoxide (DMSO; Wako, Osaka) to prepare a 10 mM stock solution, which was then diluted with distilled water to working concentrations immediately prior to experimental use. The following selective tachykinin receptor antagonists were synthesized by Takeda Pharmaceutical Company and used for pharmacological analysis of tachykinin-induced smooth muscle contraction: CP122721, (2*S*,3*S*)-2-phenyl-3-[(5-trifluoromethoxy-2-methoxy) benzylamino]piperidine (6), an NK₁-R antagonist; saredutant (SR48968), [(*S*)-*N*-methyl-*N*-[4-acetyl-amino-4-phenylpiperidino-2-(3,4-dichlorophenyl) butyl] benzamide] (7), an NK₂-R antagonist; and talnetant (SB223412), (*S*)-(-)-*N*-(alpha-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide (8), an NK₃-R antagonist. The antagonists were dissolved in DMSO. The antagonists were added to the organ baths to give a final DMSO concentration of 0.1%. Carbachol (CCh; Sigma, St. Louis, MO, USA), atropine (Wako), tetrodotoxin (TTX, Wako), and *N*^G-nitro-L-arginine methyl ester (L-NAME, Wako) were dissolved in distilled water. All

other reagents were of analytical grade.

Experimental protocol

In the first series of experiments, natural tachykinins or selective tachykinin receptor agonists were added to the organ bath in a cumulative manner from 0.001 to 10 μ M. The maximum contractile force from baseline was determined for each concentration. In this case, the contractile force measurements were determined after investigating the response to CCh (1 μ M) and maximum contractile force was used as an internal control. The relative contraction against CCh-induced contraction was then calculated.

In a second series of experiments, the effects of selective tachykinin receptor antagonists on natural tachykinin-induced contraction were investigated. First, colonic contraction was induced by a sub-maximal concentration of natural tachykinins and then washed out. Next, a selective tachykinin receptor antagonist was added to the organ bath and then tachykinin was added again 15 min later. Finally, the net contractions before and after the antagonist treatment were compared to calculate the differences.

In the pharmacological analysis of NK₂-R-mediated contraction, a submaximal concentration of β Ala⁸-NKA(4-10) (0.1 μ M) was used for the induction of colonic contraction. In this case, the same protocol was used as indicated above. All test reagents were applied 15 min before the second β Ala⁸-NKA(4-10) application.

Histological analysis

Small pieces of human colon with mucosa were fixed with 10% buffered formalin solution (Wako) for 3 nights and mounted in paraffin. Then, the tissue was sectioned (4 μ m) and hematoxylin-eosin staining was conducted using Mayer's hematoxylin (Wako) and 1% Eosin Y solution (Wako). In the case of NK₂-R, the avidin-biotin conjugated method was also applied. The section was incubated overnight at 4°C with rabbit anti-NK₂-R antibody (1:200, LS-1288; MBL, Nagoya) after deparaffinization and blocking (Vectastain ABC rabbit IgG Kit, RK-4001; Vector Laboratories, Burlingame, CA, USA). The section was washed 3 times and incubated with a secondary antibody (biotin-labeled anti-rabbit IgG, Vectastain ABC Rabbit IgG kit, PK-4001; Vector Laboratories) for 30 min at room temperature. NK₂-R immune-reactivity was visualized by diaminobenzidine (DAB Substrate Kit, SK-4100; Vector Laboratories). After completion of the above procedure, counter staining was conducted using hematoxylin. The images stained by the above procedure were captured by means of an optical microscope (BX50, DP70; Olympus,

Tokyo).

In another series of experiments, double-immunostaining against NK₂-R and nNOS was performed. Furthermore, PGP 9.5 was also stained in order to visualize the neuronal cells. The section (10 μ m) was incubated with rabbit anti-NK₂-R antibody (1:200, LS-1288; MBL) and mouse anti-nNOS antibody (1:200, MONX10768; SANBIO BV, Uden, Netherlands), or rabbit anti-PGP 9.5 antibody (1:200, RB-9202-R7; Thermo Scientific, Fremont, CA, USA) and mouse anti-nNOS antibody (1:200) as the primary antibodies overnight at 4°C. After washing, the section was incubated again with secondary antibodies: Alexa568-labeled goat anti-rabbit IgG (A11011; Invitrogen, Carlsbad, CA, USA) and Alexa488 labeled goat anti-mouse IgG (A11001, Invitrogen). The slide image was analyzed using confocal laser scanning microscopy (LSM 510 and LSM Image Browser 4.2.0.0; Carl Zeiss Japan, Tokyo). In order to check the specificity of staining, the same procedure except primary antibody was performed as a negative control (indicated NC in the figure).

Data analyses

The data are presented as the mean $\pm$ S.E.M. from the data of 5 – 10 donors. The averaged data from the same donor, containing 2 – 3 strips was used as $n = 1$. For the binding assay and tachykinin reactivity determination, data were analyzed by regression against a three-parameter logistic equation using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and IC₅₀ values or EC₅₀ values with confidence limit were calculated. For statistical analysis, the paired Student's *t*-test or Williams' test was conducted and $P < 0.05$ was recognized as a significant result.

Results

Binding affinity of natural tachykinins and selective tachykinin receptor agonists and antagonists against each tachykinin receptor

Binding assays were conducted on the pharmacological tools in this study (Table 1). With regard to NK₁-R, SP and the NK₁-R-selective ligand Sar⁹-Met¹¹-SP showed pM order affinities and the natural ligands NKA and NKB showed nM order affinities. NKA and its analogue, β Ala⁸-NKA(4-10) and NKB also showed nM order potencies to NK₂-R. Only NKB and its analogue Met-Phe⁷-NKB possessed nM order affinities to NK₃-R. The NK₁-R antagonist CP122721 showed potent affinity to NK₁-R. Similarly, the NK₂-R antagonist saredutant and NK₃-R antagonist talnetant showed potent affinities to NK₂-R and NK₃-R, respectively.

Table 1. Binding affinity of natural tachykinins and selective tachykinin receptor agonists and antagonists against each tachykinin receptors

	NK ₁ -R	NK ₂ -R	NK ₃ -R
SP	0.086	120	> 10000
NKA	17	0.48	> 10000
NKB	32	13	25
Sar ⁹ -Met ¹¹ -SP	0.15	> 10000	> 10000
β Ala ⁸ -NKA(4-10)	> 10000	9.8	> 10000
Met-Phe ⁷ -NKB	3800	3600	1.9
CP122721	0.007	-	-
Saredutant	-	0.45	-
Talnetant	-	-	6.8

Data are presented as IC₅₀ values (nM) calculated from the regression curve.

Effects of natural tachykinins on circular smooth muscle contraction in the human colon

Cumulative treatment with SP induced smooth muscle contraction in a concentration-dependent manner (Fig. 1). The maximal contractile force by SP was equipotent to that of CCh (1 μ M). A similar effect was observed in the case of NKA and NKB, but the sensitivity for contraction differed among the tachykinins. The rank order potency was NKA > NKB > SP, and their EC₅₀ values were 11 nM (4.46 – 62.73) for NKA, 51 nM (11.2 – 161.1) for NKB, and 453 nM (85.9 – 2067) for SP, respectively.

Effects of selective tachykinin receptor antagonists on tachykinin-induced circular smooth muscle contraction in the human colon

Smooth muscle contraction was induced using a submaximal concentration of SP (1 μ M), (Fig. 2A). Pretreatment with saredutant (0.01 – 1 μ M) suppressed SP-induced smooth muscle contraction in a concentration dependent manner. Significant inhibition was observed at 0.1 μ M. However, both CP122721 and talnetant had no effect on SP-induced smooth muscle contraction even at concentrations as high as 10 μ M.

In the case of NKA (0.1 μ M)-induced contraction, saredutant inhibited smooth muscle contraction in a concentration-dependent manner (Fig. 2B). Significant inhibition was observed at 1 μ M. However, both CP122721 and talnetant had no effect on NKA-induced contraction. The same effect was observed in the case of NKB (1 μ M)-induced contraction (Fig. 2C). Again, saredutant inhibited NKB-induced smooth muscle contraction, but CP122721 and talnetant had no effect on it.

Fig. 1. Effects of natural tachykinins SP, NKA, and NKB on smooth muscle contraction in the human colon. Natural tachykinin-induced smooth muscle contractions were monitored. Tachykinin contraction relative to CCh vs. concentration. Data are presented as the mean $\pm$ S.E.M. of the data from 10 donors. Note that all tachykinins produced contraction and the order of potency was NKA > NKB > SP.

Effects of selective tachykinin receptor agonists on circular smooth muscle contraction in the human colon

We further examined the effects of tachykinin receptor-selective agonists on smooth muscle contraction. The NK₂-R-selective agonist β Ala⁸-NKA(4-10) produced smooth muscle contraction in a concentration dependent manner and the EC₅₀ was 20 nM (6.87 – 310.92) (Fig. 3). The NK₃-R-selective agonist Met-Phe⁷-NKB also produced smooth muscle contraction at concentrations much greater than that of the corresponding natural TK and NKB. On the other hand, the NK₁-R-selective agonist Sar⁹-Met¹¹-SP did not produce smooth muscle contraction at concentrations up to 10 μ M.

Figure 4 shows typical traces of colonic smooth muscle tension after treatment with natural tachykinins and the NK₂-R-selective agonist β Ala⁸-NKA(4-10). Any typical differences were observed in their contractile patterns.

Pharmacological analysis of β Ala⁸-NKA(4-10)-induced circular smooth muscle contraction in human colon

The effects of various pharmacological agents on NK₂-R-mediated contraction of the smooth muscle in human colon were studied. For this purpose, a submaximal concentration of β Ala⁸-NKA(4-10) was used for induction of smooth muscle contraction. Pretreatment with saredutant inhibited β Ala⁸-NKA(4-10)-induced smooth muscle contraction in a concentration-dependent manner (Fig. 5). On the other hand, β Ala⁸-NKA(4-10)-induced contraction was not influenced by atropine (Fig. 6). TTX or L-NAME enhanced β Ala⁸-NKA(4-10)-induced smooth muscle contraction up to 110% of the initial contraction. In this case, no baseline change was observed after TTX or L-NAME treatment (data not shown).

Fig. 2. Effects of selective tachykinin receptor antagonists on natural tachykinin-induced circular smooth muscle contraction in the human colon. Natural tachykinin-induced smooth muscle contractions were monitored. Selective tachykinin receptor antagonists CP-122721 (NK₁), saredutant (NK₂), and talnetant (NK₃) were applied 15 min before ligand application. A, SP-induced contraction; B, NKA-induced contraction; and C, NKB-induced contraction. Data are presented as the mean $\pm$ S.E.M. of the data from 5 different donors. *Significant difference from the DMSO-treated control at $P < 0.05$. Note that saredutant inhibited contraction induced by all tachykinins, while CP-122721 and talnetant did not inhibit contractions of any tachykinins.

Histological analysis

Hematoxylin-eosin staining showed that the colonic tissue used in this study was histologically normal (Fig. 7A). No inflammation or carcinoma-like signs were observed. Immunostaining showed broad NK₂-R-positive signals in circular and longitudinal muscle layers, and strong signals were observed in the myenteric plexus (Fig. 7: B, C).

Fluoro-staining pictures also showed broad NK₂-R-positive signals in circular and longitudinal muscle layers, and strong signals were observed in the myenteric plexus (Fig. 8). On the other hand, nNOS-positive signals were observed mainly in the myenteric plexus and they were co-expressed with PGP 9.5. Some of the NK₂-R-positive signals merged with the nNOS-positive signals in the myenteric plexus.

Fig. 3. Effects of selective tachykinin receptor agonists on smooth muscle contraction in the human colon. Selective tachykinin receptor agonist-induced smooth muscle contractions were monitored. Data are presented as the mean $\pm$ S.E.M. of the data from 5 donors. Note that the NK₂-R-selective agonist produced marked contraction, while the NK₁-R- and NK₃-R-selective agonists did not.

Fig. 4. Typical trace of natural tachykinins and β Ala⁸-NKA(4-10)-induced smooth muscle contraction in the human colon. After a stable baseline was obtained, each ligand was applied into the organ bath and muscle tone was monitored. The agonist concentrations were as follows: A, SP (1 μ M); B, NKA (0.1 μ M); C, NKB (1 μ M); and D, β Ala⁸-NKA(4-10) (0.1 μ M). Note that every contraction exhibited a similar contractile pattern and no typical difference was observed.

Fig. 5. Effect of saredutant on β Ala⁸-NKA(4-10)-induced smooth muscle contraction in the human colon. β Ala⁸-NKA(4-10)-induced smooth muscle contractions were monitored. Saredutant was applied 15 min before β Ala⁸-NKA(4-10). Data are presented as the mean $\pm$ S.E.M. of the data from 5 donors. *Significant difference from the vehicle-treated control at $P < 0.05$.

Fig. 6. Pharmacological characterization of β Ala⁸-NKA(4-10)-induced smooth muscle contraction in the human colon. β Ala⁸-NKA(4-10)-induced smooth muscle contractions were monitored. Atropine (10 μ M), L-NAME (100 μ M), or TTX (10 μ M) were applied 15 min before β Ala⁸-NKA(4-10). Data are presented as the mean $\pm$ S.E.M. of the data from 5 donors. *Significant difference from the vehicle-treated control at $P < 0.05$.

Discussion

Tachykinins and their receptors are abundantly expressed in gut tissue (9), and it is thought that tachykinins play an important role in regulating gut motility. Although many studies have been conducted on gut smooth muscle contractility induced by tachykinins, it is still not well understood. We conducted the present study to further clarify the role of tachykinin receptors on motor response in the human intestine.

The natural tachykinins SP, NKA, and NKB, produced circular smooth muscle contraction. The maximal con-

tractile forces were equipotent to 1 μ M of CCh. However, the relative potencies were different showing NKA (EC_{50} = 11 nM) > NKB (51 nM) > SP (453 nM). Our results confirm the reports from other groups of similar spasmogenic effects for human colonic circular smooth muscles (18–20).

We also examined the effects of selective antagonists for tachykinin receptors. We found that all natural tachykinin-induced contractions were suppressed by pretreatment with the NK₂-R antagonist but not by the NK₁-R or NK₃-R antagonist. In the present study, we used CP122721 as an NK₁-R antagonist, saredutant as an

Fig. 7. Localization of NK₂-R in the human colon. The immunoreactivity of NK₂-R in the human colon was investigated by immunostaining. Hematoxylin-eosin staining (A), immunostaining for NK₂-R (B, C). A high magnification image of the dotted square in panel B is shown in panel C. Note that the tissue used in this study was histologically normal and NK₂-R immunoreactivity was found in the smooth muscle layers, the myenteric plexus, and some parts of the submucosal layers. Arrows indicate the myenteric plexus. Abbreviations: M, mucosa; SM, submucosal layer; CM, circular muscle; MP, myenteric plexus; LM, longitudinal muscle.

NK₂-R antagonist, and talnetant as an NK₃-R antagonist. We did not determine the affinities of them comprehensively, but it was reported that the pIC₅₀ of CP122721 was 9.8 for NK₁-R and < 5 for both NK₂-R and NK₃-R (6), and K_i values of saredutant and talnetant were 454 and > 100,000 nM for NK₁-R, 0.23 and 144 nM for NK₂-R, and 707 and 1.0 nM for NK₃-R, respectively (8). Therefore, the probability of crossover and blocking of other tachykinin receptors was negligible and the receptor blockade activities of each compound against the corresponding receptors were confirmed in our study. It is well known that the selectivity of natural tachykinins is limited (2, 3). In fact, not only SP but also NKA and NKB possessed high affinities to NK₁-R and NK₂-R with nanomolar order in our binding assay, although they preferentially bound to corresponding each receptor. Therefore, it is suggested that natural tachykinin induced smooth muscle contraction in human colon via NK₂-R.

This is supported by the following findings: 1) only the NK₂-R-selective agonist β Ala⁸-NKA(4-10) produced smooth muscle contraction with reactivity similar to that of the corresponding natural NKA ligand, and 2) the responsibilities of natural ligands and selective tachykinin receptor agonists in colonic contraction were highly correlated with the affinities of these agents to NK₂-R in binding assays. In this way, it is reasonable that similar contractile patterns were observed.

In contrast to our findings in human tissues, different effects have been reported in experimental animals. It was reported that selective agonists for NK₁-R, NK₂-R, and NK₃-R can induce contraction in the guinea-pig ileum with high sensitivity, that is, 10 nM was enough to induce the maximum response, although the contractile patterns of each agonist were slightly different: the NK₁-R agonist produced tonic contractions with superimposed phasic contractions, the NK₂-R agonist gave phasic contractions,

Fig. 8. Co-localization of NK₂-R and nNOS immunoreactivities in the myenteric plexus of the human colon. Upper indicates H&E staining of human colon smooth muscle layers including the myenteric plexus. The immunoreactivities of NK₂-R and nNOS in the human colon were investigated by immunostaining. PGP 9.5 or NK₂-R was stained by red, while nNOS was stained by green. Merged picture is shown on the right side. Bar indicates 100 µm. Note that nNOS immunoreactivities were found in the myenteric plexus and some were merged with the NK₂-R immunoreactivity. Abbreviations: NC, negative control (primary antibody free); CM, circular muscle; MP, myenteric plexus; LM, longitudinal muscle.

and the NK₃-R agonist produced both tonic and phasic contractions (12). These contractions were blocked by the corresponding selective antagonists (12). Furthermore, both NK₁-R and NK₂-R agonists induced colonic contraction in anesthetized rats (13). In this way, it is possible that the contribution of tachykinin would depend on species, and attention should be paid to such a species difference when discussing the role of tachykinin in the gut. Jaafari et al. (22) reported that TACR2 alpha mRNA encoding NK₂-R was predominantly expressed in the normal human colon. Furthermore, Lordal et al. (23, 24) demonstrated that intravenous infusion of NKA in healthy volunteers could induce marked contraction of the small intestine and was inhibited by the NK₂-R-selective antagonist nepadutant. These results strongly support our findings and suggest that our in vitro findings could translate into an in vivo clinical setting.

We also examined the nature of the NK₂-R-mediated motor response using a variety of pharmacological tools. Since saredutant completely inhibited the β Ala⁸-NKA(4-10)-induced colonic contraction, it is thought that the β Ala⁸-NKA(4-10)-induced smooth muscle contraction was mediated by NK₂-R. Consistent with Croci's findings (18), we confirmed that NK₂-R-mediated smooth muscle contraction was not inhibited by atropine and TTX, suggesting that NK₂-R-mediated smooth muscle contraction of the human colon is a result of direct action on smooth muscle and is not mediated by indirect components such as a cholinergic component for contraction. It has been reported that β Ala⁸-NKA(4-10)-induced colonic contractions are sensitive to atropine blockade in the rat (13). In contrast, the NK₂-R agonist-induced contractions in mice (14) and the human colon (18) were resistant to atropine and TTX. Lecci et al. (25, 26) summarized the role of NK₂-R on gut smooth muscle contraction and suggested that the neuronal components partly contribute to the smooth muscle contractions in tachykinin-induced contraction. However, it seems to be not applicable at least in our preparation of human colonic smooth muscles. These data suggest that the mechanisms involved in NK₂-R-mediated smooth muscle contraction may be species-dependent.

Interestingly, TTX significantly enhanced but did not suppress β Ala⁸-NKA(4-10)-induced colonic contraction in the human colon. Immunohistochemical analysis demonstrated that NK₂-R immunoreactivity was found not only in the smooth muscle layers but also in the myenteric plexus. Since it was reported that NK₂-R was expressed with PGP 9.5 in the myenteric plexus of the human colon (27), it is likely that some neuronal modulation via NK₂-R is present. Thus, we speculate that some inhibitory neuronal components may be involved in the NK₂-R-mediated response in the human colon. In gen-

eral, nitric oxide (NO) neurons are considered one of the major relaxing components in gut tissues (28). Therefore, we further examined the effect of the NO synthase inhibitor L-NAME on β Ala⁸-NKA(4-10)-induced colonic contraction of human colonic smooth muscle preparations. As expected, pretreatment with L-NAME enhanced β Ala⁸-NKA(4-10)-induced colonic contraction. Furthermore, immunohistochemical observation demonstrated that some of the NK₂-R-positive signals were co-localized with nNOS-positive signals. These results suggest that the activation of NO neurons may be involved in NK₂-R-mediated relaxation of the smooth muscle preparations of the human colon. Regarding inhibitory components via NK₂-R, we only looked at NO neurons in this study. It has been speculated that the vasoactive intestinal polypeptide (VIP) neuron interacts with NK₂-R (29). Also, Gallego et al. (30) reported that the P2Y₁ receptor is also involved in relaxation of smooth muscle in the human sigmoid colon. Therefore, it is interesting to investigate whether or not VIP or purinergic neurons are involved in NK₂-R-mediated smooth muscle relaxation.

In summary, it is suggested that NK₂-R is the major contributor to tachykinin-induced smooth muscle contraction in the human colon and that the NK₂-R-mediated response is consistent with an excitatory component via a direct action on the smooth muscle with an inhibitory component possibly via neuronal NO.

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