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Novel triple neurokinin receptor antagonist CS-003 strongly inhibits neurokinin related responses

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ABSTRACT

Neurokinins are known to induce neurogenic inflammation related to respiratory diseases, though there is little information on triple neurokinin receptor antagonists. The pharmacological properties of the novel triple neurokinin 1, 2 and 3 receptor antagonist [1-{2-[(2*R*)-(3,4-dichlorophenyl)-4-(3,4,5-trimethoxybenzoyl) morpholin-2-yl]ethyl}spiro[benzo[*c*]thiophene-1(3*H*),4′-piperidine]-(2*S*)-oxide hydrochloride] (CS-003) were evaluated in this study. The binding affinities of CS-003 were evaluated with human and guinea pig neurokinin receptors. As well, the *in vivo* antagonism of CS-003 against exogenous neurokinins and effects on capsaicin-induced and citric acid-induced responses were investigated in guinea pigs. CS-003 exhibited high affinities for human neurokinin 1, neurokinin 2 and neurokinin 3 receptors with K_i values (mean±S.E.M.) of 2.3±0.52, 0.54±0.11 and 0.74±0.17 nM, respectively, and for the guinea pig receptors with K_i values of 5.2±1.4, 0.47±0.075 and 0.71±0.27 nM, respectively. Competitive antagonism was indicated in a Schild analysis of substance P-, neurokinin A- and neurokinin B-induced inositol phosphate formation with pA_2 values of 8.7, 9.4 and 9.5, respectively. CS-003 inhibited substance P-induced tracheal vascular hyperpermeability, neurokinin A- and neurokinin B-induced bronchoconstriction with ID_{50} values of 0.13, 0.040 and 0.063 mg/kg (i.v.), respectively. CS-003 also inhibited capsaicin-induced bronchoconstriction (ID_{50}: 0.27 mg/kg, i.v.), which is caused by endogenous neurokinins. CS-003 significantly inhibited citric acid-induced coughs and the effect was greater than those of other selective neurokinin receptor antagonists. CS-003 is a potent antagonist of triple neurokinin receptors and may achieve the best therapeutic efficacy on respiratory diseases associated with neurokinins compared to selective neurokinin receptor antagonists.

1. Introduction

Neurokinins (or tachykinins), which include substance P, neurokinin A and neurokinin B, are members of a family of small peptides with a common C-terminal sequence of Phe-X-Gly-Leu-Met-NH_2. Three types of G protein-coupled receptors, named neurokinin 1, neurokinin 2 and neurokinin 3, mediate the biological effects of neurokinins. Substance P interacts preferentially with neurokinin 1 receptor, neurokinin A with neurokinin 2 receptor and neurokinin B with neurokinin 3 receptor (Helke et al., 1990). Activation of the receptors induces stimulation of phospholipase C, resulting in phosphoinositide breakdown and the elevation of intracellular calcium (Maggi et al., 1993; Otsuka and Yoshioka, 1993).

Neurokinins are widely distributed in the whole body and in the airways they are found in unmyelinated pulmonary C fibers which are sensitive to capsaicin (Otsuka and Yoshioka, 1993; Maggi, 1995). Neurokinins released by mechanical and chemical stimuli elicit a wide range of biological actions in mammalian airways (Joos et al., 2003). Neurokinin 1 receptors are primarily distributed on the vascular endothelium and epithelial cells and within the mucus glands. Furthermore, various cells that have infiltrated the lungs in inflammatory diseases may also express neurokinin 1 receptors. Neurokinin 2 receptors are primarily associated with the airway smooth muscle. Epithelial cells and some inflammatory cells may also express neurokinin 2 receptors (Mazzone, 2004). Neurokinin 3 receptors have been shown to be present in airway parasympathetic ganglia, where they play an important role in regulating parasympathetic nerve excitability (Phillips et al., 2003; Myers et al., 2005). In recent years, it has become clear that immune cells are additional sources of neurokinins (Maggi, 1997) and that epithelial cells may also produce substance P (Chu et al., 2000).

Coinciding with these reports, all three neurokinin receptors are also reported to mediate respiratory responses. For example, they induce vascular hyperpermeability, bronchoconstriction, airway hyperresponsiveness, cough and mucus secretion (Chapman et al., 1998).

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Since these effects are characteristic of patients suffering from respiratory diseases such as asthma, rhinitis and chronic obstructive pulmonary disease, it has been proposed that neurokinin receptor antagonists may be useful for the treatment of these respiratory diseases (Barnes, 1986; Mazzone, 2004). Furthermore, it is reported that neurokinins and their receptors are upregulated in several respiratory diseases (Nieber et al., 1992; Adcock et al., 1993; Heaney et al., 1998; Bardelli et al., 2005).

Although there is a great deal of information available on neurokinins, only two triple neurokinin receptor antagonists have been reported (Rumsey et al., 2001; Anthes et al., 2002). However, besides the contribution of all three receptors to respiratory diseases, all three receptor subtypes are known to interact with all of the endogenous neurokinins (Severini et al., 2002; Pennefather et al., 2004). Thus, antagonizing only one neurokinin receptor subtype may not be enough for the treatment of respiratory diseases and we have developed compounds with high affinities for all of three neurokinin receptors (Nishi et al., 2000). This study describes the pharmacological properties of CS-003, a novel, triple neurokinin 1, 2 and 3 receptor antagonist.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Japan SLC, Inc., Hamamatsu, Japan) were purchased (5–6 weeks old, 301–400 g) and housed in aluminum cages in a room set at a room temperature of 23±2 °C, humidity of 55±5% and with a 12 h lighting cycle (7:00–19:00). The animals were fasted for about 24 h before the experiments. The experimental procedures employed in this study were in accordance with the guidelines of the Institutional Animal Care and Use Committee at Sankyo Research Laboratories (Tokyo, Japan).

2.2. Reagents

CS-003 [1-{2-[(2*R*)-(3,4-dichlorophenyl)-4-(3,4,5-trimethoxybenzoyl)morpholin-2-yl]ethyl}spiro[benzo[*c*]thiophene-1(3*H*),4′-piperidine]-(2*S*)-oxide hydrochloride] was synthesized by Sankyo Co., Ltd. (Tokyo, Japan). The chemical structure of CS-003 is shown in Fig. 1. FK888 (neurokinin 1 receptor antagonist, N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methly-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide) and SB 223956 (neurokinin 3 receptor antagonist, (−)-3-methoxy-2-phenyl-*N*-[(1*S*)-phenylpropyl]quinoline-4-carboxamide) were synthesized by Sankyo Co., Ltd. SR 48968 (neurokinin 2 receptor antagonist, (*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) was synthesized by Chemtech Labo., Inc. (Tokyo, Japan). In the *in vivo* experiments, all the test compounds were intravenously injected (i.v.) to the animals 5 min before administration of the stimulating agents.

[^{3}H]Substance P, [^{3}H]SR 48968 and *myo*-[^{3}H]inositol were purchased from Amersham Pharmacia Biotech Ltd. (Tokyo, Japan). [^{3}H]Senktide was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). Substance P, neurokinin A and neurokinin B were obtained from Peptide Institute Inc. (Osaka, Japan). Succinyl-[Asp^6, N-Me-Phe^8]-substance P fragment 6–11 (senktide), gallamine triethiodide, capsaicin and citric acid were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Evans blue from Merck KGaA (Darmstadt, Germany). Pentobarbital sodium (Abbott Laboratories, Abbott Park, IL, USA) was used to anesthetize the animals.

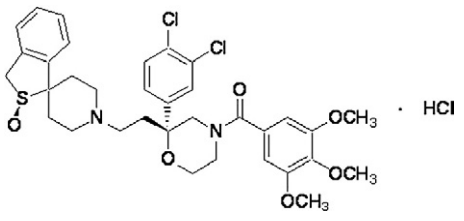

Fig. 1. Chemical structure of CS-003.

2.3. Receptor binding assay

Receptor binding assays were performed with [^{3}H]substance P, [^{3}H]SR 48968 and [^{3}H]senktide as ligands for neurokinin 1, neurokinin 2 and neurokinin 3 receptor binding assays, respectively, according to the method described by Kudlacz et al. (1996) with some modifications. For the human receptor assays, crude membranes prepared from 1.25×10^6 cells of COS cells stably expressing human neurokinin 1 receptor (GeneBank: M81797), human neurokinin 2 receptor (GeneBank: M57414) or human neurokinin 3 receptor (GeneBank: M89473) were used for each assay tube. For the guinea pig receptor assays, crude membranes containing 0.25 mg protein prepared from guinea pig lung, ileum or brain were used for the neurokinin 1, neurokinin 2 and neurokinin 3 receptor binding assays, respectively. The binding assay was initiated by incubating the membrane preparations with the radioactive ligand (0.050–15 nM) in 50 mM Tris–HCl buffer (pH 7.4). The reaction was terminated by the addition of ice cold buffer after 35 min incubation, and the radioactivity of the membrane preparation harvested on a GF/B glass microfiber filter (Whatman International Ltd., Maidstone, Kent, UK) was counted using a liquid scintillation counter (TRI-CARB 2300TR, PerkinElmer Inc., Wellesley, MA, USA). Non-specific binding was determined in the presence of 10 μM unlabeled ligand, and specific binding was calculated by subtracting the non-specific binding from the total binding. The inhibitory effects of the antagonists were evaluated with 1.0 nM of radioactive ligands. Dissociation constant (K_d) values were computed with KELL software (Biosoft, Great Shelford, Cambridge, UK) and the 50% inhibitory concentration (IC_{50}) values were determined by linear regression. The inhibition constant (K_i) values were calculated according to Cheng and Prusoff (1973) and expressed as the means of three independent experiments in triplicate.

2.4. Inositol phosphate formation

Measurement of inositol phosphate formation in cells expressing human neurokinin receptors was performed according to the method described by Kudlacz et al. (1996) with a slight modification. COS cells expressing human neurokinin receptors were cultured with Dulbecco's Modified Eagle Medium (D-MEM) containing 74 kBq/ml of *myo*-[^{3}H]inositol and incubated for 24 h before the assay. After the medium was replaced with inositol-free D-MEM, CS-003 was added and the cells were incubated for 15 min. Then they were stimulated with neurokinins and incubated for 60 min. The reaction was terminated by eluting the cell content with Triton and the cell content was then loaded onto a poly-prep prefilled chromatography column (AG 1-X8, Bio-Rad Laboratories Inc., Hercules, CA, USA). Inositol phosphate was eluted from the column with 0.1 M formic acid/0.4 M ammonium formate (1:1, v/v) and the radioactivity was counted using the liquid scintillation counter. Data are expressed as a percent of the agonist-induced maximum formation of [^{3}H]inositol phosphate over the basal levels. The pA_2 value, the negative logarithm of the molar concentration of an antagonist that produces a two-fold shift to the right of the agonist concentration curve, and the slope were determined by a regression analysis of Schild plots (Arunlakshana and Schild, 1959).

2.5. Tracheal vascular hyperpermeability in guinea pigs

The effects of the compounds on substance P-induced tracheal vascular hyperpermeability were measured according to the method of Rogers et al. (1988). Briefly, Evans blue dye (20 mg/kg) was injected

into the femoral vein of an anesthetized (pentobarbital sodium, 10 mg per body, i.p.) guinea pig and immediately after the dye injection, saline or substance P (1.0 μg/kg) was intravenously injected. The animal was killed with carbon dioxide gas 15 min after the saline or substance P injection. Each isolated tracheal tissue was weighed, and the dye was extracted with an extraction solution (acetone: 0.5% Na_2SO_4 = 7: 3, v/v) for over night. The optical density of the extraction solution of each tracheal tissue was measured with a spectrophotometer at 620 nm and the values of the optical density were converted to the concentration of the dye with a standard curve. The amount of dye per 0.1 g tracheal tissue was calculated as the intensity of vascular permeability. The number of animals used for each group was 4.

2.6. Bronchoconstriction in guinea pigs

The effects of the compounds on neurokinin A-, neurokinin B- or capsaicin-induced bronchoconstriction were evaluated by measuring the airway pressure under a constant flow according to a modified method of Konzett and Rössler (1940) using a pressure transducer (TP-200T or TP-400T, Nihon Kohden Corporation, Tokyo, Japan) connected to a side arm of the tracheal cannula of an anesthetized (pentobarbital sodium, 22.5 mg per body, s.c.) guinea pig. Following the suppression of spontaneous respiration by gallamine triethiodide (20 mg/kg, i.v.), the animal was ventilated artificially with a respiration pump (1 Hz, 10 ml/kg, Ugo Basile Biological Research Apparatus Company, Comerio, VA, Italy). After administration of neurokinin A or neurokinin B (4.0 μg/kg, i.v.), or inhalation of aerosolized capsaicin solution (0.1 mg/ml) for 1 min with an ultrasonic nebulizer (OMRON Corporation, Tokyo, Japan), the changes in the airway pressure were measured for 10 min. The increase in airway pressure from the baseline is represented as 'increase in airway pressure', and 'Δ increase in airway pressure', which is the area under the curve of 'increase in airway pressure', is expressed as an index of the bronchoconstriction. The number of animals used for each group was 4.

2.7. Citric acid-induced coughs in guinea pigs

An unanesthetized guinea pig was placed in an unrestrained chamber (Buxco Electronics, Inc., Wilmington, NC, USA), and exposed to saline or 0.4 M citric acid solution using an ultrasonic nebulizer for 10 min. Two trained observers counted the number of induced coughs for 15 min from the start of citric acid exposure. Sneezes were excluded from the data. The number of animals used for each group was 12.

2.8. Data analysis and statistics

The pA_2 values and slopes of inositol phosphate formation experiment are shown together with the corresponding 95% confidence intervals and other data are expressed as the mean ± S.E.M. The IC_{50} and 50% inhibitory dose (ID_{50}) values were calculated from the dose–response curves fitted by linear regression, as described by Fieller (1940). Statistical analyses between two groups were performed by *t*-test or Welch's test, whereas multiple comparisons were performed by Dunnett's test or Tukey's test using SAS system Release 8.2 (SAS Institute Inc., Cary, NC, USA). Probability (*P*) values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Receptor binding affinities

CS-003 exhibited high affinities for crude membrane preparations of COS cells expressing human neurokinin 1, neurokinin 2 and neurokinin 3 receptors with K_i values of 2.3 ± 0.52, 0.54 ± 0.11 and 0.74 ± 0.17 nM, respectively (Table 1). CS-003 also exhibited high affinities for guinea pig neurokinin 1, neurokinin 2 and neurokinin 3 receptors with K_i values of 5.2 ± 1.4, 0.47 ± 0.075 and 0.71 ± 0.27 nM, respectively (Table 1). We synthesized FK888, SR 48968 and SB 223956, which are subtype-selective neurokinin 1, neurokinin 2 and neurokinin 3 receptor antagonists, respectively, to compare the binding affinities for each neurokinin receptor. FK888 selectively bound to human and guinea pig neurokinin 1 receptors with K_i values of 0.15 ± 0.044 and 3.2 ± 1.2 nM, respectively. SR 48968 selectively bound to human and guinea pig neurokinin 2 receptors with K_i values of 0.32 ± 0.023 and 0.31 ± 0.050 nM, respectively. SB 223956 selectively bound to human and guinea pig neurokinin 3 receptors with K_i values of 5.3 ± 0.60 and 16 ± 3.8 nM, respectively. CS-003 showed low affinities for both mouse and rat neurokinin 1 and neurokinin 2 receptors. For mouse neurokinin 1 and neurokinin 2 receptors, K_i values of CS-003 were 4100 and 420 nM, respectively, and for rat neurokinin 1 and neurokinin 2 receptors, K_i values of CS-003 were 7100 and 120 nM, respectively. To evaluate the selectivity for the neurokinin receptors, the binding affinities of CS-003 for non-neurokinin receptors were assayed in over 100 receptors (including G protein-coupled receptors, ion-channels and transporters). At 10 μM, the inhibitory rate of CS-003 was less than 50% for almost all the receptors tested, except in the rat calcium channel L type (K_i = 5.8 μM) and rat sodium channel site 2 (K_i = 3.8 μM) assays.

3.2. Neurokinin-induced inositol phosphate formation

The activation of neurokinin receptors induces stimulation of phospholipase C, resulting in phosphoinositide breakdown and inositol phosphate formation. The *in vitro* antagonistic activity of CS-003 was investigated by measuring the inhibition of this inositol phosphate formation. Substance P, neurokinin A and neurokinin B concentration-dependently induced inositol phosphate formation in cells expressing the human neurokinin 1, neurokinin 2 and neurokinin 3 receptors, respectively (Fig. 2). CS-003 inhibited this induced inositol phosphate formation in a concentration-dependent manner and produced a rightward shift of the concentration–response curve. The pA_2 values (and corresponding 95% confidence intervals) determined by a Schild analysis were 8.7 (8.4–9.0), 9.4 (8.9–10) and 9.5 (9.3–9.7) for the neurokinin 1, neurokinin 2 and neurokinin 3 receptors, respectively, and the slope values (and corresponding 95% confidence intervals) were 1.0 (0.85–1.1), 1.2 (0.80–1.6) and 0.94 (0.84–1.0), respectively (Table 2). Since the 95% confidence intervals of the slopes

Table 1
Receptor binding affinities for neurokinin receptors

K_i (nM, human)				K_i (nM, guinea pig)		
Compound	Neurokinin 1	Neurokinin 2	Neurokinin 3	Neurokinin 1	Neurokinin 2	Neurokinin 3
CS-003	2.3 ± 0.52	0.54 ± 0.11	0.74 ± 0.17	5.2 ± 1.4	0.47 ± 0.075	0.71 ± 0.27
FK888	0.15 ± 0.044	>78	>240	3.2 ± 1.2	>130	>620
SR 48968	>79	0.32 ± 0.023	>240	>140	0.31 ± 0.050	>620
SB 223956	>74	>78	5.3 ± 0.60	>140	>130	16 ± 3.8

Crude membrane preparations of cells expressing human neurokinin receptors and tissues isolated from guinea pigs were used. [^{3}H]Substance P, [^{3}H]SR 48968 and [^{3}H]senktide were selected as ligands for neurokinin 1, 2 and 3 receptors, respectively. K_i values are expressed as mean ± S.E.M. of three independent experiments in triplicate.

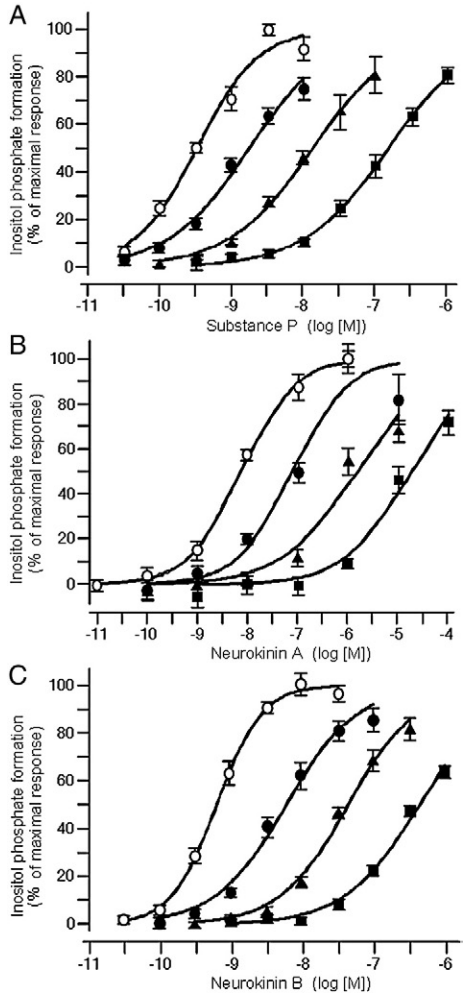

Fig. 2. Concentration–response curves of neurokinin-induced inositol phosphate formation in the absence (open circles) or presence (closed symbols) of CS-003. Cells expressing neurokinin receptors were incubated with *myo*-[^{3}H]inositol before the addition of CS-003 and inositol phosphate formation was measured, as described in Materials and methods. (A) The concentrations of CS-003 were 1.0×10^{-8} M (closed circles), 1.0×10^{-7} M (closed triangles) or 1.0×10^{-6} M (closed squares), and the neurokinin 1 receptor expressing cells were stimulated by substance P. The neurokinin 2 and neurokinin 3 receptor expressing cells were stimulate by neurokinin A (B) and neurokinin B (C), respectively, and the concentrations of CS-003 were 3.3×10^{-9} M (closed circles), 3.3×10^{-8} M (closed triangles) or 3.3×10^{-7} M (closed squares). Values (% of maximal response) are the means±S.E.M. of three independent experiments performed in duplicate.

included 1 for all three assays, it was considered that CS-003 is a competitive antagonist for all the three human neurokinin receptors.

3.3. Substance P-induced tracheal vascular hyperpermeability in guinea pigs

The *in vivo* neurokinin 1 receptor antagonistic activity were measured as the inhibition of substance P-induced tracheal vascular hyperpermeability in guinea pigs. Substance P (1.0 μg/kg, i.v.)-induced tracheal vascular hyperpermeability, and FK888 (0.10 mg/kg, i.v.) showed a significant inhibitory effect on the induced response with an inhibitory rate of 95.8% (Fig. 3A). However, neither SR 48968 (1.0 mg/kg, i.v.) nor SB 223956 (10 mg/kg, i.v.) had significant effects (data not shown). Thus, it was indicated that this substance P-induced vascular hyperpermeability is mainly mediated by neurokinin 1 receptor. CS-

Table 2
Schild analysis data of CS-003 on inositol phosphate formation antagonism

Parameter	Neurokinin 1	Neurokinin 2	Neurokinin 3
pA_2 (95% C.I.)	8.7 (8.4–9.0)	9.4 (8.9–10)	9.5 (9.3–9.7)
Slope (95% C.I.)	1.0 (0.85–1.1)	1.2 (0.80–1.6)	0.94 (0.84–1.0)

Schild plots were obtained from the data in Fig. 2 and the pA_2 values and slopes were determined by regression analysis of the Schild plots. The values were obtained from three independent experiments performed in duplicate. pA_2: negative logarithm of the molar concentration of an antagonist that produces a two-fold shift to the right of the agonist concentration curve. 95% C.I.: 95% confidence interval.

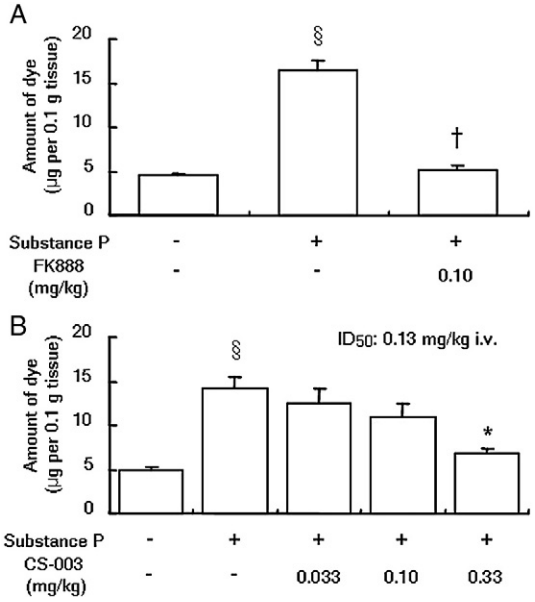

Fig. 3. Effects of neurokinin receptor antagonists on substance P-induced tracheal vascular hyperpermeability in guinea pigs. FK888 (A) and CS-003 (B) were administered (i.v.) 5 min before substance P injection. The optimal density of the extracted dye in each tracheal sample was measured and the results were expressed as the amount of dye per 0.1 g tracheal tissue (means±S.E.M., $n=4$ for each group). $^{\S}P<0.05$ versus saline control group (Welch's test). $^{\dagger *}P<0.05$ versus substance P-treated vehicle group ($^{\dagger}t$-test and *Dunnett's test).

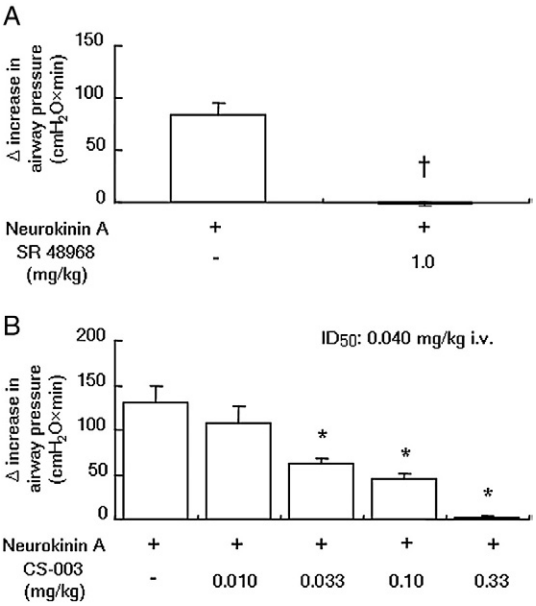

Fig. 4. Effects of neurokinin receptor antagonists on neurokinin A-induced bronchoconstriction in guinea pigs. SR 48968 (A) and CS-003 (B) were administered (i.v.) 5 min before neurokinin A injection, and 'Δ increase in airway pressure', which is the area under the time–response curve of airway pressure, is expressed as an index of the bronchoconstriction (means±S.E.M., $n=4$ for each group). $^{\dagger *}P<0.05$ versus neurokinin A-treated vehicle group (†Welch's test and *Dunnett's test).

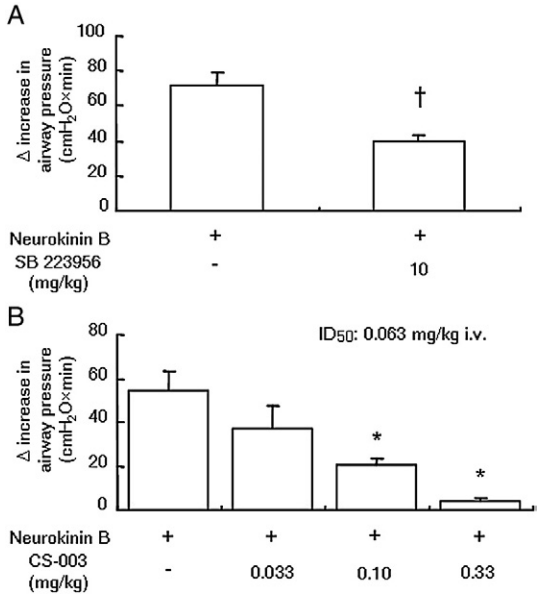

Fig. 5. Effects of neurokinin receptor antagonists on neurokinin B-induced bronchoconstriction in guinea pigs. SB 223956 (A) and CS-003 (B) were administered (i.v.) 5 min before neurokinin B injection, and 'Δ increase in airway pressure', which is the area under the time–response curve of airway pressure, is expressed as an index of the bronchoconstriction (means±S.E.M., $n=4$ for each group). $^{\dagger *}P<0.05$ versus neurokinin B-treated vehicle group ($^{\dagger}t$-test and *Dunnett's test).

003 (i.v.) dose-dependently inhibited this induced response with an ID_{50} value of 0.13 mg/kg (Fig. 3B).

3.4. Neurokinin A-induced bronchoconstriction in guinea pigs

The *in vivo* neurokinin 2 receptor antagonistic activities were measured as the inhibition of neurokinin A-induced bronchoconstriction in guinea pigs. Neurokinin A (4.0 μg/kg, i.v.)-induced significant bronchoconstriction, and SR 48968 (1.0 mg/kg, i.v.) completely inhibited the induced response (Fig. 4A). FK888 (1.0 mg/kg, i.v.), at a dosage high enough to inhibit substance P-induced vascular hyperpermeability, did not show a significant inhibitory effect on this neurokinin A-induced response. SB 223956 (10 mg/kg, i.v.) showed a significant effect with an inhibitory rate of only 20.1%. These results indicate that this neurokinin A-induced bronchoconstriction is mainly mediated by neurokinin 2 receptor. CS-003 (i.v.) inhibited this induced response in a dose-dependent manner with an ID_{50} value of 0.040 mg/kg (Fig. 4B).

3.5. Neurokinin B-induced bronchoconstriction in guinea pigs

The *in vivo* neurokinin 3 receptor antagonistic activities were measured as the inhibition of neurokinin B-induced bronchoconstriction in guinea pigs. Neurokinin B (4.0 μg/kg, i.v.)-induced significant bronchoconstriction, and SB 223956 (10 mg/kg, i.v.) showed a significant inhibitory effect on the induced response with an inhibitory rate of 44.5% (Fig. 5A). However, at high enough dosage to inhibit substance P- or neurokinin A-induced response, neither FK888 (1.0 mg/kg, i.v.) nor SR 48968 (1.0 mg/kg, i.v.) showed significant inhibitory effects on this neurokinin B-induced response (data not shown). These results indicate that this neurokinin B-induced bronchoconstriction is mainly mediated by neurokinin 3 receptor. CS-003 (i.v.) inhibited this induced response in a dose-dependent manner with an ID_{50} value of 0.063 mg/kg (Fig. 5B). The effects of CS-003 on *in vivo* methacholine-, histamine- or leukotriene D_4-induced bronchoconstriction were also examined, and CS-003 did not inhibit any of the induced responses at all (data not shown).

3.6. Capsaicin-induced bronchoconstriction in guinea pigs

In anesthetized guinea pigs, the effect of CS-003 was evaluated on capsaicin-induced bronchoconstriction, which is mainly caused by endogenous neurokinins released from sensory nerves. Exposure to aerosolized capsaicin solution (0.1 mg/ml) for 1 min induced bronchoconstriction, and CS-003 at dosages of 0.10, 0.33 and 1.0 mg/kg (i.v.) inhibited the induced response in a dose-dependent manner with inhibitory rates of 18.5%, 63.0% and 85.0%, respectively (Fig. 6A). The ID_{50} value of CS-003 was calculated to be 0.27 mg/kg (i.v.). SR 48968 at a dose of 1.0 mg/kg (i.v.) significantly inhibited the induced response with inhibitory rates of 58.6%, whereas FK888 (1.0 mg/kg, i.v.) and SB 223956 (10 mg/kg, i.v.) did not show significant effects (Fig. 6B and C).

3.7. Citric acid-induced coughs in guinea pigs

Neurokinins are reported to mediate coughs induced by citric acid, which stimulates C fibers (Karlsson, 1996). The inhibitory effects of neurokinin receptor antagonists on citric acid-induced coughs were evaluated in unanesthetized guinea pigs. Exposure to aerosolized citric acid solution (0.4 M) for 10 min induced 10±1.4 coughs. FK888 (1.0 mg/kg, i.v.), SR 48968 (1.0 mg/kg, i.v.) and SB 223956 (10 mg/kg, i.v.) significantly inhibited the number of induced coughs to 5.9±1.1, 3.3±0.62 and 6.0±0.87, respectively. CS-003 (1.0 mg/kg, i.v.) also significantly inhibited the number of induced coughs to 2.3±0.30, which was significantly greater than that of SB 223956 (Fig. 7).

4. Discussion

There are several subtype-selective neurokinin receptor antagonists with potent activities, such as FK888 (neurokinin 1 receptor antagonist), SR 48968 (neurokinin 2 receptor antagonist) and SB 223956 (neurokinin 3 receptor antagonist). However, all three neurokinin receptor subtypes are present in the airways and have been reported to contribute to many airway responses (Mazzone,

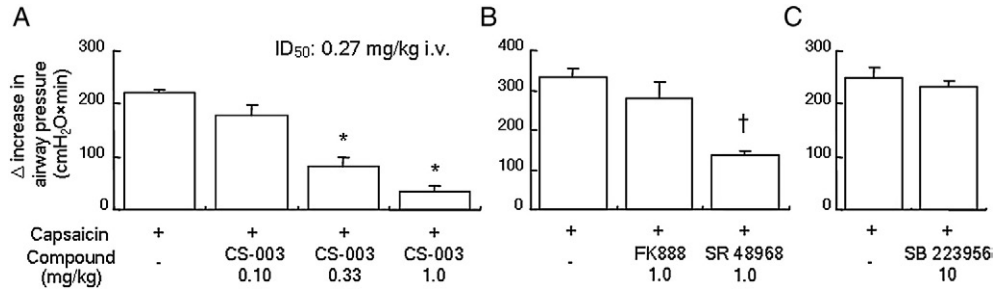

Fig. 6. Effects of neurokinin receptor antagonists on capsaicin-induced bronchoconstriction in guinea pigs. CS-003 (A), FK888 and SR 48968 (B), and SB 223956 (C) were administered (i.v.) 5 min before capsaicin inhalation (0.1 mg/ml, 1 min), and 'Δ increase in airway pressure', which is the area under the time–response curve of airway pressure, is expressed as an index of the bronchoconstriction (means±S.E.M., $n=4$ for each group). $^{\dagger *}P<0.05$ versus capsaicin-treated vehicle group ($^{\dagger}t$-test and *Dunnett's test).

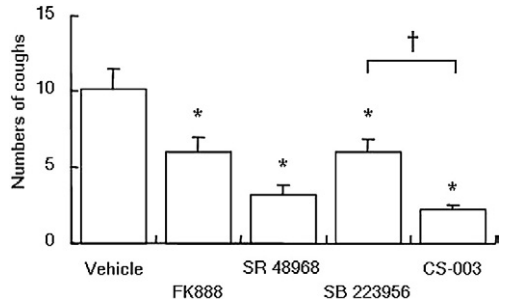

Fig. 7. Inhibitory effects of neurokinin receptor antagonists on citric acid-induced coughs in guinea pigs. Coughs were induced by aerosolized citric acid solution, and the number of coughs was counted by trained observers for 15 min (means ± S.E.M., $n = 12$ for each group). Multiple comparison was performed by Tukey's test. $^{*}P<0.05$ compared to the vehicle group and $^{\dagger}P<0.05$ compared to another compound-treated group.

2004). Neurokinin 1 receptor is primarily related to plasma extravasation, activation of inflammatory cells and mucus secretion. Neurokinin 2 receptor is primarily related to contraction of airway smooth muscles. Neurokinin 3 receptor is primarily related to mucus secretion and responses evoked by acetylcholine. Thus, blocking only one receptor subtype is considered to be insufficient and inhibition of more than one neurokinin receptor subtypes may achieve high therapeutic effects on airway diseases such as asthma and chronic obstructive pulmonary disease (Corboz et al., 2003).

In this report, we characterized the pharmacological activity of a novel antagonist of all three neurokinin receptor subtypes. In receptor binding assays, CS-003 exhibited high binding affinities for neurokinin 1, neurokinin 2 and neurokinin 3 receptors of both human and guinea pig. In comparison with other selective neurokinin receptor antagonists, the binding affinity of CS-003 was about 10 times weaker than that of FK888 for human neurokinin 1 receptor, nearly equal to that of SR 48968 for human neurokinin 2 receptor and about 10 times stronger than that of SB 223956 for human neurokinin 3 receptor (Table 1). These data are consistent with previous reports (Emonds-Alt et al., 1992; Fujii et al., 1992; Giardina and Raveglia, 1997). In the *in vivo* studies, only CS-003 showed the potent antagonism against all three agonist-induced reactions, substance P-induced tracheal vascular hyperpermeability, neurokinin A- and neurokinin B-induced bronchoconstriction. Considering that all neurokinin receptor subtypes are expressed in airway, CS-003 may show higher efficacy on airway diseases than the subtype-selective antagonists.

Two antagonists, ZD6021 and SCH 206272, have been reported as triple neurokinin receptor antagonists so far (Rumsey et al., 2001; Anthes et al., 2002). Comparing the binding affinities (K_i) for human neurokinin receptors, CS-003 shows a profile similar to that of SCH 206272. Both CS-003 and SCH 206272 show higher affinities for neurokinin 2 and neurokinin 3 receptors than those for neurokinin 1 receptor, whereas ZD6021 shows highest affinity for neurokinin 1 receptor. Among these triple neurokinin receptor antagonists, the effect of CS-003 in clinical study has been reported. CS-003 showed significant inhibition against neurokinin A-induced bronchoconstriction in asthmatic patients (Schelfhout et al., 2006). These results indicate that CS-003 is a potent antagonist for all three neurokinin 1, neurokinin 2 and neurokinin 3 receptors not only *in vitro* but also *in vivo* and that CS-003 also has potent activity in asthmatic patients.

In the neurokinin 2 antagonism assay *in vivo*, SB 223956 showed a slight but significant effect. Since neurokinin A can also bind to neurokinin 3 receptor with a low affinity (Severini et al., 2002; Pennefather et al., 2004), neurokinin A might have induced neurokinin 3 receptor-mediated weak bronchoconstriction in this study. In the neurokinin 3 antagonism assay *in vivo*, SB 223956 showed significant inhibition on neurokinin B-induced bronchoconstriction, and what is more, atropine also showed significant inhibition on the bronchoconstriction (data not shown). Neurokinin 3 receptor is reported to regulate bronchial parasympathetic ganglion neurotransmission in human airways (Myers et al., 2005). Furthermore, neurokinin 3 receptor antagonists showed inhibitory effects on airway hyperresponsiveness to methacholine in guinea pigs (Mukaiyama et al., 2004). These and our findings indicate that exogenous neurokinin B may activate neurokinin 3 receptors in the airway ganglia regulating acethylcholine release from parasympathetic nerve terminals.

Capsaicin-induced bronchoconstriction is caused by endogenous neurokinin release from the sensory nerves and is mediated mainly by neurokinin 2 receptors (Hua et al., 1986; Lou et al., 1993). CS-003 also showed significant inhibition on this bronchoconstriction and the inhibitory rate of CS-003 was higher than those of other selective neurokinin receptor antagonists. A combination of neurokinin 1 receptor and neurokinin 2 receptor antagonists blocked the capsaicin-induced bronchoconstriction to a greater extent than the response obtained with the neurokinin 1 receptor or neurokinin 2 receptor antagonist alone in guinea pigs (Corboz et al., 2003). Although a direct comparison between the effects of CS-003 and neurokinin 2 receptor antagonist on the capsaicin-induced bronchoconstriction has not been performed in this study, the high inhibitory rate of CS-003 might be the result of potentiation of inhibition by blocking all three neurokinin 1, neurokinin 2 and neurokinin 3 receptors.

Pulmonary C fibers are involved in citric acid-induced coughs (Karlsson, 1996). Selective antagonists of all three neurokinin receptors have been reported to inhibit induced coughs (Yasumitsu et al., 1996; Daoui et al., 1998). In this study, all three kinds of selective neurokinin receptor antagonists significantly inhibited citric acid-induced coughs, although the efficacies were different. The inhibitory effect of the neurokinin 2 receptor antagonist was more potent than those of the neurokinin 1 and neurokinin 3 receptor antagonists. Cough may be a secondary response of other lung responses including bronchoconstriction (Karlsson et al., 1988), and this may be the reason why a neurokinin 2 receptor antagonist which mainly mediates bronchoconstriction shows a high inhibitory rate. CS-003 showed the most potent inhibitory effect among the other selective antagonists. Since the doses of all the antagonists were high enough according to the *in vivo* antagonism with exogenous natural ligands, the highest efficacy of CS-003 may be achieved due to an additive effect by a blockade of multiple receptors, though they were not significantly different from those of the neurokinin 1 receptor and neurokinin 2 receptor antagonists. The effects of the selective neurokinin receptors on coughs in this study were different from those in the study of SCH 206272 (Anthes et al., 2002). The biggest difference is the activity of selective neurokinin 3 receptor antagonists. Although the neurokinin 3 receptor antagonists used in both studies were different, both antagonists showed efficacies in *in vitro* and *in vivo* assays. The tussigenic agents used to induce the coughs, citric acid and capsaicin, were also different between the studies. Since the mechanisms of citric acid- and capsaicin-induced coughs have been reported to be different, this may be the reason why the selective neurokinin 3 receptor antagonists showed different results (Canning et al., 2006). After all, triple neurokinin 1, neurokinin 2 and neurokinin 3 receptor antagonists showed their highest inhibitory effect on both capsaicin- and citric acid-induced coughs.

According to several studies with selective neurokinin receptors antagonists, there are some biological actions related to multiple neurokinin receptor subtypes, whereas other actions are related to specific neurokinin receptor subtypes (Mazzone, 2004). Moreover, additive or synergistic effects on inhibiting the plasma leakage and bronchoconstriction resulting from the blockade of both neurokinin 1 and neurokinin 2 receptors have been reported (Savoie et al., 1995; Rumsey et al., 2001). In the same manner, CS-003 is expected to show potent additive or synergistic blockade of all three neurokinin 1, neurokinin 2 and neurokinin 3 receptors and may achieve the better therapeutic efficacy than selective neurokinin receptor antagonists.

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