NK_1 Receptor Stimulation Causes Contraction and Inositol Phosphate Increase in Medium-size Human Isolated Bronchi

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Although contraction of human isolated bronchi is mediated mainly by tachykinin NK_2 receptors, NK_1 receptors, via prostanoid release, contract small-size ($\sim$ 1 mm in diameter) bronchi. Here, we have investigated the presence and biological responses of NK_1 receptors in medium-size (2–5 mm in diameter) human isolated bronchi. Specific staining was seen in bronchial sections with an antibody directed against the human NK_1 receptor. The selective NK_1 receptor agonist, [Sar^9, $Met(O_2)^{11}$]SP, contracted about 60% of human isolated bronchial rings. This effect was reduced by two different NK_1 receptor antagonists, CP-99,994 and SR 140333. Contraction induced by [Sar^9, $Met(O_2)^{11}$]SP was independent of acetylcholine and histamine release and epithelium removal, and was not affected by nitric oxide synthase and cyclooxygenase (COX) inhibition. [Sar^9, $Met(O_2)^{11}$]SP increased inositol phosphate (IP) levels, and SR 140333 blocked this increase, in segments of medium- and small-size ($\sim$ 1 mm in diameter) human bronchi. COX inhibition blocked the IP increase induced by [Sar^9, $Met(O_2)^{11}$]SP in small-size, but not in medium-size, bronchi. NK_1 receptors mediated bronchoconstriction in a large proportion of medium-size human bronchi. Unlike small-size bronchi this effect is independent of prostanoid release, and the results are suggestive of a direct activation of smooth muscle receptors and IP release.

The tachykinins, substance P (SP) and neurokinin A (NKA), are stored and released from peripheral terminals of a subset of primary sensory neurons (1, 2). The proinflammatory effects produced by SP and NKA are collectively referred to as "neurogenic inflammation" (3). In the airways neurogenic inflammatory responses consist of vasodilatation, plasma extravasation, leukocyte adhesion, secretion from seromucous glands, and bronchoconstriction (4–6). Three types of receptors mediate the biological effects of tachykinins, the NK_1, NK_2, and NK_3 receptors (7). All three tachykinin receptors belong to the seven-transmembrane domain receptor superfamily that are coupled to the $G_{q/11}$ proteins, and their activation causes inositol phosphate (IP) accumulation (7). NK_2 receptors mediate smooth muscle contraction in the airways of most mammalian species (5, 6, 8, 9). However, there is evidence that NK_1 receptors also contribute to tachykinin-induced bronchoconstriction in different mammals (8, 10), including guinea pigs. In guinea pigs selective agonists of NK_1 receptors, including [Sar^9, $Met(O_2)^{11}$]SP, produce bronchoconstriction in *in vivo* and *in vitro* experimental settings. The atropine-resistant contractile response to electrical field stimulation in guinea pig isolated bronchi is blocked completely, only when an NK_1 receptor antagonist is added to an NK_2 receptor antagonist (11, 12). Similar findings have been reported in *in vivo* conditions (13). Thus, in anesthetized guinea pigs complete abolition of the bronchoconstriction induced by endogenous tachykinins, released by capsaicin, is obtained only by the combination of NK_1 and NK_2 receptor antagonists (13).

Immunoreactivity for SP and NKA has been detected in nerve fibers around intramural ganglia and within the smooth muscle layer of human bronchi (14). NKA is a powerful bronchoconstrictor agent in the human airways both *in vitro* and *in vivo* (9, 14, 15). Its action appears to be exclusively mediated by NK_2 receptors (9, 14). However, in small-diameter bronchi ($\sim$ 1 mm in diameter), in addition to a robust NK_2-mediated contraction, tachykinins also cause bronchoconstriction via NK_1 receptor activation (16). NK_1-mediated contraction of small-diameter human bronchi, being abolished by indomethacin (16), appears to be caused by prostanoid release. With the use of a ribonuclease protection assay, messenger RNA for the NK_1 receptor has been demonstrated in human bronchus (17). More recently, the distribution of NK_1 receptors has been studied in sections of human bronchi by using an antiserum directed against the carboxyl terminus of the human NK_1 receptor (18). Specific staining was observed in submucosal glands, in the endothelium and smooth muscle of lung vessels and in the bronchial smooth muscle layer (18).

These findings led us to investigate whether NK_1 receptor activation may cause motor responses in medium-size (2- to 5-mm-diameter) human isolated bronchi. For this purpose the ability of the selective agonist of NK_1 receptors, [Sar^9, $Met(O_2)^{11}$]SP, to cause motor responses and increase IP levels was studied in a large number of medium-size human bronchi. Because we observed that [Sar^9, $Met(O_2)^{11}$]SP increased the tone and IP levels in most of the preparations studied, the effect of selective NK_1 receptor antagonists, CP-99,994 (19) and SR 140333 (20), on [Sar^9, $Met(O_2)^{11}$]SP-induced responses was also studied. The role of the epithelium, acetylcholine (ACh), histamine, nitric oxide (NO), and prostanoids in modulating the bronchoconstriction induced by [Sar^9, $Met(O_2)^{11}$]SP was also investigated. Results indicate that NK_1 receptor activation causes contraction of, and increases IP in, middle-size human bronchi. Unlike small-size bronchi ($\sim$ 1 mm in diameter), this effect is independent of the release of prostanoids.

METHODS

Tissues

The study samples were taken from 28 patients who were undergoing lung resection for a solitary peripheral carcinoma. Twenty-one sub-

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jects had a history of cigarette smoking. The bronchial rings (from 3 to 11 from each sample) were taken from the lobar or segmental bronchus of the lobe obtained at surgery, away from the tumor site. The study conformed to the Declaration of Helsinki and was approved by the Ethics Committee of the University of Ferrara (Ferrara, Italy).

Functional Experiments

Bronchial rings (2–5 mm in diameter) were mounted in 5-ml organ baths containing a modified Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$,1 mM $NaHPO_4$, and 11.1 mM glucose) maintained at 37° C, and oxygenated with a mixture of 95% O_2 and 5% CO_2. Tissues were fixed to the base of the organ bath and connected to an isometric force transducer. An optimal tension of 2.5 g was applied. During the initial stabilization period (90 min) tissues were washed six times. A challenge with ACh (1 mM) was performed and after washing, the tissue was allowed to equilibrate for an additional 90 min. To prevent peptide degradation captopril (1 μM) and phosphoramidon (1 μM) were added to the bath. To block NK_2 receptors, the selective NK_2 receptor antagonist SR 48968 (1 μM) was added to the bath. Viability of the tissues was tested with the response to ACh (1 mM). Responses to the tachykinin receptor agonist were expressed as a percentage to the response to ACh (1 mM). A cumulative concentration–response curve was constructed by applying increasing concentrations of [Sar^9, $Met(O_2)^{11}$]SP as soon as a plateau was reached with the previous concentration. The effect of pretreatment with atropine (1 μM, 15 min before the stimulus), pyrilamine (1 μM, 15 min before the stimulus), indomethacin (5 μM, 45 min before the stimulus), CP-99,994 (1 μM) or SR 140333 (1 μM) (each 15 min before the stimulus), or their respective vehicles was also studied. Some experiments were conducted in the presence of the NO synthase inhibitor, N^G-nitro-L-monomethylarginine (L-NMMA) or its inactive enantiomer, N^G-nitro-D-monomethyl arginine (D-NMMA) (each 100 μM, 15 min before the stimulus).

In a separate set of experiments the epithelial layer of bronchial tissue was removed with a cotton swab. To verify that the tissues were denuded of epithelium, histologic examinations were performed. The tissues were fixed by immersion in formaldehyde (4%) and embedded in paraffin blocks. Sections measuring 5 μm were cut and stained with hematoxylin and eosin for histologic evaluation. Histologic examination showed that the epithelial layer was completely removed in the preparations that were treated with the cotton swab, whereas no damage was observed to the lamina propria (data not shown). The effect of epithelium removal, receptor antagonists, or enzyme inhibitors was determined in parallel experiments in which two or more adjacent bronchial rings were used and the control preparation (pretreated with the vehicle of the drug under investigation) responded to [Sar^9, $Met(O_2)^{11}$]SP with a concentration-related contraction.

Inositol Phosphate Measurement

Total inositol phosphate accumulation was determined as previously reported (21). Briefly, cryostored human bronchi (stored at −80° C in fetal calf serum containing 1.8 M dimethyl sulfoxide [DMSO] for a maximum of 1 mo) were rapidly thawed in a 37° C water bath and rinsed in a large volume of physiological salt solution (PSS) to eliminate DMSO. Tissues were cut into small segments (about 1 mm^2), washed in PSS, with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1, $MgSO_4$ 1.2, $NaHCO_3$ 25, and glucose 11, and incubated in 25 ml of PSS containing 50 μCi of *myo*-[^{3}H]inositol for 4 h at 37° C under a stream of 95% O_2–5% CO_2. After this incubation, the tissue was washed twice with PSS.

Aliquots of washed tissue (1–1.5 g) were placed in a 2-ml final volume of PSS and preincubated at 37° C for 30 min. Just before stimulation, 20 μl of LiCl was added (final concentration, 20 mM). The samples were then stimulated with 20 μl of PSS (control), [Sar^9, $Met(O_2)^{11}$]SP (0.25 μM), NKA (0.025 μM), or ACh (100 μM, serving as reference of 100%) for 30 min at 37° C. Where indicated, indomethacin (2 μM final concentration) was added 45 min before stimulation and SR 140333 (0.1 μM final concentration) was added 15 min before stimulation. Stimulation was stopped by the addition of 3 ml of an ice-cold mixture of chloroform–methanol–12 N HCl mixture (100: 200:4, v/v/v) and shaken vigorously. The samples were centrifuged (4,000 × *g*) for 10 min at 4° C and the aqueous phases were brought to pH 4 and stored at −20° C until analysis. The separation of IP was performed by a high-performance liquid chromatography (HPLC) ion-exchange system (linear gradient of 1 M potassium phosphate, pH 3.7), and radioactivity was measured in a Flow-One on-line radioactivity detector (Packard, Meriden, CT) as described previously (21). Results are expressed as the percent increase of baseline values. Experiments were run in triplicate and pools of samples from different patients were used in each experiment. Pools of bronchial tissue were obtained from a minimum of 5 patients to a maximum of 20 patients.

Immunohistochemistry

Bronchial rings were fixed immediately in freshly prepared 1% paraformaldehyde, in phosphate-buffered saline (PBS, pH 7.4) for 6 h, washed twice (1 h) with PBS containing 15% sucrose, embedded in O.C.T. compound, snap-frozen in isopentane precooled in liquid nitrogen, and stored at −70° C to be used later in immunohistochemistry.

Crystostat sections (10 μm thick) were immunostained with an antibody to NK_1 receptors by the streptavidin–biotin complex peroxidase method, and the peroxidase activity was revealed by the nickel enhancement method, as previously described (22). Briefly, endogenous peroxidase activity was blocked by immersing slides in 0.3% hydrogen peroxide in methanol for 30 min. After washing in PBS, nonspecific binding was blocked by incubating in 3% normal swine serum in PBS containing 0.05% bovine serum albumin (BSA) and 0.1% sodium azide for 30 min. The sections were then incubated overnight at 4° C with the primary antibody. Rabbit anti-human polyclonal antibody to NK_1 receptor was used (1:1500 dilution). The ability of the antibody to recognize selectively the human NK_1 receptor is indicated by the fact that it was generated against a synthetic peptide corresponding to the last 15 amino acid residues of the carboxy terminus of the human NK_1 receptor (residues 391–406). This sequence is different from the corresponding sequences of the human NK_2 and NK_3 receptors. A description and characterization of this antibody has been reported (23). Negative controls were performed by preabsorbing the antibody with the immunogenic peptide diluted at 100 μM in the antibody 1:1000 dilution, and incubating for at least 4 h before application to the tissue. Further negative controls were performed by omission of the primary antibody and by substituting the primary antibody with rabbit preimmune serum.

After washing in PBS, the sections were incubated for 30 min with biotinylated swine anti-rabbit IgG antibody (E431; Dako, High Wycombe, UK). Sections were washed and incubated for 60 min with streptavidin–biotin complex reagent (StreptABComplex/HRP, KO377; Dako). Immunoreactivity was visualized with diaminobenzidine. Sections were dehydrated and mounted in Eukitt (Electron Microscopy Sciences, Fort Washington, PA).

Materials

[Sar^9, $Met(O_2)^{11}$]SP and [βAla^8]NKA(4–10) were purchased from Bachem (Budendorf, Switzerland). Acetylcholine, captopril, phosphoramidon, L-NMMA, D-NMMA, and indomethacin were from Sigma (St. Louis, MO). CP-99,994 was a gift from J. A. Lowe III (Pfizer, Groton, CT). SR 48968 and SR 140333 were gifts from X. Emonds-Alt (Sanofi Recherche, Montpellier, France). *myo*-[^{3}H] Inositol (specific activity, 10–20 Ci/mmol) was purchased from Amersham International (Amersham, Buckinghamshire, UK).

Statistical Analysis

All data are expressed as means ± SEM Statistical analysis was performed by analysis of variance and the Dunnett test for multiple comparisons or Student *t* test for unpaired data when applicable. Statistical significance was accepted at a level of $p < 0.05$. pEC_{50} is the negative log of the molar concentration of the agonist producing 50% of the maximum response induced by the agonist.

RESULTS

Isometric Tension Measurement

After the equilibration period, 87% of the human medium-size isolated bronchi (2–5 mm in diameter) responded to ACh (1 mM), and 58% of the preparations responded to [Sar^9,

$Met(O_2)^{11}$]SP (Figure 1). Only one preparation that responded to [Sar^9, $Met(O_2)^{11}$]SP did not respond to ACh. This preparation was excluded from the study. Contraction in response to ACh (1 mM) was 1.25 ± 0.21 g (n = 11) (Figure 1). In a small percentage (12%) of the preparations studied, exposure to [Sar^9, $Met(O_2)^{11}$]SP caused a relaxant response that was not related to the concentration used (data not shown). In the remaining preparations (30%), [Sar^9, $Met(O_2)^{11}$]SP was inactive.

In intact human bronchi [Sar^9, $Met(O_2)^{11}$]SP (0.01 nM–10 μM) caused a concentration-related contraction with a pEC_{50} of 7.02 ± 0.6 (n = 9). Maximum contraction to [Sar^9, $Met(O_2)^{11}$]SP was 40.2 ± 3.4% (n = 9) of the response to ACh (1 mM) and 44 ± 5% (n = 6) of the response to [βAla^8]NKA(4–10) (pEC_{50} 7.33 ± 0.20, E_{max} 91.7 ± 6.3% of ACh, n = 8) (Figure 1). Pretreatment with the NK_1 receptor antagonist CP-99,994 or SR 140333 (both 0.1 μM) caused a significant shift to the right of the concentration-response curve to [Sar^9, $Met(O_2)^{11}$]SP (Figure 2). Contraction to [Sar^9, $Met(O_2)^{11}$]SP was abolished in the presence (1 μM) of either CP-99,994 or SR 140333. The concentration–response curve to [Sar^9, $Met(O_2)^{11}$]SP obtained from intact human bronchial rings (pEC_{50} 7.32 ± 0.52, E_{max} 39 ± 6% of ACh, n = 12) was not significantly different from the curves obtained from epithelium-denuded (pEC_{50} 7.66 ± 0.7, E_{max} 31 ± 5% of ACh, n = 8) preparations or in preparations pretreated with indomethacin (pEC_{50} 7.41 ± 0.6, E_{max} 34 ± 5% of ACh, n = 9). The contractile response to [Sar^9, $Met(O_2)^{11}$]SP was similar in bronchial rings pretreated with either the NO synthase inhibitor L-NMMA (pEC_{50} 7.33 ± 0.6, E_{max} 38 ± 4% of ACh, n = 9) or its inactive enantiomer D-NMMA (pEC_{50} 7.51 ± 0.4, E_{max} 33 ± 4% of ACh, n = 9).

Inositol Phosphate Measurement

In segments of medium-size human bronchi (2–5 mm in diameter), [Sar^9, $Met(O_2)^{11}$]SP (0.25 μM) caused a small increase in total [^{3}H]IP that was 22 ± 3% of the response produced by ACh (100 μM) (Table 1). In the presence of the NK_1 receptor antagonist SR 140333, the increase in [^{3}H]IP level produced by [Sar^9, $Met(O_2)^{11}$]SP was virtually abolished. Incubation of the tissues with indomethacin (2 μM) did not affect the response to [Sar^9, $Met(O_2)^{11}$]SP. In segments of medium-size human bronchi the response to NKA was similar (22 ± 4% of ACh) to that of [Sar^9, $Met(O_2)^{11}$]SP (Table 1).

In segments from small human bronchi (~ 1 mm in diameter), [Sar^9, $Met(O_2)^{11}$]SP caused a small increase in [^{3}H]IP release (6 ± 0.3% of ACh 100 at μM). This response was completely abolished by SR 140333 and by indomethacin. In segments from small-size bronchi the [^{3}H]IP increase induced by NKA was about 10-fold higher (55 ± 2% of ACh at 100 μM) than the response to [Sar^9, $Met(O_2)^{11}$]SP (Table 1).

Immunohistochemistry

Intense positive immunostaining for the NK_1 receptor was observed in the bronchial smooth muscle layer of medium-size bronchi (Figure 3A). Similar findings were obtained in small-size bronchi (~ 1 mm in diameter), where the immunostaining for the NK_1 receptor was localized to the bronchial smooth muscle layer (Figure 3C). Preabsorption of the anti-NK_1 antibody with the immunogenic peptide completely abolished the staining in both medium (Figure 3B) and small (Figure 3D) bronchi. Although staining for the NK_1 receptor was observed in all the tissues examined, particularly intense staining was observed in the smooth muscle layer of 5 of the 8 medium-size and in 7 of the 11 small-size bronchi studied (data not shown). Samples for immunohistochemistry were taken from bronchi different from those used in functional experiments. For this reason a correlation study between intensity of immunostaining and magnitude of the functional response was not performed.

DISCUSSION

Tachykinin NK_2 receptor activation causes robust contraction of bronchial smooth muscle of most mammal species, including humans (8, 9, 14, 24). In most species the NK_2 receptor seems the sole tachykinin receptor subtype involved in mediating tachykinin-induced airway smooth muscle contraction (8, 9, 14, 24). There are, however, a few examples indicating that NK_1 receptors may contribute to tachykinin-induced bronchoconstriction. Thus, in guinea pigs both *in vitro* and *in vivo* tachykininergic bronchoconstriction is abolished completely by the combination of NK_2 and NK_1 receptor antagonists (11–13). In rats NK_1 receptor agonists cause a bronchocon-

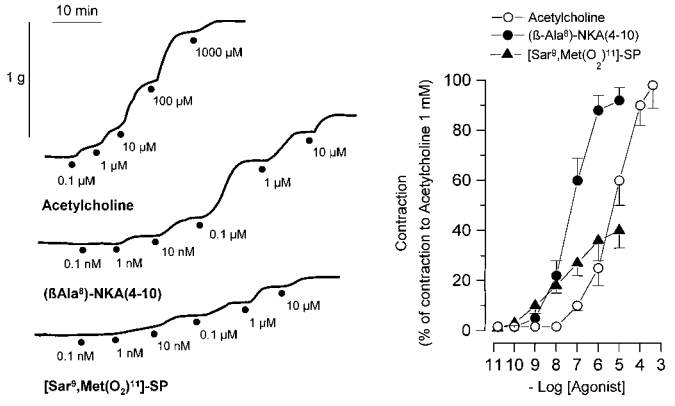

Figure 1. Typical tracings (*left*) and pooled data (*right*) of the contractile response to acetylcholine, the selective agonists of tachykinin NK_2, [βAla^8]NKA(4–10), and NK_1, [Sar^9, $Met(O_2)^{11}$]SP, receptors in human medium-size (2–5 mm in diameter) isolated bronchial rings in the presence of the tachykinin NK_2 receptor antagonist SR 48968 (1 μM).

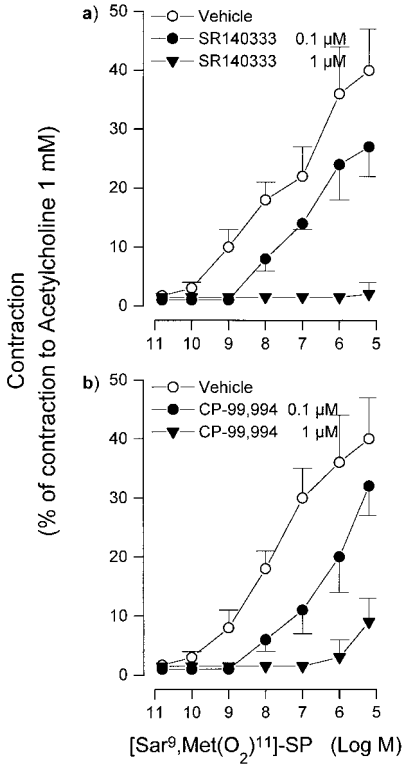

Figure 2. Effect of two different tachykinin NK_1 receptor antagonists, SR 140333 and CP-99,994, on the concentration–response curve to [Sar^9, $Met(O_2)^{11}$]SP in human medium-size (2–5 mm in diameter) isolated bronchial rings, in the presence of the tachykinin NK_2 receptor antagonist SR 48968 (1 μM). Entries represent means ± SEM of at least six experiments.

TABLE 1. INCREASE IN [^{3}H]INOSITOL PHOSPHATE INDUCED BY [Sar^9, $Met(O_2)^{11}$]SP OR NKA AS A PERCENTAGE OF THE INCREASE PRODUCED BY 100 μM ACETYLCHOLINE IN SEGMENTS OF HUMAN BRONCHI*

	NK_1 Agonist [Sar^9, $Met(O_2)^{11}$]SP (*0.25 μM*)	NK_2 Agonist NKA (*0.025 μM*)
	Medium-size Bronchi	
Vehicle	22 ± 3	22 ± 4
SR 140333 (1 μM)	1 ± 0.1†	
Indomethacin (5 μM)	21 ± 6	
	Small-size Bronchi	
Vehicle	6 ± 0.3	55 ± 2
SR 140333 (1 μM)	0.2 ± 0.06†	
Indomethacin (5 μM)	0.5 ± 0.09†	

* Each entry represents the mean ± SEM of at least six experiments.
† $p < 0.05$ versus vehicle (0.5% DMSO).

strictor response that is mediated indirectly by mediator release from mast cells (10).

In human bronchi tachykinins are amongst the most powerful bronchoconstrictor agents (15). This remarkable bronchoconstriction is mediated by NK_2 receptors, and previous evidence has suggested that this receptor subtype is the sole receptor involved in tachykinin-mediated bronchoconstriction (9, 14). However, data have shown that in small-size (∼ 1 mm in diameter) human bronchi NK_1 receptors mediate a moderate, although significant, bronchoconstriction (16). This effect, being abolished by indomethacin pretreatment, was ascribed to an indirect mechanism that consists of the activation of NK_1 receptor and the subsequent release of contractile prostanoids (16).

More recent immunohistochemical data, obtained in sections of human bronchi with an antiserum directed against the carboxyl terminus of the human NK_1 receptor (18), indicated the presence of specific staining on the smooth muscle layer of bronchi of different sizes. These observations led us to investigate whether NK_1 receptors may cause motor effects in the bronchial smooth muscle of medium-size (2–4 mm in diameter) human bronchi. Results indicate that NK_1 receptors mediate a moderate bronchoconstrictor response that is apparently due to a direct action of the agonist on receptors located on smooth muscle cells. This conclusion is based on the following observations. First, in a large proportion of the samples tested, the selective NK_1 receptor agonist [Sar^9, $Met(O_2)^{11}$]SP caused a concentration-dependent increase in tone that was blocked by two different selective and chemically unrelated NK_1 receptor antagonists CP-99,994 and SR 140333. The presence of the NK_2 receptor antagonist, SR 48968 further excluded any contribution of this receptor subtype in the response induced by [Sar^9, $Met(O_2)^{11}$]SP. Second, the bronchomotor response to [Sar^9, $Met(O_2)^{11}$]SP was unaffected by epithelium removal or by pretreatment with indomethacin. These latter findings excluded a role of the epithelium, and of epithelial or extraepithelial prostanoids, in the bronchoconstriction mediated by NK_1 receptors. Numerous tachykinin-immunoreactive nerve fibers have been localized around intrinsic neurons and local bronchial ganglia (14). Tachykinin receptors, including the NK_1 receptor, have been shown to affect cholinergic transmission (6); it may be possible that NK_1 receptor activation may stimulate bronchoconstriction indirectly via ACh release from postganglionic parasympathetic neurons. However, because atropine did not affect [Sar^9, $Met(O_2)^{11}$]SP-induced bronchoconstriction this hypothesis is excluded in human bronchi.

The data from the functional study were further corroborated by the biochemical findings. [Sar^9, $Met(O_2)^{11}$]SP was able to increase IP levels in segments taken from medium-size human bronchi. Likewise bronchoconstriction, the increase in IP induced by [Sar^9, $Met(O_2)^{11}$]SP was unaffected by indomethacin and was blocked by SR 140333. In contrast with results obtained in medium-size bronchi, the slight increase in IP lev-

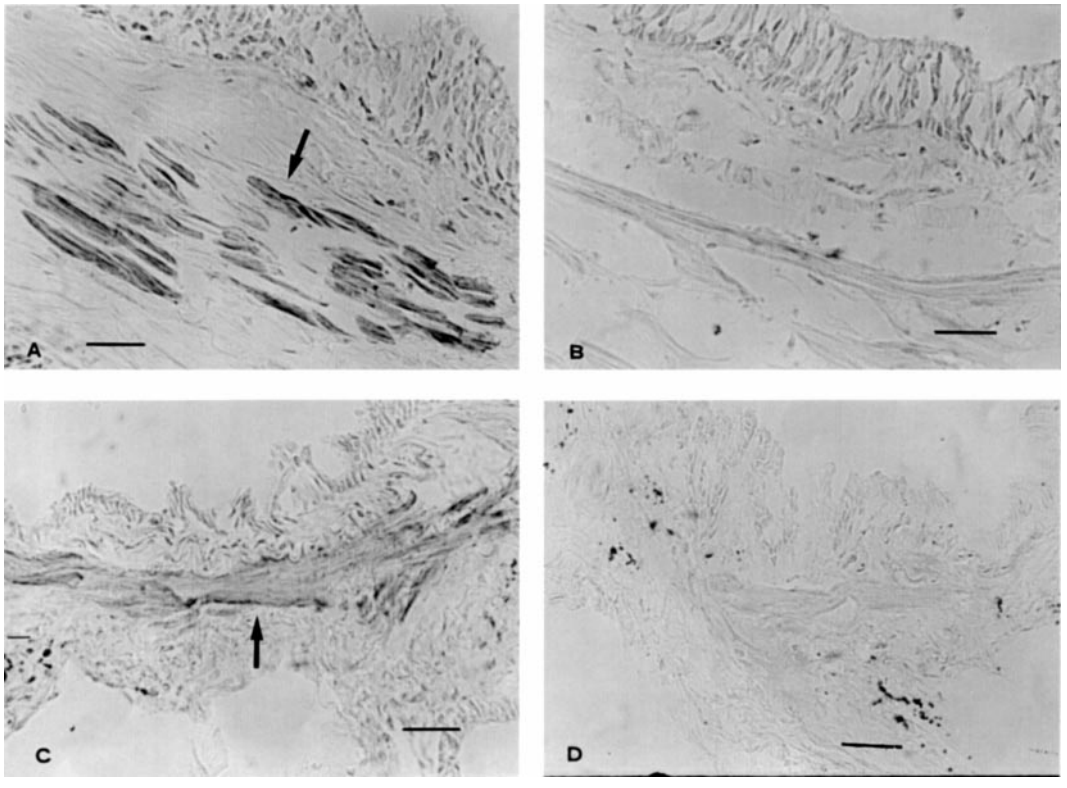

Figure 3. Microphotographs showing NK_1 receptor immunostaining in the smooth muscle layer (*arrows*) of medium-size (*A*, 2–5 mm in diameter) and small-size (*C*, ∼ 1 mm in diameter) human bronchi. Preincubation with the immunizing peptide abolished the staining in both medium (*B*) and small (*D*) bronchi. Bar: 100 μm.

els produced by [Sar^9, Met(O$_2$)^{11}]SP in small-size bronchi was blocked by indomethacin. These findings are consistent with previous observations that the contraction induced by [Sar^9, Met(O$_2$)^{11}]SP in small-size bronchi was also abolished in preparations pretreated with indomethacin (16). Biochemical and functional data of the present study are qualitatively similar. However, IP increases caused by NKA and [Sar^9, Met(O$_2$)^{11}]SP do not seem to reflect the magnitude of the contractile responses produced by these tachykinins. One possible explanation for the discrepancy is that biochemical results originated from cryopreserved samples and functional data were obtained in fresh tissues. However, previous investigation did not report any significant difference in either the contractile response or IP accumulation between cryopreserved and fresh bronchial tissues stimulated with different agents, including ACh, tachykinins, kinins, and leukotrienes (21). Thus, the reason for the discrepancy remains unresolved.

The present results indicate that different bronchoconstrictor mechanisms are activated by NK_1 receptors in proximal (medium-size) and distal (small-size) portions of human intrapulmonary bronchi. In distal (small-size) bronchi, NK_1 receptors mediated an indirect, prostanoid-dependent contraction (16), whereas in proximal (medium-size) bronchi, NK_1 receptors mediated a prostanoid-independent contraction that is likely due to a direct effect on bronchial smooth muscle cells. Contraction in response to NK_1 receptor stimulation was observed in a majority (60%) of the samples tested, but not in all of them. In addition to the variability, intrinsic to pathological human samples, one possible explanation for this finding is that NK_1 receptor expression may vary greatly between different samples. This hypothesis may find some support from the observation that staining with the NK_1 receptor antiserum in the bronchial smooth muscle was less pronounced in a significant proportion (about 60%) of tissues tested. However, because immunohistochemistry and functional experiments were done in different bronchi, often derived from different patients, no correlation study was performed between the intensity of the staining and the magnitude of the contractile response.

A direct bronchoconstrictor effect produced by NK_1 receptors on bronchial smooth muscle cells via an increase in IP level is not unique to human preparations. There is evidence in guinea pigs that stimulation by [Sar^9, Met(O$_2$)^{11}]SP produces bronchoconstriction directly, via activation of smooth muscle NK_1 receptors (11-13). However, in guinea pigs NK_1 receptor agonists may also cause bronchodilatation indirectly, via activation of epithelial NK_1 receptors that release nitric oxide (NO) (25). This complex motor mechanism does not seem to occur in human bronchi, as the NO synthase inhibition did not affect the bronchoconstrictor response to [Sar^9, Met(O$_2$)^{11}]SP.

In contrast with present findings, previous studies (9, 14) have not reported a significant contraction produced by NK_1 receptor stimulation in human isolated bronchi. The reasons for the discrepancy may be due to the fact that in the present study a large number of samples have been investigated systematically, in the presence of a selective NK_2 receptor antagonist with the precise purpose to determine whether an NK_1-mediated response was present. In addition, it is worth mentioning that in the present investigation a significant proportion of bronchi (about 40%) failed to respond to the NK_1 agonist.

Tachykinin receptor antagonists are under scrutiny as potential bronchodilators and antiinflammatory drugs in airway disease (5, 6, 26). Traditionally, NK_1 receptor antagonists are regarded as antiinflammatory drugs because they may reduce vasodilatation and plasma protein extravasation (27, 28), whereas NK_2 receptor antagonists are regarded as bronchodilators, because they have the potential to inhibit tachykinin-induced airway smooth muscle contraction (29).

Present data show the bronchoconstrictor role of NK_1 receptors in human bronchi of different sizes, emphasizing the potential of NK_1 receptor antagonists as bronchodilator agents. The presence of tachykinin immunoreactivity in nerve fibers in the bronchial smooth muscle layer (14) supports a role for endogenous tachykinins to activate contractile NK_2 and NK_1 receptors. However, final proof that endogenous tachykinins may exert a motor function in human airways has not yet been presented. In addition, it is not known whether the present *in vitro* data may be of significance in *in vivo* conditions. In this respect, it might be of interest to investigate whether in asthma selective NK_1 receptor agonists cause bronchoconstriction *in vivo*, and whether the combination of NK_1 and NK_2 receptor antagonists or the more recently developed dual NK_1 and NK_2 receptor antagonists (30, 31) may afford better protection than selective NK_2 receptors antagonists against bronchoconstriction induced by nonselective naturally occurring tachykinins.

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References

1. Maggi C. The pharmacology of the efferent function of sensory nerves. *J Auton Pharmacol* 1991;11:173–208.
2. Holzer P. 1988. Local effector functions of capsaicin-sensitive sensory nerves endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 1988;24:739–768.
3. Geppetti P, Holzer P. Neurogenic inflammation. Boca Raton, FL: CRC Press; 1996.
4. Geppetti P. Sensory neuropeptide release by bradykinin: mechanisms and pathophysiological implications. *Regul Pept* 1993;47:1–23.
5. Geppetti P, Tognetto M, Trevisani M, Amadesi S, Bertrand C. Tachykinins and kinins in airway allergy. *Exp Opin Invest Drugs* 1999;8:947–956.
6. Advenier C, Lagente V, Boichot E. The role of tachykinin receptor antagonists in the prevention of bronchial hyperresponsiveness, airway inflammation and cough. *Eur Respir J* 1997;10:1892–1906.
7. Regoli D, Boudon A, Fauchere J-L. Receptors and antagonists for substance P and related peptides. *Pharmacol Rev* 1994;46:551–599.
8. Sheldrick RL, Ball DI, Coleman RA. Characterization of the neurokinin receptor mediating contraction of isolated tracheal preparations from a variety of species. *Agents Actions Suppl* 1990;31:205–210.
9. Advenier C, Naline E, Toty L, Bakdach H, Emonds-Alt X, Vilain P, Breliere JC, Le Fur G. Effects on the isolated human bronchus of SR 48968, a potent and selective nonpeptide antagonist of the neurokinin A (NK_2) receptors. *Am Rev Respir Dis* 1992;146:1177–1181.
10. Joos GF, Kips JC, Pauwels RA. In vivo characterization of the tachykinin receptor involved in the direct and indirect bronchoconstrictor effect of tachykinins in two inbred rat strains. *Am J Respir Crit Care Med* 1994;149:1160–1166.
11. Maggi CA, Patacchini R, Rovero P, Santicioli P. Tachykinin receptors and noncholinergic bronchoconstriction in the guinea-pig isolated bronchi. *Am Rev Respir Dis* 1991;144:363–367.
12. Boni P, Ballati L, Evangelista S. Tachykinin NK_1 and NK_2 receptors mediate the non-cholinergic bronchospastic response to capsaicin and vagal stimulation in guinea–pigs. *J Auton Pharmacol* 1995;15:49–54.
13. Bertrand C, Nadel JA, Graf PD, Geppetti P. Capsaicin increases airflow resistance in guinea pigs in vivo by activating both NK_2 and NK_1 tachykinin receptors. *Am Rev Respir Dis* 1993;148:909–914.
14. Sheldrick RL, Rabe KF, Fischer A, Magnussen H, Coleman RA. Further evidence that tachykinin-induced contraction of human isolated bronchus is mediated only by NK_2-receptors. *Neuropeptides* 1995;29:281–292.
15. Joos GF, Pauwels RA, Van der Straeten ME. The effect of inhaled substance P and neurokinin A on the airways of normal and asthmatic subjects. *Thorax* 1987;42:779–782.
16. Naline E, Molimard M, Regoli D, Emonds-Alt X, Bellamy JF, Advenier C. Evidence for functional tachykinin NK1 receptors on human isolated small bronchi. *Am J Physiol* 1996;271:L763–L767.

17. Bai TR, Zhou D, Weir T, Walker B, Hegele R, Hayashi S, McKay K, Bondy GP, Fong T. Substance P (NK_1)- and neurokinin A (NK_2)-receptor gene expression in inflammatory airway diseases. *Am J Physiol* 1995;269:L309–L317.
18. Mapp C, Miotto D, Braccioni M, Saetta M, Turato G, Mestrelli P, Krause JE, Karpinskiy V, Boyd N, Geppetti P, Fabbri LM. The distribution of neurokinin-1 and neurokinin-2 receptors in human central airways. *Am J Respir Crit Care Med* 2000;161:207–215.
19. McLean S, Ganong A, Seymour PA, Snider RM, Desai MC, Rosen T, Bryce DK, Longo KP, Reynolds LS, Robinson G, *et al*. Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *J Pharmacol Exp Ther* 1993;267:472–479.
20. Emonds-Alt X, Doutremepuich JD, Heaulme M, Neliat G, Santucci V, Steinberg R, Vilain P, Bichon D, Ducoux JP, Proietto V, *et al.* In vitro and in vivo biological activities of SR140333, a novel potent non-peptide tachykinin NK1 receptor antagonist. *Eur J Pharmacol* 1993;250: 403–413.
21. Sarria B, Naline E, Cortijo J, Moreau J, Cerda JM, Morcillo EJ, Advenier C. Functional, biochemical and morphological studies on human bronchi after cryopreservation. *Br J Pharmacol* 1995;116:2569–2574.
22. Shu SY, Ju G, Fan LZ. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988;85: 169–171.
23. MacDonald D, Silberman SC, Lowe JA III, Drozda SE, Leeman SE, Boyd ND. Photoaffinity labeling of the human substance P (neurokinin-1) receptor with [$^3H_2$]azido-CP-96,345, a photoreactive derivative of a nonpeptide antagonist. *Mol Pharmacol* 1996;49:808–813.
24. Geppetti P, Betrand C, Bacci E, Huber O, Nadel JA. Characterization of tachykinin receptors in the ferret trachea by peptide agonists and non-peptide antagonists. *Am J Physiol* 1993;265:L164–L169.
25. Figini M, Emanueli C, Bertrand C, Javdan P, Geppetti P. Evidence that tachykinins relax the guinea-pig trachea via nitric oxide release and by stimulation of a peptide-insensitive NK_1 receptor. *Br J Pharmacol* 1996;115:128–132.
26. Barnes PJ, Belvisi MG, Rogers DF. Modulation of neurogenic inflammtion: novel approaches to inflammatory diseases. *Trends Pharmacol Sci* 1990;11:185–189.
27. Lundberg JM. Tachykinins, sensory nerves, and asthma—an overview. *Can J Physiol Pharmacol* 1995;73:908–914.
28. Piedimonte G, Hoffman JI, Husseini WK, Snider RM, Desai MC, Nadel JA. NK1 receptors mediate neurogenic inflammatory increase in blood flow in rat airways. *J Appl Physiol* 1993;74:2462–2468.
29. Van Schoor J, Joos GF, Chasson B, Brouard RJ, Pauwels RA. The effect of SR 48968, a nonpeptide neurokinin-2 receptor antagonist on neurokinin A-induced bronchocostriction in asthmatics. *Eur Respir J* 1998; 12:17–23.
30. Robineau P, Lonchampt M, Kucharczyk N, Krause JE, Regoli D, Fauchere JL, Prost JF, Canet E. In vitro and in vivo pharmacology of S 16474, a novel dual tachykinin NK_1 and NK_2 receptor antagonist. *Eur J Pharmacol* 1995;294:677–684.
31. Kudlacz EM, Knippenberg RW, Logan DE, Burkholder TP. The effect of MDL 105,172A, a nonpeptide NK_1/NK_2 receptor antagonist, in an allergic guinea pig model. *J Pharmacol Exp Ther* 1996;279:732–739.