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Tachykinin-Induced Phosphoinositide Breakdown in Airway Smooth Muscle and Epithelium: Relationship to Contraction

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SUMMARY

We have studied the contractile response and phosphoinositide hydrolysis induced by substance P (SP), neurokinin A (NKA), neurokinin B (NKB), and Alp-Phe-Phe(R)-Gly[ANC-2]-Leu-Met-NH_2 (L 363851), a selective NK_2-receptor agonist, in guinea pig tracheal smooth muscle. The four tachykinins elicited a concentration-dependent contraction in tracheal smooth muscle devoid of epithelium, with the following order of potency: NKA>L 363851>NKB>SP, (EC_{50} 1.0×10^{-9} M, 3.2×10^{-9} M, 7.5×10^{-9} M and 1.2×10^{-7} M, respectively), which suggests that NK_2 receptors predominate in airway smooth muscle. In the presence of epithelium, the sensitivity of airway smooth muscle to tachykinins was decreased, and the concentration response curves to tachykinins were shifted rightward by 30-fold for SP, 9-fold for NKA, and 5-fold for NKB. The concentration response curve to L 363851 was not significantly shifted in the presence of epithelium. This suggests that epithelium may release a relaxant factor in response to tachykinins via an NK_1 receptor. In airway smooth muscle, we found that tachykinins elicited phosphoinositide breakdown with an order of potency similar to that for contractile response (EC_{50} 2.2×10^{-5} M, 3.6×10^{-5} M, 4.4×10^{-5} M, and 5.9×10^{-5} M). In epithelium, SP alone elicited a significant phosphoinositide breakdown, suggesting that epithelial receptors to tachykinins may be of the NK_1 subtype. Since it is established that phosphoinositide derivatives can elicit mobilization of intracellular calcium, our results suggest that phosphoinositide breakdown is the coupling mechanism for tachykinin-induced contraction of airway smooth muscle.

Substance P (SP) is localized in the sensory nerve endings in lung of various species (1). This neuropeptide has several effects on airway function including contraction of airway smooth muscle *in vitro* and *in vivo* in various species including man (2–6), stimulation of airway mucus secretion (7), and increased airway microvascular permeability (8). The effect of SP may be mediated through the stimulation of specific receptors, which have recently been visualized in guinea pig and human lung by autoradiographic technique (9). The cellular mechanism by which SP induces contraction has not yet been determined, however. Recent evidence suggests that many bronchoconstrictors stimulate the breakdown of membrane phosphoinositides (PPI) whose derivatives, such as inositol phosphates (10, 11), are involved in intracellular calcium mobilization. SP induces PPI hydrolysis in salivary glands (12, 13) and bladder (14).

Two novel tachykinins, NKA and NKB, have recently been identified in the mammalian nervous system (15, 16). Both NKA and NKB are present in lung (17) and may have different effects from SP which might be explained by activation of different receptors. Three distinct tachykinin receptors have been identified, based on the relative potencies of tachykinins (18). NK_1 receptors are mainly activated by SP, whereas NK_2 and NK_3 receptors may be activated by NKA and NKB, respectively. NKA is more potent than SP in contracting guinea pig and human airways *in vitro* (5, 6) and *in vivo* (19, 20), suggesting the presence of NK_2 receptors in airway smooth muscle. Recently, another neuropeptide, Alp-Phe-Phe(R)-Gly[ANC-2]-Leu-Met-NH_2 (L 363851), has been synthetized as a highly selective agonist of NK_2 receptors, which has proved useful in studying tachykinin receptor subtypes (21). We have investigated the effects of SP, NKA, NKB, and L 363851 on contractile responses and PPI hydrolysis in guinea pig tracheal smooth muscle. Because airway epithelium modulates the contractile response of airway smooth muscle (22, 23), we have also studied the effects of these tachykinins on airway smooth muscle responses, in the presence or absence of epithelium.

Materials and Methods

Tissue preparation. Male Dunkin-Hartley guinea pigs (300–500 g) were sacrificed by cervical dislocation, and the trachea and lung were immediately removed. Tracheas were opened longitudinally through cartilage rings and placed in KH medium pH 7.4, gassed with 95% O_2/5% CO_2. In some experiments, airway epithelium was removed by gentle rubbing with a cotton swab as previously described (24).

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ABBREVIATIONS: SP, substance P; PPI, phosphoinositide; NK, neurokinin; KH, Krebs-Henseleit (buffer); IP, inositol monophosphate.

Contraction studies. Segments of guinea pig trachea (four cartilaginous rings), with or without epithelium, were mounted in 10-ml organ baths containing KH solution gassed continuously with 95% O_2/5% CO_2 at 37° for isometric recording of contraction. A tension of 1 *g* was first applied and the rings were washed three times at 15-min intervals. The tension was then increased to 2 *g*, which was optimal for recording changes in tension. Tension was measured with Grass FT.03 transducers (Grass Instruments, Quincy, MA) coupled to a polygraph (Grass 7D). Cumulative concentration response curves were constructed for SP, NKA, NKB, and L 363851 (10^{-10}–10^{-5} M) by incremental addition of 0.5 log unit (M) tachykinin, in the presence of indomethacin (3×10^{-6} M) to inhibit the formation of cyclooxygenase products. Each ring was used to perform one concentration response curve to tachykinin only. Preliminary experiments showed that preincubation of tissue for 15 min with 10^{-7} M SP and subsequent washing did not significantly modify the concentration response curve to tachykinins, thus demonstrating that there was no significant tachyphylaxis to this agonist.

EC_{50} values for the concentration response curve were determined by linear regression of all points between 20 and 80% of the maximal response to each agonist. Maximal contraction was expressed as percentage of the maximal response to SP.

PPI breakdown. In order to reduce the basal level of PPI breakdown, guinea pigs were injected with indomethacin (15 mg, dissolved in 1 ml of Tris buffer, pH 10–11) 13 hr and 1 hr before the experiment, and with phentolamine (10 mg) 1 hr before the experiment, to reduce stimulation by cyclooxygenase products and norepinephrine, respectively.

PPI breakdown was determined using the method of Berridge *et al.* (25), as previously described (10, 11), with lithium chloride to inhibit inositol phosphate degradation and allow direct measurement of tachykinin-stimulated phospholipase C-specific phospholipid hydrolysis. Under such conditions, all inositol phosphates collected arise from PPI breakdown (25, 26). For smooth muscle studies, whole trachea was preincubated for 2 hr in KH solution containing 10^{-5} M indomethacin, during which time buffer was changed three times. Tissue was then washed and incubated for 90 min with 0.3 μM [^{3}H]inositol in order to label membrane PPI. Tracheas were then washed and cut in four to six pieces of the same weight. Each piece was placed in 1 ml of KH solution containing 10 mM lithium chloride while continuously gassed with 95% O_2/5% CO_2, prior to addition of tachykinins for 30 min. Final incubation KH solution contained 0.1% bovine serum albumin, 5 mg/liter chymostatin, 4 mg/liter leupeptin, 40 mg/liter bacitracin, and 5000 units/ml aprotonin to prevent enzymatic degradation of tachykinins. The incubation was stopped by addition of 3.75 ml of a chloroform/methanol/HCl (1 M) (v/v, 100:200:2) solution (27), because acid improves the recovery of the more polar inositol phosphates (28). Tissue was then homogenized with a Polytron (Kinematica, Switzerland) at speed 5 for 60 sec in this mixture. Chloroform (1.25 ml) and water (1.75 ml) was then added to separate the phases. The methanol/water phase was then applied to a Dowex resin in the formate form, and inositol phosphates were collected in buffers of increasing strength, which were used to wash the columns. Inositol was eluted with 5 ml of water, glycerophosphoinositol was eluted with 5 ml of 60 mM sodium formate and 5 mM sodium tetraborate, IP was eluted with 8 ml of 0.2 M ammonium formate and 5 mM sodium tetraborate, inositol bisphosphate was eluted with 6 ml of 0.3 M ammonium formate and 0.1 M formic acid, and inositol trisphosphate was eluted with 5 ml of 0.75 M ammonium formate and 0.1 M formic acid. In the presence of lithium, IP accumulated more than other inositol phosphates, and we measured IP accumulation to monitor PPI hydrolysis. A total of 0.5 ml of the third elution buffer (0.2 M ammonium formate and 5 mM sodium tetraborate) was collected and added to 4.5 ml of scintillation fluid (Hydrofluor, National Diagnostics, London, UK) and counted in a scintillation counter at an efficiency of 53% (LKB Instruments, Croydon, UK).

For studies of epithelial cells, epithelium was gently scraped with a spatula and immediately resuspended in KH solution, preincubated in KH solution, pH 7.4, continuously gassed with 95% O_2/5% CO_2 for 1 hr, and then incubated with 0.2 μM [^{3}H]inositol in KH solution containing 10 mM LiCl for 90 min at 37°. Cells were then filtered through a gauze, adjusted at a concentration of approximately 10^{-6} cells/ml, and aliquoted, and tachykinins were added for 30 min. Incubation was stopped as for smooth muscle. Because guinea pig trachea is devoid of submucosal glands (29), this technique results in the isolation of epithelial cells predominantly.

Each incubation was performed in triplicate. Data was expressed as means ± standard errors. Statistical significance was determined by Student's *t* test.

Drugs and chemicals. SP was purchased from Sigma, (Poole, Dorset, UK), NKA and NKB were from Bachem (Saffron Walden, Essex, UK), and Dowex resin and sulpholane were from British Drug Houses (Poole, Dorset, UK). L 363851 was a generous gift from Dr. L. L. Iversen (Merck, Sharp and Dohme Research Laboratories, Harlow, UK). [^{3}H]Inositol (specific activity, 30 Ci/mmol) was purchased from Amersham International. SP and NKA were prepared in distilled water and NKB in sulpholane, containing 0.1% bovine serum albumin. KH medium had the following composition: 118 mmol NaCl, 5.9 mmol KCl, 1.2 mmol $MgSO_4\cdot7H_2O$, 26 mmol $NaHCO_3$, 2.5 mmol $CaCl_2\cdot6H_2O$, 1.2 mmol $NaPO_4\cdot H_2O$, 11 mmol glucose.

Results

Contraction studies. In guinea pig tracheal smooth muscle, after removal of epithelium, tachykinins elicited contraction in a concentration-dependent manner with the following order of potency: NKA>L 363851>NKB>SP [EC_{50}, 1.0 (±0.8) × 10^{-9} M, 3.2 (±0.3) × 10^{-9}M, 7.5 (±0.7) × 10^{-9}M, 1.2 (±0.2) × 10^{-7} M for NKA, NKB, L 33851 and SP, respectively; means SE; n = 7]. Concentration response curves to SP and NKB were flattened as compared to response curves to NKA and L 363851, suggesting the contribution of more than one receptor subtype. These results suggested that tachykinin receptors in tracheal smooth muscle were predominantly of the NK_2 type (Fig. 1). Maximal contractile responses to NKA, L 363851, and NKB were 124±5%, 120±6%, and 87±8% of that to SP, respectively (n = 7). Half-maximal contraction under the experimental conditions described above was obtained after a 15- to 20-min incubation time for NKA and a 20- to 30-min incubation time with SP or NKB.

In the presence of epithelium, concentration response curves to SP, NKA, and NKB were shifted to the right. The EC_{50} value was shifted to a greater extent with SP (30-fold) than with NKA and NKB (9- and 5-fold, respectively); in the presence of epithelium, the EC_{50} values were 2.9×10^{-6} M, 9.6×10^{-9} M, and 5.8×10^{-8} M for SP, NKA, and NKB, respectively. For L 363851, there was no significant shift in the presence of epithelium (1.4-fold leftward) with an EC_{50} value of 6×10^{-9} M (Fig. 2).

PPI breakdown. SP, NKA, NKB, and L 363851 elicited concentration-dependent PPI breakdown. All inositol phosphates were increased by tachykinins (Fig. 3). However, in the presence of lithium, IP accumulation was more enhanced than that of other inositol phosphates, as previously reported (10, 11, 24, 25). The order of potency was NKA>L 363851>NKB>SP, with EC_{50} values for IP accumulation of 2.2 (±0.3) × 10^{-5} M, 3.6 (±0.2) × 10^{-5} M, 4.4 (±0.4) × 10^{-5} M and 5.9 (±0.5) × 10^{-5} M for NKA, NKB, L 363851, and SP, respectively (mean ± SE; n = 6). The maximal IP accumulation above basal value was in the range of 100 to 220% for the four tachykinins (Fig. 1). Half-maximal PPI breakdown for tachy-

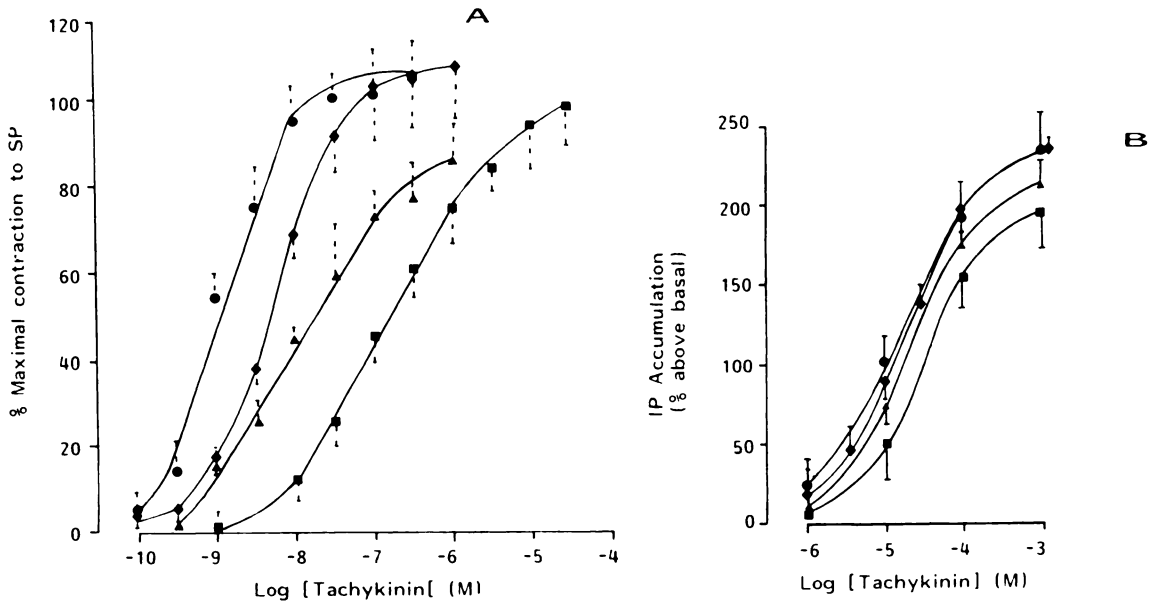

Fig. 1. Effect of tachykinins on guinea pig tracheal smooth muscle. A. Contractile concentration response curve to NKA (●), L 363851 (♦), NKB (▲), and SP (■) after epithelium removal. Results are expressed as percentage of maximal response to SP. Each point is the mean (±standard error) of seven experiments. B. IP accumulation in response to NKA (●), L 363851 (♦), NKB (▲), and SP (■) after epithelium removal. Results are expressed as percentage of IP accumulation above basal level. Each point is the mean (±standard error) of six experiments.

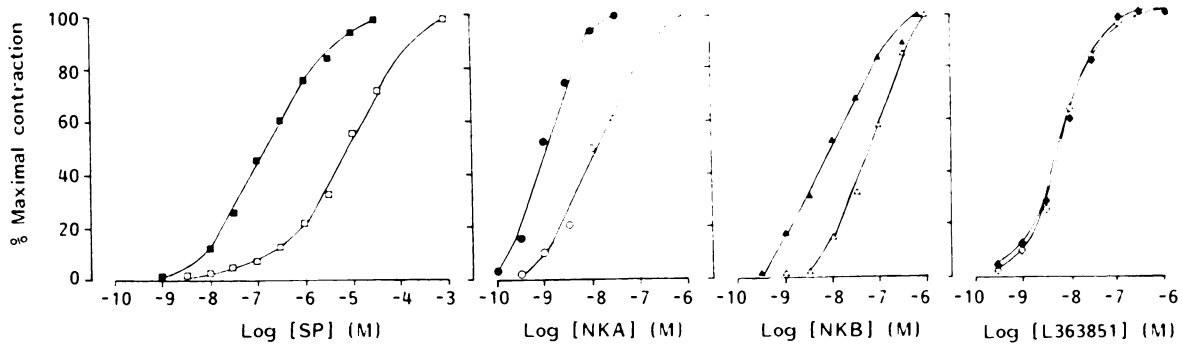

Fig. 2. Effect of airway epithelial removal on contractile response of guinea pig tracheal smooth muscle to tachykinins. Contractile concentration response curves to tachykinins in the presence (□, ○, △, ◇) or absence (■, ●, ▲, ♦) of epithelium. Each point is the mean of seven experiments. Each point is calculated as the percent of maximal response to a given tachykinin.

kinins at a concentration of 10^{-4} M was obtained after a 15- to 20-min incubation for the four tachykinins.

In epithelium, 10^{-5} M SP, but not the other tachykinins, elicited a significant increase (85%±12; $n = 8$) in IP accumulation above basal value (Fig. 4).

Discussion

We have shown that in guinea pig trachea, tachykinins cause a concentration-dependent contraction with the order of potency NKA>L 363851>NKB>SP. Our results are in agreement with previous data in this species *in vivo* (20) and *in vitro* (17), which suggest that NK_2 receptors are predominant. This is confirmed by the fact that L 363851, a highly selective NK_2 agonist (21), was potent in eliciting contraction.

The mechanisms by which tachykinins cause bronchoconstriction are not certain. A previous study of guinea pig trachea suggests that SP stimulated an increase in cyclic GMP (30), but this may be a secondary event in response to elevated calcium ions.

In guinea pig trachea, SP and related tachykinins stimulated PPI breakdown in a concentration-dependent manner. Again, the order of potency was NKA>L 363851>NKB>SP, suggesting that an NK_2 receptor was predominantly involved. This is presumably a direct effect of tachykinins, since all the experiments were performed in the presence of indomethacin to prevent formation of cyclooxygenase products. Tachykinin-induced PPI hydrolysis has been demonstrated in other tissues, such as salivary glands (12, 13) and urinary bladder (14). This indicates that PPI hydrolysis is a possible coupling mechanism for tachykinin-induced contraction, since PPI derivatives such as diacylglycerol and inositol phosphates are directly involved in contraction through activation of protein kinase C (31) and release of calcium from intracellular stores (32). A similar tachykinin-induced time course of PPI breakdown and contraction in our preparation suggests that PPI breakdown might be a coupling mechanism of tachykinin-induced concentration. The differences between tachykinins were far greater for contractile effect than for stimulation of PPI hydrolysis, however. This may be because of the presence of cellular elements other than airway smooth muscle cells in this preparation, such as

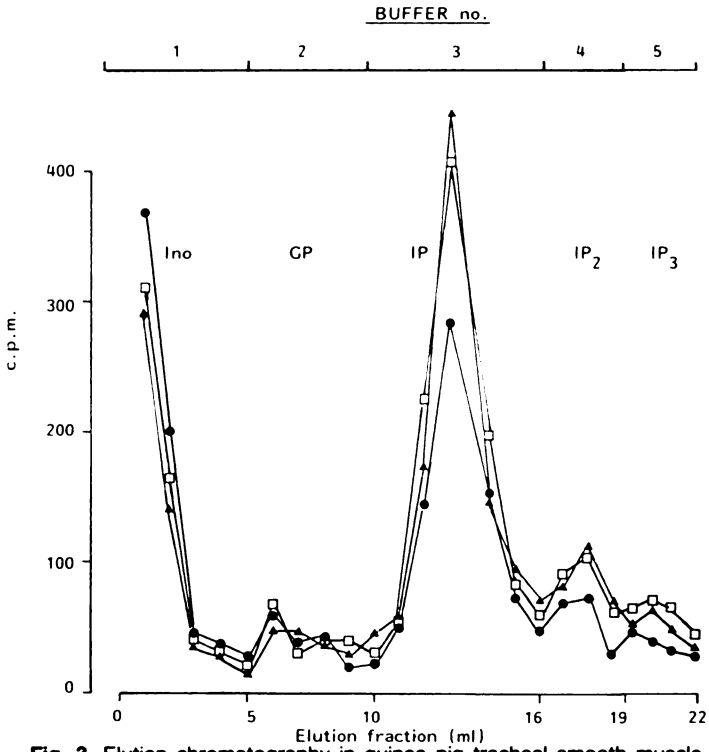

Fig. 3. Elution chromatography in guinea pig tracheal smooth muscle incubated with [^{3}H]inositol in the absence (●) or presence of NKA (▲) and SP (□), 10^{-5} M. Inositol (*Ino*) was collected in peak *1*, glycerophosphoinositol (*GP*) in peak *2*, IP in peak *3*, inositol bisphosphate (IP_2) in peak *4*, and inositol trisphosphate (IP_3) in peak 5. Results are expressed as cpm. Each point represents the mean of three separate incubations in one experiment. Similar results were obtained in five other experiments. An elution chromatography pattern similar to that with NKA and SP was obtained with NKB and L 363851. Mean (±standard error; cpm) IP accumulation above basal level in six different experiments was in the range of values shown in Fig. 1. Mean (±SE) inositol bisphosphate (IP_2) accumulation (cpm) values are: control, 30 ± 5; NKA, 93 ± 6;* L363851, 98 ± 7;* SP, 86 ± 10;* NKB, 75 ± 9.* Mean (±SE) inositol trisphosphate (IP_3) accumulation values are: control, 72 ± 15; NKA, 149 ± 14;* L 363851, 103 ± 16;* SP, 118 ± 19;* NKB, 90 ± 10* (*, *t* test, $p < 0.01$, compared to control value).

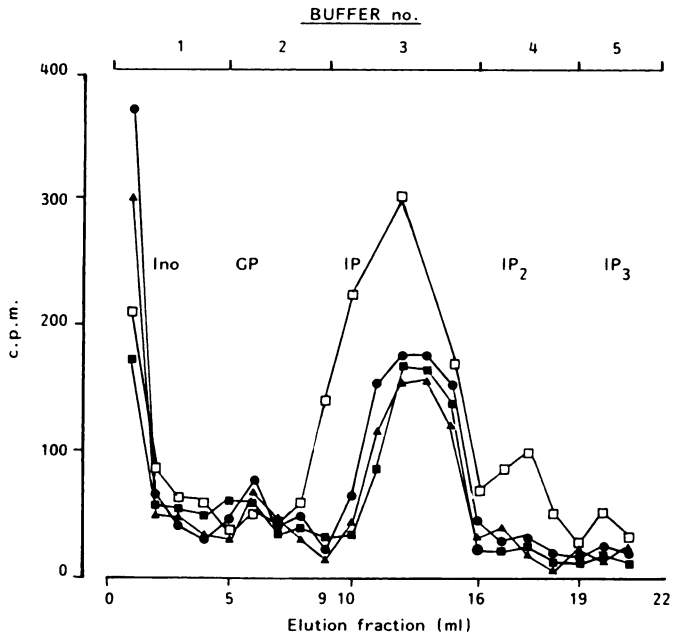

Fig. 4. Elution chromatography in guinea pig airway epithelial cells incubated with [^{3}H]inositol in the absence (▲) or presence of SP (□), NKA (●), and L 363851 (■), 10^{-6} M. Mean (±SE) inositol monophosphate (IP) accumulation in six different experiments was: control, 160 ± 15; NKA, 142 ± 17; L 363851, 150 ± 32; SP, 295 ± 24.* Mean inositol bisphosphate (IP_2) accumulation was: control 38 ± 6; NKA, 33 ± 5; L 363851, 41 ± 7; SP, 123 ± 13.* Mean inositol trisphosphate accumulation (IP_3) was: control, 65 ± 21; NKA, 72 ± 7; L 363851, 70 ± 14; SP, 112 ± 16 (*, $p < 0.01$). An elution chromatography pattern similar to that with NKA and L 363851 was obtained with NKB and showed no significant increase of any inositol phosphate above basal level.

vascular structures, which may have different receptors. It is possible that the densities of NK_1, NK_2, and NK_3 receptors are similar in airways, and since the magnitude of PPI turnover is related to receptor density (10, 11), inositol phosphate accumulation induced by the various tachykinins might therefore be similar. The maximal contractile response to tachykinins might therefore be similar. The maximal contractile response to tachykinins was obtained at concentrations lower than those required to elicit PPI breakdown, suggesting the presence of numerous "spare" receptors. Indeed, we have already shown that muscarinic contraction in airway smooth muscle requires less than 20% muscarinic receptor occupancy (11). The presence of SP "spare" receptors has been demonstrated in guinea pig ileal smooth muscle (33).

Tachykinin-induced contraction was enhanced by epithelial removal. These findings are in agreement with those of others who reported an enhancement of the contractile effect of several spasmogens in bovine (22), canine (23), and guinea pig trachea (34). There was a difference in the effect of epithelial removal on the contraction induced by SP, NKA, and NKB but not L 363851. Thus, for SP the concentration response curve was shifted to the right by 30-fold, but for NKA and NKB, the shift was less than 10 times, and was not significant for L 363851. This discrepancy is unlikely to be explained by differences in diffusion between the tachykinins, since their molecular weights are very close, and a nonspecific diffusion barrier effect cannot account for the differences between SP and the other tachykinins. It is possible that catabolism of peptides by epithelial enkephalinases depends on the peptide (35), and that SP is degraded more rapidly than other tachykinins by epithelium. It is also possible that a relaxant factor is released from epithelial cells, following stimulation of epithelial specific receptors, in a manner similar to the release of endothelium-derived relaxing factor from vascular endothelial cells (36), although such a factor has not yet been identified. The presence of epithelial removal was greatest for SP and did not occur with the highly selective NK_2 agonist L 363851. Therefore, our results suggest the presence of epithelial NK_1 receptors (SP-P type of receptor), since SP, but not NKA or NKB, stimulated PPI breakdown in airway epithelial cells. That NK_1 receptors predominate on airway epithelial cells is further supported by the demonstration that SP is more potent than NKA in stimulating epithelial ion transport in dog trachea (37).

Tachykinins are potent bronchoconstrictors of airway smooth muscle and their release from sensory nerve endings during chronic inflammation of airway disease might be of relevance in the pathogenesis of asthma (38). We have shown that tachykinins induce contraction and PPI hydrolysis in airway smooth muscle via an NK_2 receptor, and that these effects may be modified by an NK_1-receptor-mediated effect of airway epithelium.

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