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Role of 5-hydroxytryptamine and mast cells in the tachykinin-induced contraction of rat trachea in vitro

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Abstract

The in vivo bronchoconstrictor effect of tachykinins in Fisher 344 rats is accompanied by release into the airways of 5-hydroxytryptamine (5-HT). 5-HT is possibly derived from mast cells. In the present study the presumed mast cell–tachykinin interaction was studied in isolated trachea from Fisher 344 rats. Contractions induced by neurokinin A were largely reduced by the 5-HT antagonist methysergide, partially reduced by atropine, but not affected by hexamethonium or tetrodotoxin. Methysergide also inhibited the contractions induced by substance P, the tachykinin NK_1 receptor agonist Ac[Arg^6, Sar^9, $Met(O_2)^{11}$]substance P-(6–11) and the mast cell depleting compound 48/80. Methysergide had no effect on contractions induced by carbachol or electrical field stimulation. Atropine significantly reduced contractions to 5-HT and completely inhibited contractions induced by electrical field stimulation. Histamine had no contractile effect. In vivo pretreatment with compound 48/80 significantly reduced the in vitro contractions to neurokinin A. Contractions to capsaicin were inhibited by methysergide and the tachykinin NK_1 receptor antagonist (±)-RP67580 ((3*aR*,7*aR*)-(7,7-diphenyl-2-(1-imino-2-(2-methoxyphenylethyl)-perhydraisoinotol-4-one))). Substance P and neurokinin A caused 5-HT release in the organ bath, in a concentration- and time-dependent way. Atropine did not affect 5-HT release. Morphometric analysis showed that substance P and neurokinin A, but not carbachol, caused a significant increase in the number of degranulating mast cells in the muscular/submuscular region. In conclusion, tachykinins contract Fisher 344 rat trachea by releasing 5-HT from mast cells, an effect mediated by a tachykinin NK_1 receptor. © 1997 Elsevier Science B.V.

Keywords: Tachykinin; Bronchoconstriction; Substance P; Neurokinin A; Mast cell; 5-HT (5-hydroxytryptamine, serotonin); Tachykinin NK_1 receptor

1. Introduction

The tachykinins substance P and neurokinin A are present within airway sensory nerves and have been implicated as neurotransmitters of noncholinergic bronchoconstriction and neurogenic inflammation (Lundberg and Saria, 1987; Barnes et al., 1991a,b; Solway and Leff, 1991). Substance P and neurokinin A cause bronchoconstriction in normal persons and patients with asthma, asthmatics being hyperresponsive. The bronchoconstrictor effect of substance P and neurokinin A in asthmatics is prevented by pretreatment with sodium cromoglycate or nedocromil sodium. These studies indicate that exogenously administered substance P and neurokinin A are indirect bronchoconstrictors of human airways, an effect which can occur through the stimulation of inflammatory cells (e.g., mast cells) and/or nerves (Joos et al., 1989).

The mechanism of airway contraction induced by tachykinins has been mainly studied in animals. In guinea pigs tachykinins contract airway smooth muscle directly (Lundberg and Saria, 1982), whereas in rabbits cholinergic mechanisms largely contribute to airway narrowing (Tanaka and Grunstein, 1984). We previously reported that inbred rat strains may differ in the mechanisms and receptors involved in the bronchial response towards tachykinins. For instance the in vivo bronchoconstrictor effect of tachykinins in BDE rats was found to be a direct effect on airway smooth muscle by stimulation of tachykinin NK_2 receptors. In the more sensitive Fisher 344 rat strain, however, in vivo studies suggested that the bronchoconstrictor effect of tachykinins is indirect and mediated by

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5-HT and stimulation of tachykinin NK_1 receptors (Joos et al., 1988, 1994a). 5-HT is the principle contractile mast cell mediator in the rat (Church, 1975). The aim of the present study was to investigate in detail a possible contribution of mast cells in tachykinin-induced airway contraction by using isolated Fisher 344 rat trachea.

2. Materials and methods

2.1. Animals

Inbred, specific pathogen free Fisher 344 rats were purchased from Harlan CPB (Zeist, The Netherlands). They were male, aged between 3 and 4 months and weighed 250–300 g. After arrival at the Department, the animals were maintained in a conventional animal house for at least one week before they were tested.

2.2. Measurement of tracheal contraction

The rats were killed by an intraperitoneal injection of an overdose of pentobarbital (Nembutal®, 350 mg/kg body weight). The animals were exsanguinated and the trachea and lungs were quickly removed. From the distal part of the trachea 2 cylindrical segments, each containing 3 to 4 cartilaginous rings, were prepared. Each cylindrical segment was placed in a 2 ml organ bath. The two segments displayed a similar contractile response to carbachol and serotonin (Joos et al., 1994b). The bathing medium was Krebs solution (composition in mM: NaCl, 118; KCl, 4.6; $CaCl_2$, 2.5; $MgSO_4$, 1.15; $NaHCO_3$, 24.9; KH_2PO_4, 1.15 and glucose, 5.5), which was maintained at 37°C and bubbled with carbogen (5% CO_2 in oxygen). The tracheal segments were allowed to stabilize for 90 to 120 min with exchange of the bathing solution every 15 min. The optimal resting tension was 0.75 g as previously determined (Joos et al., 1994b). Contractions were measured isometrically with Grass FT03 transducers and recorded on a Graphtec Linearcorder type WR3701. After the stabilization period, carbachol 3.3×10^{-5} M was added to the organ bath. This procedure was repeated once or twice at an interval of 30 min, until contractions were stable (less than 10% variation).

2.3. Contractions induced by carbachol, 5-HT, neurokinin A, substance P and the tachykinin NK_1 receptor agonist $Ac[Arg^6, Sar^9, Met(O_2)^{11}]$substance P-(6–11)

Concentration–response curves to carbachol, 5-HT and histamine were made in a cumulative way (10^{-8} to 10^{-4} M in steps of 0.5 log units). Increasing concentrations of neurokinin A, substance P or the tachykinin NK_1 receptor agonist $Ac[Arg^6, Sar^9, Met(O_2)^{11}]$substance P-(6–11) were added to the organ bath in a non-cumulative way (10^{-8} to 10^{-5} M in steps of 0.5 log units). The contact time for each concentration was at least 5 min. Between administrations, an interval of at least 30 min was allowed with changing of the bathing medium every 10 min. The influence of methysergide (10^{-7} M), atropine (10^{-6} M), hexamethonium (5.0×10^{-4} M) and tetrodotoxin (3.0×10^{-6} M) were studied by incubating the antagonists for 20 min before administering the contractile agonists or applying electrical field stimulation. All antagonists were used in sufficiently high concentrations as determined in previous studies (Farmer et al., 1975; Munoz et al., 1989).

2.4. Contractions induced by compound 48/80 and capsaicin

To evaluate the in vitro contractile effect of compound 48/80 and capsaicin, only one concentration of each agent was administered per preparation. In preliminary experiments capsaicin was shown to give a maximal contraction at 10^{-6} M, while contractions to compound 48/80 were found to be maximal at 0.125 mg/ml.

The influence of the tachykinin NK_1 receptor antagonist RP67580 (10^{-7} M) and the tachykinin NK_2 receptor antagonist SR48968 (10^{-7} M) on the contractions by 10^{-6} M capsaicin and 0.125 mg ml^{-1} compound 48/80 was tested by incubating the antagonists for 20 min before administering the contractile agents. The concentrations of RP67580 and SR48968 were determined in a previous study (Joos et al., 1994b).

2.5. In vivo pretreatment with compound 48/80 and capsaicin

To deplete histamine and serotonin from mast cells, rats were pretreated with compound 48/80. Compound 48/80 was given intraperitoneally in increasing doses: day 1, 1 mg/kg; day 2 and 3, 2 mg/kg; day 4, 5 and 8, 3 mg/kg; day 10, 4 mg/kg; day 11 and 15, 5 mg/kg (Joos and Pauwels, 1993). Control animals received saline intraperitoneally. In vitro tracheal contraction to neurokinin A was evaluated on day 16.

To deplete the sensory neuropeptides of adult rats, capsaicin 50 mg/kg was administered subcutaneously 24 h before in vitro testing (Holzer, 1991). Control animals received the solvent for capsaicin. To prevent acute respiratory distress all animals were pretreated with atropine (0.05 mg/kg i.p.) and terbutaline (0.1 mg/kg s.c.) 30 min before injection of compound 48/80 or capsaicin. Control animals received the same pretreatment.

2.6. Transmural nerve stimulation

The tracheal rings were placed between two platinum electrodes. Electrical field current was delivered by a Grass S44 stimulator. Parameters used were: supramaximal voltage, pulse duration 0.5 ms, frequency 1 to 50 Hz. 30 s

trains at increasing frequency were applied at 5 min intervals.

2.7. In vitro release of 5-HT

At different time points during the contraction induced by neurokinin A or substance P, 100 μl was taken from the organ bath to measure 5-HT. 5-HT was measured using an enzymo-immunoassay which is based on the competition of acylated 5-HT and the acylated enzyme-conjugate 5-HT-acetylcholinesterase for an antibody against acyl-5-HT (Immunotech, Marseille, France). The sensitivity of this assay is 0.5 nM.

In a first protocol the effect of substance P, 10^{-6} M, and neurokinin A, 10^{-6} M, was compared by sampling 100 μl from the organ bath at the moment of maximal contraction. In control experiments carbachol was used as a contractile agonist. Then the concentration–response relationship was studied for neurokinin A (10^{-8}, 10^{-7} and 10^{-6} M). The time course was studied by taking 100 μl of the bathing fluid before and 1, 3 and 10 min after the administration of neurokinin A (10^{-6} M). Finally the effect of pretreatment with atropine (10^{-6} M), given 10 minutes before neurokinin A (10^{-6} M) was studied.

2.8. Processing of tissue for light microscopy and morphometry

Two rings of the trachea were processed for light microscopy following either carbachol treatment (3.3×10^{-7} M) or stimulation with substance P or neurokinin A (for both 10^{-6} M, i.e., the maximal effective concentration). The segments were removed from the organ bath and placed immediately in fixative (4% paraformaldehyde, pH 7.2 in 0.1 M phosphate buffer at room temperature) and left overnight. The tissue was then thoroughly rinsed before being dehydrated and embedded in Technovit 8100 (Kulzer, Germany). Sections were cut through the tissue block at 3–5 μm intervals, each section being 0.5 μm thick. They were then stained with 0.125% toluidine blue in 1% borax (pH 9.3) for 20 s at 60°C for demonstration of metachromatic cells.

For morphometry, the sections were viewed in a Zeiss Axiophot microscope linked to the Alcatel Impact software system for analysis. The area evaluated included the muscular region between the ends of the cartilaginous rings. All morphometry was performed in a blinded fashion by one of the investigators (G.B.). Two regions, the epithelial and subepithelial (designated subepithelial) and the muscu-

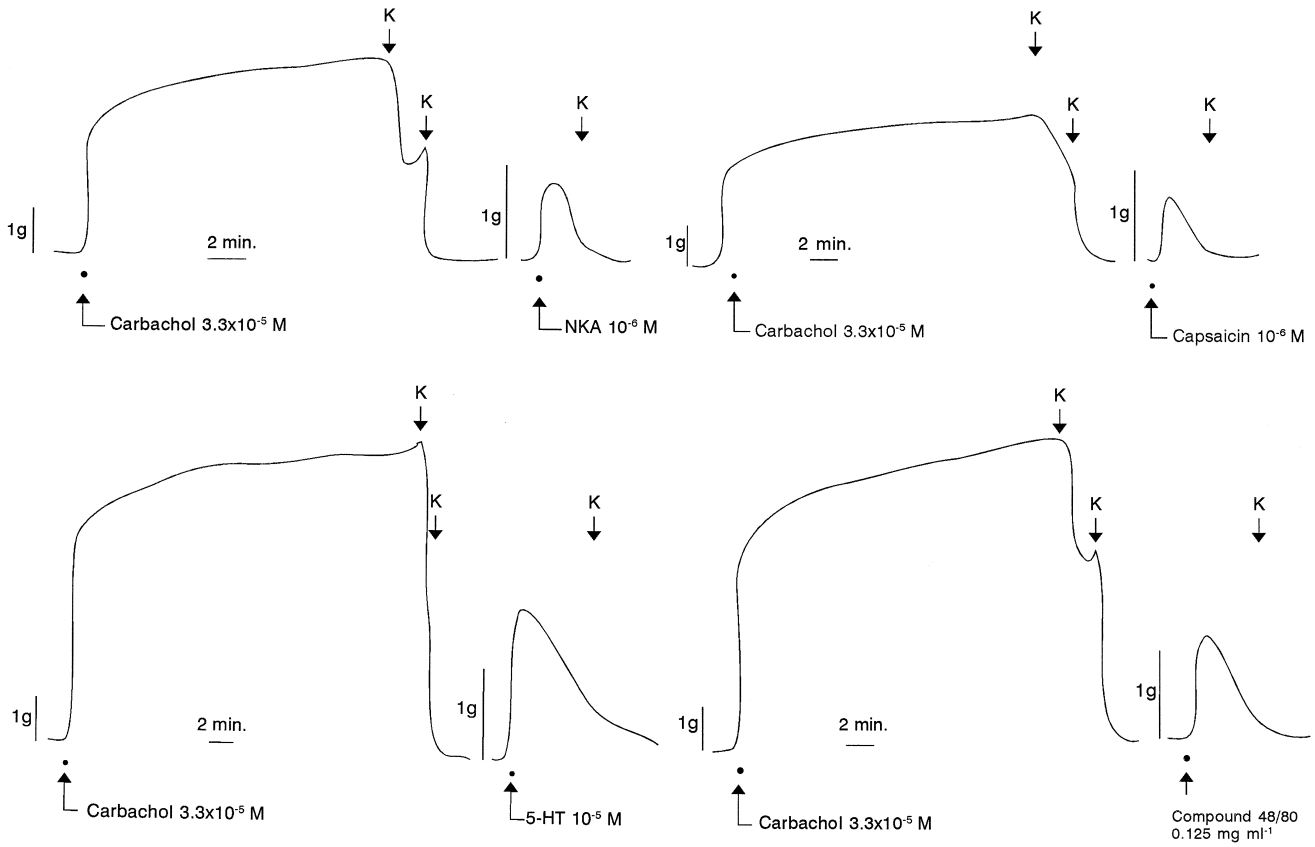

Fig. 1. Example of individual tracings depicting the effect of carbachol (3.3×10^{-5} M), neurokinin A (NKA, 10^{-6} M), 5-HT (10^{-5} M), compound 48/80 (0.125 mg/ml) and capsaicin (10^{-6} M) on Fisher 344 rat trachea. K indicates replacing of the bathing medium with fresh Krebs solution.

lar/submuscular (designated submuscular) were delineated and both the total number of cells (normal and degranulating) and the relevant number per μm^2 tissue were calculated. Cells were deemed to be degranulating when the granular density had altered, the cells were swollen and a shift in the metachromasia was apparent.

2.9. Chemicals and drugs

Neurokinin A and the specific tachykinin NK_1 receptor agonist Ac[Arg^6, Sar^9, Met(O_2)^{11}]substance P-(6–11) were obtained from Peninsula (St. Helens, UK). Substance P was obtained from UCB (Brussels, Belgium). The tachykinin NK_1 receptor antagonist, (±)-RP67580 ((3*aR*,7*aR*)-(7,7-diphenyl-2-(1-imino-2-(2-methoxyphenylethyl)-perhydraisoinotol-4-one))), was given by Rhone-Poulenc Rorer (Vitry-sur-Seine, France). The tachykinin NK_2 receptor antagonist, SR48968 ((*S*)-*N*-methyl-*N*[4-(4-acetylamine-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butylbenzamide), was a gift from Sanofi Recherche (Montpellier, France). Pentobarbital was obtained from Ceva (Brussels, Belgium).

Atropine, capsaicin, carbachol, compound 48/80, histamine, 5-hydroxytryptamine and tetrodotoxin were obtained from Sigma (Sigma-Aldrich, Belgium). Methysergide was obtained from Sandoz-Wander (Brussels, Belgium), hexamethonium from Janssen Chimica (Beerse, Belgium) and terbutaline from Astra Pharmaceuticals (Brussels, Belgium).

Most drugs and chemicals were dissolved in Krebs medium; (±)-RP67580 was dissolved in 10% dimethylsulfoxide and SR48968 in 20% of 0.1 M HCl before further dilution in Krebs. A stock solution of 10^{-2} M capsaicin was prepared in a 50/50 solution of ethanol 70% and Tween 80 and was further diluted with Krebs to 10^{-4} M. Stock solutions of the peptides, 10^{-3} M, were kept at −20°C. The control solutions consisted of the solvent in the appropriate concentrations. The dilutions of the peptides were freshly made each day and kept on ice during the experiments.

2.10. Data analysis

The contractile responses were measured as g isometric tension per mg of tissue wet weight (g/mg) and expressed as percentages of the maximal contraction induced by carbachol. To determine wet weight, the tracheal rings were blotted on filter paper at the end of the experiments and then weighed.

The EC_{50} was determined by linear interpolation from a log concentration–response curve, as the concentration required to produce 50% of the maximal contraction induced by the agonist. The results are reported as the mean ± S.E.M. Concentration–response curves for contractile agents in the absence and the presence of antagonists were compared by two-way analysis of variance (ANOVA). When significance was reached, a two-tailed *t*-test was used to assess the statistical significance of the results at each concentration of the contractile agent. Differences were regarded as significant when $P < 0.05$.

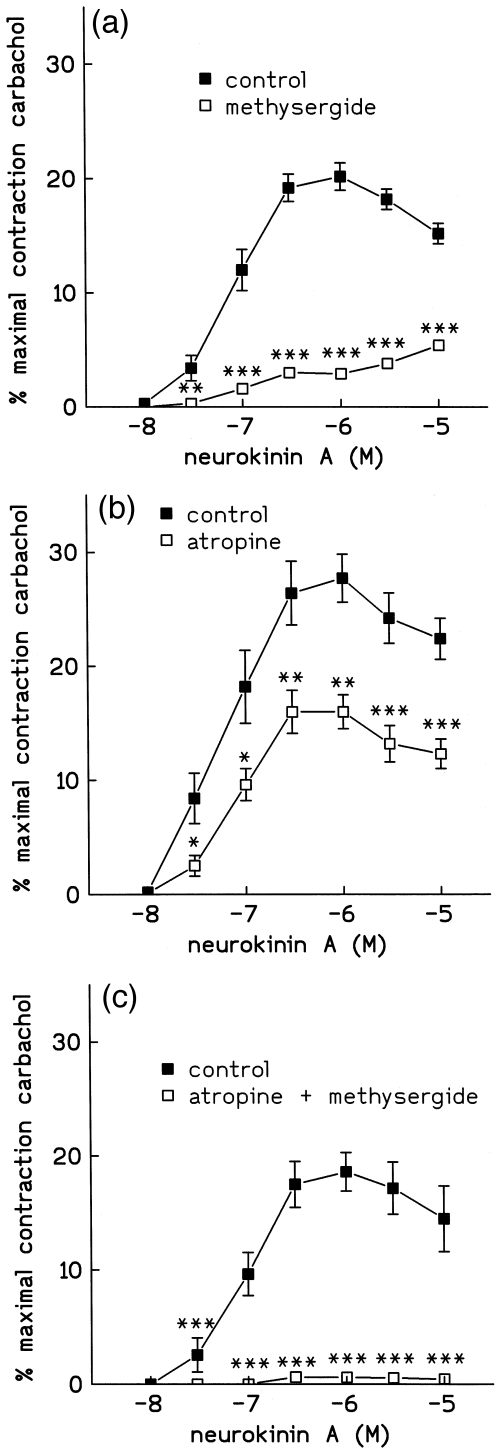

Fig. 2. Non-cumulative concentration–response curves for the contractile effect of neurokinin A. Each point represents the mean ± SEM. (a) In the presence of methysergide (10^{-7} M) versus control ($n = 9$) (ANOVA: $P < 0.005$). (b) In the presence of atropine (10^{-6} M) versus control ($n = 9$) (ANOVA $P < 0.005$). (c) In the presence of atropine (10^{-6} M) + methysergide (10^{-7} M) versus control ($n = 4$) (ANOVA $P < 0.005$). Two-tailed *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

3. Results

3.1. Contractions induced by neurokinin A, substance P, Ac[Arg^6, Sar^9, Met(O$_2$)^{11}]substance P-(6–11), carbachol, histamine, 5-HT and electrical field stimulation; effect of pretreatment with methysergide, atropine, hexamethonium and tetrodotoxin

Representative examples of the contractions induced by carbachol, 5-HT and neurokinin A are shown in Fig. 1. In control rings neurokinin A, 10^{-8} to 10^{-5} M, caused a concentration-dependent contraction, with an EC_{50} around 10^{-7} M and a maximal contraction up to 30% of the maximal contraction to carbachol. The 5-HT antagonist methysergide, 10^{-7} M, largely reduced the contractile effect of neurokinin A (Fig. 2a). Methysergide also largely depressed the concentration–response curve to substance P and the specific tachykinin NK_1 receptor agonist Ac[Arg^6, Sar^9, Met(O$_2$)^{11}]substance P-(6–11). For substance P, the maximal effect declined from $12.1 \pm 2.8\%$ of the maximal contraction to carbachol ($n = 6$) to $1.4 \pm 0.3\%$ ($n = 6$) in the presence of methysergide ($P < 0.005$). For Ac[Arg^6, Sar^9, Met(O$_2$)^{11}]substance P-(6–11) the maximal effect declined from $15.9 \pm 1.9\%$ ($n = 4$) to $1.4 \pm 0.2\%$ ($n = 4$) of the maximal contraction to carbachol ($P < 0.005$). Atropine, 10^{-6} M, partially reduced the maximal contractile effect of neurokinin A (Fig. 2b). The combination of methysergide, 10^{-7} M, and atropine, 10^{-6} M, abolished the effect of neurokinin A (Fig. 2c). Hexamethonium, 5.0×10^{-4} M ($n = 8$), and tetrodotoxin, 3.0×10^{-6} M ($n = 4$), did not affect the concentration–response curve for neurokinin A.

The cumulative administration of both carbachol and 5-HT (10^{-8} to 10^{-4} M) caused a concentration-dependent contraction. Histamine, in concentrations up to 10^{-3} M had no effect ($n = 4$). Methysergide, 10^{-7} M, completely

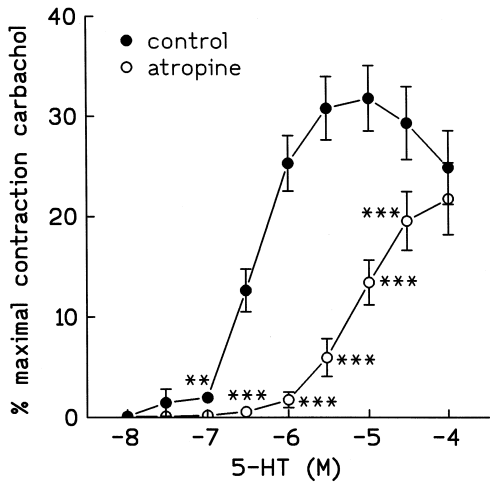

Fig. 3. Cumulative concentration–response curves for 5-HT ($n = 10$): control response versus response after pretreatment with atropine (10^{-6} M) (ANOVA: $P < 0.005$). Two-tailed *t*-test: $^{**}P < 0.01$; $^{***}P < 0.005$.

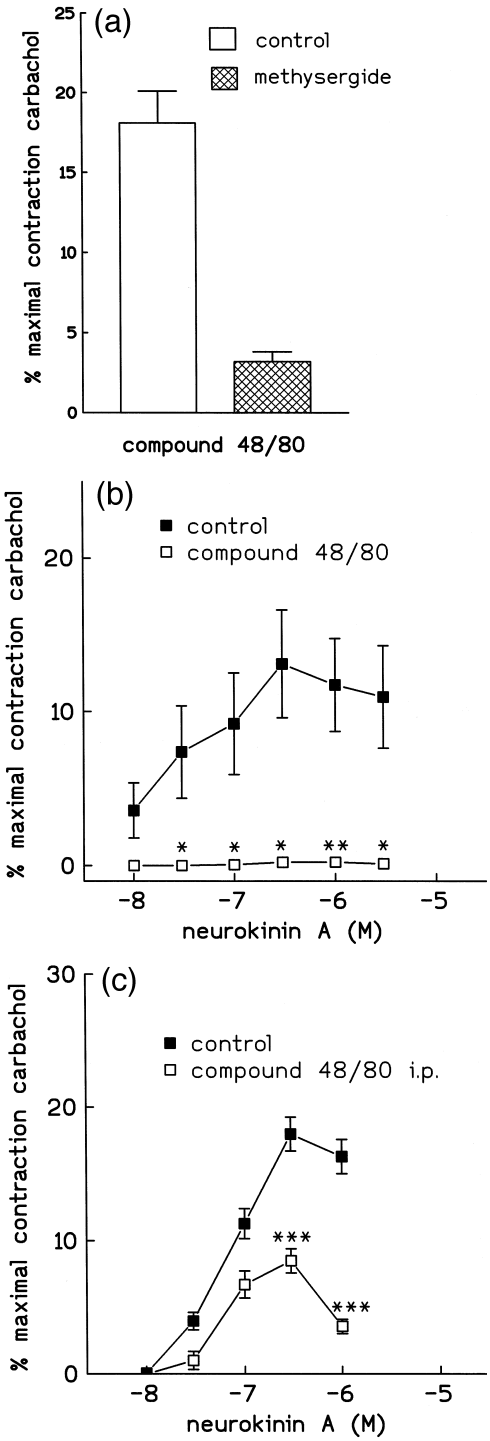

Fig. 4. (a) Contractile effect of compound 48/80 (0.125 mg/ml) administered directly into the organ bath and the effect of pretreatment with methysergide (10^{-7} M) (mean ± SEM) ($n = 6$ for control and for methysergide) ($^{***}P < 0.005$). (b) Effect of in vitro pretreatment with compound 48/80 on the non-cumulative concentration–response curve to neurokinin A (mean ± SEM) ($n = 4$ for control and compound 48/80) (control versus pretreatment ANOVA: $P < 0.005$). (c) Effect of in vivo pretreatment with compound 48/80 on the in vitro non-cumulative concentration–response curve to neurokinin A (mean ± SEM) ($n = 8$ for control and compound 48/80) (control versus pretreatment ANOVA: $P < 0.005$). Two-tailed *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

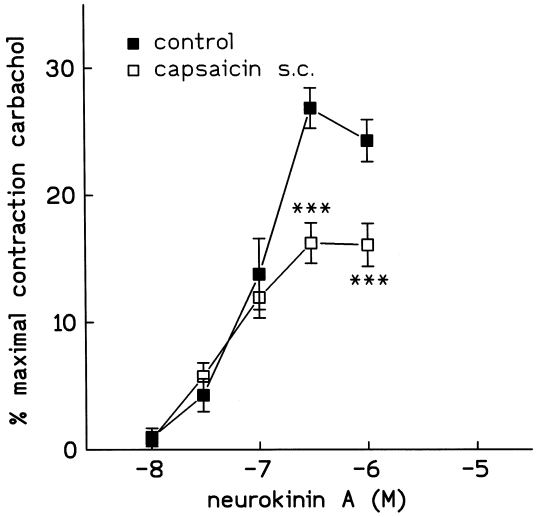

Fig. 5. Effect of in vivo pretreatment with capsaicin (50 mg/kg s.c.) on the in vitro concentration-response curve for neurokinin A (control versus capsaicin ANOVA $P < 0.05$). Two-tailed *t*-test: $^{***}P < 0.005$.

inhibited the contractile effect of 5-HT ($n = 4$), but had no effect on the concentration–response curve for carbachol ($n = 6$). Atropine, 10^{-6} M, completely inhibited the contractile effect of carbachol ($n = 4$). It also significantly reduced the contractions to 5-HT (Fig. 3).

Electrical field stimulation (1–50 Hz) caused frequency-dependent contractions, a maximum being obtained at 30 Hz ($46.6 \pm 5.7\%$ of the maximal contraction to carbachol, $n = 8$). These contractions were abolished by atropine, 10^{-6} M ($n = 7$), as well as by tetrodotoxin, 3.0×10^{-6} M ($n = 8$). Methysergide, 10^{-7} M, had no effect on these contractions ($n = 4$). No evidence for an atropine-resistant contraction was found.

3.2. Tracheal contractions caused by compound 48/80 and capsaicin

In vitro administration of single concentrations of capsaicin caused a contraction of the Fisher 344 rat trachea

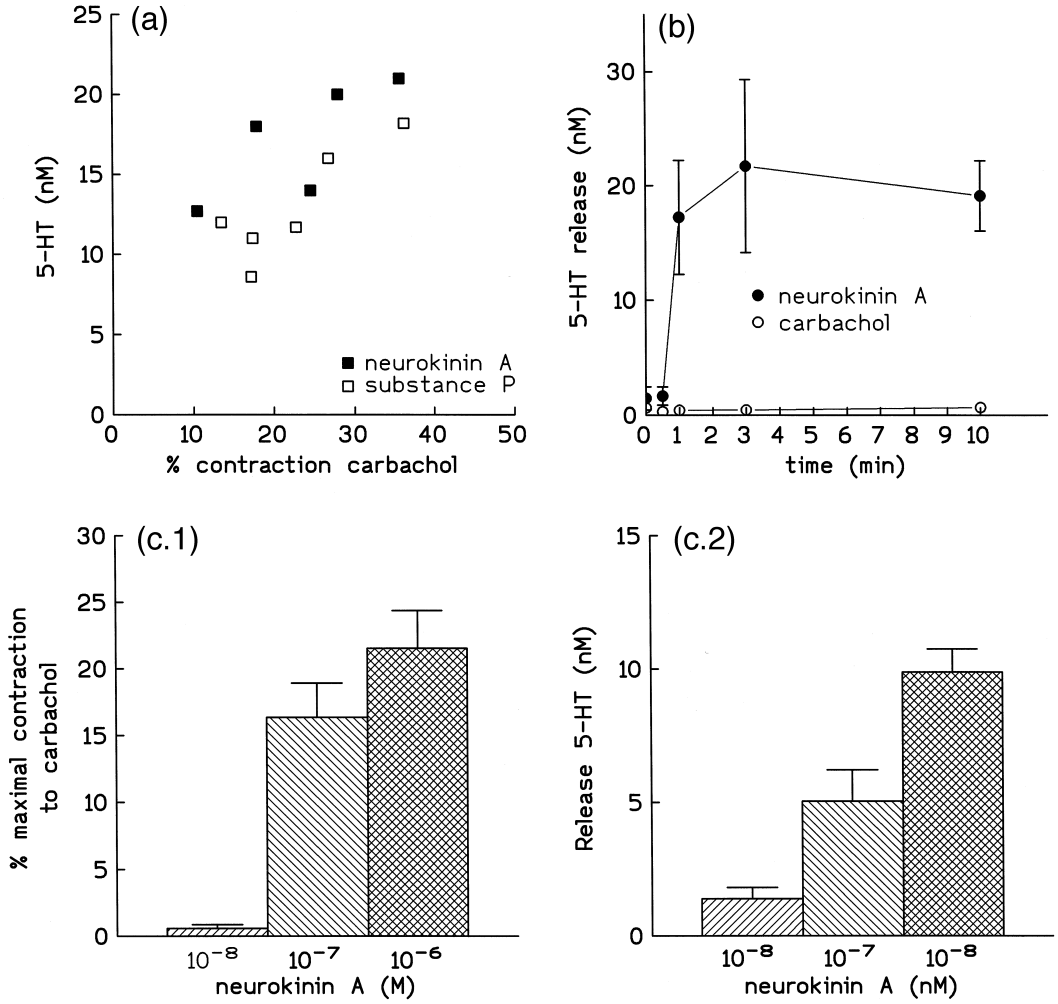

Fig. 6. (a) Correlation of the contraction (expressed as % of the maximal contraction to carbachol) and the 5-HT release induced by neurokinin A (10^{-6} M) and substance P (10^{-6} M) ($r = 0.81$; $P = 0.0038$). (b) Time–response curve for neurokinin A (10^{-6} M)-induced 5-HT release ($n = 4$). Carbachol, 3.3×10^{-7} M served as control ($n = 4$). (c) Concentration–response curve for contraction (c1) and for 5-HT release (c2) induced by neurokinin A. Each bar represents the mean of 8 animals. Two-tailed *t*-test: $^{***}P < 0.005$ (versus value in the presence of carbachol).

which was dependent on the concentration of capsaicin. Maximal contractions, up to 15% of the maximal contraction to carbachol, were observed at 10^{-6} M. The tachykinin NK_1 receptor antagonist RP67580 (10^{-7} M) abolished the contraction to 10^{-6} M capsaicin (control: $15.2 \pm 2.5\%$ versus RP67580: $0.6 \pm 0.6\%$ of the maximal contraction to carbachol; $n = 7$; $P < 0.005$). The tachykinin NK_2 receptor antagonist SR48968 (10^{-7} M) had no effect on the contraction induced by capsaicin, 10^{-6} M (control: $15.2 \pm 2.1\%$ versus SR48968: $12.9 \pm 2.8\%$ of the maximal contraction to carbachol; $n = 7$; NS). Methysergide (10^{-7} M) nearly abolished the contraction induced by capsaicin, 10^{-6} M ($0.8 \pm 0.2\%$ of the maximal contraction to carbachol; $n = 8$; $P < 0.005$ versus control). Compound 48/80, 0.125 mg/ml, administered directly into the organ bath caused a contraction which was largely inhibited by pretreatment with methysergide, 10^{-7} M (Fig. 4a). These contractions were not affected by pretreatment with the tachykinin NK_1 receptor antagonist RP67580 (10^{-7} M) (control: $12.4 \pm 3.1\%$ versus RP67580: $16.7 \pm 2.7\%$ of the maximal contraction of carbachol; $n = 8$; NS).

3.3. In vivo and in vitro pretreatment with compound 48/80 and capsaicin

The administration of compound 48/80, 0.125 mg/ml, into the organ bath, completely prevented the contractile response to neurokinin A administered 30 min later (Fig. 4b).

After in vivo pretreatment with compound 48/80 (i.p.) the in vitro contractile response to neurokinin A was significantly, but not completely inhibited (maximal effect of neurokinin A: $8.5 \pm 0.9\%$ after compound 48/80 versus $17.6 \pm 1.0\%$ of the maximal contraction to carbachol in control animals; $n = 8$; $P < 0.005$) (Fig. 4c). In vivo pretreatment with capsaicin, 50 mg/kg s.c., partially reduced the contractile effect of 3×10^{-7} and 10^{-6} M neurokinin A (Fig. 5) while it inhibited the in vitro contractile effect of capsaicin (capsaicin 10^{-6} M in control rats $10.3 \pm 2.2\%$ versus in rats pretreated with capsaicin $2.7 \pm 1.6\%$ of the maximal contraction to carbachol; $n = 10$ preparations from 5 rats in each group, $P < 0.05$).

3.4. Release of 5-HT in the organ bath

The contractions induced by substance P (10^{-6} M) and neurokinin A (10^{-6} M) were associated with a significant release of 5-HT in the organ bath (Fig. 6a; $r = 0.81$; $P = 0.0038$). The 5-HT release induced by neurokinin A (10^{-6} M) was time-dependent, a maximal effect occurring from 1 min after application of neurokinin A (Fig. 6b). With carbachol no significant increases in 5-HT levels were obtained. The 5-HT release was dependent on the concentration of neurokinin A as was the contraction (Fig. 6c).

Atropine (10^{-5} M) reduced the contractile effect of neurokinin A (10^{-6} M) (control: $29.3 \pm 2.3\%$ versus atropine: $16.5 \pm 1.2\%$ of the maximal contraction to carbachol, $P < 0.01$, $n = 9$); however, it had no effect on the 5-HT release induced by neurokinin A (control: 15.3 ± 5.2 nM 5-HT versus atropine: 13.9 ± 4.1 nM 5-HT, NS). Compound 48/80, 0.125 mg/ml, induced a significant 5-HT release (9.3 ± 1.4 nM; $n = 8$) in contrast to carbachol, 2×10^{-7} M (1.6 ± 0.5 nM; $n = 8$).

3.5. Microscopy and morphometry

Following Technovit 8100 embedding and toluidine blue staining of the sections, degranulating mast cells were clearly seen. A range of structural changes were visible from a swollen apparently intact cell with the granules very distinct and already showing a color change to completely degranulated cells surrounded by a halo of pink granules.

Tracheal tissue which had been exposed to carbachol showed a very high percentage of degranulating mast cells in the sub-epithelial region (77% of the total) (30.4 ± 9.7 degranulating versus 9.8 ± 3.0 normal mast cells per μm^2). The submuscular zone had just over 50% degranulating mast cells (36.6 ± 4.1 degranulating versus 34.3 ± 5.7 normal mast cells per μm^2). Initially the high percentage of degranulating mast cells in carbachol treated tissue was puzzling. However, comparison with untreated material showed us that a relatively high percentage of these resulted from the mechanical contraction of the tissue during or after removal. In view of the high percentage of degranulating mast cells in the subepithelial layer before treatment with neurokinin A or substance P, only the submuscular region was evaluated. Here the percentage degranulation steadily increased to 74.8% for 10^{-6} M substance P (from 15.0 ± 6.9 to 47.1 ± 9.8 degranulating mast cells per

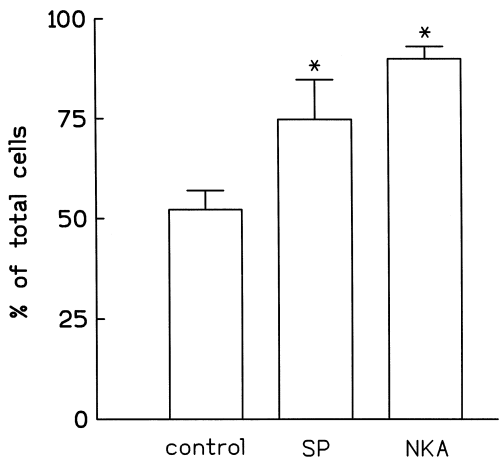

Fig. 7. Morphometric analysis. Percentage degranulated mast cells per μm^2 in the submuscular area of tissues stimulated with carbachol (3.3×10^{-7} M) (control), substance P (10^{-6} M) (SP) or neurokinin A (10^{-6} M) (NKA) ($n = 6$ preparations for each group). Two-tailed t-test: $^{*}P < 0.05$.

μm^2) and to 90% for 10^{-6} M neurokinin A (from 8.2 ± 2.7 to 72.0 ± 3.1 degranulating mast cells per μm^2 for neurokinin A) (Fig. 7).

4. Discussion

The manifest inhibitory influence of the 5-HT antagonist methysergide on the contractile effect of neurokinin A, substance P and Ac[Arg^6, Sar^9, Met$(O_2)^{11}$]substance P-(6–11) indicate that 5-HT is involved in the contraction induced by these tachykinins. These in vitro findings extend our earlier in vivo observations made in this rat strain (Joos et al., 1994a). We have chosen to focus in this study our attention on neurokinin A: we previously showed that in the Fisher 344 rat trachea contractions induced by neurokinin A and substance P were similar, both agonists being equipotent and exerting their effect by interaction with a tachykinin NK_1 receptor (Joos et al., 1994b).

In the present study we demonstrate that the 5-HT involved has a direct effect on airway smooth muscle and an additional indirect action, i.e., activation of postganglionic cholinergic nerves. Indeed, atropine reduced the contractile effect of 5-HT as well as neurokinin A. The release of acetylcholine from cholinergic nerves, induced by 5-HT, does not involve propagation of action potentials as the neurokinin-induced contractions were not influenced by the sodium channel blocker tetrodotoxin. In contrast, the latter neurotoxin abolished the cholinergic contraction induced by electrical stimulation. A sequential link where acetylcholine leads to release of 5-HT can be excluded as methysergide did not influence the contractions to carbachol.

Capsaicin, which releases tachykinins from sensory nerves (Holzer, 1991), caused a sizeable contraction of the Fisher 344 airway preparation. The inhibitory effect of the tachykinin NK_1 receptor antagonist RP67580 suggests that endogenous tachykinins mediate the contractile response to capsaicin by interaction with tachykinin NK_1 receptors corroborating previous in vivo findings with exogenous tachykinins (Joos et al., 1994b). As for neurokinin A and substance P the contraction induced by capsaicin was inhibited by methysergide, indicating 5-HT as the responsible contractile mediator. In vivo pretreatment with capsaicin completely abolished the in vitro contraction to capsaicin and partially inhibited the in vitro contraction to neurokinin A 24 hours later. It is highly likely that this treatment released tachykinins stimulating the airway mast cells in our preparation. Partial depletion of mast cells would then offer an explanation for the reduced in vitro contractile response to neurokinin A.

5-HT is the contractile mast cell mediator in rats. 5-HT contracted the Fisher 344 rat trachea, while histamine had no contractile effect on the Fisher 344 rat trachea, which is similar to findings obtained in the Wistar rat (Church, 1975). The mast cell depleting agent compound 48/80 caused contractions of the Fisher 344 rat trachea which were mediated by 5-HT. Compound 48/80 has long been used as a mast cell depleting agent (Lagunoff et al., 1983). In some experimental systems, compound 48/80 has also been found to activate capsaicin-sensitive sensory nerves. For example, in rat urinary bladder compound 48/80 caused a Ca^{2+}-dependent release of calcitonin gene-related peptide. The contractions induced by 48/80 in that preparation were not affected by pretreatment with methysergide (Eglezos et al., 1992). The contractions induced by compound 48/80 in guinea pig bronchi were partially inhibited by a histamine receptor antagonist and partially by in vitro capsaicin desensitization, suggesting an effect of compound 48/80 not only on mast cells but also on sensory nerves (Mapp et al., 1993). Finally, an inhibitor of neutral endopeptidase, phosphoramidon, potentiated compound 48/80 induced contraction of the guinea-pig trachea (Tudoric et al., 1994). However, in our study the in vitro contractile effect of compound 48/80 was completely inhibited by the 5-HT antagonist methysergide and not affected by pretreatment with a tachykinin receptor antagonist, suggesting that compound 48/80 does not activate sensory nerves in the Fisher 344 rat trachea. We are not aware of any effect of compound 48/80 on other 5-HT containing cells such as platelets and endocrine cells. In vitro and in vivo pretreatment with compound 48/80 reduced the in vitro contractile effect of neurokinin A, suggesting that neurokinin A stimulates mast cells in the isolated Fisher 344 rat trachea.

It is well known that isolated mast cells of the rat from peritoneum, pleura, skin and the gastrointestinal tract respond to tachykinins (for review see Marshall and Waserman, 1995). The stimulation of the airway mast cells by tachykinins in the isolated Fisher 344 rat trachea is further corroborated by 2 observations. First, the contractions of the Fisher 344 rat tracheal preparation induced by neurokinin A, substance P and compound 48/80 were associated with release of 5-HT in the organ bath. The release of 5-HT was concentration-dependent and followed a time course very similar to the previously reported substance P-induced mast cell degranulation (Fewtrell et al., 1982). The neurokinin A-induced 5-HT release was not influenced by atropine illustrating that the cholinergic component of the contraction by neurokinin A can be completely ascribed to the 5-HT-mediated activation of cholinergic nerves. The concentrations of 5-HT measured in the organ bath (up to 20 nM; Fig. 6a) are definitely lower than the concentrations of 5-HT required upon exogenous administration to obtain tracheal contraction (a clear effect being observed from 300 nM onwards, Fig. 3). However, in the organ bath only the overflow of 5-HT is measured and the concentrations of 5-HT are probably much higher in the tissue, in the immediate vicinity of the mast cells. Second, the contractions of the Fisher 344 rat trachea by substance P and neurokinin A were associated with a significant degranulation of the mast cells located in the

muscular/submuscular region. Moreover, our control data compare well with those obtained in another study on rat trachea (Kiernan, 1990). Finally, the 5-HT antagonist methysergide did not inhibit tracheal contractions induced by carbachol, indicating that the epithelial mast cell degranulation is functionally not relevant.

The present study illustrates that mast cells in rat airways can be degranulated both by exogenously applied tachykinins and by endogenously released tachykinins. Recently other data have been presented that support the importance of the interaction between sensory neuropeptides and mast cells in the airways. A combination of electrical field stimulation and substance P (10^{-7} M) induced an enhanced recovery of chymotryptic activity, histamine and rat mast cell protease I and II in the isolated perfused trachea obtained from Sprague–Dawley rats (Tam et al., 1994). Hua et al. (1996) subsequently showed that this effect of substance P in the isolated perfused rat trachea was mimicked by neurokinin A (10^{-6} M) and capsaicin (10^{-6} M) and was blocked by a tachykinin NK_1 receptor antagonist. In isolated tracheally perfused guinea pig lungs, substance P and capsaicin caused histamine release which added up to 25% of the total histamine release following allergen challenge and which was dependent on the stimulation of both tachykinin NK_1 and NK_2 receptors. These effects were associated with an increase in the number of degranulating mast cells. However, in this model histamine release did not contribute to the contractile effects of substance P (Lilly et al., 1995). The recent demonstration that substance P can release histamine from bronchoalveolar mast cells suggests that in human airways tachykinin–mast cell interaction can occur (Heaney et al., 1995).

In conclusion, both the exogenously applied and endogenously released tachykinins contract Fisher 344 rat trachea by an indirect mechanism. This indirect mechanism involves the activation of a tachykinin NK_1 receptor and the release of 5-HT, which then contracts airway smooth muscle directly and indirectly, by stimulation of postganglionic cholinergic nerves. The experiments with compound 48/80 and the morphometric analysis point to mast cells as the source of 5-HT. Our study thus demonstrates that tachykinins can constrict airways by interaction with mast cells.

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References

Barnes, P.J., Baraniuk, J.N., Belvisi, M.G., 1991a. Neuropeptides in the respiratory tract (Part 1). Am. Rev. Respir. Dis. 144, 1187–1196.

Barnes, P.J., Baraniuk, J.N., Belvisi, M.G., 1991b. Neuropeptides in the respiratory tract (Part 2). Am. Rev. Respir. Dis. 144, 1391–1399.

Church, M.K., 1975. Response of rat lung to humoral mediators of anaphylaxis and its modification by drugs and sensitization. Br. J. Pharmacol. 55, 423–430.

Eglezos, A., Lecci, A., Santicioli, P., Guiliani, Tramontana, M., Del Bianco, E., Maggi, C.A., 1992. Activation of capsaicin-sensitive primary afferents in the rat urinary bladder by compound 48/80: A direct action on sensory nerves?. Arch. Int. Pharmacodyn. T. 315, 96–109.

Farmer, J.B., Richards, I.M., Sheard, P., Woods, A.M., 1975. Mediators of passive lung anaphylaxis in the rat. Br. J. Pharmacol. 55, 57–64.

Fewtrell, C.M.S., Foreman, J.C., Jordan, C.C., Oehme, P., Renner, H., Stewart, J.M., 1982. The effects of substance P on histamine and 5-hydroxytryptamine release in the rat. J. Physiol. 330, 393–411.

Heaney, L.G., Cross, L.J.M., Stanford, C.F., Ennis, M., 1995. Substance P induces histamine release from human pulmonary mast cells. Clin. Exp. Allergy 25, 179–186.

Holzer, P., 1991. Capsaicin: Cellular targets, mechanisms of action and selectivity for thin sensory neurons. Pharmacol. Rev. 43, 143–201.

Hua, X.-Y., Back, S.M., Tam, E.K., 1996. Substance P enhances electrical field stimulation-induced mast cell degranulation in rat trachea. Am. J. Physiol. (Lung Cell. Mol. Physiol.) 14, L985–L991.

Joos, G.F., Pauwels, R.A., 1993. The in vivo effect of tachykinins on airway mast cells in the rat. Am. Rev. Respir. Dis. 148, 922–926.

Joos, G.F., Pauwels, R.A., Van Der Straeten, M.E., 1988. The mechanism of tachykinin-induced bronchoconstriction in the rat. Am. Rev. Respir. Dis. 137, 1038–1044.

Joos, G., Pauwels, R., Van Der Straeten, M., 1989. The effect of nedocromil sodium on the bronchoconstrictor effect of neurokinin A in subjects with asthma. J. Allergy Clin. Immunol. 83, 663–668.

Joos, G.F., Kips, J.C., Pauwels, R.A., 1994a. In vivo characterization of the tachykinin receptors involved in the direct and indirect bronchoconstrictor effect of tachykinins in the rat. Am. J. Respir. Crit. Care Med. 149, 1160–1166.

Joos, G.F., Lefebvre, R.A., Kips, J.C., Pauwels, R.A., 1994b. Tachykinins contract trachea from Fisher 344 rats by interaction with a NK_1 receptor. Eur. J. Pharmacol. 271, 47–54.

Kiernan, J.A., 1990. Degranulation of mast cells in the trachea and bronchi of the rat following stimulation of the vagus nerve. Int. Arch. Allergy Appl. Immunol. 91, 398–402.

Lagunoff, D., Martin, T.A., Read, G., 1983. Agents that release histamine from mast cells. Annu. Rev. Pharmacol. Toxicol. 23, 331–351.

Lilly, C.M., Hall, A.E., Rodger, I.W., Kobzik, L., Haley, K.J., Drazen, J.M., 1995. Substance P-induced histamine release in tracheally perfused guinea pig lungs. J. Appl. Physiol. 78, 1234–1241.

Lundberg, J.M., Saria, A., 1982. Bronchial smooth muscle contraction induced by stimulation of capsaicin sensitive sensory neurons. Acta Physiol. Scand. 116, 473–476.

Lundberg, J.M., Saria, A., 1987. Polypeptide-containing neurons in airway smooth muscle. Annu. Rev. Physiol. 49, 557–572.

Mapp, C.E., Boniotti, A., Papi, A., Chitano, P., Coser, E., Di Stefano, A., Saetta, M., Ciaccia, A., Fabbri, L.M., 1993. The effect of compound 48/80 on contractions induced by toluene diisocyanate in isolated guinea-pig bronchus. Eur. J. Pharmacol. 248, 67–73.

Marshall, J.S., Waserman, S., 1995. Mast cells and the nerves-potential interactions in the context of chronic disease. Clin. Exp. Allergy 25, 102–110.

Munoz, N.M., Chang, S.-W., Murphy, T.M., Stimler-Gerard, N.P., Blake, J., Mack, M., Irvin, C., Voelkel, N.F., Leff, A.R., 1989. Distribution of bronchoconstrictor responses in isolated-perfused rat lung. J. Appl. Physiol. 66, 202–209.

Solway, S., Leff, A.R., 1991. Sensory neuropeptides and airway function. J. Appl. Physiol. 71, 2077–2087.

Tam, E.K., Aufderheide, J., Hua, X.-Y., 1994. Chymotryptic activity in perfusates of isolated rat trachea: Correlation with mucosal and connective tissue mast cell secretion. Am. J. Respir. Cell. Mol. Biol. 11, 321–328.

Tanaka, D.T., Grunstein, M.M., 1984. Mechanisms of substance P induced contraction of rabbit airway smooth muscle. J. Appl. Physiol. 57, 1551–1557.

Tudoric, N., Coon, R.L., Bosnjak, Z.J., 1994. Inhibition of neutral endopeptidase potentiates compound 48/80-induced constriction of guinea-pig tracheal smooth muscle. Pulmon. Pharmacol. 7, 343–347.