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Potentiation by viral respiratory infection of ovalbumin-induced guinea-pig tracheal hyperresponsiveness: role for tachykinins

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1 We investigated whether virus-induced airway hyperresponsiveness in guinea-pigs could be modulated by pretreatment with capsaicin and whether viral respiratory infections could potentiate ovalbumin-aerosol-induced tracheal hyperresponsiveness.

2 Animals were inoculated intratracheally with bovine parainfluenza-3 virus or control medium 7 days after treatment with capsaicin (50 mg kg^{-1}, s.c.). Four days after inoculation, tracheal contractions were measured to increasing concentrations of substance P, histamine and the cholinoceptor agonist, arecoline.

3 In tracheae from virus-infected guinea-pigs, contractions in response to substance P, histamine and arecoline were significantly enhanced ($P<0.01$) by 144%, 46% and 77%, respectively. Capsaicin pretreatment inhibited the hyperresponsiveness to substance P partly (62%) and to histamine and arecoline completely.

4 In another series of experiments animals were first sensitized with ovalbumin (20 mg kg^{-1}, i.p.). After 14 days animals were exposed to either saline or ovalbumin aerosols for 8 days. After 4 aerosol exposures (4 days) animals were inoculated with either parainfluenza-3 virus or control medium. One day after the last ovalbumin aerosol, tracheal contraction in response to increasing concentrations of substance P, histamine and arecoline was measured.

5 Tracheae from ovalbumin-aerosol-exposed control inoculated animals showed a similar degree of airway hyperresponsiveness to saline-aerosol-exposed virus-treated guinea-pigs. Virus inoculation of ovalbumin-treated animals significantly potentiated the tracheal contractions to substance P compared to either of the treatments alone. The contractions in response to histamine and arecoline were only slightly enhanced.

6 In conclusion, sensory nerves and/or tachykinins are involved in virus-induced airway hyperresponsiveness in guinea-pigs and viral respiratory infections can potentiate the increase in tracheal responsiveness to bronchoconstrictor agonists after ovalbumin exposure.

Keywords: Tracheal hyperresponsiveness; capsaicin; virus infection; ovalbumin; allergy; airway reactivity

Introduction

Characteristic features of asthma include reversible airway constriction and nonspecific airway hyperresponsiveness. Respiratory virus infections can exacerbate asthma and enhance the airway hyperresponsiveness in healthy human subjects and asthma patients (Ouellette & Reed, 1965; MacIntosh *et al.*, 1973; Empey *et al.*, 1976). The mechanisms involved in airway hyperresponsiveness remain unclear. Respiratory virus infection in guinea-pigs enhance airway smooth muscle responses to histamine, the cholinoceptor agonist, arecoline (Folkerts *et al.*, 1992; 1993a,b) and substance P (Saban *et al.*, 1987). It has been shown that viral infections decrease the activity of neutral endopeptidase, the main degradative enzyme of the tachykinins, and this action may account for the virus-induced airway hyperresponsiveness to these agents (Jacoby *et al.*, 1988, Dusser *et al.*, 1989). Histamine can release multiple tachykinins from capsaicin-sensitive sensory nerves in the lung and therefore, the histamine hyperresponsiveness could also be explained by this mechanism (Saria *et al.*, 1988). Whether, such a pathway also exists for cholinoceptor stimulation is unknown.

Repeated antigen aerosol exposure of sensitized guinea-pigs has been shown to cause a prolonged airway hyperresponsiveness (Ishida *et al.*, 1989). Previous studies have shown that depletion of neuropeptides with the neurotoxin, capsaicin, inhibits the induction of airway hyperresponsiveness after repeated aerosolized antigen in sensitized guinea-pigs (Matsuse *et al.*, 1991) and toluene diisocyanate-induced airway hyperresponsiveness (Thompson *et al.*, 1987). In addition, capsaicin aerosols, causing endogenous release of neuropeptides, could increase airway responsiveness to histamine (Umeno *et al.*, 1992) and acetylcholine and neurokinin A (Hsiue *et al.*, 1992) in guinea-pig *in vivo* acutely. Also, the intravenous administration of the tachykinin, substance P, resulted in potentiation of histamine responses in the airways (Umeno *et al.*, 1992). These studies suggest that the release of sensory neuropeptides such as tachykinins may be responsible for the induction of airway hyperresponsiveness. Thus, inhibition of neutral endopeptidase, lengthening the residence time of tachykinins in the airways may lead to increased airway responses to nonspecific agents.

The purpose of the present study was to test the hypothesis that sensory neuropeptides are important for the development of the nonspecific tracheal reactivity seen after virus infection and to investigate whether virus infection could exacerbate the tracheal hyperresponsiveness induced by repeated antigen aerosol in sensitized guinea-pigs.

Methods

Animals

Male Hartley-strain guinea-pigs (weight range 250–300 g) of SPF quality were obtained from Harlan CPB (Zeist, The Netherlands). After delivery, animals were housed in metal cages and allowed 1 week to recuperate after transport. Ani-

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mals were fed normal guinea-pig chow and allowed water *ad lib*. Indicator animals were housed under identical circumstances and were regularly screened for respiratory infections by the Central Laboratory Animal Institute (Utrecht, The Netherlands). All experiments were approved by the ethics committee of Utrecht University.

Capsaicin treatment

Guinea-pigs were anaesthetized with Nembutal (30 mg kg^{-1}, i.p.). A capsaicin (12.5% solution in equal part of 95% ethanol and Tween-80, diluted to 25 mg ml^{-1} with saline) injection was given subcutaneously every hour in the following doses (in mg ml^{-1}): 0.25, 0.25, 0.5, 1.0, 3.0, 5.0, 10.0, 10.0, 20.0 for a total dose of 50 mg kg^{-1} as described previously (Ladenius & Nijkamp, 1993). Animals receiving the above mentioned solution without capsaicin will be referred to as vehicles. All animals were treated with atropine (1 mg kg^{-1}, s.c.) and mepyramine (1 mg kg^{-1}, s.c.) 30 min prior to the first capsaicin injection and atropine and mepyramine were re-administered at the same dose 4 h later. Salbutamol (10 μg kg^{-1}, s.c.) was injected 10 min before each capsaicin injection. Before each vehicle or capsaicin injection the level of anaesthesia was adjusted. After recovery from the anaesthetic animals appeared normal.

Virus inoculation

Bovine Parainfluenza 3 virus (PI-3) (kindly provided by Duphar B.V., Weesp, The Netherlands) was grown on Madin Darby Bovine Kidney cells and harvested 3–5 days (dependent on the cytopathic effects) after the initial inoculation. Accordingly, the tissue culture infective dose (TCID) of the virus suspension was determined. The virus suspension ($TCID_{50} = 10^{8.9}$ ml^{-1}) was centrifuged at 100,000 *g*, the supernatant removed and the virus-pellet resuspended in only 0.1 ml sterile saline to minimize side effects of the inoculation procedure. Growth medium was treated identically. Previous experiments have shown that PI-3 virus could be isolated from the airways of all guinea-pigs 2 days after infection (Folkerts *et al.*, 1993a).

The animals were anaesthetized with ether and placed in a supine position on a small table. The jaws were kept apart by 2 elastic bands and a needle with a bulbous tip was inserted to just behind the glottis. Then, 0.1 ml of inoculum was gently injected in the trachea, the bands were removed and the chest was gently massaged. Guinea-pigs receiving the above mentioned control solution are from now on referred to as controls. To prevent cross-infection animals were housed in separate isolators.

Sensitization and challenge

Animals were injected with ovalbumin (20 mg kg^{-1}, i.p., injection volume of 0.2 ml). After 14 days animals were exposed to either saline or 2% ovalbumin-aerosols daily for 8 consecutive days. Animals were placed under a plastic bell which was attached to the nebulizer and the aerosol started. Animals were watched closely for the appearance of respiratory signs (coughing, wheezing). As soon as animals reacted the nebulizer was turned off and the chamber cleared. Animals were observed until respiratory signs disappeared. At the end of the sensitization period *all* animals reacted upon ovalbumin exposure between breaths 5 and 20. Animals that had been exposed daily to an aerosol with saline for 30 s did not show any respiratory reaction. Aerosols were delivered with a Medix 8001 ultrasonic nebulizer which yields particles 3–5 μm in size.

Treatment protocols

In the first series of experiments animals were inoculated with either control medium or virus, 7 days after vehicle or capsaicin-treatment. Four days later experiments were performed. In the second set of experiments animals were sensitized to ovalbumin and challenged as mentioned above. After recovery from the 4th aerosol challenge, animals were inoculated with virus or control medium and 24 h after the 8th aerosol exposure, experiments were performed.

Experiments

Guinea-pigs were killed with an overdose of pentobarbitone sodium (300 mg kg^{-1}, i.p.). The trachea was removed and placed in ice-cold Krebs-bicarbonate buffer (pH = 7.4) of the following composition (mmol l^{-1}): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 8.3, gassed with 5% CO_2 and 95% O_2. Under a dissecting microscope the trachea was cleared of excess fat and connective tissue and opened by cutting the cartilaginous rings opposite the smooth muscle. Tracheae were then cut into 3 pieces of 3 rings of cartilage and each end tied with 2–0 cotton thread. Tissues were mounted in organ baths filled with Krebs (37°C) and attached to an isotonic transducer (Harvard Bioscience, Kent, UK). Througout the experiments tracheal strips were kept under a constant optimal load of 0.5 g. Using different preloads it was found that with 0.5 g the tissues demonstrated the largest contraction upon stimulation with histamine and the largest relaxation with the β-receptor agonist, salbutamol. Tissues were washed 3 times at 15 min intervals and allowed to stabilize.

When a stable tone was reached cumulative concentration-response curves to substance P, histamine, the muscarinic receptor agonist, arecoline were constructed. The 3 tissues of 3 rings each were exposed to one agent only. The isotonic transducers were connected to an analog-digital converter (Intelligent International PCI System, Burr Brown Company, Tucson, Arizona, U.S.A.) integrating the organ baths in a semi-automatic setup. This enabled continuous sampling, on-line equilibrium detection, and real-time display of the responses on a computer screen of up to 12 baths. At the end of experiments in which animals had been pretreated with either capsaicin or vehicle, all strips were exposed to capsaicin (3×10^{-7} M) to confirm depletion of tachykinins in capsaicin-pretreated animals. If the tissues did not contract after capsaicin, tissues were exposed to 30 mM KCl to ensure viability.

Chemicals

Compounds used: arecoline bromide, egg albumin (ovalbumin) (Grade V), substance P (Sigma, St. Louis, MO, U.S.A.); atropine sulphate, histamine phosphate (OPG, Utrecht, The Netherlands); mepyramine maleate (Specia, Paris, France). All chemicals used for Krebs-bicarbonate buffer were purchased from Merck. All compounds were of reagent grade or higher. Substance P, histamine and arecoline were dissolved in Krebs. Mepyramine, atropine and ovalbumin were dissolved in 0.9% saline.

Statistics

The cumulative concentration-response curves are expressed in μm contraction and presented as mean ± standard error of the mean (s.e.mean). Differences after cumulative concentration-response curves with substance P, histamine, or arecoline on isolated tissues from the medium and virus-inoculated groups were tested with a Bonferroni adjustment. The maximal contractions are expressed as percentage of the maximal contraction of the control groups. Changes between the control, virus, control-ovalbumin and virus-ovalbumin groups were first tested with ANOVA followed by a multiple range test (Student-Newman-Keuls). *P*-values < 0.05 were considered to reflect a statistically significant difference.

Results

Effects of capsaicin on virus-induced tracheal hyperresponsiveness

In the control group, concentration-response curves to substance P did not reach a clear maximal response (169.0 ± 13.6 μm, Figure 1), which is consistent with findings of Warner *et al.* (1990). The maximal contraction induced in the control group by histamine (274.0 ± 13.1 μm) was higher ($P<0.01$) than for substance P but lower ($P<0.01$) than for arecoline (371.9 ± 15.7 μm, Figure 1).

Virus infection caused a significant (Bonferroni) enhancement in the concentration-response curves to substance P, histamine and arecoline (Figure 1): the maximal responses were increased by 144% ($P<0.01$), 46% ($P<0.05$) and 77% ($P<0.01$), respectively, compared to the control groups (Figure 2a). The pD_2 values did not differ between the experimental groups after the histamine or arecoline concentration-response curves.

Capsaicin pretreatment did not influence the substance P concentration-response curve of the control group. In the virus-treated guinea-pigs the maximal contraction was increased by 53% compared to the vehicle-control group (Figure 2b) and reduced by 62% compared to virus group treated with the vehicle solution (Figure 2a). These differences did not reach the level of significance (Bonferroni). Capsaicin completely inhibited the virus-induced tracheal hyperresponsiveness to histamine and arecoline (Figure 2a and b).

Tracheal strips from vehicle-treated animals showed a small contraction when exposed to capsaicin (about 75 μm) while strips from capsaicin-pretreated ones gave no contraction but did react when exposed to KCl (results not shown).

Interactions of virus and ovalbumin-induced tracheal hyperresponsiveness

In saline-aerosol exposed control inoculated guinea-pigs the maximal tracheal contraction after a substance P concentration-response curve was 143.0 ± 11.4 μm. Virus infection and ovalbumin-aerosol exposure alone induced a similar degree of

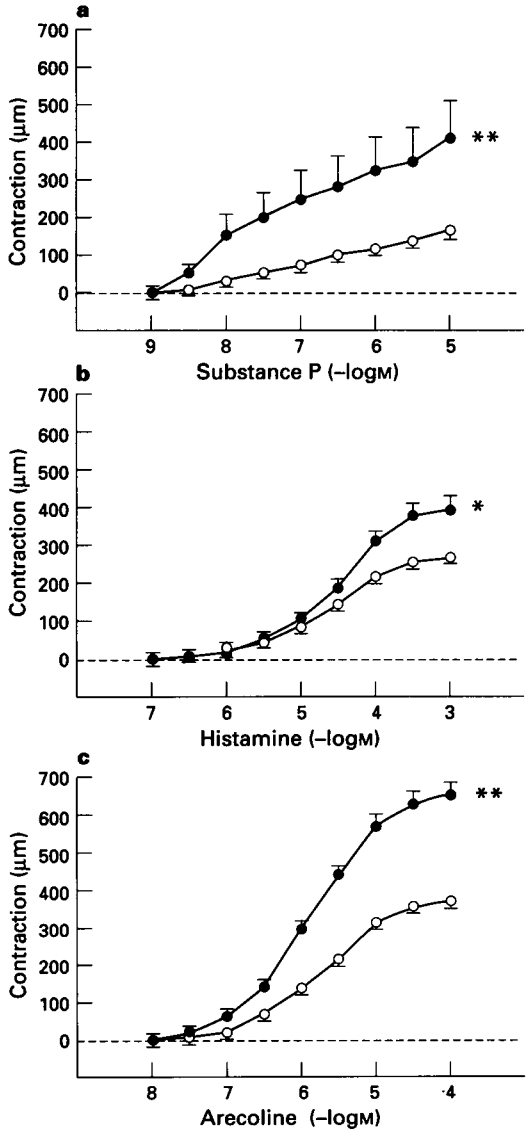

Figure 1 Concentration-response curves to (a) substance P, (b) histamine and (c) arecoline: (○) vehicle-control group; (●) vehicle-virus group. Curves were constructed 4 days after inoculation. Each data point represents the mean ± s.e.mean of 8 tracheal strips. *$P<0.05$; **$P<0.01$ (Bonferonni) compared to the control inoculated group.

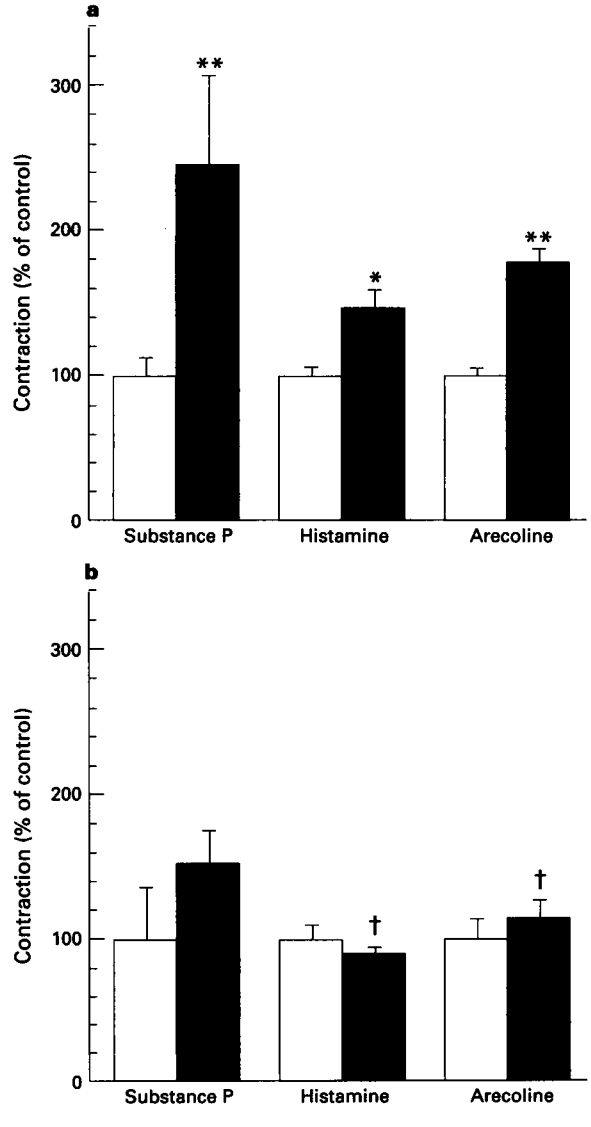

Figure 2 Maximal tracheal contractions (as % of control) in response to substance P, histamine and arecoline 4 days after inoculation with control solution (open columns) or PI-3 virus (solid columns) of animals (a) pretreated with vehicle (b) pretreated with capsaicin. *$P<0.05$; **$P<0.01$ (Bonferonni) compared to the control inoculated group. †$P<0.05$ (Bonferonni) compared with the virus inoculated groups in Figure 2a. The data are presented as mean ± s.e.mean ($n=8$).

tracheal hyperresponsiveness to substance P (Figure 3). A combination of ovalbumin-aerosol exposure and virus infection induced a tracheal hyperresponsiveness to substance P which was increased significantly compared to either treatment alone (Figure 3, $P<0.01$, Student-Neuman-Keuls).

The maximal tracheal contraction of the control group in response to histamine was 368.8 ± 38.2 μm. In virus- or ovalbumin-treated animals the maximal contractions were significantly enhanced; however, the combination had only a slight additional effect (Figure 3).

In the control group, arecoline induced a maximal contraction of 392.0 ± 32.0 μm. Pretreatment of the animals with virus or ovalbumin significantly increased the maximal responses (Figure 3). The ovalbumin-aerosol exposed guinea-pigs with or without a respiratory infection significantly enhanced the tracheal contraction compared to virus-treatment alone ($P<0.01$, Student-Neuman-Keuls). The respiratory infection in the ovalbumin-aerosol exposed animals had a small additional effect compared to ovalbumin treatment alone (Figure 3).

The pD_2 values did not differ between the experimental groups.

Discussion

In the present study it is shown that virus-induced tracheal hyperresponsiveness can be inhibited by capsaicin pretreatment. In addition, virus infection was able to potentiate responses to substance P and to a lesser extent to histamine and arecoline in ovalbumin-aerosol exposed guinea-pigs.

Airway hyperresponsiveness is a main characteristic of the pathophysiology of asthma. Although the mechanism(s) underlying the hyperresponsiveness are not clear, increasing attention has been focused on the role of the sensory innervation in the induction of airway hyperresponsiveness. In guinea-pigs respiratory virus infection has been shown to enhance airway smooth muscle responses to histamine, arecoline (Folkerts *et al.*, 1993a,b; 1995) and substance P (Saban *et al.*, 1987). The present study confirms these findings. Virus infection in guinea-pigs has been shown to cause epithelial damage (Folkerts *et al.*, 1992; 1993b). The epithelial damage could expose sensory nerve endings and cause excessive release of sensory neuropeptides (Barnes *et al.*, 1991). Several studies have now shown that endogenous release of neuropeptides or exogenously applied tachykinins can induce airway hyperresponsiveness (Umeno *et al.*, 1992; Hsiue *et al.*, 1992). Exogenously administered substance P potentiated acetylcholine-induced bronchospasm in the guinea-pig (Omini *et al.*, 1989). Tamura *et al.* (1989) reported that neurokinin A augmented methacholine responses for as long as 4 weeks in Japanese monkeys. Moreover, it has been shown that substance P antibodies can inhibit the ovalbumin-induced airway hyperresponsiveness in guinea-pigs (Ladenius *et al.*, 1991). In the present study capsaicin pretreatment depleted the sensory nerves of neuropeptides, as demonstrated by the lack of responses to exogenously applied capsaicin. Therefore, the suppression of the airway hyperresponsiveness might be due to a diminished release of sensory neuropeptides after a viral respiratory infection. This idea is supported by the finding that destruction of sensory nerves with capsaicin inhibits the induction of airway hyperresponsiveness by (a) repeated antigen inhalation, (b) toluene diisocyanate and (c) a delayed-type hypersensitivity reaction (Thompson *et al.*, 1987; Ladenius & Biggs, 1989; Matuse *et al.*, 1991; Ladenius & Nijkamp, 1993; Buckley & Nijkamp, 1994).

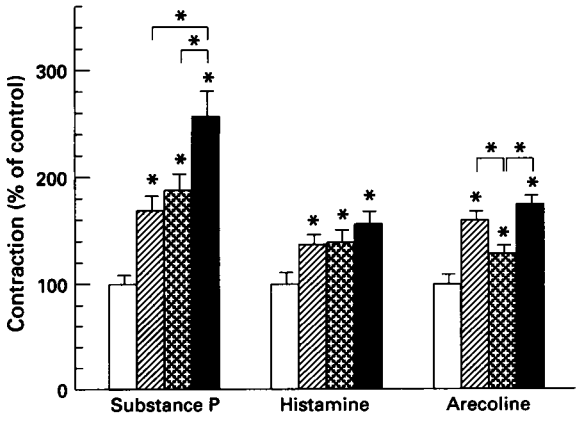

Figure 3 Maximal tracheal contractions (as % of control) in response to substance P, histamine and arecoline of guinea pigs: inoculated with control solution and exposed to a saline aerosol (open columns), inoculated with control solution and exposed to an ovalbumin-aerosol (hatched column), inoculated with PI-3 virus and exposed to a saline aerosol (cross hatched columns) or inoculated with PI-3 virus and exposed to an ovalbumin-aerosol (solid columns). *$P<0.01$ (ANOVA followed by Student-Neumann-Keuls) compared to the control inoculated group or indicated group. The data are presented as mean ± s.e.mean ($n=8$).

Part of the hyperresponsiveness to substance P was still present after capsaicin pretreatment. Interestingly, Jacoby *et al.* (1988) and Dusser *et al.* (1989) showed that the activity of the neutral endopeptidase, the main degradative enzyme for substance P, decreases in the respiratory tract after a viral infection. The increasing residence time of substance P might be responsible for the increased responsiveness in virally infected capsaicin pretreated animals.

Parainfluenza-3 virus expresses large quantities of neuraminidase on its viral coat (Scheid *et al.*, 1972). This enzyme is known to reduce the activity of neutral endopeptidase (Dusser *et al.*, 1989) but can also degrade muscarinic M_2 receptors (Fryer *et al.*, 1990). This decrease in the M_2-receptor subtype has been shown to increase bronchoconstriction induced by vagal stimulation due to a decrease in the autoinhibition of acetylcholine release. However, the decrease in M_2-receptor number can not account for an increase in tracheal reactivity to exogenously applied histamine or the muscarinic agonist, arecoline.

In ovalbumin-treated guinea-pigs PI-3 virus only moderately affected the contraction in response to histamine or arecoline. This might indicate that the pathogenesis of airway hyperresponsiveness due to viral respiratory infections or due to ovalbumin is similar. Capsaicin pretreatment inhibits both the virus-induced (this study) and the ovalbumin-induced changes in airway responsiveness (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991; Ladenius & Nijkamp, 1993). Therefore, a likely explanation for the induction of the increase in tracheal responsiveness to histamine and arecoline is that the concentration of tachykinins is enhanced in the respiratory tract of both models. The possibility cannot be excluded that the shortening capacity of the smooth muscle was already maximal to these agonists after ovalbumin aerosol exposure and thus viral infection could not further increase the shortening. Maximal contraction was not achieved for substance P. Interestingly, the increased tracheal responsiveness to substance P after virus- or ovalbumin-treatment was significantly enhanced when both treatments were combined. There is growing evidence that the release of neuropeptides is increased after ovalbumin exposure (Hsiue *et al.*, 1993, Kawano *et al.*, 1993, Mosimann *et al.*, 1993). It is tempting to speculate that the enhanced tracheal responsiveness in the ovalbumin-exposed animals is due to an increased release of tachykinins which induces a non-specific airway hyperresponsiveness and that in virus-infected animals the hyperresponsiveness is due to a decreased activity of neutral endopeptidase activity. Both treatments could lead to an additional increase in airway hyperresponsiveness.

It is concluded that tachykinins contribute to the virus-induced airway hyperresponsiveness and that these neuropeptides could be involved in the enhancement of asthmatic exacerbations due to viral respiratory infections.

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