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The involvement of sensory neuropeptides in toluene diisocyanate-induced tracheal hyperreactivity in the mouse airways

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1 Recently, we developed a murine model to investigate toluene diisocyanate (TDI)-induced occupational asthma. After skin-sensitization and intranasal challenge with TDI (1%) mice exhibited tracheal hyperreactivity 24 h after the challenge.

2 The aim of the present study was to investigate the possible role for sensory neuropeptides in the development of this tracheal hyperreactivity.

3 First, we demonstrated that direct application of TDI *in vitro* induced the release of tachykinins from the sensory nerves in the mouse isolated trachea. Second, capsaicin pretreatment, resulting in the depletion of sensory neuropeptides, completely abolished the TDI-induced tracheal hyperreactivity 24 h after the challenge. Third, the selective neurokinin$_1$ (NK_1)-receptor antagonist RP 67580 (0.2 μmol kg^{-1}) also inhibited tracheal hyperreactivity when it was administered before the challenge. However, administration of RP 67580 during the sensitization phase did not result in a suppression of the TDI-induced tracheal hyperreactivity 24 after the challenge.

4 When TDI-sensitized mice were topically challenged with TDI a marked ear swelling response was observed. The cutaneous response after TDI application was not affected by capsaicin pretreatment or RP 67580 administration.

5 These results clearly show that sensory neuropeptides, particularly tachykinins, are essential for the development of TDI-induced tracheal hyperreactivity during the effector phase. The differences between the airways and skin with respect to the sensory neuropeptides is intriguing and could suggest a local action for the tachykinins in the airways.

Keywords: Toluene diisocyanate; tracheal hyperreactivity; sensory neuropeptides

Introduction

Toluene diisocyanate (TDI), a low molecular weight compound, is a well known cause of occupational asthma (Bernstein, 1982). Sensitized subjects exhibit specific airway hyperresponsiveness and a marked inflammation of the airways characterized by an influx of neutrophils and eosinophils (Mapp *et al.*, 1987; Boschetto *et al.*, 1987). The mechanisms underlying these symptoms are controversial. In only 20% of the subjects with TDI-induced asthma an increase in TDI-specific IgE antibodies has been found (Butcher *et al.*, 1980). Recently, we developed a murine model to investigate the mechanisms of action of TDI (Scheerens *et al.*, 1996). After skin-sensitization on two consecutive days and intranasal challenge 7 days later with 1% TDI no increase in serum IgE was observed. However, 24 h after the challenge a marked increase in tracheal reactivity to carbachol was measured in TDI-sensitized mice when compared to non-sensitized mice.

The sensory nerves which are part of the noncholinergic, nonadrenergic (NANC) system, are thought to play an important role in the skin and airways. In the respiratory tract, sensory nerves are found in abundance around pulmonary blood vessels and in the epithelium of the trachea and bronchi of many species (Ghatei *et al.*, 1982; Barnes *et al.*, 1991). They contain a range of neuropeptides including the tachykinins (substance P and neurokinin A) (Lundberg *et al.*, 1984) and calcitonin gene-related peptide (CGRP) (Mak & Barnes, 1988). The tachykinins can induce bronchoconstriction via activation of the neurokinin$_2$ (NK_2)-receptor (Lundberg *et al.*, 1983a) and mucus production (Rogers *et al.*, 1989), plasma-protein leakage and vasodilatation (Lundberg *et al.*, 1983b) in the airways via the activation of NK_1-receptors. However, in mice airways only NK_1-receptors have been demonstrated (Manzini, 1992). Additionally, substance P has been shown to degranulate mast cells by a non-receptor mediated mechanism, thus promoting the release of vasoactive amines such as histamine and 5-hydroxytryptamine (5-HT) (Fewtrell *et al.*, 1982; Barnes *et al.*, 1986). The most important biological action of CGRP is the regulation of airway blood flow; it is a potent vasodilator (Brain *et al.*, 1985). Interestingly, several studies have already demonstrated that TDI is capable of inducing the release of sensory neuropeptides from capsaicin-sensitive nerves *in vivo* and *in vitro* (Mapp *et al.*, 1990; 1991; Kitajiri *et al.*, 1993; Chitano *et al.*, 1994).

More recent data have revealed the ability of various sensory neuropeptides to modulate immune functions. The tachykinins have been shown to induce proliferation of mitogen-stimulated T-lymphocytes (Payan *et al*, 1983; Stanisz *et al.*, 1986; Casini *et al.*, 1989), migration of leukocytes (Marasco *et al.*, 1981; Moore, 1984), and activation of macrophages (Koff & Dunegan, 1985). In contrast, CGRP has been found to inhibit the proliferation of mitogen-stimulated T-lymphocytes (Umeda *et al.*, 1988; Casini *et al.*, 1989), although several studies also suggest that CGRP may be pro-inflammatory on its own (Gamse & Saria, 1985; Buckley *et al.*, 1991; 1992). In the literature the role for sensory neuropeptides in the induction of airway hyperreactivity has been investigated in several studies (Boichot *et al.*, 1995; Sakamoto *et al.*, 1994). In addition studies in our own laboratory have recently demonstrated the importance of sensory neuropeptides in a murine model for pulmonary delayed-type hypersensitivity (DTH) reaction (Buckley & Nijkamp, 1994a). The aim of the present study was

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to investigate the role for sensory nerves and the tachykinins in the sensitization and effector phase of TDI-induced tracheal hyperreactivity in the mouse.

Methods

Animals

Mice (male BALB/c 6–8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Isometric measurement of tracheal reactivity

Tracheal reactivity was measured by the method of Garssen *et al.* (1990). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg kg^{-1} i.p.). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. An intact piece of 9 tracheal rings (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (mM: NaCl 118, KCl 4.7, $CaCl_2 \cdot 6H_2O$ 2.5, $MgCl_2 \cdot 6H_2O$ 0.5, $NaHCO_3$ 25.0, $NaHPO_4 \cdot H_2O$ 1.0 and glucose 11.1). The trachea was directly slipped onto 2 supports of an organ bath one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95%: 5%; O_2: CO_2) at a constant temperature (37°C). Isometric measurements were made by a force displacement transducer (Harvard Bioscience, Boston, MA) and a 2 channel recorder (Servogar type SE-120) and were expressed as changes in mg force. Optimal pre-load for the mouse trachea was determined to be 1 g. The trachea was allowed to equilibrate for at least 1 h before further manipulations. During the equilibrium phase the fluid in the bath was changed every 15 min. To assess reactivity concentration-response curves to carbachol or 5-HT were determined 24 h after challenge with TDI. In some experiments the direct effect of TDI and substance P on the isolated mouse trachea was measured *in vitro*. After precontraction with carbachol (3×10^{-7} M bath concentration) one single concentration of TDI (ranging from 10^{-6}–10^{-4} M) or substance P (ranging from 10^{-9}–10^{-6} M) was added to the organ bath. TDI was dissolved in dimethyl sulphoxide (DMSO); however, the concentration of DMSO did not exceed 0.1% in the organ bath and had no effect on its own. To study the effect of RP 67580, the trachea was incubated for 30 min with RP 67580 (10^{-8}–10^{-6} M bath concentrations) before precontraction with carbachol and subsequent addition of TDI or substance P. In separate experiments, isoprenaline (10^{-7} M bath concentration), which is also a relaxant on murine smooth muscle, was added to the precontracted trachea to establish the specificity of RP 67580 for the NK_1-receptor.

Sensitization procedure

Mice were sensitized twice daily on day 0 and day 1 either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (non-sensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl). During the sensitization procedure the mice were anaesthetized with sodium pentobarbitone (50 µl; 30 mg kg^{-1}, i.p.).

Challenge procedure

TDI-sensitized and non-sensitized groups were challenged with 1% TDI dissolved in ethylacetate:olive oil (1:4) on day 8. Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg kg^{-1}, i.p.). Furthermore, mice were also challenged on the ears; TDI (20 µl; 0.5%; dissolved in acetone) or vehicle control (20 µl; acetone) was applied topically to both sides of the ears.

Capsaicin pretreatment

Mice were anaesthetized with sodium pentobarbitone (100 µl; 30 mg kg^{-1}) and injected s.c. (2×50 µl) in the neck region with a capsaicin suspension (25 mg kg^{-1}) or vehicle control (alcohol:Tween 80:saline; 2:1:7) on two consecutive days. The capsaicin pretreatment was performed before the mice were 6 weeks of age, after which the animals were left to recover for 14 days before being used further. The reduction in function of the sensory nerves in the capsaicin-pretreated and saline-pretreated groups was evaluated *in vitro* in the isolated trachea. These tracheal preparations were precontracted with a single dose of carbachol (3×10^{-7} M bath concentration). After a steady level was achieved, capsaicin (100 nM) was added to the bath.

Drug administration in vivo

The effect of RP 67580 was tested either during the sensitization or challenge procedure or both. TDI-sensitized mice and non-sensitized mice were injected i.v. with RP 67580 (0.2 µmol kg^{-1}) 5 min before each application of TDI during the sensitization and/or challenge procedure. This dose has been shown to be most effective (Buckley & Nijkamp, 1994b; Kraneveld *et al.*, 1995). As a control group, TDI-sensitized and non-sensitized mice were injected with the inactive enantiomer of RP 67580, RP 68651 at the same dose. In additional experiments TDI-sensitized and non-sensitized mice were treated with calcitonin gene-related peptide (CGRP) or the CGRP-antagonist, $CGRP_{8-37}$, around the TDI challenge. CGRP was injected i.p. (10^{-7} M) 12 h before the challenge and i.v. (10^{-9} M) 1 h before and 5 h after the challenge. The specific CGRP-antagonist, $CGRP_{8-37}$ (0.2 µmol kg^{-1}) was administered 5 min before and 1 h after the challenge (Buckley & Nijkamp, 1994b).

Measurement of cutaneous reactions

An increase in ear thickness was measured 24 h after topical challenge with 0.5% TDI in acetone. Mice were injected i.p. with an overdose of sodium pentobarbitone and the thickness of the TDI-treated ear and the vehicle-treated ear were measured with an engineers micrometer immediately after injection (Mitutoyo, Japan, No. 293–561) (Buckley & Nijkamp, 1994b). Results are expressed as the difference in ear thickness (Δ ear thickness, mm) between the two ears.

Chemicals

Toluene diisocyanate, olive oil and $CGRP_{8-37}$ were purchased from Sigma Chemical Co., St. Louis, U.S.A. Capsaicin and Tween 80 were purchased from Fluka Chemie, AG, Buchs, Switzerland. Substance P and CGRP were purchased from Bachem Feinchemikalien AG, Bubendorf, Switzerland. RP 67580 ((3aR, 7aR)-7,7-diphenyl-2-(1-imino-2-(2-methoxyphenyl)ethyl)perhydroisoindol-y-one) and RP 68651 ((3a5, 7aS)-7,7-diphenyl-2-(1-imino-2-(2-methoxyphenyl)ethyl)perhydroisoindol-y-one) were generous gifts of Rhone-Poulenc Rorer, (Dr C. Garret) France. Carbachol and isoprenaline were purchased from Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands, Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands.

Statistics

All experiments were designed as completely randomized multifactorials with 6–14 mice per group. EC_{50} and E_{max} values for the carbachol and 5-HT-induced tracheal con-

tractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol or 5-HT concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analyzed by two-way analysis of variance followed by a *post-hoc* comparison between groups. In the figures and tables group means ± s.e.mean are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, analyses of variance and *post-hoc* comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

Results

Direct effect of TDI and substance P on the mouse isolated trachea

Tracheal preparations taken from naive mice were tested for their reactivity to directly applied TDI and substance P. TDI (10^{-4} M) and substance P (10^{-7} M) had no effect on the resting basal tone of the mouse isolated trachea. However, after precontraction with carbachol (3×10^{-7} M bath concentration) TDI produced a concentration-dependent relaxation of the mouse trachea with a maximal effect at 10^{-4} M bath concentration (Figure 1a). A similar concentration-dependent relaxation was found after administration of substance P (3×10^{-10}–3×10^{-7} M bath concentration) to the precontracted trachea (Figure 1b). In further experiments the effect of the NK_1-receptor antagonist, RP 67580, on the relaxation induced by TDI and substance P was examined. Incubation with RP 67580 for 30 min did not have any effect on the precontraction induced by carbachol. Figure 2a and b show that the relaxation induced by TDI (10^{-5} M) and substance P (3×10^{-9} M) were both concentration-dependently inhibited by RP 67580 (10^{-8}–10^{-6} M), with a maximal effect at 10^{-6} M (90% inhibition). To demonstrate the specificity for the NK_1-receptor we tested the effect of RP 67580 on isoprenaline-induced relaxation. RP 67580 (10^{-6} M) had no effect on isoprenaline (10^{-7} M)-induced relaxation of the mouse isolated trachea (isoprenaline; 92 ± 3% relaxation; isoprenaline + RP 67580: 94 ± 2% relaxation, mean ± s.e.mean for $n=6$ mice/group). Moreover, capsaicin pretreatment completely inhibited TDI-induced relaxation, whereas substance P-induced relaxation was unaltered (Table 1).

Effect of capsaicin pretreatment on tracheal hyperreactivity

First, the effectiveness of sensory neuropeptide depletion by capsaicin was assessed by adding capsaicin (100 nM) to trachea precontracted with carbachol (3×10^{-7} M bath concentration). Direct application of capsaicin induced a relaxation of 17 ± 4% in capsaicin-pretreated mice in comparison to a 60 ± 8% relaxation in vehicle-pretreated mice. These results are in agreement with previous studies where depletion of sensory neuropeptides was tested in the airways and the skin (Buckley & Nijkamp, 1994a). Once sensory nerve depletion had been established tracheal preparations taken from TDI-sensitized and non-sensitized mice, pretreated with capsaicin or vehicle, were tested for their reactivity to carbachol and 5-HT (10^{-8}–10^{-4} M) 24 h after intranasal challenge with TDI. Twenty four hours after the challenge the vehicle-pretreated TDI-sensitized mice exhibited hyperreactivity to either of the chemically dissimilar compounds, carbachol and 5-HT, when compared to the vehicle-pretreated non-sensitized mice (Figure 3, Table 2). TDI-induced tracheal hyperreactivity to carbachol was completely inhibited by capsaicin pretreatment (Figure 3, Table 2). Interestingly, capsaicin pretreatment itself increased the tracheal reactivity to carbachol in non-sensitized mice but this was not significant (Figure 3, Table 2). The sensitivity (EC_{50}) to carbachol and 5-HT remained unchanged in all groups tested (Table 2).

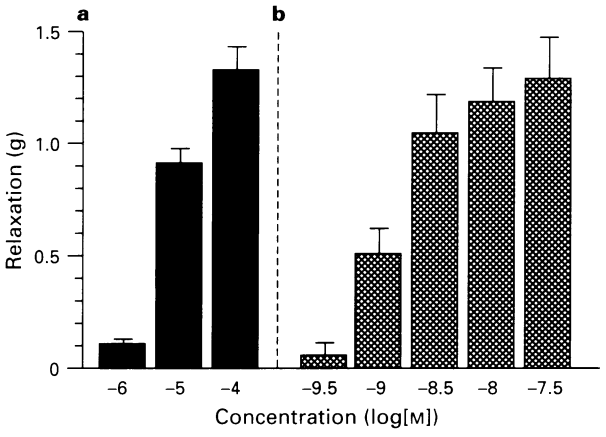

Figure 1 Direct effect of (a) toluene diisocyanate (TDI) and (b) substance P on the mouse isolated trachea. Tracheal preparations were first precontracted with carbachol (3×10^{-7} M bath concentration) whereafter TDI (10^{-6}–10^{-4} M) or substance P (3×10^{-10}–3×10^{-7} M) was added. Results are expressed as mean ± s.e.mean for $n=4$–6 mice/group.

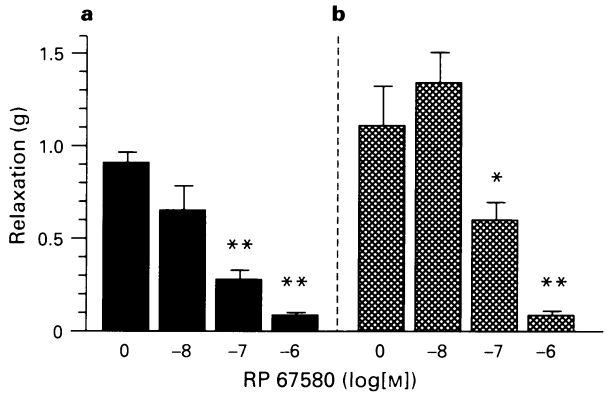

Figure 2 Effect of RP 67580 on the (a) toluene diisocyanate (TDI)- and (b) substance P-induced tracheal relaxation. Before precontraction with carbachol (3×10^{-7} M bath concentration) and subsequent relaxation with TDI (10^{-5} M) or substance P (3×10^{-9} M) the tracheal preparations were incubated for 30 min with RP 65780 (10^{-8}–10^{-6} M). Results are expressed as means ± s.e.mean for $n=4$–6 mice/group. Significant differences between values are denoted by $^*P<0.05$ and $^{**}P<0.01$.

Table 1 Effect of capsaicin pretreatment on TDI- and substance P-induced relaxation in the mouse isolated trachea

Pretreatment	*Relaxing agent*	*% relaxation*
Vehicle	Capsaicin	65 ± 6
Capsaicin	Capsaicin	20 ± 5**
Vehicle	Substance P	67 ± 8
Capsaicin	Substance P	66 ± 8
Vehicle	TDI	73 ± 3
Capsaicin	TDI	12 ± 6**

Results are expressed as mean ± s.e.mean for $n=4$–7 mice/group. Significant differences are denoted by $^{**}P<0.01$, when compared to the vehicle-pretreated mice.

Effect of NK_1-receptor antagonist on tracheal hyperreactivity

The effects of RP 67580, the specific NK_1-receptor antagonist and its inactive enantiomer RP 68651 were measured both during the sensitization and effector phases on the TDI-induced tracheal hyperreactivity 24 h after intranasal challenge with TDI. RP 67580 (0.2 μmol kg^{-1}, i.v.) had no effect on the TDI-induced tracheal hyperreactivity when it was administered 5 min before each application of TDI during the sensitization phase (Table 3). However, when RP 67580 (0.2 μmol kg^{-1}, i.v.) was administered 5 min before the TDI challenge, tracheal hyperreactivity was suppressed and there was no significant difference between the TDI-sensitized and non-sensitized mice (Table 3). Furthermore, RP 67580 (0.2 μmol kg^{-1}, i.v.) abolished the TDI-induced tracheal hyperreactivity when administered 5 min before each application of TDI during both the sensitization and effector phases (Table 3). Neither of the RP 67580 treatment regimes had a significant effect on the sensitivity (EC_{50} value) of control or TDI-sensitized mice (Table 3). In all these experiments RP 68651 had no effect on the tracheal hyperreactivity observed in TDI-sensitized mice (Table 3).

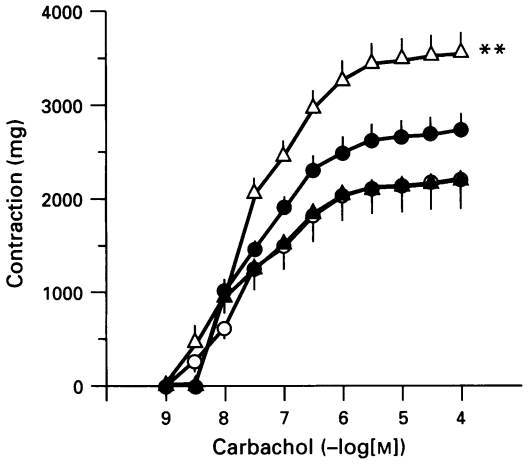

Figure 3 Tracheal reactivity after capsaicin pretreatment in TDI-sensitized and non-sensitized mice. Concentration-response curves to carbachol were measured in the trachea of non-sensitized (○,●) and TDI-sensitized (△,▲) mice either pretreated with capsaicin (●,▲) or vehicle (○,△) 24 h after intranasal challenge with 1% TDI. Results are expressed as mean for $n=6$ mice/group; vertical lines show s.e.mean. Significant differences between the vehicle-pretreated-TDI-sensitized group and the capsaicin-pretreated-TDI-sensitized groups are denoted by $^{**}P<0.01$.

Effect of CGRP and $CGRP_{8-37}$ on tracheal hyperreactivity

The effect of CGRP on the TDI-induced tracheal hyperreactivity 24 h after the challenge was measured. Administration of CGRP around the time of challenge (−12, −1 and +5 h) had no significant effect on tracheal hyperreactivity induced by exposure to TDI (Figure 4). The TDI-sensitized groups, treated with saline or CGRP, exhibited hyperreactivity to carbachol when compared to the relevant non-sensitized groups (E_{max}: non-sensitized-saline-treated 2150±97 mg; TDI-sensitized-saline-treated 3027±287 mg; non-sensitized-CGRP-treated 2214±251 mg; TDI-sensitized-CGRP-treated 2995±305 mg; mean±s.e.mean, for $n=7-8$ mice/group, $P<0.05$). $CGRP_{8-37}$ (0.2 μmol kg^{-1}, i.v.) did not influence the TDI-induced tracheal hyperreactivity either (E_{max}: non-sensitized-saline-treated 2182±86 mg; TDI-sensitized-saline-treated 2837±177 mg; non-sensitized-$CGRP_{8-37}$-treated 1907±101 mg; TDI-sensitized-$CGRP_{8-37}$-treated 2769±312 mg; mean ± s.e.mean for $n=6-12$ mice/group, $P<0.05$).

Table 2 EC_{50} and E_{max} values derived from concentration-response curves to carbachol and 5-hydroxytrytamine (5-HT) ($10^{-8}-10^{-4}$ M) in non-sensitized and TDI-sensitized mice after vehicle or capsaicin pretreatment

Pretreatment	*Sensitization*	*Contractile agent*	*EC_{50} (10^{-7} M)*	*E_{max} (mg)*
Vehicle	Control	Carbachol	0.274±0.041	2225±266
Vehicle	TDI	Carbachol	0.263±0.044	3564±240*
Vehicle	Control	5-HT	3.76±1.05	1837±118
Vehicle	TDI	5-HT	2.53±0.27	2430±197*
Capsaicin	Control	Carbachol	0.215±0.024	2630±171
Capsaicin	TDI	Carbachol	0.352±0.192	2115±280

Results are expressed as mean±s.e.mean for $n=6$ mice/group. Significant differences are denoted by $^{*}P<0.05$ between the non-sensitized and TDI-sensitized mice.

Table 3 EC_{50} and E_{max} values derived from concentration-response curves to carbachol ($10^{-8}-10^{-4}$ M) in non-sensitized and TDI-sensitized mice after RP 67580 treatment

Sensitization	*Treatment*	*Administration (day)*	*EC_{50} ($\times 10^{-7}$ M)*	*E_{max} (mg)*
Control	RP 68651	0-1-8	0.364±0.052	2248±263
TDI	RP 68651	0-1-8	0.339±0.164	3120±298*
Control	RP 67580	0-1-8	0.398±0.062	2148±85
TDI	RP 67580	0-1-8	0.345±0.098	2223±133¶
Control	RP 68651	0-1	0.409±0.065	2160±183
TDI	RP 68651	0-1	0.504±0.095	2770±131*
Control	RP 67580	0-1	0.500±0.125	2279±143
TDI	RP 67580	0-1	0.674±0.116	2770±131*
Control	RP 68651	8	0.324±0.065	1886±139
TDI	RP 68651	8	0.554±0.351	2930±238**
Control	RP 67580	8	0.277±0.050	2186±235
TDI	RP 67580	8	0.273±0.062	2235±226

Results are expressed as mean±s.e.mean for $n=4-8$ mice/group. Significant differences are denoted by $^{*}P<0.05$ or $^{**}P<0.01$, between the non-sensitized and TDI-sensitized mice, or by $\P P<0.05$ between the RP 68651 and RP 67580-treated mice.

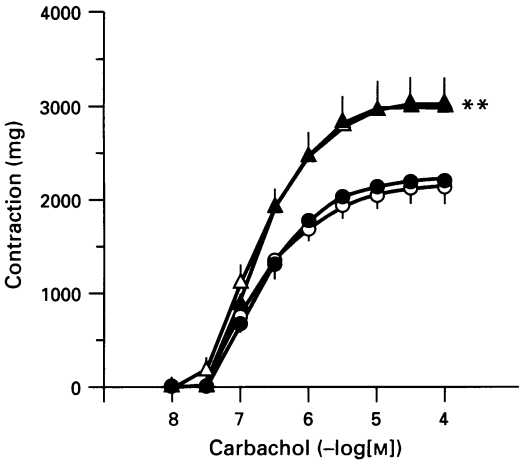

Figure 4 Tracheal reactivity after calcitonin gene-related peptide (CGRP) treatment around the time of the challenge. Concentration-response curves to carbachol were measured in trachea of non-sensitized (○,●) and TDI-sensitized (△,▲) mice after CGRP (●,▲) or vehicle (○,△) treatment during the challenge (−12 h, −1 h and +5 h). Results are expressed as mean for $n=7-8$ mice/group; vertical lines show s.e.mean. Significant differences between the non-sensitized and TDI-sensitized curves are denoted by $^{**}P<0.01$.

Cutaneous responses

In addition to tracheal reactivity, the development of cutaneous responses was also followed. TDI sensitization followed by topical challenge with TDI resulted in a marked and significant increase in ear swelling 24 h after the challenge when compared to the non-sensitized mice (Table 4). In contrast to the effects on tracheal hyperreactivity, none of the treatments (i.e. capsaicin-pretreatment, RP 67580 administration, CGRP and $CGRP_{8-37}$ treatment) had a significant effect on the TDI-induced ear swelling response (Table 4).

Table 4 Increase in ear swelling (10^{-2} mm) of TDI-sensitized and non-sensitized mice 24 h after the challenge and the effect of capsaicin, RP 67580, CGRP and $CGRP_{8-37}$ treatment

Treatment	*Non-sensitized*	*TDI-sensitized*
Vehicle	0.071±0.011	0.260±0.007**
Capsaicin	0.065±0.002	0.200±0.023**
RP 68651	0.077±0.012	0.334±0.038**
RP 67580	0.085±0.008	0.389±0.043**
Vehicle	0.062±0.010	0.299±0.048**
CGRP	0.063±0.007	0.284±0.027**
Vehicle	0.077±0.031	0.238±0.034*
$CGRP_{8-37}$	0.018±0.008	0.311±0.022**

Results are expressed as mean±s.e.mean for $n=4-6$ mice/group. Significant differences between TDI-sensitized and non-sensitized mice are denoted by $^{*}P<0.05$ and $^{**}P<0.01$.

Discussion

In a previous study we developed a model to study occupational asthma that is induced by repeated exposure to TDI (sensitization phase and a challenge phase) (Scheerens *et al.*, 1996). In the present study we demonstrated the important role for sensory neuropeptides, particularly the tachykinins, in the development of tracheal hyperreactivity associated with this model. Direct application of TDI induced a concentration-dependent relaxation of the mouse trachea *in vitro*. Substance P also induced a concentration-dependent relaxation of the mouse trachea *in vitro*. Both these responses were completely inhibited by the selective NK_1-receptor antagonist, RP 67580. Additionally, capsaicin pretreatment blocked the TDI-induced relaxation whereas the substance P-induced relaxation remained equal. These results clearly demonstrate that TDI is capable of releasing a tachykinin in the murine trachea. It has been demonstrated that *in vitro* in the rat bladder (Mapp *et al.*, 1990) and in guinea-pig airway smooth muscle (Mapp *et al.*, 1991; 1993) TDI induced a contraction through the activation of the efferent function of capsaicin-sensitive sensory nerves. In these latter studies it was also shown that tachykinin release from the peripheral endings resulted in mast cell degranulation and the subsequent release of bioactive mediators (Mapp *et al.*, 1993). Mapp and coworkers showed that TDI was able to either contract or relax human bronchial smooth muscle (Chitano *et al.*, 1994). They showed that capsaicin provoked the same responses in the human bronchi suggesting that TDI and capsaicin act similarly in human isolated airways. From these studies it can be concluded that TDI can stimulate sensory nerves. However, a discrepancy exists between species in the effect sensory neuropeptides have on smooth muscle activity. In man, guinea-pig and rat, release of sensory neuropeptides results in contraction of the airway smooth muscle whereas tachykinins produce a relaxation in the mouse airway smooth muscle (Manzini, 1992).

We developed a murine model to investigate TDI-induced asthma (Scheerens *et al.*, 1996). This model exhibits some of the important features of clinical TDI-induced asthma. After skin-sensitization with TDI (1%) followed by an intranasal challenge (1% TDI) non-specific tracheal hyperreactivity to carbachol and 5-HT was found in TDI-sensitized mice compared to non-sensitized mice (Scheerens *et al.*, 1996). The results from the present study clearly show that TDI is able to release tachykinins from sensory nerves in the tracheal smooth muscle of the mouse. Moreover, we found that after capsaicin pretreatment TDI was no longer able to induce tracheal hyperreactivity. These results suggest an important role for sensory nerves and neuropeptides in the induction of tracheal hyperreactivity after exposure to TDI. When capsaicin pretreatment, resulting in sensory neuropeptide depletion, was performed before sensitization, the different phases (i.e. sensitization and effector phases) could not be examined separately. Therefore, we tested the effect of a selective NK_1-antagonist, RP 67580, during the sensitization and effector phases. RP 67580 was found to inhibit the TDI-induced tracheal hyperreactivity when administered before the challenge. However, the TDI-induced tracheal hyperreactivity was not suppressed when RP 67580 was administered during the sensitization phase. Takeda and co-workers showed, in a model for nasal allergy in the guinea-pig, that sensory neuropeptide depletion before sensitization with TDI resulted in suppression of nasal allergy-like symptoms (Takeda *et al.*, 1993). Thompson and co-workers demonstrated that TDI-induced airway hyperresponsiveness in spontaneously breathing guinea-pigs was prevented by capsaicin pretreatment and the tachykinin receptor antagonist (D-Arg^1, D-Pro^2, D-$Trp^{7,9}$, Leu^{11}) substance P (Thompson *et al.*, 1987). This TDI-induced airway hyperresponsiveness was also inhibited by another tachykinin receptor antagonist, spantide, and potentiated by an inhibitor of tachykinin metabolism, phosphoramidon (Sheppard & Scypinski, 1988). However, both these studies differed quite considerably from our mouse model because the responses were measured 1−2 h after a 1 h exposure to TDI and, in our opinion, in this latter model the irritant effect after TDI exposure and not the effect of repeated exposure to TDI was investigated.

While it is clear from this present study that the tachykinins are involved in TDI-induced tracheal hyperreactivity, it is not known which cells are the target for these mediators. A direct effect of tachykinins in the induction of tracheal hyperreactivity in smooth muscle is not likely because they have been shown to induce relaxation in mouse airways. Interestingly, in our previous studies it has been proposed that T-cells play an important role in TDI-induced tracheal hyperreactivity

(Scheerens *et al.*, 1996). It is known that tachykinins can concentration-dependently enhance the proliferation of mitogen-stimulated T-lymphocytes (Payan *et al.*, 1983; Stanisz *et al.*, 1986; Casini *et al.*, 1989). In addition, substance P has been found to activate macrophages (Koff & Dunegan, 1985), induce the production of cytokines (Lotz *et al.*, 1988) and facilitate the migration of macrophages, neutrophils, and T-lymphocytes (Marasco *et al.*, 1981; Moore, 1984). Finally, it has been demonstrated that substance P activates mast cells of the respiratory system (Heaney *et al.*, 1995), resulting in the release of bioactive mediators. It is therefore possible that sensory neuropeptides indirectly modulate tracheal smooth muscle reactivity during the effector phase via one of these mechanisms. We have clearly shown that upon intranasal challenge with TDI tachykinins are released from the sensory nerve endings in the airways. We hypothesize that the tachykinins then stimulate antigen-specific T-lymphocytes, macrophages, mast cells or a combination of these cell populations. The subsequent release of bioactive mediators (i.e. cytokines, histamine) could lead to the observed tracheal hyperreactivity in the mouse airways.

Several studies have suggested a pro-inflammatory activity for CGRP (Gamse & Saria, 1985; Buckley *et al.*, 1991; 1992). However, in our model $CGRP_{8-37}$ was unable to inhibit the TDI-induced tracheal hyperreactivity and the cutaneous responses. Recently, CGRP has been shown to inhibit a cutaneous delayed-type hypersensitivity (DTH) reaction when administered before the induction (sensitization) phase (Asahina *et al*, 1995). In light of these experiments it was thought that CGRP itself might suppress the TDI-induced tracheal hyperreactivity or cutaneous responses during the effector phase. However, CGRP did not influence the TDI-induced responses in either the trachea or the skin and we conclude that CGRP does not play a role in these reactions.

In contrast to the tracheal hyperreactivity the cutaneous hypersensitivity responses induced by TDI were not influenced by sensory neuropeptides. Neither capsaicin, RP 67580, CGRP nor $CGRP_{8-37}$ treatment had any effect on the TDI-induced ear swelling response 24 h after the challenge. These results are in agreement with a previous study from our group (Buckley & Nijkamp, 1994a). In this study it was demonstrated that DTH reactions in the skin were not affected by capsaicin pretreatment whereas tracheal hyperreactivity was abolished. Interestingly, in the same study leukocyte accumulation and mucosal exudation in the bronchoalveolar lavage fluid were markedly enhanced after the systemic depletion of sensory neuropeptides, leading to the possibility that it was not a difference between the airways and skin that was important but a difference between airway smooth muscle reactivity and inflammation (leukocyte accumulation and oedema). In agreement, Thompson and co-workers also found a discrepancy between TDI-induced airway hyperresponsiveness and airway oedema. Capsaicin pretreatment abolished the TDI-induced increase in airway hyperresponsiveness in contrast to both the PMN accumulation and the tracheal oedema which were slightly increased after capsaicin pretreatment (Thompson *et al.*, 1987).

In summary, we have shown that sensory neuropeptides and particularly tachykinins are involved in the effector phase of TDI-induced tracheal hyperreactivity. The tachykinins do not act directly on the tracheal smooth muscle but their effects are probably mediated through the activation of other cells (i.e. T-lymphocytes or mast cells). Clarification of the mechanism of action of the tachykinins will undoubtedly lead to a better understanding of the pathophysiological events prevalent to occupational asthma and may offer new targets for therapeutic intervention.

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