Modulatory role of tachykinin NK₁ receptor in cholinergic contraction of mouse trachea

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ABSTRACT: The role of the NK₁ receptor in airway contraction induced by electrical field stimulation (EFS) was evaluated by comparing the response in NK₁ receptor knockout mice (NK₁R^{-/-}) with that of NK₁ receptor wild-type controls (WT).

A frequency/response curve on tracheas from NK₁R^{-/-} mice and NK₁R WT littermates was constructed. After incubation with [³H]choline, [³H]acetylcholine release upon EFS was measured by high-performance liquid chromatography and liquid scintillation counting. The effects of atropine (1×10⁻⁶ M), tetrodotoxin (1×10⁻⁶ M) and a specific NK₁R antagonist (SR140333, 1×10⁻⁸ M) were studied, as well as the effects of substance P (1×10⁻⁵ M) on precontracted tracheas.

Upon EFS, NK₁R^{-/-} mice had a significant lower trachea contractility than the NK₁R WT animals, accompanied with less [³H]acetylcholine release. Pretreatment with atropine or tetrodotoxin abolished the EFS-induced contraction in both strains. Pretreatment with the NK₁R antagonist SR140333 significantly reduced the contractility in the NK₁R WT but not in the NK₁R^{-/-} mice. Substance P caused a small contraction in both NK₁R WT and NK₁R^{-/-} mice. Substance P induced a relaxation in precontracted tracheas in NK₁R WT but not in NK₁R^{-/-} mice.

The data presented here provide direct evidence that the NK₁ receptor augments cholinergic neurotransmission in mouse trachea.

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Substance P is a member of the tachykinin peptide family and is localised to the sensory nerves of the airways in various species including mice. This peptide has effects that are potentially relevant in the pathogenesis of asthma, such as bronchoconstriction, bronchial vasodilatation, plasma extravasation, augmentation of airway secretions and effects on inflammatory and immune cells [1]. The bronchoconstrictor effects of substance P are mediated *via* a direct effect on airway smooth muscle and *via* indirect effects on nerves and inflammatory cells.

Direct and indirect evidence exists that substance P has a facilitatory role in the release of acetylcholine from postganglionic cholinergic nerves in guinea pig [2–4], rabbit [5, 6] and human [7, 8] airways. In guinea pigs, both exogenous and endogenous tachykinins may facilitate cholinergic neurotransmission. Through the use of specific antagonists, it has been suggested that NK₁ receptors may be involved in this facilitation [9].

Conversely, tachykinins also exhibit additional biological effects that may protect against pro-asthmatic actions. In mouse and rat airways, substance P is involved in the tachykinin NK₁ receptor-mediated airway relaxations due to epithelium-derived prostaglandin (PG)E₂ [10–12].

The present study was designed to evaluate the effects of endogenous tachykinins acting through the NK₁ receptor [13, 14] on the cholinergic neurotransmission in mouse trachea *in vitro*. Electrical field stimulation (EFS)-induced contractions and acetylcholine release were measured in tracheas of tachykinin NK₁ receptor knockout mice (NK₁R^{-/-}) *versus* NK₁ receptor wild-type control mice (WT). The results provide direct evidence that tachykinins facilitate cholinergic transmission in mouse trachea *in vitro* through the NK₁ receptor. Furthermore, this study has confirmed and extended previous findings that NK₁ receptors are also involved in the substance P-induced relaxation of precontracted smooth muscle cells [10–12, 15].

Materials and methods

Animals

NK₁R^{-/-} and NK₁R WT mice were derived from the mating of heterozygous NK₁R^{+/-} mice, as described previously [16]. The targeting construct was derived from a mouse 129/sv strain genomic library and targeted

clones were injected into C57BL/6 blastocysts. Chimaeric males were mated with C57BL/6 females. The mice were bred from successive generations of sibling knockout and NK₁R WT mice and can be thought of as representing a recombinant inbred strain. The respective NK₁R^{-/-} and WT breeding pairs were provided by the lab of S. Hunt (Cambridge, UK). The animals were bred locally and maintained under germ-free conditions in a conventional animal house in the animal research facilities of the Dept of Respiratory Diseases of the University Hospital, Ghent, Belgium, and received food and water *ad libitum*.

General procedure

The mice were killed by an intraperitoneal injection of pentobarbital (100 mg·kg⁻¹ Nembutal®; Sanofi, Libourne, France). The trachea was gently excised and the adhering connective tissue was carefully dissected and removed under microscopy guidance. The whole tracheas were mounted in 2 mL organ baths containing Krebs solution (KS) (composition in mM: NaCl 118, KCl 4.6, CaCl₂ 2.5, MgSO₄ 1.15, NaHCO₃ 24.9, KH₂PO₄ 1.15 and glucose 5.5), which was maintained at 37°C and bubbled with carbogen (5% carbon dioxide, 95% oxygen (O₂)). The optimal resting tension on the trachea of this mouse strain was 0.7 g, as determined by length-tension experiments. The tracheas were allowed to stabilise for 15 min at this resting tension. After this stabilisation period, increasing concentrations of carbachol were added to the organ bath in a noncumulative way (3.3×10⁻⁷, 1×10⁻⁶ and 3.3×10⁻⁶ M) in order to stabilise the tissues. The contact time for each concentration was 15 min. An interval of 10 min was inserted between the administrations while the bathing medium was changed. The contractions were measured isometrically with Grass FT03 transducers (Grass Instruments Co., Quincy, MA, USA) and recorded on a Graphtec Linearecorder type WR3701 (Graphtech Corp., Tokyo, Japan).

Transmural nerve stimulation

The tracheal rings were suspended between two platinum field bar electrodes (15 mm distance) in 2 mL organ baths containing KS. Responses to EFS were studied by applying monophasic square wave pulses from a Grass S44 stimulator (Grass Instruments Co.), using a supramaximal voltage of 50 V, 230 mA and a pulse duration of 0.5 ms. Frequency/response curves were generated using a frequency range of 0.1–30 Hz. One 10-s train at increasing frequency was given at 2-min intervals, while the Krebs solution was refreshed after two stimulus trains to prevent disturbances of the ion balance. Preliminary experiments showed that, both in NK₁R WT and NK₁R^{-/-} mice, two frequency/response curves could be constructed, with a 30-min interval, without the occurrence of tachyphylaxis. The effect of atropine (1×10⁻⁶ M), tetrodotoxin (TTX, 1×10⁻⁶ M; Alomone Labs, Jerusalem, Israel) and SR140333 (a specific NK₁R antagonist, 1×10⁻⁸ M; X. Emonds-Alt, Sanofi

Recherche, Montpellier, France) [17] was evaluated by incubating tracheal tissue from both mouse strains for 20 min before the construction of the second frequency/response curve.

[³H]acetylcholine release

Two tracheas were mounted vertically between 2 platinum wire electrodes (40×0.5 mm, distance 5 mm) in a 2 mL organ bath containing KS supplemented with cholinechloride (1×10⁻⁶ M) and ascorbic acid (57×10⁻⁶ M). During a 30-min equilibration period, the tissues were superfused at a rate of 2 mL·min⁻¹, using a peristaltic pump (Gilson Minipuls, Villers Le Bel, France). During the last 10 min, the tracheas were subjected to a continuous EFS (Grass S88 Stimulator; Grass Instruments Co.), characterised by 0.5 ms monophasic square wave pulses of 0.1 Hz at 40 V to liberate the stores of neurotransmitter. After this equilibration period, the superfusion was stopped. The neuronal stores of acetylcholine were labelled with [³H]choline (5 µCi·mL⁻¹; [methyl-³H]-choline chloride (2775 GBq·mmol⁻¹); NEN, Boston, USA) under field stimulation delivered at 0.1 Hz during 30 min (40 V, 0.5 ms). To remove loosely bound radioactive choline, the tissues were superfused (2 mL·min⁻¹ for 60 min) with KS supplemented with hemicholinium-3 (1×10⁻⁷ M; hemicholinium-3-bromide; RBI, USA), physostigmine (1×10⁻⁷ M; physostigmine salicylate; Federa, Brussels, Belgium) and atropine (1×10⁻⁶ M). These substances were added to prevent the re-uptake of choline, the hydrolysis of acetylcholine and the presynaptic autoinhibition of acetylcholine release. Presynaptic muscarinic autoreceptors have indeed been demonstrated in the trachea of different species [18–20] and preliminary experiments in the absence or presence of atropine during stimulation phase 3 (S₃, see below) showed that no acetylcholine release could be detected in the mouse trachea without atropine. After this 60-min washout period, the content of the organ baths (1 mL from now on) was collected and replaced every 3 min for a total of 30 collections. The tracheal rings were stimulated three times (S₁, S₂ and S₃) for 30 s (10 Hz at 40 V with 0.5 ms pulses). S₁ started 13 min (5th sample), S₂ 43 min (15th sample) and S₃ 73 min (25th sample) after the end of the washout period. A total of 500 µL of all collected samples was fixed with 2 mL Ultima Gold (Canberra Packard, Meriden, CT, USA). The radioactivity of all samples was measured by liquid scintillation counting (Packard Tri-Carb TR; Canberra Packard) (fig. 1a). The stimulation-induced increase in tritium overflow was calculated by subtracting the basal tritium overflow. The basal tritium overflow during the period of stimulation was calculated by fitting a regression line through the values of the four samples before S₁ and through the values of the samples starting from where the overflow had returned to basal values after S₁, S₂ and S₃. The amounts of [³H]acetylcholine, [³H]choline and [³H]phosphorylcholine were measured by reverse-phase high-performance liquid chromatography (HPLC) (Hyperchrome-HPLC-column, 250×4.6 mm, prepacked with hypersil-ods 5.0 µm; Bischoff Chromatography, Leonberg, Germany), as previously described

Fig. 1. – Example of acetylcholine release measurements. a) Measurement of the released radioactivity in samples collected every 3 min for 90 min. Tracheas were stimulated three times (#; S₁; S₂; and +; S₃). b) Measurement of the stimulation-induced efflux of choline (§), phosphorylcholine (§') and acetylcholine (§§) after high-performance liquid chromatography separation of sample no. 25. The fractions 6–11 contained the peaks with phosphorylcholine and choline while the fractions 14–24 contained acetylcholine. dpm: disintegrations per minute.

[21]. Briefly, a 0.1 M phosphate buffer (pH 4.7) containing methanol (8 vol %; Lab-Scan, Dublin, Ireland) and tetramethylammonium (0.2 mM; Merck-Schuchardt, Hohenbrunn, Germany) was used with a flow of 0.5 mL·min⁻¹. The collection of the effluent was in 1-min fractions. HPLC was performed on one basal fraction before S₁, S₂ and S₃ and on the fractions with the highest radioactivity after the stimulations. A total of 100 µL of the samples was injected into the HPLC and 27 fractions were collected and mixed with 2.5 mL Ultima Gold. The fractions 6–11 contained the peaks with phosphorylcholine and choline, while fractions 14–24 contained the acetylcholine (fig. 1b) (proven by the preliminary HPLC on the standards, results not shown). The real amount of choline, phosphorylcholine and acetylcholine expressed as percentages was calculated by subtraction of the background counting. The background counting was estimated by fitting a regression line through the values of the first five fractions and fractions 26–27 [22].

Effects of carbachol, substance P and Sar⁹,Met(O₂)¹¹-substance P on the tracheal ring tension

Increasing concentrations of carbachol (1×10⁻⁸ to 1×10⁻⁴ M) were added to the organ bath in a cumulative way (in steps of 0.5 log[concentration]), whereby the contact time for each concentration was ≥4 min. Concentration/response curves to substance P (Peninsula Laboratories Ltd, St Helens, UK) and Sar⁹,Met(O₂)¹¹-substance P (a selective NK₁R agonist; Peninsula Laboratories Ltd) were constructed by adding increasing concentrations to the organ bath in a noncumulative way (1×10⁻⁸ M to 1×10⁻⁴ M) after the stabilisation period with carbachol. The contact time for each concentration was ≥4 min. Isolated mouse airways do not exhibit intrinsic tone, thus airway segments were contracted to 50–75% of carbachol maximum with 3.3×10⁻⁷ M carbachol to allow a possible relaxation to substance P and Sar⁹,Met(O₂)¹¹-substance P (1×10⁻⁵ M). Whether the observed relaxation effects of substance P were mediated through the production of nitric oxide (NO) or prostaglandin E₂ was analysed by measuring the effect of a pretreatment with 2×10⁻⁴ M N^o-nitro-L-arginine methyl ester (L-NAME) or 1×10⁻⁶ M indomethacin (Indocid ®; MSD, Brussels, Belgium) on the substance P-induced relaxation in carbachol precontracted tissue. A positive control of the relaxation in indomethacin pretreated tracheal tissue was performed by means of the β-adrenergic compound isoproterenol (Isuprel®; Abbott, Ottignies, Belgium) at 1×10⁻⁵ M.

Statistical analysis

The results of the *in vitro* analyses of airway responsiveness and acetylcholine release are reported as mean±SEM. Frequency/response curves in the absence and presence of antagonists, atropine and TTX (multiple comparisons) were compared by analysis of variance (ANOVA). The differences in acetylcholine release were evaluated using the nonparametric Mann-Whitney U-test. Differences were regarded as significant when *p*<0.05.

Results

Comparison of carbachol- and electrical field stimulation-induced tracheal contractions in NK₁R^{-/-} and corresponding NK₁R WT mice

No differences in tracheal contractions were observed between NK₁R^{-/-} and NK₁R WT mice during a cumulative concentration response curve to carbachol (fig. 2). In contrast, the EFS-induced contraction of the trachea was significantly lower in the NK₁R^{-/-} mice (fig. 3). Pretreatment with SR140333 at 1×10⁻⁸ M was able to reduce the EFS-induced contraction in the NK₁R WT mice (*n*=5) (fig. 4a). At a concentration of 1×10⁻⁸ M, SR140333 had no effect on the EFS-induced contraction of the NK₁R^{-/-} trachea (*n*=4) (fig. 4b). At a higher concentration (1×10⁻⁷ M) of SR140333, a small decrease of EFS-induced

Fig. 2. – *In vitro* response to carbachol of tracheal smooth muscle from NK₁ receptor knockout mice (----) versus NK₁ receptor wild-type control mice (—) (n=6). Data are presented as mean±SEM. There were no differences between the two strains in terms of their *in vitro* responses to this agonist (NS).

Fig. 3. – *In vitro* response to electrical field stimulation (EFS; 50 V, 230 mA, 0.5 ms) of tracheal smooth muscle from NK₁ receptor knockout mice (NK₁R^{-/-}; ----) versus NK₁ receptor wild-type control mice (NK₁R WT; —) (n=12). Data are presented as mean±SEM. Tracheas from NK₁R WT mice responded significantly more to EFS than tracheas from NK₁R^{-/-} mice (p<0.001).

contractions was observed in the NK₁R^{-/-} mice (results not shown). The EFS-induced contractions were completely abolished by pretreatment with TTX (n=2) (fig. 5a) as well as by pretreatment with atropine (n=6) (fig. 5b).

Comparison of acetylcholine release of tracheas from NK₁R^{-/-} and corresponding NK₁R WT mice

In NK₁R WT as well as NK₁R^{-/-} mice, the authors studied whether activation of the NK₁R pathway resulted in differences of acetylcholine release. After labelling with radioactive choline, EFS of tracheas of both NK₁R^{-/-} and NK₁R WT mice resulted in the release of a radioactive signal in the organ bath. The

Fig. 4. – a) *In vitro* response to electrical field stimulation (EFS; 50 V, 230 mA, 0.5 ms) of tracheal smooth muscle from NK₁ receptor wild-type control mice in the presence (—) or absence (----) of 1×10^{-8} M SR140333 (n=5). Data are presented as mean±SEM. p<0.05. b) *In vitro* response to EFS (50 V, 230 mA, 0.5 ms) of tracheal smooth muscle from NK₁ receptor knockout mice in the presence (—) or absence (----) of 1×10^{-8} M SR140333 (n=4) (NS).

first stimulation released more radioactive signal into the organ bath than the second. No significant difference was observed between the second and the third stimulation (results not shown). The tracheas of the NK₁R WT mice released 36% more radioactive signal than the NK₁R^{-/-} mice ($2,306 \pm 305$ versus $1,595 \pm 260$ disintegrations per min·mg wet weight (ww)⁻¹, NS, n=6) during the third stimulation. Performing HPLC on a sample of the organ bath taken just before S₃ revealed a similar basal leakage of [³H]choline and [³H]phosphorylcholine in the tracheas of both strains, but no detectable basal [³H]acetylcholine release. In contrast, during S₃, tracheas of both strains released a detectable amount of [³H]acetylcholine. The NK₁R WT mice released significantly more acetylcholine than the NK₁R^{-/-} counterparts during S₃ (fig. 6).

Effect of substance P and the NK₁R agonist Sar⁹,Met(O₂)¹¹-substance P on tracheal ring tension

Tracheas of both mouse strains were exposed to increasing doses of substance P (1×10^{-8} – 1×10^{-4} M).

Fig. 5.—*In vitro* response to electrical field stimulation (EFS; 50 V, 230 mA, 0.5 ms) of tracheal smooth muscle from NK₁ receptor knockout mice (NK₁R^{-/-}; ----) versus NK₁ receptor wild-type control mice (NK₁R WT; —) in the presence (□) or absence (■) of a) tetrodotoxin (n=2) or b) atropine (n=6). Responses are fully abrogated in the presence of these substances. $p < 0.001$.

Both NK₁R WT and NK₁R^{-/-} mice reacted with a comparable small, but significant contractile response only at the highest concentration of substance P

Fig. 6.—Acetylcholine (ACh) release from tracheas of NK₁ receptor knockout mice (NK₁R^{-/-}) versus NK₁ receptor wild-type control mice (NK₁R WT) caused by stimulation 3 (10 Hz at 40 V with 0.5 ms pulses) after high-performance liquid chromatography separation. Tracheas from NK₁R WT mice released significantly larger amounts of ACh, compared with tracheas from NK₁R^{-/-} mice. **: $p < 0.01$. dpm: disintegrations per minute.

Fig. 7.—Individual tracings showing a) *in vitro* response to substance P (1×10^{-4} M) in NK₁ receptor wild-type control mice (NK₁R WT), b) *in vitro* response to substance P (1×10^{-4} M) in NK₁ receptor knockout mice (NK₁R^{-/-}), c) *in vitro* response to substance P (1×10^{-5} M) of precontracted tracheas from NK₁R WT mice and d) *in vitro* response to substance P (1×10^{-5} M) of precontracted tracheas from NK₁R^{-/-} mice. Solid arrow: carbachol; open arrow: substance P.

(fig. 7). In contrast, no contractile response was observed upon administration of the specific tachykinin NK₁ receptor agonist Sar⁹,Met(O₂)¹¹-substance P in either mouse strain (n=6, data not shown). In a second set of experiments, the effect of these molecules on the trachea, precontracted by carbachol (3.3×10^{-7} M) was evaluated. Upon administration of substance P, a relaxing response was observed in the NK₁R WT mice (fig. 7), averaging $78.52 \pm 8.70\%$ of the carbachol-induced contraction (n=4). In contrast, the tracheas of NK₁R^{-/-} mice (fig. 7) failed to relax ($0.0 \pm 0.0\%$) (n=4). The NK₁R agonist, Sar⁹,Met(O₂)¹¹-substance P induced a relaxation in precontracted trachea (carbachol 3.3×10^{-7} M) of NK₁R WT mice ($86.6 \pm 44.62\%$) but not in precontracted trachea (carbachol 3.3×10^{-7} M) of NK₁R^{-/-} mice ($0.0 \pm 0.0\%$) (n=4).

Two potential mediators of the substance P-induced relaxation were investigated in this respect. Pretreatment of the tracheas of the NK₁R WT mice with L-NAME (2×10^{-4} M), a specific NO-synthase inhibitor, did not inhibit the relaxing effect of substance P (results not shown). In contrast, pretreatment of the NK₁R WT tracheas with the cyclooxygenase inhibitor indomethacin (1×10^{-6} M) completely abolished the substance P-induced relaxation (from $78.52 \pm 8.70\%$ to $0.03 \pm 0.03\%$ in the presence of indomethacin, $p < 0.05$) (n=6). The β_2 -adrenergic-mediated isoprenaline-induced relaxation was not affected by indomethacin pretreatment (data not shown).

Discussion

In this study, the role of the NK₁R in cholinergic neurotransmitter-mediated contraction of mouse trachea has been evaluated *in vitro*. In mice lacking the NK₁R, the contractile response of the trachea to EFS was significantly decreased. An NK₁R antagonist partially reduced the EFS-induced responses in the NK₁R WT mice. The responses to EFS were completely

blocked by the neurotoxin TTX and by atropine, establishing that stimulation of cholinergic neural elements within the airways mediated these contractile responses. Upon EFS, the NK₁R^{-/-} mice produced less acetylcholine compared to the WT littermates. These results suggest that endogenous tachykinins, coreleased during EFS, signal through the NK₁R on cholinergic nerve endings to augment acetylcholine release.

The lower tracheal contraction to electrical stimulation in the NK₁R^{-/-} mice cannot be explained by a lower sensitivity to cholinergic agonists. A cumulative concentration response curve to carbachol revealed no differences between the trachea of the NK₁R WT and NK₁R^{-/-} mice. The EC₅₀ values of both curves were very similar for both strains. Moreover, the maximal response to carbachol was somewhat higher in the NK₁R^{-/-} mice (compared to NK₁R WT) (fig. 2).

Endogenous tachykinins (*e.g.* substance P) do not seem to be involved in mediating direct contractile responses through the NK₁R, as only high concentrations of exogenous substance P were able to induce a small contraction in both strains. Moreover, as substance P-induced contraction in both NK₁R WT and NK₁R^{-/-} mice, it is likely that this effect is mediated through another tachykinin receptor, probably the NK₂R. This is supported by the lack of a contractile effect for the specific NK₁R agonist, Sar⁹,Met(O₂)¹¹-substance P. Moreover, pretreatment with atropine completely abolished the EFS-induced contractions, suggesting that these contractions were mediated only through acetylcholine release, excluding a direct effect by endogenously released tachykinins on smooth muscle cells.

In the NK₁R^{-/-} mice a significantly lower release of acetylcholine was observed after EFS, in comparison to the WT mice. These results provide direct evidence that the NK₁R is involved in the augmentation of cholinergic neurotransmission in the mouse *in vitro*. These present findings are in agreement with data obtained in other species, such as rabbits and guinea pigs. In rabbits, a significant increase in acetylcholine release *in vitro* was observed upon application of substance P, which could be abolished by the addition of a specific NK₁R antagonist [6]. In guinea pigs, functional evidence exists for the presence of facilitatory tachykinin NK₁ receptors on postganglionic nerve terminals [2–4, 9]. In humans, NK₁ receptors were occasionally found in nerves by the use of immunostaining with specific antibodies [23]. NK₁ receptor immunoreactivity has also been found in the epithelium, particularly on cell surfaces of the upper half of the epithelial layer [24].

The present findings contrast with data from a recently published study in which it was shown that tachykinin NK₂ receptors are involved in the facilitation of acetylcholine release from guinea pig trachea [25]. These differing results may be the consequence of species specificity or due to limitations of the experimental methods used. The study on guinea pig trachea was carried out with the use of specific agonists for the NK₁, NK₂ and NK₃ receptors. However, only an NK₂ receptor antagonist was used to evaluate the involvement of the NK₂ receptor. The present results do not

exclude a possible involvement of the NK₂ receptor, but the involvement of the NK₁ receptor is incontestable by the use of an NK₁ receptor knockout model. Moreover, the functional results observed upon EFS in the NK₁R^{-/-} trachea were mimicked by application of a specific NK₁ receptor antagonist (SR140333) [17] on NK₁R WT tracheas. However, the reduction of the EFS-induced contractions by SR140333 in NK₁R WT mice seemed less pronounced than the decrease in response to EFS in the NK₁R^{-/-} mice. It is possible that the concentration of SR140333 used (1×10^{-8} M) did not fully block the NK₁ receptor. Indeed, higher concentrations, such as 1×10^{-7} M, are needed to inhibit NK₁-agonist-induced effects in a human astrocytoma cell line [26]. Higher concentrations are also used in the brain tissue of mice [27, 28]. However, higher concentrations of SR140333 induced nonspecific effects in the preparation reported here, which precluded the performance of a concentration/response curve for SR140333.

Besides its pro-asthmatic effects, substance P also exhibits the capacity to exert a protective effect. Elevated levels of substance P may play a protective role in the responses of rat airways to chronic exposure of inhaled irritants like sulphur dioxide and ozone [29–31]. A relaxing effect of substance P on the airways has been demonstrated in Sprague-Dawley rats and mice [10–12, 15, 32–34]. In rat trachea, the importance of the presence of the epithelium and the NK₁ receptor has been demonstrated. Through the use of specific inhibitors and measurements of mediator release, PGE₂ was found to be the relaxing factor [10, 15, 33]. This phenomenon has also been described in mice. A specific NK₁ receptor agonist, [Pro⁹]-substance P sulphone, and exogenously administered as well as endogenously released substance P mediate relaxation of precontracted mouse main bronchi. The specific NK₁ receptor antagonist, CP96345, can block this effect. The relaxation induced by substance P was inhibited by indomethacin [11]. This hypothesis was further confirmed by the use of EP₂ (one of the four PGE₂ receptor subtypes)-deficient mice. Mice lacking the EP₂ receptor have a defect in PGE₂-mediated relaxation of smooth muscle tissue. Relaxation to substance P was significantly reduced in tracheas of these mice. However, the release of PGE₂ induced by substance P was not impaired [12]. The current study in the NK₁R^{-/-} mice and their corresponding NK₁R WT littermates confirm that substance P-induced relaxation in mouse trachea is mediated solely through the NK₁ receptor. Moreover, the specific NK₁R agonist, Sar⁹,Met(O₂)¹¹-substance P was able to induce relaxation of the precontracted NK₁R WT tracheas. The relaxation of precontracted tracheas of NK₁R WT animals could be blocked by the addition of indomethacin, suggesting that an inhibitory metabolite of arachidonic acid derived from cyclooxygenase is involved. As the relaxing capacity of the tracheas, as measured by application of isoprenaline, was not influenced by indomethacin, the present observations, together with previous findings of other groups [10, 11], suggest that PGE₂ is the relaxing factor. In addition, it was reported that guinea pig tracheal tube preparations

relax by stimulation of epithelial NK₁ receptors *via* NO release [35, 36].

It is important to note that relaxation occurred 30 s after the addition of substance P in the NK₁R WT tracheas. As in the functional studies, the tracheas were stimulated during 10 s whereby an immediate contractile response was observed, and therefore relaxation cannot interfere with these EFS-induced contractions.

In conclusion, by using NK₁ receptor knockout mice, the authors have evaluated the modulatory role of the NK₁ receptor pathway in cholinergic contractions of mouse trachea. Through functional studies and direct acetylcholine release experiments in an NK₁ receptor knockout model, the authors conclude that tachykinin NK₁ receptor activation augments cholinergic neurotransmission upon electrical field stimulation. Exogenously applied NK₁ receptor agonists do not induce contraction but can influence tracheal tone by induction of relaxation of precontracted tracheas.

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