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Tachykinin NK_3 and NK_1 receptor activation elicits secretion from porcine airway submucosal glands

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1 We presently characterized the tachykinin receptor subtypes, using tachykinin receptor agonists and selective antagonists, that induce submucosal gland fluid flux (J_G) from porcine tracheal explants with the hillocks technique. We also investigated the effects of the tachykinin receptor agonists on the electrophysiologic parameters of the tracheal epithelium in Ussing chambers.

2 The NK_1 tachykinin receptor agonist substance P (SP, 1 μM) and the NK_3 tachykinin receptor agonist [MePhe^7]neurokinin B ([MePhe^7]NKB, 1 μM) induced gland fluid fluxes of 0.29 ± 0.03 μl min^{-1} cm^{-2} ($n=26$) and 0.36 ± 0.05 μl min^{-1} cm^{-2} ($n=24$), respectively; while the NK_2 tachykinin receptor agonist [βAla^8]neurokinin A (4-10) ([βAla^8]NKA (4-10), 1 μM) had no effect on J_G ($n=10$).

3 The NK_1 receptor antagonist CP99994 (1 μM, $n=9$) blocked 93% of the SP-induced J_G, whereas the NK_3 receptor antagonist SB223412 (1 μM, $n=12$) had no effect on the SP-induced J_G. However, SB223412 (1 μM, $n=9$) blocked 89% of the [MePhe^7]NKB-induced J_G while CP99994 (1 μM, $n=10$) did not affect the [MePhe^7]NKB-induced J_G. The NK_2 receptor antagonist SR48968 (1 μM) did not block the J_G induced by either the NK_1 ($n=4$) or NK_3 ($n=13$) receptor agonists.

4 The nicotinic ganglionic acetylcholine receptor antagonist hexamethonium (1 μM) and the muscarinic acetylcholine receptor antagonist atropine (1 μM) also decreased the NK_3 receptor agonist-induced J_G by 67% ($n=10$) and 71% ($n=12$), respectively.

5 The potential difference (PD), short-circuit current (I_{SC}), and membrane resistance (R_M) of the porcine tracheal epithelial membranes were not significantly affected by any of the neurokinin agonists or antagonists (1 μM, basolateral) used in this study, although SP and [βAla^8]NKA (4-10) induced a slight transient epithelial hyperpolarization.

6 These data suggest that NK_1 and NK_3 receptors induce porcine airway gland secretion by different mechanisms and that the NK_3 receptor agonists induced secretion is likely due to activation of prejunctional NK_3 receptors on parasympathetic nerves, resulting in acetylcholine-release. We conclude that tachykinin receptor antagonists may have therapeutic potential in diseases with pathophysiological mucus hypersecretion such as asthma and chronic bronchitis.
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Abbreviations: DMSO, dimethyl sulphoxide; HBSS, Hanks' Balance Salt Solution; I/V, current/voltage; I_{SC}, short-circuit current; J_G, airway submucosal gland fluid flux; N_H, number of hillocks; NKA, neurokinin A; NKB, neurokinin B; PD, potential difference; R_M, membrane resistance

Introduction

The tachykinin peptides substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) display preferential affinity for the tachykinin NK_1, NK_2 and NK_3 receptors, respectively (Maggi, 1995). However, each tachykinin peptide can act as a full agonist on all three receptors, if present at sufficiently high concentrations (Regoli *et al*., 1994). Activation of airway C-fibre afferent nerves leads to a local release of tachykinins that elicit many biological effects, including submucosal gland secretion (Joos *et al*., 2001). These tachykinins may, therefore, play a role in diseases with characteristic pathophysiological mucus hypersecretion (Rogers, 2002) such as asthma (Joos *et al*., 2000; Spina & Page, 1996; Spina *et al*., 1998) whereas chronic bronchitis (Barnes, 2001).

In human airways, it is generally agreed that mucus secretion, microvascular leakage, and increased blood flow are mediated by the NK_1 receptors (Piedimonte, 1995) whereas the NK_2 receptors directly mediate bronchoconstriction (Advenier *et al*., 1992a; Rizzo *et al*., 1999) and potentiate cholinergic bronchomotor tone (Hey *et al*., 1996). Tachykinin NK_3 receptors have yet to be identified in the human airways by immunohistochemical techniques (Bai *et al*., 1995; Baluk *et al*., 1996), although there is evidence that functional NK_3 receptors are localized on guinea-pig airway parasympathetic ganglion neurons (Myers & Undem, 1993). The literature also suggests that airway peripheral NK_3 receptors modulate airway hyperresponsiveness (Daoui *et al*., 2000) and cough (Daoui *et al*., 1998) in guinea-pigs, and inflammatory cell recruitment in mice (Nenan *et al*., 2001).

Previous studies in mucus secretion with selective tachykinin receptor antagonists on ferret (Geppetti *et al*., 1993; Meini *et al*., 1993), rat (Wagner *et al*., 1999), and human (Rogers *et al*., 1989) tissues showed that the tachykinin-induced mucus secretory response is mostly mediated *via*

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tachykinin NK_1 receptors. In these studies, the secretagogue effects elicited by activation of NK_2 and NK_3 receptors were not significant. However, Nagaki *et al.* (1994) have shown that activation of NK_2 receptors can induce secretion from isolated cat airway glands, but not from tracheal explants.

The submucosal glands present in the proximal airways of large animal species play a critical role in the airway defense mechanisms (Donaldson *et al.*, 2002). In the present study, we determined the tachykinin receptor subtypes mediating submucosal gland secretion from pig tracheal epithelium using tachykinin agonists and selective antagonists. Furthermore, the effects of acetylcholine receptor antagonists on tachykinin-induced gland secretion and the effects of tachykinin receptor agonists on transepithelial ion transport were examined. The tissues used in this study were from pigs, large mammals with a dense network of upper airway submucosal glands (Goco *et al.*, 1963; Jones *et al.*, 1975) similar to what is observed in humans (Jeffery, 1983; Choi *et al.*, 2000) but absent in many smaller animal species (Choi *et al.*, 2000). Pig and human airway submucosal glands are also similar with respect to morphology (Goco *et al.*, 1963), density (Phipps, 1981; Phillips *et al.*, 2002a), and the distribution of the different types of mucin (acidic and neutral glycoprotein) they contain (Jones *et al.*, 1975).

Methods

Tissue preparation

The tracheae from 53 pigs weighing 77 ± 5 kg were obtained from a local abattoir and used within 2 h after removal from the pigs. Adventitious tissue was dissected from the external surface of the trachea and the part of the trachea between the larynx and the lobar bronchus before the carina was cut into five or more tubes of approximately 2.5 cm length. The tubes were then longitudinally cut through the anterior and posterior aspects, leaving a small piece of tissue as a tether to assure a paired control tissue from the same location in the trachea, and resulting in two tissues with exposed epithelium. Submucosal gland fluid flux from randomly selected tissues with exposed epithelia was then measured by the hillocks technique (Davis *et al.*, 1976; Nadel & Davis, 1977). The epithelium from one tube of each trachea was used to assess the viability of the trachea and to measure agent-induced changes in electrophysiological parameters *via* the Ussing technique (Ussing, 1949).

Hillocks Technique: Submucosal Grand Fluid Flux

The membrane preparation and subsequent gland fluid flux measurements were carried out as described in detail previously (Phillips *et al.*, 2002a, b). Briefly, the pieces of each trachea were submerged in a tissue bath (Radnoti, Monrovia, CA, U.S.A.) filled with Hanks' Balance Salt Solution (HBSS) continuously bubbled with 95% O_2 and 5% CO_2. The composition of HBSS in mM is: NaCl, 136.8; dextrose, 5.6; KCl, 5.4; $NaHCO_3$, 4.2; $CaCl_2$, 1.3; $MgSO_4$, 0.8; KH_2PO_4, 0.4; and Na_2HPO_4, 0.3. To determine the effects of tachykinin agonists on submucosal gland secretion from porcine tracheae, the tissues were then pretreated for 20 min with 1 μM of either the tachykinin NK_1 receptor antagonist CP99994 (McLean *et al.*, 1993), the tachykinin NK_2 receptor antagonist SR48968 (Advenier *et al.*, 1992b), the tachykinin NK_3 receptor antagonist SB223412 (Sarau *et al.*, 1997), the nicotinic ganglionic acetylcholine receptor antagonist hexamethonium, the muscarinic acetylcholine receptor antagonist atropine, or the appropriate vehicle HBSS or dimethyl sulphoxide (DMSO). After pretreatment, segments of trachea were removed from the bath and the epithelial surface was blotted with a tissue wiper (Kimwipes; Kimberly-Clark Co., Roswell, GA, U.S.A.) to remove any airway secretions present, and then evenly coated with aerosolized tantalum from an aerosol generator. The inert tantalum powder captures the gland secretions above the gland duct openings in the epithelium and also acts as a contrast agent. The tissue was then replaced in the bath containing HBSS, with 1 μM of one of the following: the tachykinin receptor agonist SP, the tachykinin NK_2 receptor agonist [βAla^8]NKA (4-10), the tachykinin NK_3 receptor agonists [MePhe^7]NKB or senktide and pretreatment agent, if any, bathing only the basolateral (cartilage) side with the tantalum-coated epithelium exposed to room air. Also, a [MePhe^7]NKB concentration-response curve (logarithmic intervals between 0.01 to 10 μM) was generated from the tracheal tissues of six pigs. Each tissue was only exposed to one concentration of [MePhe^7]NKB. A microscope was used to capture $25\times$ images of 8 mm^2 of the epithelial surface area, 3 min after the epithelium was coated with tantalum. Preliminary experiments showed that the initial gland fluid flux induced by SP in this preparation was completed in 3 min, consistent with published observations of cholinergically-induced gland secretion in bovine (Wu *et al.*, 1998), ovine (Joo *et al.*, 2001), and porcine (Phillips *et al.*, 2002b) airways.

Elevations or hillocks caused by submucosal gland fluid secretion induced by tachykinin receptor agonists are trapped above the gland ducts. The areas of the hillocks within the image were interactively measured by computer assisted digital image processing and converted to volumes (Phillips *et al.*, 2002a), and the number of hillocks present per image (N_H) was also determined. The results were expressed as submucosal gland fluid flux J_G (μl min^{-1} cm^{-2}) by dividing the total calculated volume of the hillocks by the 3 min data acquisition time interval and the digitized epithelial surface area.

Ussing technique: Epithelial electrophysiology

The electrophysiological parameters of the epithelia mounted in Ussing chambers (Ussing, 1949) were measured to determine the viability of the tissues and to monitor agent-induced changes in epithelial potential difference (PD) and short-circuit current (I_{SC}, the current required to clamp the PD to 0 mV), indicators of transepithelial ion transport. The membrane preparation and subsequent electrophysiological measurements were carried out as described in detail previously (Phillips *et al.*, 2002b). Briefly, one tube from each trachea was cut longitudinally through the anterior side to expose and dissect the posterior epithelium from the underlying cartilage. The epithelium was retained in a plastic slider that was mounted between the half-chambers of an Ussing chamber system (P2300; Physiologic Instruments Inc., San Diego, CA, U.S.A.). Electrodes (Ag^+/AgCl) connected

to a voltmeter and ammeter with current/voltage (I/V) clamp capabilities (VCC-MC2-HV; Physiologic Instruments Inc.) measure the membrane's PD and I_{SC}, respectively. The membrane's resistance (R_M) was calculated using Ohm's law $R_M(\Omega \cdot cm^2) \cdot 10^{-3} = PD(mV)/I_{SC}(\mu A/cm^2)$. Each trachea was required to exhibit a baseline epithelial PD greater than 3 mV for inclusion of gland fluid flux or electrophysiological data in the present study. The effects of basolateral administration of 1 μM SP, [βAla^8]NKA (4-10), senktide, or [MePhe^7]-NKB on airway epithelial electrophysiological parameters were then measured.

Data analysis

The J_G, N_H, PD, I_{SC}, and R_M were summarized as mean$\pm$s.e.mean and n refers to the number of tissues tested. With the hillocks technique, no more than three tissues from each trachea were used with the same experimental protocol and only one tissue per trachea was used with the Ussing technique. Paired, two-tailed, Student's t-tests were performed to determine whether the changes in gland fluid flux or number of hillocks were significantly different after different treatments. The null hypothesis was rejected at $P<0.05$.

Drugs and chemicals

The tachykinin receptor antagonists SR48968 ((S)-N-methyl-N((4-acetylamino-4-phenylpiperidino-2-(3,4)di-chlorophenyl)-butyl)benzamide), CP99994 ((2S,3S)-3-(2-methoxybenzyl)-amino-2-phenyl-piperidine), and SB223412 ((S)-N-(α-ethyl-benzyl)-3-hydroxy-2-phenylquinoline-4-carbox-amide), were synthesized by the Chemical Research Department, Schering-Plough Research Institute (Kenilworth, NJ, U.S.A.) and the tachykinin receptor agonists SP, [βAla^8]NKA (4-10), [MePhe^7]NKB, and senktide were obtained from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.). The nicotinic ganglionic acetylcholine receptor antagonist hexamethonium bromide and the muscarinic acetylcholine receptor antagonist atropine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Concentrated stock solutions were prepared by dissolving the agents in HBSS before addition to the tissue bath, except [βAla^8]NKA (4-10) and [MePhe^7]NKB dissolved in 5% DMSO in HBSS, and SR48968 and SB223412 dissolved in DMSO. The final concentrations of DMSO did not exceed 0.1% by volume.

Results

The average baseline submucosal gland fluid flux ($n=53$ tissues from 53 porcine tracheae) was not significantly greater than zero and no hillocks were apparent on 83% of the control tissues 3 min after addition of tantalum powder. The epithelium from 53 porcine tracheae had a baseline PD of 8.2 ± 0.7 mV (lumen negative) and generated an I_{SC} of 63 ± 3 $\mu A/cm^2$ resulting in an R_M of 134 ± 11 $\Omega \cdot cm^2$.

The tachykinin receptor agonist SP (1 μM) administered to the basolateral (cartilage) side increased J_G to 0.29 ± 0.03 $\mu l\ min^{-1}\ cm^{-2}$ and N_H to 3.4 ± 0.3 hillocks ($n=26$ from 13 pigs). The SP-induced J_G was significantly inhibited by the tachykinin NK_1 receptor antagonist CP99994 (Figure 1) at 0.1 μM ($J_G=0.14\pm0.02$ $\mu l\ min^{-1}\ cm^{-2}$ and $N_H=3.6\pm0.8$ hillocks, $n=5$ from two pigs) and at 1 μM ($J_G=0.02\pm0.01$ $\mu l\ min^{-1}\ cm^{-2}$ and $N_H=1.1\pm0.5$ hillocks, $n=9$ from five pigs). The SP-induced J_G was not significantly inhibited by pretreatment with either 1 μM tachykinin NK_2 receptor antagonist SR48968 ($J_G=0.18\pm0.07$ *vs* 0.19 ± 0.08 $\mu l\ min^{-1}\ cm^{-2}$ and $N_H=5.0\pm1.2$ *vs* 4.3 ± 1.3 hillocks, respectively, $n=4$ from two pigs *versus* paired SP treated control tissues) or 1 μM tachykinin NK_3 receptor antagonist SB223412 ($J_G=0.27\pm0.03$ $\mu l\ min^{-1}\ cm^{-2}$ and $N_H=2.4\pm0.3$ hillocks, $n=12$ from six pigs, Figure 1). With the Ussing chamber, SP (1 μM) administered to the basolateral side caused a slight transient hyperpolarization of 0.5 ± 0.4 mV, which reached its maximum in 350 ± 200 s, without causing a significant change in the electrophysiological parameters PD, I_{SC}, or R_M ($n=6$, Table 1).

The tachykinin NK_2 receptor agonist [βAla^8]NKA (4-10) (1 μM) administered to the basolateral (cartilage) side did not induce gland secretion ($n=10$ from five pigs). With the Ussing chamber, 1 μM [βAla^8]NKA (4-10) administered to the basolateral side caused a slight transient hyperpolarization of 0.3 ± 0.1 mV, which reached its maximum in 300 ± 150 s, without causing a significant change in the PD, I_{SC}, or R_M electrophysiological parameters ($n=5$, Table 1).

The tachykinin NK_3 receptor agonist [MePhe^7]NKB (0.01, 0.1, 1, and 10 μM) caused concentration-dependent increases in J_G (Figure 2a, $n=12$ tissues from six pigs) and N_H (1.5 ± 0.4, 2.6 ± 0.6, 3.8 ± 0.5, and 6.1 ± 0.8 hillocks). 1 μM [MePhe^7]NKB or another tachykinin NK_3 receptor agonist senktide induced similar J_G (0.36 ± 0.06 $\mu l\ min^{-1}\ cm^{-2}$ and 0.40 ± 0.06 $\mu l\ min^{-1}\ cm^{-2}$, respectively) and N_H (5.3 ± 0.4 and 5.9 ± 0.6 hillocks, respectively) in 21 tissues from 11 pigs. CP99994 (1 μM) and SR48968 (1 μM) had no significant effect on the J_G induced by [MePhe^7]NKB ($n=10$ from six pigs) or senktide ($n=13$ from seven pigs, Figure 2b). However, the tachykinin NK_3 receptor antagonist SB223412 (1 μM) significantly decreased the J_G (Figure 2b) and N_H induced by [MePhe^7]NKB ($J_G=0.04\pm0.01$ $\mu l\ min^{-1}\ cm^{-2}$ and $N_H=2.8\pm0.6$ hillocks, $n=9$ from five pigs) and senktide ($J_G=0.06\pm0.02$ $\mu l\ min^{-1}\ cm^{-2}$ and $N_H=2.0\pm0.5$ hillocks, $n=11$ from six pigs). The [MePhe^7]NKB-induced J_G (0.27 ± 0.05 $\mu l\ min^{-1}\ cm^{-2}$) and N_H (4.6 ± 0.6 hillocks) were also decreased by 1 μM atropine (0.09 ± 0.04 $\mu l\ min^{-1}\ cm^{-2}$ and 2.0 ± 0.6 hillocks, respectively, $n=12$ from six pigs) and 1 μM hexamethonium (0.07 ± 0.02 $\mu l\ min^{-1}\ cm^{-2}$ and 4.4 ± 1.0 hillocks, $n=10$ from five pigs, Figure 3). With the Ussing

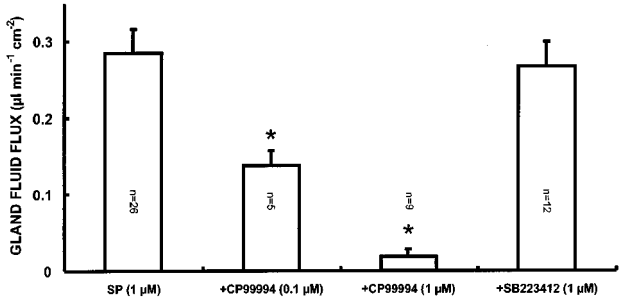

Figure 1 Effect of SP on submucosal gland secretion. The SP-induced gland secretion is inhibited in a dose-dependent manner by the NK_1-receptor antagonist CP99994 at 0.1 μM and 1 μM but was unaffected by the NK_3 antagonist SB224312. *$P<0.05$ compared to SP alone.

Table 1 Effect of basolaterally administered neurokinin receptor agonists (1 μM) on epithelial electrophysiological parameters

Agonist	*Parameter*	*Baseline*	*After Treatment*
SP			
($n=6$)	PD (mV)	5.0 ± 3.9	5.5 ± 4.2
	I_{SC} (μA cm^{-2})	61.9 ± 4.8	62.5 ± 4.7
	R_m (Ω cm^2)	85 ± 62	94 ± 67
[β Ala^8]NKA (4–10)			
($n=5$)	PD (mV)	8.8 ± 1.7	9.1 ± 1.7
	I_{SC} (μA cm^{-2})	80.4 ± 10.1	81.0 ± 10.2
	R_m (Ω cm^2)	116 ± 25	120 ± 27
[MePhe^7]NKB			
($n=12$)	PD (mV)	9.7 ± 1.5	9.7 ± 1.5
	I_{SC} (μA cm^{-2})	56.3 ± 5.5	56.3 ± 5.5
	R_m (Ω cm^2)	183 ± 34	186 ± 36
Senktide			
($n=6$)	PD (mV)	10.6 ± 2.8	10.7 ± 2.8
	I_{SC} (μA cm^{-2})	69.0 ± 5.1	68.9 ± 4.7
	R_m (Ω cm^2)	149 ± 36	150 ± 35

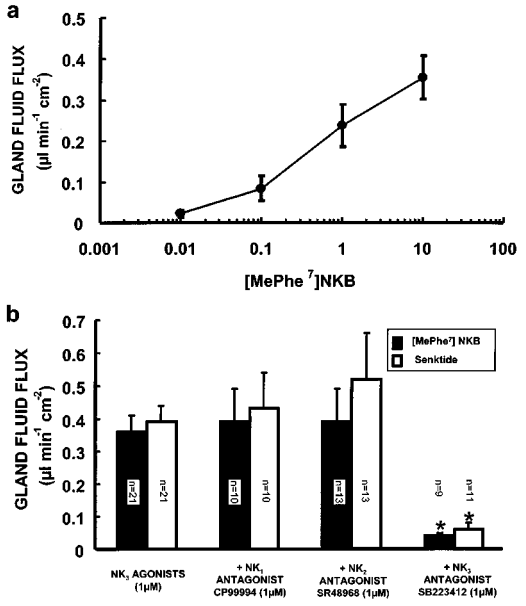

Figure 2 Effect of [MePhe^7] NKB and senktide on porcine tracheal tissues. (a) Concentration-response curve to [MePhe^7] NKB on porcine tracheal submucosal gland secretion ($n=12$ from six pigs at each concentration). (b) Effects of tachykinin NK_1 receptor antagonist CP99994 (1 μM), tachykinin NK_2 receptor antagonist SR48968 (1 μM), and tachykinin NK_3 receptor antagonist SB223412 (1 μM) on submucosal gland secretion induced by (1 μM) [MePhe^7] NKB or senktide. The data are presented as mean$\pm$s.e.mean and were compared using paired student's t-tests. Asterisks indicate significant difference ($P<0.05$) from tissues of the same trachea treated with the respective tachykinin NK_3 receptor agonist alone.

chamber, neither [MePhe^7]NKB or senktide (1 μM) administered to the basolateral (Table 1) or luminal side caused a change in the PD, I_{SC}, or R_m electrophysiological parameters ($n=12$ and 6, respectively). Treatment with 1 μM CP99994, SR48968, SB223412, or 0.1% DMSO had no effect on baseline J_G or electrophysiological parameters (data not shown).

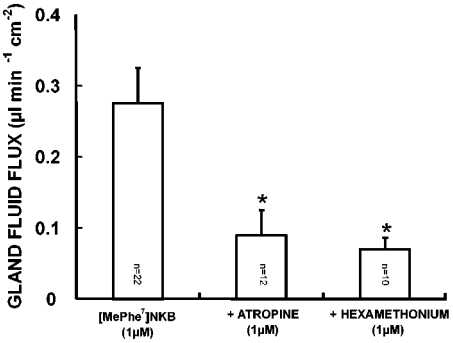

Figure 3 Effect of atropine and hexamethonium on the NK_3 receptor agonist [MePhe^7]NKB-induced gland secretion. *$P<0.05$ compared to [MePhe^7]NKB alone.

Discussion

To our knowledge, this is the first study to report that tachykinin NK_3 agonists induce submucosal gland fluid secretion from porcine trachea (Figure 2). Our study demonstrates that functional tachykinin NK_3 receptors are present within the porcine trachea, and stimulation of these receptors induces submucosal gland secretion. This secretion was significantly reduced by the tachykinin NK_3 receptor antagonist SB223412, whereas the tachykinin NK_1 receptor antagonist CP99994 and the tachykinin NK_2 receptor antagonist SR48968 had no effect (Figure 2b). The [MePhe^7]NKB induced J_G was also decreased by the nicotinic ganglionic acetylcholine receptor antagonist hexamethonium and by the muscarinic acetylcholine receptor antagonist atropine (Figure 3). Furthermore, we confirmed that the tachykinin receptor agonist SP (Figure 1) induces gland secretion by an NK_1 receptor dependent mechanism, whereas the tachykinin NK_2 receptor agonist [βAla^8]NKA (4-10) had no effect, as shown previously in different species.

Previous studies on ferret (Geppetti *et al*., 1993; Meini *et al*., 1993), rat (Wagner *et al*., 1995) and human (Rogers *et al*., 1989) tracheae have shown that [MePhe^7]NKB or NKB have a modest response or no effect on airway gland secretion. Recent studies suggest that only the NK_1 receptor is responsible for capsaicin-sensitive 'sensory-efferent' nerve stimulation of mucus output in ferret (Khawaja *et al*., 1999) and rat (Wagner *et al*., 1999), whereas in our preparation, activation of either NK_1 (Figure 1) or NK_3 (Figure 2) receptors increase gland secretion. The apparent species differences between these studies may be due to differences in tachykininergic regulation of airway fluid secretion, as shown for the tachykinin regulation of bronchial muscle contraction (Belvisi *et al*., 1994), or due to the temporal resolution differences between the radiolabeled mucin ^{35}S tracer technique in the previous studies (Geppetti *et al*., 1993; Meini *et al*., 1993; Wagner *et al*., 1999) and hillocks technique in the present study.

To date, NK_3 receptors have not been detected by immunohistochemical techniques in the airways (Baluk *et al*., 1996) but, peripheral NK_3 receptors have been found in submucosal neurons in the rat gut by immunofluorescence (Grady *et al*., 1996). Also, it has been shown that the tachykinin NK_3 receptor agonist NKB depolarized guinea-pig bronchial ganglion neurons (Myers & Undem, 1993),

and that functional activation of NK_3 receptors by senktide induces release of ACh from guinea-pig ileum (Guard & Watson, 1991). Taken together, these data and our results suggest that release of endogenous NK_3 receptor agonists *in vivo* may induce porcine airway gland secretion by activation of prejunctional NK_3 receptors on parasympathetic nerves although a peripheral local afferent-parasympathetic reflex (Undem & Myers, 1997) cannot be ruled out. The small residual gland secretion from the hexamethonium ($0.07\ \mu l\ min^{-1}\ cm^{-2}$) and atropine ($0.09\ \mu l\ min^{-1}\ cm^{-2}$)-treated tissues challenged with [MePhe^7]NKB (Figure 3) is not likely due to non-selective actions of [MePhe^7]NKB on NK_1 receptors because the [MePhe^7]NKB-induced gland secretion from SB223412 pretreated tissues ($0.04\ \mu l\ min^{-1}\ cm^{-2}$, Figure 2b) suggests that the secretion is NK_3 receptor specific and because CP99994 at a concentration that significantly inhibited SP-induced gland secretion (Figure 1) had no effect on NK_3 agonist-induced gland secretion (Figure 2b).

We also showed by using the hillocks technique that SP is a potent airway submucosal gland secretagogue confirming reports using the same technique in pig (Haxhiu *et al*., 1990) and other techniques in different species such as human (Rogers *et al*., 1989), dog (Haxhiu *et al*., 1988), ferret (Khan *et al*., 2001), rat (Wagner *et al*., 1999), and also in pig (Trout *et al*., 2001) airways. The concentration of SP (1 μM) is commonly used in many studies of gland secretion (Rogers *et al*., 1989; Haxhiu *et al*., 1990; Wagner *et al*., 1999; Trout *et al*., 2001). The SP-induced secretion was dose-dependently inhibited by CP99994 (Figure 1), indicating that this secretion was specifically mediated by the NK_1 receptors. The inhibitory action of an NK_1 antagonist on SP-induced gland secretion has also been shown in rats (Wagner *et al*., 1999) and ferrets (Khan *et al*., 2001). The measured J_G-induced by 1 μM SP of $0.29\ \mu l\ min^{-1}\ cm^{-2}$ in the present study is similar to the value reported by Trout *et al*. (2001) of $0.30\ \mu l\ min^{-1}\ cm^{-2}$ in a whole excised pig bronchi preparation, but greater than methacholine (1 μM)-induced gland secretion of $0.03 \pm 0.01\ \mu l\ min^{-1}\ cm^{-2}$ (Phillips *et al*., 2002b), an observation already reported in ferret trachea (Khan *et al*., 2001). SP likely induces mucus secretion by a direct effect on gland NK_1 receptors as Trout *et al*. (2001) have shown that atropine has no effect on SP-induced porcine airway fluid secretion.

No airway submucosal gland secretion was obtained upon addition of the tachykinin NK_2 receptor agonist [βAla^8]NKA (4-10), confirming other studies in different species (Ramnarine *et al*., 1994; Khawaja *et al*., 1999; Wagner *et al*., 1999). Secretion from isolated cat airway glands has been demonstrated in the presence of the NK_2 agonist NKA but was absent in whole tissue preparations (Nagaki *et al*., 1994).

Our baseline electrophysiological parameters for porcine tracheal epithelium for PD (8.2 ± 0.7 mV, lumen negative) and I_{SC} ($63 \pm 3\ \mu A/cm^2$) are in agreement with previous values reported by Ballard *et al*. (1992) and our group (Phillips *et al*., 2002b) in porcine tracheal epithelia (PD of 9.7 mV and 7.5 ± 0.5 mV and I_{SC} of $83\ \mu A/cm^2$ and $73 \pm 4\ \mu A/cm^2$, respectively). The rank order of potency for increasing porcine tracheal epithelial absolute PD among basolaterally administered tachykinins and their analogues (Table 1) was SP (0.5 mV) > [βAla^8]NKA (4-10) (0.3 mV) > Senktide (0.1 mV) > [MePhe^7]NKB (0 mV). The tachykinin receptor antagonists CP99994, SR48968, and SB223412 (1 μM, basolateral) had no effect on epithelial electrophysiological parameters. Our measured increase in absolute PD induced by SP is smaller than that observed in canine tracheal epithelium by SP of $\sim$3 mV (Rangachari & McWade, 1985). Accompanying this small increase in PD is a significant increase in J_G that suggests SP induces an electrically silent process. It has been shown using radioactive ions in ferret trachea, that basolateral administration of SP is a potent secretagogue of both Na^+ and Cl^- ions under short circuit conditions with most of this secretion electrically silent (NaCl) and not detected by transepithelial electrophysiologic measurements (Mizoguchi & Hicks, 1989). The fluid and ion secretion processes are likely taking place in the submucosal glands particularly rich in NK_1 receptors as demonstrated in cat (Lundgren *et al*., 1989), guinea-pig (Hoover & Handcock, 1987), human (Castairs & Barnes, 1986) and ferret (Meini *et al*., 1993).

The present studies show that activation of NK_3 and NK_1 receptors, but not NK_2 receptors, can induce porcine tracheal gland secretion. The mechanism of the NK_1 receptor-induced gland secretion is likely a direct effect of SP on the glands, whereas the NK_3 receptor mechanism likely involves activation of airway submucosal parasympathetic ganglia, as demonstrated by inhibition of gland secretion by atropine and hexamethonium. Moreover, the NK_3 agonist-induced gland secretions in our pig preparation indicate that tachykinins are more potent gland secretagogues than the muscarinic acetylcholine receptor agonist methacholine (Phillips *et al*., 2002b), suggesting that tachykinin receptor antagonists may have therapeutic potential in diseases with pathophysiological mucus hypersecretion such as asthma and chronic bronchitis.

References

ADVENIER, C., NALINE, E., TOTY, L., BAKDACH, H., EMONDS-ALT, X., VILAIN, P., BRELIERE, J.-C. & LEFUR, G. (1992a). Effects on the isolated human bronchus of SR48968, a potent and selective nonpeptide antagonist of the neurokinin A (NK_2) receptors. *Am. Rev. Respir. Dis.,* **146,** 1177 – 1181.

ADVENIER, C., ROUISSI, N., NGUYEN, Q.T., EMONDS-ALT, X., BRELIERE, J.-C., NELIAT, G., NALINE, E. & REGOLI, D. (1992b). Neurokinin A (NK_2) receptor revisited with SR48968, a potent non-peptide antagonist. *Biochem. Biophys. Res. Commun.,* **184,** 1418 – 1424.

BAI, T.R., ZHOU, D., WEIR, T., WALKER, B., HEGELE, R., HAYASHI, S., MCKAY, K., BONDY, G.P. & FONG, T. (1995). Substance P and Neurokinin A-receptor gene expression in inflammatory airway diseases. *Am. J. Physiol.,* **269,** L309 – L317.

BALLARD, S.T., SCHEPENS, S.M., FALCONE, J.C., MEININGER, G.A. & TAYLOR, A.E. (1992). Regional bioelectric properties of porcine airway epithelium. *J. Appl. Physiol.,* **73,** 2021 – 2027.

BALUK, P., BUNNETT, N.W. & MCDONALD, D.M. (1996). Localization of tachykinin NK_1, NK_2, and NK_3 receptors in airways by immunohistochemistry. *Am. J. Respir. Crit. Care Med.,* **153,** A161.

BARNES, P.J. (2001). Neurogenic inflammation in the airways. *Respir. Physiol.,* **125,** 145–154.

BELVISI, M.G., PATACCHINI, R., BARNES, P.J. & MAGGI, C.A. (1994). Facilitatory effects of selective agonists for tachykinin receptors on cholinergic neurotransmission: evidence for species differences. *Br. J. Pharmacol.,* **111,** 103–110.

CASTAIRS, J.R. & BARNES, P.J. (1986). Autoradiographic mapping of substance P receptors in the lung. *Eur. J. Pharmacol.,* **127,** 295–296.

CHOI, H.K., FINKBEINER, W.E. & WIDDICOMBE, J.H. (2000). A comparative study of mammalian tracheal mucous glands. *J. Anat.,* **197,** 361–372.

DAOUI, S., COGNON, C., NALINE, E., EMONDS-ALT, X. & ADVENIER, C. (1998). Involvement of tachykinin NK_3 receptors in citric acid-induced cough and bronchial responses in guinea pigs. *Am. J. Respir. Crit. Care Med.,* **158,** 42–48.

DAOUI, S., NALINE, E., LAGENTE, V., EMONDS, X. & ADVENIER, C. (2000). Neurokinin B- and specific tachykinin NK_3 receptor agonists-induced airway hyperresponsiveness in the guinea-pig. *Br. J. Pharmacol.,* **130,** 49–56.

DAVIS, B., MARIN, M., FISHER, S., GRAF, P., WIDDICOMBE, J. & NADEL, J. (1976). New method for study of canine mucous gland secretion *in vivo*: Cholinergic regulation. *Am. Rev. Respir. Dis.,* **113,** 257.

DONALDSON, S.H., PICHER, M. & BOUCHER, R.C. (2002). Secreted and cell-associated adenylate kinase and nucleoside diphosphokinase contribute to extracellular nucleotide metabolism on human airway surfaces. *Am. J. Respir. Cell Mol. Biol.,* **26,** 209–215.

GEPPETTI, P., BERTRAND, C., BACCI, E., HUBER, O. & NADEL, J.A. (1993). Characterization of tachykinin receptors in ferret trachea by peptide agonists and nonpeptide antagonists. *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **265,** L164–L169.

GOCO, R.V., KRESS, M.B. & BRANTIGAN, O.C. (1963). Comparison of mucus glands in the tracheobronchial tree of man and animals. *Ann. N.Y. Acad. Sci.,* **106,** 555–571.

GRADY, E.F., BALUK, P., BOHM, S., GAMP, P.D., WONG, H., PAYAN, D.G., ANSEL, J., PORTBURY, A.L., FURNESS, J.B., MCDONALD, D.M. & BUNNETT, N.W. (1996). Characterization of antisera specific to NK_1, NK_2, and NK_3 neurokinin receptors and their utilization to localize receptors in the rat gastrointestinal tract. *J. Neurosci.,* **16,** 6975–6986.

GUARD, S. & WATSON, S.P. (1991). Tachykinin receptor types: Classification and membrane signaling mechanisms. *Neurochem. Int.,* **18,** 149–165.

HAXHIU, M.A., HAXHIU-POSKURICA, B., MORACIC, V., CARLO, W.A. & MARTIN, R.J. (1990). Reflex and chemical responses of tracheal submucosal glands in piglets. *Respir. Physiol.,* **82,** 267–278.

HAXHIU, M.A., TSENG, H.C. & DAVIS, B. (1988). Thiorphan enhances baseline and substance P induced tracheal gland secretion in dogs. *Clin. Res.,* **36,** 506A.

HEY, J.A., DANKO, G., DELPRADO, M. & CHAPMAN, R.W. (1996). Augmentation of neurally evoked cholinergic bronchoconstrictor responses by prejunctional NK_2 receptors in the guinea-pig. *J. Auton. Pharmacol.,* **16,** 41–48.

HOOVER, D.B. & HANDCOCK, J.C. (1987). Autoradiographic localization of substance P binding sites in guinea-pig airways. *J. Auton. Nerv. Syst.,* **19,** 171–174.

JEFFERY, P.K. (1983). Morphologic features of airway surface epithelial cells and glands. *Am. Rev. Respir. Dis.,* **128,** S14–S20.

JONES, R., BASKERVILLE, A. & REID, L. (1975). Histochemical identification of glycoproteins in pig bronchial epithelium. *J. Pathol.,* **116,** 1–11.

JOO, N.S., WU, J.V., KROUSE, M.E., SAENZ, Y. & WINE, J.J. (2001). Optical method for quantifying rates of mucus secretion from single submucosal glands. *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **281,** L458–L468.

JOOS, G.F., DESWERT, K.O. & PAUWELS, R.A. (2001). Airway inflammation and tachykinins: prospects for the development of tachykinin receptor antagonists. *Eur. J. Pharmacol.,* **429,** 239–250.

JOOS, G.F., GERMONPRE, P.R. & PAUWELS, R.A. (2000). Role of tachykinins in asthma. *Allergy,* **55,** 321–337.

KHAN, S., LIU, Y.-C., KHAWAJA, A.M., MANZINI, S. & ROGERS, D.F. (2001). Effect of the long-acting tachykinin NK_1 receptor antagonist MEN11467 on tracheal mucus secretion in allergic ferrets. *Br. J. Pharmacol.,* **132,** 189–196.

KHAWAJA, A.M., LIU, Y.-C. & ROGERS, D.F. (1999). Effect of non-peptide tachykinin NK_1 receptor antagonists on non-adrenergic, non-cholinergic neurogenic mucus secretion in ferret trachea. *Eur. J. Pharmacol.,* **384,** 173–181.

LUNDGREN, J.D., WIEDERMANN, C.J., LOGUN, C., PLUTCHOK, J., KALINER, M. & SHELHAMER, J.H. (1989). Substance P receptor-mediated secretion of respiratory glycoconjugate from feline airways in vitro. *Exp. Lung Res.,* **15,** 17–29.

MAGGI, C.A. (1995). The mammalian tachykinin receptors. *Gen. Pharmac.,* **26,** 911–944.

MCLEAN, S., GANONG, A., SEYMOUR, P.A., SNIDER, R.M., DESAI, M.C., ROSEN, T., BRYCE, D.K., LONGO, K.P., REYNOLDS, L.S., ROBINSON, G., SCHMIDT, A.W., SIOK, C. & HEYM, J. (1993). Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin neurokinin-1 receptor. *J. Pharmacol. Exp. Ther.,* **267,** 472–479.

MEINI, S., MAK, J.C., ROHDE, J.A. & ROGERS, D.F. (1993). Tachykinin control of ferret airways: Mucus secretion, bronchoconstriction and receptor mapping. *Neuropeptides,* **24,** 81–89.

MIZOGUCHI, H. & HICKS, C.R. (1989). Effects of neurokinins on ion transport and sulfated macromolecule release in the isolated ferret trachea. *Exp. Lung Res.,* **15,** 837–848.

MYERS, A.C. & UNDEM, B.J. (1993). Electrophysiological effects of tachykinins and capsaicin on guinea-pig bronchial parasympathetic ganglion neurones. *J. Physiol.,* **470,** 665–679.

NADEL, J.A. & DAVIS, B. (1977). Autonomic regulation of mucus secretion and ion transport in airways. In *Asthma: Physiology, Immunopharmacology, and Treatment*. eds. Lichtenstein, L.M. & Austen, K.F. pp. 197–210. New York: Academic Press.

NAGAKI, M., ISHIHARA, H., SHIMURA, S., SASAKI, T., TAKISHIMA, T. & SHIRATO, K. (1994). Tachykinins induce a $[Ca^{2+}]_i$ rise in the acinar cells of feline tracheal submucosal gland. *Respir. Physiol.,* **98,** 111–120.

NENAN, S., GERMAIN, N., LAGENTE, V., EMONDS-ALT, X., ADVENIER, C. & BIOCHOT, E. (2001). Inhibition of inflammatory cell recruitment by the tachykinin NK_3-receptor antagonist, SR 142801, in a murine model of asthma. *Eur. J. Pharmacol.,* **421,** 201–205.

PHILLIPS, J.E., HEY, J.A. & CORBOZ, M.R. (2002a). Automated measurement of airway gland secretions by the hillocks technique. *Comput. Methods Programs Biomed.,* **68,** 215–222.

PHILLIPS, J.E., HEY, J.A. & CORBOZ, M.R. (2002b). Effects of ion transport inhibitors on methacholine-mediated secretion from porcine airway submucosal glands. *J. Appl. Physiol.,* **93,** 873–881.

PHIPPS, R.J. (1981). The airway mucociliary system. In *International Review of Physiology*. ed. Widdicombe, J.G. pp. 216–260. Baltimore: University Park Press.

PIEDIMONTE, G. (1995). Tachykinin peptides, receptors and peptidases in airway disease. *Exp. Lung Res.,* **21,** 809–834.

RAMNARINE, S.I., HIRAYAMA, Y., BARNES, P.J. & ROGERS, D.F. (1994). 'Sensory-efferent' neural control of mucus secretion: characterization using tachykinin receptor antagonists in ferret trachea *in vitro*. *Br. J. Pharmacol.,* **113,** 1183–1190.

RANGACHARI, P.K. & MCWADE, D. (1985). Effects of tachykinins on the electrical activity of isolated canine tracheal epithelium. *Regul. Pept.,* **12,** 9–19.

REGOLI, D., BOUDON, A. & FAUCHERE, J.-L. (1994). Receptors and antagonists for substance P and related peptides. *Pharmacol. Rev.,* **46,** 551–599.

RIZZO, C.A., VALENTINE, A.F., EGAN, R.W., KREUTNER, W. & HEY, J.A. (1999). NK_2-receptor mediated contraction in monkey, guinea-pig and human airway smooth muscle. *Neuropeptides,* **33,** 27 – 34.

ROGERS, D.F. (2002). Mucoactive drugs for asthma and COPD: any place in therapy? *Exp. Opin. Invest. Drugs,* **11,** 15 – 35.

ROGERS, D.F., AURSUDKIJ, B. & BARNES, P.J. (1989). Effects of tachykinins on mucus secretion in human bronchi *in vitro*. *Eur. J. Pharmacol.,* **174,** 283 – 286.

SARAU, H.M., GRISWOLD, D.E., POTTS, W., FOLEY, J.J., SCHMIDT, D.B., WEBB, E.F., MARTIN, L.D., BRAWNER, M.E., ELSHOURBAGY, N.A., MEDHURST, A.D., GIARDINA, G.A. & HAY, D.W. (1997). Nonpeptide tachykinin receptor antagonists: I. Pharmacological and pharmacokinetic characterization of SB223412, a novel, potent, and selective neurokinin-3 receptor antagonist. *J. Pharmacol. Exp. Ther.,* **281,** 1303 – 1311.

SPINA, D. & PAGE, C.P. (1996). Airway sensory nerves in asthma – targets for therapy? *Pulm. Pharmacol.,* **9,** 1 – 18.

SPINA, D., SHAH, S. & HARRISON, S. (1998). Modulation of sensory nerve function in the airways. *Trends Pharmacol. Sci.,* **19,** 460 – 466.

TROUT, L., CORBOZ, M.R. & BALLARD, S.T. (2001). Mechanism of substance P-induced liquid secretion across bronchial epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **281,** L639 – L645.

UNDEM, B.J. & MYERS, A.C. (1997). Autonomic Ganglia. In *Autonomic control of the respiratory system*. ed. Barnes, P.J. pp. 87 – 118. Amsterdam: Harwood Academic.

USSING, H.H. (1949). The active ion transport through the isolated frog skin in the light of tracer studies. *Acta Physiol. Scand.,* **17,** 1 – 37.

WAGNER, U., FEHMANN, H.C., BREDENBROKER, D., KLUBER, D., LANGE, A. & VON WICHERT, P. (1999). Effects of selective tachykinin-receptor antagonists on tachykinin-induced airway mucus secretion in the rat. *Neuropeptides,* **33,** 55 – 61.

WAGNER, U., FEHMANN, H.C., BREDENBROKER, D., YU, F., BARTH, P.J. & VON WICHERT, P. (1995). Galanin and somatostatin inhibition of neurokinin A and B induced airway mucus secretion in the rat. *Life Sci.,* **57,** 283 – 289.

WU, D.X., LEE, C.Y., UYEKUBO, S.N., CHOI, H.K., BASTACKY, S.J. & WIDDICOMBE, J.H. (1998). Regulation of the depth of surface liquid in bovine trachea. *Am. J. Physiol. Lung Cell. Mol. Physiol.,* **274,** L388 – L395.

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