Influence of Influenza A Infection on Capsaicin-Induced Responses in Murine Airways

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ABSTRACT
The principal aim of the study was to determine the influence of influenza A virus infection on capsaicin-induced relaxation responses in mouse isolated tracheal segments and clarify the underlying mechanisms. Anesthetized mice were intranasally inoculated with influenza A/PR-8/34 virus (VIRUS) or vehicle (SHAM), and 4 days later tracheal segments were harvested for isometric tension recording and biochemical and histologic analyses. Capsaicin induced dose-dependent relaxation responses in carbachol-contracted SHAM trachea (e.g., 10 μM capsaicin produced 66 ± 4% relaxation; n = 11), which were significantly inhibited by capsazepine [transient receptor potential vanilloid type 1 (TRPV1) antagonist], (2S,3S)-3-[[3,5-bis(trifluoromethyl)phenyl]methoxy]-2-phenylpiperidine hydrochloride (L-733,060) [cyclooxygenase (COX) inhibitor], and the combination of 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809) and 7-[[1S, 1α(Z)-biphenyl]-4-ylmethoxy]-3-oxocyclopentyl]-4-heptenoic acid, calcium salt, hydrate (AH23848) [E-prostanoid (EP)2 and EP4 receptor antagonists, respectively], indicating that capsaicin-induced relaxation involved the TRPV1-mediated release of substance P (SP), activation of epithelial NK1 receptors, and production of COX products capable of activating relaxant EP2/EP4 receptors. Consistent with this postulate, capsaicin-induced relaxation was associated with the significant release of SP and prostaglandin E2 (PGE2) from mouse tracheal segments. As expected, influenza A virus infection was associated with widespread disruption of the tracheal epithelium. Tracheal segments from VIRUS mice responded weakly to capsaicin (7 ± 3% relaxation) and were 25-fold less responsive to SP than tracheas from SHAM mice. In contrast, relaxation responses to exogenous PGE2 and the β-adrenoceptor agonist isoprenaline were not inhibited in VIRUS trachea. Virus infection was associated with impaired capsaicin-induced release of PGE2 but the release of SP was not affected. In summary, influenza A virus infection profoundly inhibits capsaicin- and SP-induced relaxation responses, most likely by inhibiting the production of PGE2μ.

Introduction
Sensory nerves innervate a range of key structures of the respiratory tract, including the airway epithelium, smooth muscle band, and submucosal glands (Verhein et al., 2009). Afferent sensory nerves located within the airway epithelium serve to detect the presence of noxious stimuli and initiate a reflex bronchoprotective response (Martling, 1987). Accumulating and compelling evidence indicates this sensory function is mediated primarily by members of the transient receptor potential (TRP) family of ion channels, which are highly expressed on sensory nerve endings. For example, TRP vanilloid type 1 (TRPV1) is a calcium ion channel, which is activated by high temperature (>40°C), low pH, and exogenous vanilloids such as the chili plant extract capsaicin (Manzini, 1992; Bevan and Geppetti, 1994; Caterina et al., 1997; Szallasi and Blumberg, 1999).

Upon TRPV1 activation by capsaicin, Ca2+ ions enter the highly energized sensory nerve ending and promote its depolarization. The newly formed nerve impulse initiates a centrally mediated parasympathetic reflex that culminates in smooth muscle contraction and mucous gland secretion (Lee and Pisarri, 2001). However, of more relevance to this study, the nerve impulse also travels in an antidromic manner, leading to local release of neuropeptides, such as substance P (SP), from sensory nerve endings. SP is capable of exerting a wide range

ABBREVIATIONS: TRP, transient receptor potential; TRPV1, TRP vanilloid type 1; VIRUS, influenza A/PR-8/34 virus; SHAM, vehicle; PG, prostaglandin; SP, substance P; EP, E-prostanoid; CDRC, cumulative dose-response curve; NK, neurokinin; COX, cyclooxygenase; EC40, effective concentration of agonist inducing a 40% relaxation response to baseline; Emax, maximum response; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; L-733,060, (2S,3S)-3-[[3,5-bis(trifluoromethyl)phenyl]methoxy]-2-phenylpiperidine hydrochloride; AH6809, 6-isopropoxy-9-oxooxanthene-2-carboxylic acid; AH23848, 7-[[1S, 1α(Z)-biphenyl]-4-ylmethoxy]-2β-(4-morpholinoxy)-3-oxocyclopentyl]-4-heptenoic acid, calcium salt, hydrate. 
of biological effects within the airways, although these vary depending on species and tissue type (Stewart et al., 2008). Application of SP (or capsaicin) to mouse isolated bronchial preparations induces airway smooth muscle relaxation (Manzini, 1992). In mouse and rat airways, SP activates neurokinin-1 (NK1) receptors on epithelial cells, leading to the activation of cyclooxygenase (COX) and production of prostaglandin E2 (PGE2) (Devillier et al., 1992; Szarek et al., 1998; Fortner et al., 2001). In turn, PGE2 activates adenylate cyclase-linked EP2/4 receptors on airway smooth muscle, increasing intracellular cAMP levels and promoting relaxation (Narumiya et al., 1999; Sheller et al., 2000). To date, no study has fully characterized the capsaicin-induced relaxation pathway. Hence a primary aim of this study was to apply pharmacological approaches to clarify and confirm the mechanisms that underpin capsaicin-mediated relaxation pathways in mouse isolated trachea.

An equally important aim of this study was to determine the effect of influenza A infection on airway sensory nerve function, in particular the capsaicin-induced relaxation pathway. It is currently unknown whether an influenza A infection modulates sensory nerve function, although circumstantial evidence exists that supports this postulate. First, it is well established that influenza A infects and destroys the pseudo-stratified columnar epithelium and as such may inhibit the epithelium-dependent, capsaicin-induced relaxation response. Second, certain neurotrophic influenza A strains can infect the central nervous system via the sensory nervous system (Shinya et al., 2000), and this may affect sensory nerve function. Finally, influenza A infection can alter the density, distribution, and function of an array of receptors involved in controlling bronchomotor tone (Henry et al., 1991; Carr et al., 1996; Lan et al., 2004). This study tested the hypothesis that influenza A infection inhibits the capsaicin-induced relaxation pathway by altering sensory nerve, epithelial cell, and smooth muscle function.

Materials and Methods

Animals and Viral Inoculation. Groups of specified pathogen-free male BALB/c mice aged 7 to 8 weeks (Animal Resources Centre, Murdoch, WA) were lightly anesthetized with inhaled methoxyflurane (VIRUS) or diluted allantoic fluid (SHAM). Four days later mice were cut into two segments, upper and lower, each approximately 2 cm in length. Each tracheal segment was loaded onto a pair of force transducers (Grass Instruments, Quincy, MA) connected to a Powerlab data acquisition and analysis system (ADInstruments Pty Ltd., Castle Hill, Australia). After a 30-min equilibration period, the viability of each segment was determined by sequential exposure to a submaximal dose of carbachol (0.2 μM) and a maximal dose of carbachol (10 μM). After a 15-min washout and rest period, segments were re-exposed to sequential doses of carbachol (0.2 and 10 μM). Segments that responded weakly to 10 μM carbachol (<600 mg of tension) were deemed unviable and discarded. Viable segments were washed and left to rest for an additional 15 min before a carbachol cumulative dose-response curve (CDRC; 0.01–10 μM using half-log dose increments) was performed. The maximum response obtained to the 10-μM dose of carbachol was deemed the maximum contractile response (Cmax). Mouse isolated tracheal segments do not exhibit intrinsic airway tone; hence precontraction with 1 μM carbachol was required to reveal relaxation responses to agents such as capsaicin. Contractile responses to 1 μM carbachol plateaued at approximately 70 to 80% Cmax. Upon reaching a plateau, a single bolus dose of capsaicin (0.01, 0.1, or 10 μM) was added to the carbachol-contracted segment. Capsaicin rapidly depletes the stores of sensory neuropeptides in isolated trachea; therefore only a single dose of capsaicin was administered to each preparation (Manzini, 1992). Once the peak relaxation response to capsaicin was observed (within 10 min), preparations were washed and left to equilibrate for 15 min with fresh Krebs’ solution. This process of precontraction, testing of an active agent, and washing out then resting was repeated several times per preparation by using an array of agents: SP (0.01–100 nM, bolus dosing, whole-log increments), PGE2 (3–3000 nM CDRC in half-log increments or 30 nM bolus), and isoprenaline (1–1000 nM CDRC in half-log increments). In separate mouse isolated tracheal segments, this protocol was repeated in the presence of various antagonists/inhibitors (or vehicles) including: capsazepine (TRPV1 antagonist; 10 μM; Ellis and Undem, 1994), indomethacin (nonsselective COX inhibitor; 5 μM; Manzini, 1992), 6-isopropoxy-9-oxo-10-oxoanthene-2-carboxylic acid (AH6809) (EP receptor antagonist; 3 μM; Lan et al., 2001), 7-[5-[3,5-bis(trifluoromethyl)phenyl]methoxy]-2-phenylperidine (AH23848) (EP4 receptor antagonist; 10 μM; Davis et al., 2004), and (2S,3S)-3-[3,5-bis(trifuoromethyl)phenyl]methoxy)-2-phenylpiperidine hydrochloride (L-733,060) (NK receptor antagonist; 10 μM; Kubo et al., 2007). The antagonists/inhibitors were added to the baths after each rest period and allowed to equilibrate for at least 10 min before each precontraction.

Bronchoalveolar Lavage and Inflammatory Cell Influx. After exsanguination, the trachea was cannulated, and the airways were washed out six times with 0.5 ml of sterile phosphate-buffered saline (PBS). The extracted fluid was pooled for each mouse and centrifuged at 4°C for 5 min at 400 g (Rotina 35R centrifuge; Hettich, Tuttingen, Germany). The pellet was resuspended (1% bovine serum albumin in PBS), and total inflammatory cell numbers were determined via an improved Neubauer hemocytometer (ProSciTech, Thuringowa, Qld, Australia). Each sample was then cytocentrifuged (Shandon cytocentrin 4; Thermo Fisher Scientific, Waltham, MA) onto a microscope slide and stained with a modified Wright’s stain (aqueous stain Rapid I and Rapid II; Amber Scientific, Perth, WA). Cells were differentially counted under a light microscope, counting 400 leukocytes per slide.

Histology. For morphological examination, mouse tracheas obtained on day 4 postinoculation (SHAM and VIRUS) were immersion-fixed (2% paraformaldehyde/0.2% wt/vol saturated picric acid in PBS) for 48 h at 4°C. After fixation, the tracheas were dehydrated through graded alcohols and embedded in paraffin wax. Five-micron transverse sections of trachea were cut and stained with hematoxylin and eosin.

PGE2 and SP ELISA Studies. As described above (isometric tension recordings), viable VIRUS and SHAM tracheal segments were washed with Krebs’ solution and precontracted with 1 μM carbachol for 15 min. Segments were then exposed to either 10 μM capsaicin or vehicle (final bath concentration of ethanol <0.1%) for 10 min, and the bathing fluid was collected and frozen (~80°C) for storage.
subsequent analysis of PGE$_2$ and SP content. Tissue SP was then extracted from these tracheal segments by using an amended protocol from Erin and Ulusoy (2009). Capsaicin- or vehicle-exposed tracheal segments were washed repeatedly (>20 times) with Krebs’ solution and transferred to tubes containing 1 ml of 2% acetic acid at 95°C for 5 min. The supernatant was collected and concentrated (Savant Instruments Speedvac SPD121P; Thermo Fisher Scientific) at 50°C for 3 h. The Instruments dry remnants were solubilized in 130 µl of enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI) and stored at −80°C for subsequent analysis of SP content.

Levels of SP and PGE$_2$ were determined by using commercially available ELISA kits (Cayman Chemical) in accordance with the manufacturer’s instructions.

**Materials.** Capsaicin, carbachol, PGE$_2$, isoprenaline-hydrochloride, indomethacin, capsazepine, AH6809, and AH23848 were obtained from Sigma-Aldrich (St Louis, MO). SP and L-733,060 were purchased from Tocris Bioscience (Ellisville, MO). SP and PGE$_2$ ELISA plates were obtained from Cayman Chemical. Sodium pentobarbitone was obtained from Virbac Animal Health (Peakhurst, Australia), and methoxyflurane was from Medical Developments International Ltd (Springvale, Australia).

![Fig. 1. Characterization of capsaicin-induced relaxation responses in SHAM trachea. A, bolus dose-response curves to capsaicin in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on capsaicin-induced relaxations are presented graphically only for the lower tracheal segments. B to E, effects of 10 µM capsazepine (B), 10 µM L-733,060 (C), 5 µM indomethacin (D), and 3 µM AH6809 plus 10 µM AH23848 (E) on capsaicin-induced relaxation responses in lower tracheal segments. Black bars indicate capsaicin-induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 5 to 15 (regional dose-response curves) or 3 to 7 (antagonist studies) lower tracheal segments. ***, p < 0.001 compared with the lower segment (vehicle). Comparable data were obtained for upper tracheal segments (data not shown).](image1)

![Fig. 2. Characterization of SP-induced relaxation responses in SHAM trachea. A, bolus dose-response curves to SP in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on SP-induced relaxations are presented graphically only for the lower tracheal segments. B to E, effects of 10 µM capsazepine (B), 10 µM L-733,060 (C), 5 µM indomethacin (D), and 3 µM AH6809 plus 10 µM AH23848 (E) on SP-induced relaxation responses in lower tracheal segments. Black bars indicate capsaicin-induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 5 to 15 (regional dose-response curves) or 3 to 7 (antagonist studies) lower tracheal segments. ***, p < 0.001; †††, p < 0.001, SP $E_{max}$ compared with lower segment (vehicle). †††, p < 0.001, SP $-\log EC_{40}$ compared with lower segment (vehicle). Comparable data were obtained for upper segments in the presence of the antagonists (Table 1).](image2)
Solutions. Capsaicin and capsazepine stock solutions were made up in 100% ethanol and serially diluted in sterile saline. The resulting bath concentrations of ethanol were ≤0.1%, which is sufficiently low to minimize ethanol-induced effects on TRPV1 (Trevisani et al., 2002). Isoprenaline stock solution and serial dilutions were made in 0.1 mM ascorbic acid. Indomethacin and AH6809 stock solutions were made up in 0.1 mM Na₂CO₃ and serially diluted in sterile saline. All other agents (carbachol, PGE₂, SP, and L-733,060) were dissolved and diluted in sterile saline.

Statistical Analysis. The two main parameters used in the statistical analysis of the isometric tension data were the logEC₄₀ (the logarithm of the concentration of agent required to produce 40% relaxation of the carbachol-induced contraction) and the Eₘₐₓ (the maximal relaxation response reached in percentage of relaxation of the carbachol-induced contraction). Parametric tests (one- and two-way analysis of variance) with post hoc comparisons (Holm-Sidak method) were the prioritized statistical tests used to analyze the isometric tension recording data, BAL fluid cell counts, and ELISA data. Differences between groups were considered statistically significant at p < 0.05.

Results

Characterization of Capsaicin-Induced Relaxation Responses in SHAM Trachea. Capsaicin elicited dose-dependent relaxation responses in SHAM mouse isolated trachea, and responses were similar in both the upper and lower segments (Fig. 1A). In the lower tracheal segments, the response to 10⁻⁸ M capsaicin (66 ± 4%; n = 11) was abolished by 10⁻⁶ M capsazepine (TRPV1 antagonist; -1 ± 2%; p < 0.001; n = 4–5; Fig. 1B) and 5⁻⁵ M indomethacin (COX inhibitor; 0 ± 1%; p < 0.001; n = 4; Fig. 1D). Relaxation responses to capsaicin were also significantly inhibited by 10⁻⁶ M AH6809 plus 10⁻⁶ M AH23848 (Eₘₐₓ 28 ± 3% vs. 0 ± 0% of control; p < 0.001; Fig. 1E). Comparable data were obtained for upper segments in the presence of the antagonists (Table 1).

Fig. 3. Characterization of PGE₂-induced relaxation responses in SHAM trachea. A, cumulative dose-response curves to PGE₂ in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on PGE₂-induced relaxations are presented graphically only for the lower tracheal segments. B to E, effects of 10⁻⁶ M capsazepine (B), 10⁻⁶ M L-733,060 (C), 5⁻⁶ M indomethacin (D), and 3⁻⁶ M AH6809 plus 10⁻⁶ M AH23848 (E) on PGE₂-induced relaxation responses in lower tracheal segments. Black bars or symbols indicate PGE₂-induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 15 to 16 (regional dose-response curve) lower tracheal segments or 3 to 10 (antagonist studies) lower tracheal segments. **, p < 0.001, PGE₂ logEC₄₀ compared with lower segment (vehicle); ***, p < 0.001, PGE₂ logEC₄₀ compared with lower segment (vehicle). Comparable data were obtained for upper segments in the presence of the antagonists (Table 1).

Fig. 4. Characterization of the isoprenaline-induced relaxation responses in SHAM trachea. A, cumulative dose-response curves to isoprenaline in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on isoprenaline-induced relaxations are presented graphically only for the lower tracheal segments. ***, p < 0.001, isoprenaline logEC₄₀ compared with lower segment. B and C, effects of 5⁻⁶ M indomethacin (B) and 3⁻⁶ M AH6809 plus 10⁻⁶ M AH23848 (C) on isoprenaline-induced relaxation responses in lower tracheal segments. Black bars or symbols indicate isoprenaline-induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 12 (regional dose-response curve) lower tracheal segments or 3 to 4 (antagonist studies) lower tracheal segments. Comparable data were obtained for upper segments in the presence of the antagonists (Table 1).
μM L-733,060 (NK1 receptor antagonist; $p < 0.001$; $n = 5$; Fig. 1C) and 3 μM AH6809 and 10 μM AH23848 (EP$_2$ and EP$_4$ receptor antagonists, respectively, $n = 6$; Fig. 1E). Capsaicin-induced relaxation responses were also blocked by capsazepine, indomethacin, L-733,060, or the combination of AH6809 and AH23848 in upper tracheal segments (data not shown).

SP elicited dose-dependent relaxation responses in SHAM tracheal segments (Fig. 2A), although it was almost 10-fold more potent in the lower segments ($-\log EC_{40} 10.0 \pm 0.1; n = 29$) than the upper segments ($-\log EC_{40} 9.1 \pm 0.1; n = 21; p < 0.01$). As expected, the potency of SP was not affected by 10 μM capsazepine (Fig. 2B) but was significantly reduced by 10 μM L-733,060 (Fig. 2C). SP-induced relaxation responses were also suppressed by 5 μM indomethacin (Fig. 2D) and the combination of 3 μM AH6809 and 10 μM AH23848 ($p < 0.001$; $n = 7$; Fig. 2E).

Cumulative addition of PGE$_2$ elicited dose-dependent relaxation in the upper and lower segments of SHAM trachea ($-\log EC_{40} 6.6 \pm 0.1; n = 16$; lower segment; Fig. 3A). Relaxation responses to PGE$_2$ in lower segments were not inhibited by capsazepine, L-733,060, or indomethacin (Fig. 3, B–D), but they were significantly inhibited by the combination of AH6809 and AH23848 (Fig. 3E).

Cumulative addition of isoprenaline elicited dose-dependent relaxation in SHAM trachea (Fig. 4A). The maximum response ($E_{\text{max}} 79 \pm 2\%$; $n = 12$; lower segment) and potency ($-\log EC_{40} 7.6 \pm 0.1; n = 12$) were not significantly inhibited by 5 μM indomethacin (Fig. 4B) or the combination of 3 μM AH6809 and 10 μM AH23848 (Fig. 4C).

**Influenza A Infection.** At day 4 postinoculation prominent pathological changes were observed in tracheal sections from VIRUS mice (Fig. 5B) compared with SHAM mice (Fig. 5A). Examination of hematoxylin and eosin-stained transverse sections of VIRUS mouse trachea revealed extensive denudation of the epithelium (Fig. 5B). Furthermore, a number of inflammatory cell infiltrates, particularly neutrophils,

![Fig. 5.](image-url)  
A and B, hematoxylin and eosin-stained photomicrographs of SHAM (A) and VIRUS (B) mouse trachea at 4 days postinoculation, illustrating marked disruption of airway epithelium during influenza A virus infection. Scale bar, 20 μm. C, levels of inflammatory cell infiltrates found in BAL fluid from SHAM (empty bars) and VIRUS (filled bars) mice at 4 days postinoculation. Shown are mean levels of cells $\pm$ S.E.M from 9 to 10 BAL samples. ***, $p < 0.001$ compared with SHAM-infected cell count.

![Fig. 6.](image-url)  
Relaxation responses to capsaicin (A), substance P (B), PGE$_2$ (C), and isoprenaline (D) in SHAM (○) and VIRUS (●) lower tracheal segments. Shown are mean responses $\pm$ S.E.M from 5 to 15 (capsaicin), 29 to 32 (substance P), 16 (PGE$_2$), and 12 (isoprenaline) lower tracheal segments. ***, $p < 0.001$, maximal response in VIRUS-infected compared with SHAM-infected tracheal segments. †††, $p < 0.001$, $-\log EC_{40}$ in VIRUS-infected compared with SHAM-infected tracheal segments. Comparable data were obtained for upper tracheal segments (Table 1).
were evident within the lumen, blood vessels, and interstitial space of VIRUS mice but not SHAM mice. The inflammatory response was further investigated by using BAL fluid cell counts, which revealed an 8-fold increase in the total number of cells infiltrating the airways \( (p < 0.001; n = 9–10; \text{Fig. 5C}) \). The inflammatory cells in the BAL fluid from SHAM mice consisted primarily of macrophages \( (0.7 \pm 0.1 \text{ million cells/BAL}; p < 0.001; n = 10) \) and a minor population of lymphocytes. The infiltrates from VIRUS mice consisted of significantly elevated numbers of macrophages \( (3.5 \pm 0.3 \text{ million cells/BAL}; p < 0.001; n = 10) \) and neutrophils \( (2.2 \pm 0.1 \text{ million cells/BAL}; p < 0.001; n = 10) \).

**Characterization of Capsaicin-Induced Relaxation Responses in VIRUS Trachea.** In stark contrast to segments from SHAM mice, both upper and lower tracheal segments from VIRUS mice responded very weakly, with a 71% contraction in SHAM mice; 25-fold less responsive to exogenous SP \( (p < 0.001; n = 29; \text{Fig. 6B}) \). On the other hand, the potencies of exogenous PGE\(_2\) \( (\text{Fig. 6C}) \) and isoprenaline \( (\text{Fig. 6D}) \) were not reduced in VIRUS trachea. Indeed, PGE\(_2\) was 2.5-fold more potent in VIRUS trachea compared with SHAM trachea \( (p < 0.001; n = 16; \text{lower segment}; \text{Fig. 6C}) \).

Carbachol-induced contractions were marginally reduced in tracheal segments from VIRUS mice compared with SHAM mice \( (e.g., 1 \mu \text{M carbachol produced 71 } \pm 1\% \text{ contraction in lower tracheal segments; } n = 11; \text{Fig. 6A}) \). Furthermore, VIRUS trachea were 25-fold less responsive to exogenous SP \( (p < 0.001; n = 10) \) and neutrophils \( (2.2 \pm 0.1 \text{ million cells/BAL}; p < 0.001; n = 10) \).

**Capsaicin-Induced Changes in PGE\(_2\) and SP Levels in SHAM and VIRUS Trachea.** Exposure of SHAM tracheal segments to 10 \( \mu \text{M} \) capsaicin for 10 min caused a significant release of PGE\(_2\) into the surrounding media \( (\text{Fig. 7A}) \). In stark contrast, capsaicin caused no significant release of PGE\(_2\) from VIRUS tracheal segments \( (\text{Fig. 7A}) \). Basal levels of PGE\(_2\) release were also lower in VIRUS mice than in SHAM mice \( (\text{Fig. 7A}) \). The levels of SP in media surrounding VIRUS or SHAM trachea after exposure to capsaicin or vehicle were below the level of detection of the ELISA kit \((<3.9 \text{ pg/ml})\). However, capsaicin caused a significant reduction in the tissue levels of SP recovered from mouse tracheal segments \( (\text{Fig. 7B}) \). It is noteworthy that there was no significant difference in the magnitude of the capsaicin-induced reduction in tracheal SP levels between SHAM and VIRUS mice \( (\text{Fig. 7B}) \).

**Discussion**

Our pharmacological studies indicate that capsaicin relaxes mouse isolated tracheal segments through the sequential activation of sensory nerve TRPV1, epithelial NK1 receptors and COX, and smooth muscle EP\(_{2,4}\) receptors \( (\text{Fig. 8}) \). Consistent with this schema, complementary biochemical assays showed that capsaicin induced the rapid release of SP and PGE\(_2\) from mouse tracheal segments. Of particular interest, both capsaicin-induced relaxation and PGE\(_2\) generation were dramatically reduced in airways obtained from influenza A virus-infected mice. Capsaicin-induced concentration-dependent relaxation responses in mouse isolated trachea, consistent with previ-

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**TABLE 1**

Effect of selected antagonists and inhibitors on the relaxant activity of substance P, PGE\(_2\), and isoprenaline in upper and lower tracheal segments from SHAM and VIRUS mice

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Treatment</th>
<th>Region (mm)</th>
<th>SHAM Mice</th>
<th>VIRUS Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-\log EC_{40})</td>
<td>(E_{max})</td>
</tr>
<tr>
<td>Substance P</td>
<td>None, vehicle</td>
<td>Upper (21–31)</td>
<td>9.1 (\pm) 0.1</td>
<td>68 (\pm) 2</td>
</tr>
<tr>
<td></td>
<td>L-733,060, 10 (\mu M)</td>
<td>Upper (5)</td>
<td>10.0 (\pm) 1.0 (^b)</td>
<td>81 (\pm) 1</td>
</tr>
<tr>
<td></td>
<td>Indomethacin, 5 (\mu M)</td>
<td>Lower (4–5)</td>
<td>9.3 (\pm) 0.2</td>
<td>73 (\pm) 3</td>
</tr>
<tr>
<td>Capsazepine vehicle</td>
<td>Upper (7–10)</td>
<td>9.1 (\pm) 0.1</td>
<td>71 (\pm) 3</td>
<td>8.2 (\pm) 0.2 (^a)</td>
</tr>
<tr>
<td>Capsazepine, 10 (\mu M)</td>
<td>Upper (5–7)</td>
<td>9.4 (\pm) 0.2</td>
<td>78 (\pm) 3</td>
<td>8.2 (\pm) 0.1 (^a)</td>
</tr>
<tr>
<td>PGE(_2)</td>
<td>None, vehicle</td>
<td>Upper (14–15)</td>
<td>6.3 (\pm) 0.1</td>
<td>75 (\pm) 3</td>
</tr>
<tr>
<td></td>
<td>L-733,060, 10 (\mu M)</td>
<td>Upper (5)</td>
<td>6.5 (\pm) 0.1</td>
<td>87 (\pm) 2</td>
</tr>
<tr>
<td></td>
<td>Indomethacin, 5 (\mu M)</td>
<td>Lower (3–5)</td>
<td>6.5 (\pm) 0.1</td>
<td>86 (\pm) 3</td>
</tr>
<tr>
<td>Capsazepine vehicle</td>
<td>Upper (4)</td>
<td>6.7 (\pm) 0.1 (^a)</td>
<td>83 (\pm) 7</td>
<td>6.7 (\pm) 0.1</td>
</tr>
<tr>
<td>Capsazepine, 10 (\mu M)</td>
<td>Upper (9–10)</td>
<td>6.8 (\pm) 0.1</td>
<td>88 (\pm) 2</td>
<td>7.2 (\pm) 0.1</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>None, vehicle</td>
<td>Upper (11–12)</td>
<td>7.3 (\pm) 0.1</td>
<td>76 (\pm) 2</td>
</tr>
<tr>
<td></td>
<td>Indomethacin, 5 (\mu M)</td>
<td>Upper (4)</td>
<td>7.6 (\pm) 0.1</td>
<td>79 (\pm) 2</td>
</tr>
</tbody>
</table>

\(n\)-D., \(-\log EC_{40}\) not determined because 40% \(E_{max}\) value not achieved.

\(^a\) \(P < 0.05\) in comparison with respective SHAM segment.

\(^b\) \(P < 0.05\) in comparison with respective upper segment.

\(^c\) \(P < 0.05\) in comparison with respective vehicle.
Holm-Sidak post hoc comparison). and exogenous SP were inhibited by an NK1 receptor antagonist (Ichikawa et al., 1995). This postulate is supported by our elevated production of the COX-derived prostanoid PGE2. This capsaicin-induced relaxation responses were associated with elevated production of the COX-derived prostanoïd PGE2. This agrees with studies in rat trachea showing that PGE2 is the primary COX product generated as a result of SP-induced NK1 receptor stimulation (Devillier et al., 1992).

PGE2-induced relaxation responses in airway smooth muscle are typically mediated by the EP2 or EP4 receptor subtype, via signaling through Gs, adenylate cyclase, and intracellular cAMP. In the current study, the combined application of an EP2 receptor antagonist, AH6809, and an EP4 receptor antagonist, AH23848, produced a more effective inhibition of relaxation responses to capsaicin, SP, and PGE2 than the use of either EP receptor antagonist alone, indicating that both EP receptor subtypes may be mediating the relaxation response. Previous studies have confirmed a role for the EP4 receptor in SP- and PGE2-induced relaxations (Sheller et al., 2000; Fortner et al., 2001). Further experiments are warranted to more definitively establish the role of EP4 receptors. In summary, capsaicin-induced relaxation in the mouse isolated trachea seems to involve the activation of TRPV1 on sensory nerve endings, NK1 receptors on the epithelium, and subsequent generation of relaxant COX products such as PGE2, which promote relaxation via smooth muscle EP2/4 receptors (Fig. 8).

A unique finding of this study was the observation that tracheal segments from influenza A-infected mice were markedly hyporesponsive to the relaxant actions of capsaicin and SP. Given the likely sequential involvement of sensory nerves, epithelial cells, and smooth muscle cells in capsaicin-induced relaxation responses, the attenuated responses to capsaicin (and SP) may well be explained by virus-induced changes in the function of these key cellular components.

One possibility worthy of consideration is that influenza A virus infection damages sensory nerves, thereby reducing their capacity to respond to capsaicin. For example, certain neurotrophic strains of influenza A virus have been reported to infect the central nervous system through the sensory nerves in murine airways (Shinya et al., 2000) and cause neuronal damage (Jang et al., 2009). However, H1N1 strains of influenza A virus (such as A/PR-8/34) do not seem to be neurotrophic (Kobasa et al., 2007), which reduces the likelihood that the current findings can be explained by virus-induced damage of the nerves leading to dysfunction. Consistent with this, the capacity of capsaicin to cause release of SP from tracheal tissue was not affected by influenza virus infection. These key findings indicate that the virus-induced attenuation of the capsaicin relaxation response is unlikely to be caused by an impaired ability of the sensory nerves to release SP in response to capsaicin. Rather, it indicates the locus of the lesion lies downstream from sensory nerve activation.

Histological examination of tracheal segments from influenza A-infected mice revealed widespread and extensive damage to the epithelium, which may reduce its capacity to generate relaxant factors such as PGE2 in response to capsaicin and SP. Previously published studies have used epithelial denudation techniques to establish the importance of the epithelium in relaxation responses to capsaicin (Szarek et al., 1995, 1998) and SP (Szarek et al., 1995, 1998; Kao et al., 1999; Liu et al., 2006) in rat and mouse isolated airway preparations. Moreover, Szarek et al. (1998) demonstrated that epithelium removal by mechanical disruption blocked both capsaicin- and SP-induced release of PGE2. Consistent with this postulate, capsaicin did not induce the release PGE2 from tracheal segments of VIRUS mice. Thus, the reduced sensitivity of VIRUS trachea to exogenously admin-

![Fig. 7. A, levels of PGE2 in media surrounding SHAM and VIRUS mouse isolated whole tracheas after 10-min exposure to 10 μM capsaicin (filled bars) and vehicle (empty bars). B, levels of residual SP in SHAM and VIRUS trachea after exposure to 10 μM capsaicin (filled bars) and vehicle (empty bars). Shown are mean responses ± S.E.M from nine (VIRUS) and nine (SHAM) whole tracheas. ***, P < 0.01 (two-way analysis of variance, Holm-Sidak post hoc comparison).](image-url)
Influenza A virus infection has been associated with reduced relaxation responses in airway smooth muscle (Henry et al., 1991; Ashraf et al., 2001). However, virus-induced reductions in relaxation responses were observed only for capsaicin and SP, which cause relaxation indirectly via the release of COX products such as PGE$_2$. In contrast, responses to exogenous PGE$_2$ and isoprenaline, which cause relaxation by directly acting on EP receptors and $\beta$-adrenoceptors located on smooth muscle cells, were not reduced in tracheal preparations from influenza A-infected mice. Thus, virus-induced loss of responsiveness to capsaicin and SP cannot be explained by hypofunction of relaxation pathways within tracheal smooth muscle cells and indicate that the locus of the dysfunction lies upstream, principally at the level of the epithelium.

In addition to attenuating capsaicin-induced generation of PGE$_2$, influenza A viral infection significantly reduced the basal release of PGE$_2$ from mouse tracheal segments. A potential homeostatic response to reducing basal PGE$_2$ levels seems to be primarily responsible for suppressed responsiveness to capsaicin and substance P. Also, influenza A viral infection significantly reduced the release of COX products such as PGE$_2$.

The underlying mechanism for virus-induced hypersensitivity to PGE$_2$ has not been determined, a driving factor may be the reduced capacity of tracheal epithelial cells to release basal PGE$_2$ at the levels observed before influenza infection.

In conclusion, this study has characterized capsaicin-induced relaxation in mouse isolated trachea and demonstrated that these responses are greatly attenuated by influenza A/PR-8/34 virus infection (Fig. 8). The mechanism through which influenza A attenuates this response is likely to involve a reduced ability to generate PGE$_2$ in response to SP. Although this phenomenon has not yet been described in humans and requires further characterization, it is tempting to speculate that the loss of this sensory nerve-mediated bronchoprotective response may be responsible for compounding viral-induced asthma exacerbations.

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Authorship Contributions

Participated in research design: Taylor and Henry.
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References


Fig. 8. Influenza A-induced modulation of capsaicin-induced smooth muscle relaxation pathway in mouse airways. In trachea from uninfected mice, capsaicin activates TRPV1 ion channels on sensory nerve endings evoking a "local axonal reflex," resulting in the release of substance P, which acts on epithelial NK$_1$ receptors to activate COX, leading to the generation of PGE$_2$. In turn, PGE$_2$ activates EP$_{2/4}$ receptors on airway smooth muscle, leading to an increase in cAMP and subsequent relaxation. Likewise, isoprenaline mediates a cAMP-dependent airway smooth muscle relaxation through its actions on $\beta$-adrenoceptors ($\beta$$_1$-AR). In contrast, tracheal segments from influenza A-infected mice did not respond to capsaicin (neither PGE$_2$, release nor relaxation response) and were hyporesponsive to substance P. Influenza A infection was not associated with impaired relaxation responses to either PGE$_2$ or isoprenaline. Thus, influenza A-induced modulation of the function of epithelial cells, rather than sensory nerves or smooth muscle cells, seems to be primarily responsible for suppressed responsiveness to capsaicin and substance P.


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