

Mast cell-cholinergic nerve interaction in mouse airways

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We addressed the mechanism by which antigen contracts trachea isolated from actively sensitized mice. Trachea were isolated from mice (C57BL/6J) that had been actively sensitized to ovalbumin (OVA). OVA ($10 \mu\text{g ml}^{-1}$) caused histamine release ($\sim 70\%$ total tissue content), and smooth muscle contraction that was rapid in onset and short-lived ($t_{1/2} < 1 \text{ min}$), reaching approximately 25% of the maximum tissue response. OVA contraction was mimicked by 5-HT, and responses to both OVA and 5-HT were sensitive to $10 \mu\text{M}$ -ketanserin (5-HT₂ receptor antagonist) and strongly inhibited by atropine ($1 \mu\text{M}$). Epithelial denudation had no effect on the OVA-induced contraction. Histological assessment revealed about five mast cells/tracheal section the vast majority of which contained 5-HT. There were virtually no mast cells in the mast cell-deficient (*sash*^{−/−}) mouse trachea. OVA failed to elicit histamine release or contractile responses in trachea isolated from sensitized mast cell-deficient (*sash*^{−/−}) mice. Intracellular recordings of the membrane potential of parasympathetic neurons in mouse tracheal ganglia revealed a ketanserin-sensitive 5-HT-induced depolarization and similar depolarization in response to OVA challenge. These data support the hypothesis that antigen-induced contraction of mouse trachea is epithelium-independent, and requires mast cell-derived 5-HT to activate 5-HT₂ receptors on parasympathetic cholinergic neurons. This leads to acetylcholine release from nerve terminals, and airway smooth muscle contraction.

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Abbreviations Atro, atropine; BSA, bovine serum albumin; DMPP, dimethylphenylpiperazinium; Ket, ketanserin; OVA, ovalbumin.

Antigen challenge in isolated smooth muscle preparations from previously sensitized animals leads to immediate and strong contractions (Aitken *et al.* 1975; Lulich *et al.* 1976; Chand & Eyre, 1978; Adams & Lichtenstein, 1979; Chand & Eyre, 1979; Koppel *et al.* 1981; Bjorck & Dahlen, 1993; Sigurdsson *et al.* 1995; Eum *et al.* 1999). Since its discovery nearly a century ago (Schultz, 1910; Dale, 1913) this response came to be known as the Schultz–Dale reaction, and is most commonly studied in isolated airway preparations because it mimics the immediate bronchial response to inhaled allergen *in vivo*, and therefore has direct relevance to allergic asthma reactions.

The Schultz–Dale reaction is now understood to involve the production of reagenic antibodies (IgE in most mammals), during the sensitization phase, that bind to high affinity Fcε receptors on mast cells. Antigen binds and cross-links the IgE, leading to mast cell activation, and release of contractile mediators onto airway smooth muscle cells. The Schultz–Dale reaction has most commonly been studied in human and guinea pig airways where the response has been shown to be due to the combined effect of histamine and cys-leukotrienes

(Adams & Lichtenstein, 1979). It should be stated that an obligatory role for mast cells in these reactions has been assumed, but has not been conclusively proven.

In the past decade, the mouse has become the most frequently used animal model in the study of allergic asthma. Koppel *et al.* (1981) have noted that, as with other species, the trachea isolated from sensitized mice contract in response to the sensitizing antigen. Others have shown that the Schultz–Dale reaction in the mouse involves both 5-hydroxytryptamine and acetylcholine (Eum *et al.* 1999). Here we address the hypothesis that mast cells are obligatory for the Schultz–Dale reaction in mouse, and that the response selectively involves the direct interaction of mast cell-derived 5-HT with cholinergic parasympathetic nerves.

Methods

Ethical approval

All protocols were approved by the Johns Hopkins Animal Care and Use Committee.

Mice and immunization protocol

Male mice (C56BL/6J or *sash*^{-/-}) obtained from The Jackson Laboratory (Bar Harbor, ME, USA) were actively sensitized by injecting 0.2 ml of an ovalbumin (OVA) solution ($3.75 \mu\text{g ml}^{-1}$) mixed with aluminum hydroxide (13 mg ml^{-1}) three times at an interval of 2 days. Experiments were conducted on animals 8–12 weeks of age beginning 2 weeks following the first injection.

Organ bath studies

Mice were killed by CO₂ asphyxiation. Whole trachea were dissected out and placed in oxygenated Krebs–Ringer bicarbonate solution containing (in mM): 118 NaCl, 5.4 KCl, 1 NaH₂PO₄, 1.2 MgSO₄, 1.9 CaCl₂, 25 NaHCO₃, 11.1 dextrose. Trachea were cleaned of connective tissue, and in some preparations, the epithelium was disrupted by gently rubbing the luminal surface with the flanged end of a short length of polyethylene tubing (1.5 mm outer diameter). Tracheal rings (whole or laterally divided in half), were suspended between two tungsten stirrups in 10 ml organ chambers filled with Krebs solution that was warmed to 37°C and bubbled with 95% O₂–5% CO₂ to maintain a pH of 7.4. One stirrup was connected to a strain gauge (model FT03; Grass Instruments, Quincy, MA, USA), and tension was recorded on a Grass Model 7 polygraph (Grass Instruments). Preparations were stretched to a resting tension of 0.2 g, and washed with fresh Krebs buffer at 15 min intervals during a 60 min equilibration period. After equilibration, trachea were challenged with either OVA ($10 \mu\text{g ml}^{-1}$), or cumulative concentrations of 5-HT (10 nM – $100 \mu\text{M}$) in the presence or absence of either ketanserin ($10 \mu\text{M}$), atropine (100 nM or $1 \mu\text{M}$), or tetrodotoxin (TTX; $3 \mu\text{M}$). At the end of each experiment, all trachea were maximally contracted with either BaCl₂ (30 mM), methacholine ($1 \mu\text{M}$), or carbachol ($1 \mu\text{M}$). All results are expressed as a percentage of maximum contraction.

Histamine release studies

Whole trachea (4–8 mg damp weight) were dissected out, cleaned of connective tissue, and incubated in 1 ml of Krebs solution (described above) for 60 min, with the buffer being exchanged for fresh solution at 15 min intervals. The tissue was then treated with OVA ($10 \mu\text{g ml}^{-1}$) for 15 min, following which, the bath solution was assayed for antigen-induced histamine release. Finally, trachea were incubated for 15 min with 1 ml of 8% perchloric acid in a 37°C water-bath, and the supernatant was collected and assayed to measure the remaining tissue histamine content. Histamine was assayed by the automated fluorometric technique described pre-

viously described (Siraganian, 1974). Histamine release is expressed as a percentage of the total histamine content.

Histological verification of epithelium removal

To verify epithelium removal, at the end of each organ bath experiment, trachea were fixed in 10% formalin overnight, rinsed in phosphate-buffered saline (PBS) for 30 min, and cryoprotected in 18% sucrose solution for 24 h. Trachea were then divided laterally into thirds, embedded in OCT, frozen, and sectioned serially ($12 \mu\text{m}$). Sections were visualized by hematoxylin and eosin (H&E) staining, and epithelial disruption was verified by examination with light microscopy. Denuded areas from rostral, mid-trachea, and caudal sections were defined as those in which epithelial cells were absent, and the basement membrane was clearly visible.

Histological identification of mast cells

In a separate set of experiments, non-sensitized mice (C57BL/6J or *sash*^{-/-}) were anaesthetized with sodium pentobarbital (3 mg per mouse). Trachea were cannulated, and the lungs fixed by tracheal instillation of 10% formalin while maintaining a constant tracheal pressure of 20 cmH₂O. The heart and lungs were removed *en bloc*, and stored in 10% formalin for sectioning. Prior to immunostaining, tissues were rinsed for 30 min in PBS, and cryoprotected overnight in 18% sucrose solution. Whole trachea with oesophagus attached were dissected free of the heart and lungs, embedded in OCT, frozen, and serially sectioned ($10 \mu\text{m}$). Sections were washed with water and PBS, and endogenous mouse antibodies were blocked for 1 h at room temperature with a mouse-on-mouse blocking kit (Vector Laboratories, Burlingame, CA, USA), followed by a 1 h incubation in goat blocking solution (10% goat serum, 1% bovine serum albumin (BSA), and 0.5% Tween 20 in PBS). Sections were then incubated overnight at 4°C with primary antibody (combination rabbit anti-5-HT, diluted 1 : 200; mouse anti-mast cell tryptase, diluted 1 : 100 both from Chemicon, Billerica, MA, USA), diluted in Triton/BSA PBS containing 0.3% Triton X-100 and 1% BSA). The following day, sections were washed twice in Triton/BSA PBS (10 min each), and incubated at room temperature for 2 h with a secondary antibody combination (Alexa 488-conjugated goat anti-rabbit, diluted 1 : 100; Alexa 594-conjugated goat anti-mouse, diluted 1 : 100; Invitrogen, Carlsbad, CA, USA). Slides were then washed two times with PBS (10 min each), once in pH 8.6 PBS, and coverslipped with pH 8.6 glycerol. Sections were visualized using an Olympus microscope (Olympus, Center Valley, PA, USA) using appropriate filter sets for Alexa-488 and -594.

Tissue preparation and electrophysiological recording

OVA-sensitized C57BL/6J mice were anaesthetized with sodium pentobarbital (3 mg per mouse). The heart and lungs were removed *en bloc*, and placed in oxygenated Krebs solution containing atropine (100 nM). The trachea was dissected free of the heart, lungs, and oesophagus, cleaned of connective tissue, and opened longitudinally along the ventral surface. The trachea was pinned flat, dorsal side up, and ganglia were visualized without staining, and isolated by fine dissection. The portion of the airway containing the ganglion was excised, and tightly pinned to the floor of a Sylgard-coated recording chamber, and equilibrated with flowing ($2\text{--}4\text{ ml min}^{-1}$) Krebs solution for 30 min at $35\text{--}37^\circ\text{C}$ in the recording chamber before intracellular recordings were performed. The trachea was challenged with 5-hydroxytryptamine ($10\text{ }\mu\text{M}$) in the presence or absence of ketanserin ($1\text{ }\mu\text{M}$), and/or with OVA ($10\text{ }\mu\text{g ml}^{-1}$) and the electrophysiological properties of the parasympathetic ganglion neurons were monitored.

Electrophysiological procedures

Intracellular micropipettes were fashioned from thick-walled capillary stock (1 mm; World Precision Instruments, Sarasota, FL, USA) by a Brown–Flaming microelectrode puller (P-87, Sutter Instruments, San Rafael, CA, USA). Electrodes were filled with a solution of potassium chloride (3 M), and connected by a Ag–AgCl pellet embedded in an electrode holder (E.W. Wright, Guilford, CT, USA) to an electrometer (Axoclamp model 2, Axon Instruments, Union City, CA, USA). The electrode DC resistance in Krebs solution ranged between 60 and 90 M Ω . A Ag–AgCl pellet in the bath was aided by a brief ($<2\text{ ms}$) overcompensation (i.e. buzz) of the capacitance neutralization circuit of the Axoclamp amplifier. Before drug or antigen application, stability of the recording (i.e. resting potential and input resistance) was confirmed, and the action potential accommodative properties (i.e. tonic or phasic firing pattern) were evaluated as previously described (Myers *et al.* 1990).

Drugs and reagents

All reagents were purchased from Sigma Aldrich (St Louis, MO, USA), except TTX (Alomone Laboratories, Jerusalem, Israel) and ketanserin (Tocris Bioscience, Ellisville, MO, USA). Concentrated stock solutions (1 mM–1 M) were prepared in distilled water and stored at -20°C . On the day of the experiment, working solutions of 5-HT were prepared in warmed, oxygenated Krebs solution.

Statistical analysis

Results are presented as means $\pm$ S.E.M. Sample means were compared using Student's *t* test for two means, paired or unpaired as indicated by the experimental parameters. For these studies four different groups ($n=8\text{--}12$) of animals were actively sensitized. For all series of experiments the control and treated tissues were obtained from the same batch of sensitized mice. Control and experimental mean values were considered to differ significantly if $P < 0.05$.

Results

OVA contraction kinetics in isolated mouse airway

Contractile responses to OVA ($10\text{ }\mu\text{g ml}^{-1}$) were measured in trachea isolated from previously sensitized C57BL/6J mice. In all preparations studied, OVA caused tracheal smooth muscle contraction that was rapid in onset, as it commenced within 30 s of addition to the tissue bath, and short-lived ($t_{1/2} < 1\text{ min}$; Fig. 1 inset). In 18 experiments using untreated, epithelium-intact trachea from sensitized mice, OVA induced contractions that ranged from 13.5 to 41.0% of tissue maximum, with mean responses reaching $26.5 \pm 2.2\%$ of the maximum response.

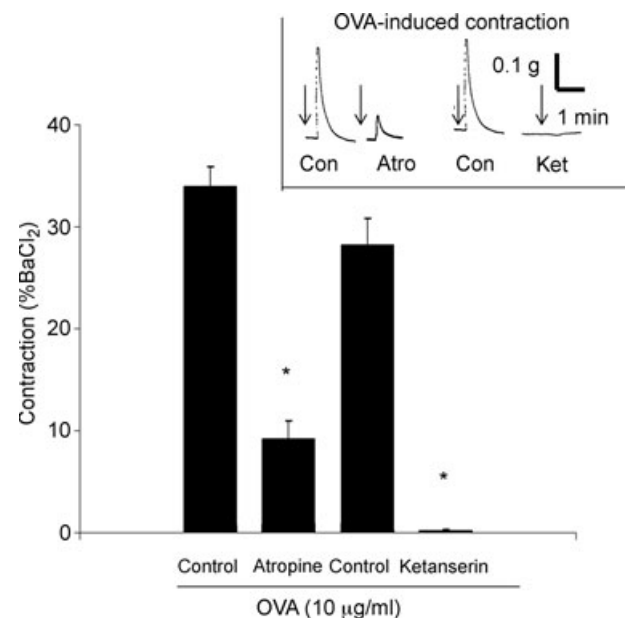


Figure 1. Ovalbumin-induced contractions of trachea isolated from actively sensitized mice in the absence (control) and presence of atropine ($1\text{ }\mu\text{M}$, $n=4$) or ketanserin ($10\text{ }\mu\text{M}$, $n=5$) The bar graph depicts mean $\pm$ S.E.M. contraction as a percentage of the maximal obtainable contraction. An asterisk (*) denotes $P < 0.01$. Inset, representative trace of airway smooth muscle contraction in response to $10\text{ }\mu\text{g ml}^{-1}$ ovalbumin (Con) in the presence and absence of atropine (Atro) or ketanserin (Ket). Arrows indicate the addition of OVA to the tissue bath.

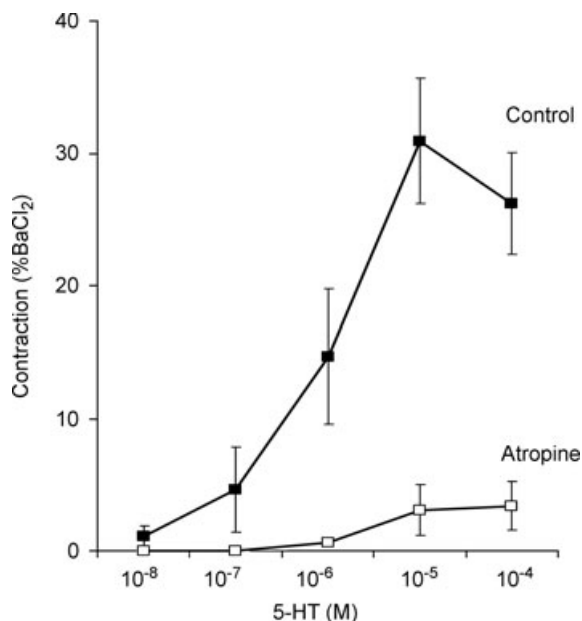


Figure 2. Cumulative concentration–response curves for 5-HT in the absence (filled squares) and presence (open squares) of atropine (100 nM)

Data are means $\pm$ S.E.M., $n = 5$.

Effect of 5-HT₂ and muscarinic receptor inhibition on bronchoconstriction

Six experiments were carried out in which one trachea served as control and a second trachea was treated with the 5-HT₂ receptor antagonist ketanserin (10 μ M). In these experiments the control responses averaged $28 \pm 3\%$ of maximum. Ketanserin abolished the contractile responses to OVA (Fig. 1).

Another set of studies was carried out in which a control response was compared to the response in trachea pretreated with atropine (1 μ M). In the control tissues OVA induced contractions that averaged $34 \pm 2\%$ of maximum. Atropine reduced the OVA response by an average of 75% (Fig. 1). Pretreatment with atropine (100 nM) also inhibited contractile responses to exogenously applied 5-HT, shifting the concentration–response curve to the right, and reducing the maximum contraction (Fig. 2).

Endogenous source of 5-HT

To examine the role of mast cells as a source of 5-HT in OVA-induced contraction, trachea from OVA-sensitized *Kit^{W-sh}/Kit^{W-sh}* *sash* (*sash*^{−/−}) mice were studied. *Sash*^{−/−} mice have an inversion mutation in a segment of the chromosome containing the *c-Kit* gene which disturbs gene regulatory elements, resulting in reduced expression of the Kit receptor in animals that are homozygous for the mutation. Consequently, *sash*^{−/−} mice are profoundly mast cell-deficient (Grimbaldeston *et al.* 2005; Wolters *et al.* 2005). Mast cells were present in the airways of C57BL/6J animals (Fig. 3). A total of 33 tracheal sections from four wild-type animals were studied. On average about five tryptase- and 5-HT-positive mast cells per section were observed (Table 1). The mast cells were concentrated in the smooth muscle layer of the airway, with relatively few cells localized near to the epithelial basement membrane, and virtually no mast cells seen in the cartilaginous portion of the trachea (Fig. 3). All 5-HT positive cells in the trachea were also positive for tryptase;

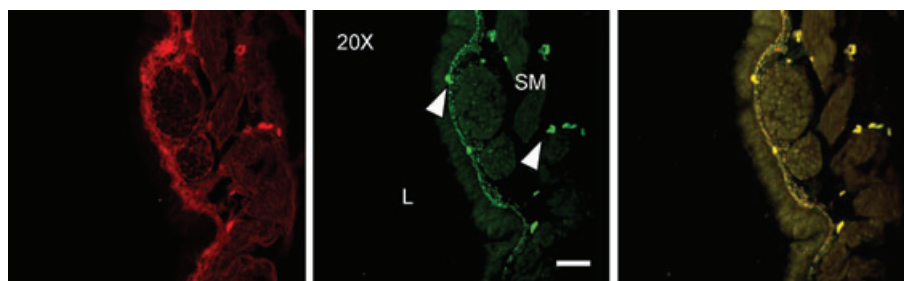


Figure 3. Immunohistochemical localization of mast cells in a tracheal section taken from the airway of a C57BL/6J mouse at 20 $\times$ magnification (L = lumen, SM = airway smooth muscle; white bar = 50 μ m)

Shown is a section stained independently with antibodies against mast cell tryptase (red) and 5-HT (green), and a photomicrograph of the two images overlaid (yellow), demonstrating the localization of 5-HT within mast cells. All 5-HT-positive mast cells identified in 33 tracheal sections from 4 mice were mast cell tryptase-positive. Mast cells were undetectable in 22 sections stained for 5-HT and mast cell tryptase from the trachea of 3 *sash*^{−/−} mice (data not shown). The arrowheads indicate one of two labelled mast cells localized to the basement membrane (left), and one of several associated with the airway smooth muscle (right). In all histological sections examined, the majority of mast cells were concentrated within the smooth muscle layer, facilitating their interaction with tracheal ganglia and nerve terminals. Mast cells were virtually absent from the cartilaginous ventral portion of the airway.

Table 1. Mast cell numbers and histamine content and release in mouse trachea

	Mast cells (no./section)	Histamine content (ng mg ⁻¹)	OVA-induced release (%)
C57BL/6J	5.18 ± 0.7	7.2 ± 1.1	74.2 ± 4.2
<i>sash</i> ^{-/-}	0.09 ± 0.06*	0.48 ± 0.5*	undetectable

Mast cell no. is the average number of mast cells per tracheal section as detected by double-labelling immunofluorescence with antibodies against 5-HT and mast cell tryptase (mean ± S.E.M., * $P < 0.01$, $n = 5$). In 33 sections from 4 C57BL/6J mice, all 5-HT-positive mast cells were also tryptase-positive, however 26% of cells were positive for tryptase only. In contrast to the airways of C57BL/6J animals, mast cells and histamine was virtually absent in the tracheas of *sash*^{-/-} mice (* denotes $P < 0.01$ comparing wild type with *sash*^{-/-}). Ova-induced histamine release ($n = 6$) is expressed as a percentage of the total tissue content. All data are presented as mean ± S.E.M.

however approximately 25% (44/169) of tryptase-positive mast cells did not stain positive for 5-HT. Mast cells were virtually absent in trachea isolated from *sash*^{-/-} mice. Only two mast cells were observed in 22 sections from three mast cell-deficient mice.

Antigen challenge of trachea resulted in the release of >70% of the tissue histamine stores in OVA-sensitized C57BL/6J mice. In sensitized *sash*^{-/-} mice, OVA failed to elicit histamine release (Table 1), or contractile responses (Fig. 4), while contraction in response to 5-HT in the same preparations was preserved (Fig. 4).

We attempted to 'reconstitute' the mast cells in the trachea of *sash*^{-/-} mice by intravenous injection of 10×10^6 bone marrow derived cultured mast cells. We evaluated 10 mice and found, consistent with the findings of others, that the mast cells began to repopulate the lung parenchyma of *sash*^{-/-} mice by 90 days post-reconstitution, but failed to repopulate the trachea (data not shown).

Effect of epithelium removal on OVA-induced contraction

Epithelium removal from trachea isolated from OVA-sensitized C56BL/6J mice was verified histologically by H&E staining. In control trachea the basement membrane was entirely covered by an intact epithelium. In mechanically denuded trachea only the presence of a few scattered cells remaining on the basement membrane was observed (data not shown). The kinetics and magnitude of OVA-induced contractions were not different between control and epithelium denuded tissues (Fig. 5).

Effect of TTX

In epithelium-denuded trachea, TTX (3 μ M) reduced, but did not abolish, the contractile responses to OVA from $32 \pm 3\%$ to $13 \pm 3\%$, $P < 0.05$ of the maximum response (data not shown).

Effect of antigen challenge and 5-HT on ganglion neurons

We next directly examined, using intracellular electrophysiological techniques, the effects of antigen challenge on parasympathetic neurons situated in the tracheal parasympathetic ganglia. The parasympathetic ganglia were visualized under $20\times$ microscopy. They were found mainly situated in the serosal aspect of the smooth muscle layer. A typical ganglion contained 6–30 neurons (see Fig. 6). Verification of the neuronal nature of the impaled cell was obtained by the rapid response to the nicotinic receptor agonist dimethylphenylpiperazinium (DMPP) often reaching action potential threshold (see inset of Fig. 6).

OVA caused an immediate and reversible depolarization of the resting membrane potential from -49 ± 4.5 to -41 ± 6.8 mV (range 2–18 mV, $P < 0.05$; Fig. 6).

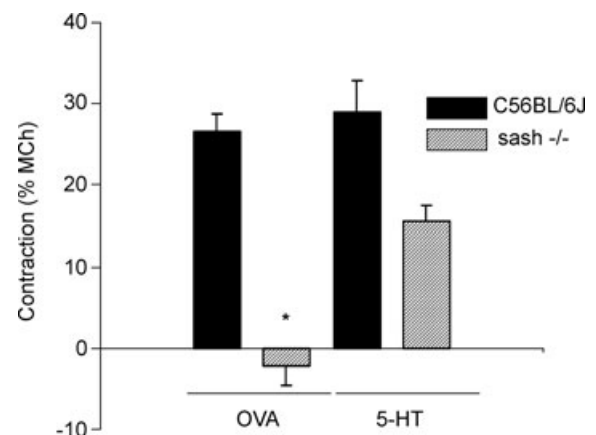


Figure 4. The maximum contractile response to ovalbumin (OVA) and 5-HT in trachea isolated from OVA-sensitized wild type and mast cell-deficient *sash*^{-/-} mice

The trachea from wild type mice ($n = 18$) contracted to OVA, whereas trachea isolated from *sash*^{-/-} mice ($n = 3$) did not. The trachea obtained from wild type ($n = 11$) and *sash*^{-/-} mice ($n = 3$) contracted in response to 5-HT (100 μ M). The bar graphs represent means ± S.E.M., * $P < 0.01$.

In a subset of two neurons, the antigen-induced depolarization was followed by a membrane hyperpolarization. In one neuron the OVA-induced membrane depolarization reached the threshold for continuous, repetitive action potential discharge throughout the exposure. 5-HT ($10 \mu\text{M}$) mimicked the effect of OVA on the ganglion neurons. 5-HT reversibly depolarized the neurons (-53 ± 2.4 to -45 ± 3.2 mV; range 5–20 mV; $P < 0.05$ Fig. 6). The same two neurons that responded to OVA with a transient depolarization followed by a hyperpolarization also responded to 5-HT in the same fashion. In addition the neuron in which the OVA-induced depolarization reached the threshold for action potential discharge responded with action potential discharge to treatment with 5-HT. The effect of 5-HT on the resting membrane potential of the neurons was blocked by ketanserin (Fig. 6).

Discussion

The data provide evidence that antigen-induced contraction of mouse trachea requires direct activation of 5-HT₂ receptors on parasympathetic cholinergic neurons

by mast cell-derived 5-HT. This leads to acetylcholine release from nerve terminals that stimulates smooth muscle contraction via activation of muscarinic receptors.

That the antigen-induced airway contractions involved both 5-HT and acetylcholine is consistent with the findings of Eum *et al.* (1999). In both the present study and the study by Eum *et al.* it was found that the antigen-induced

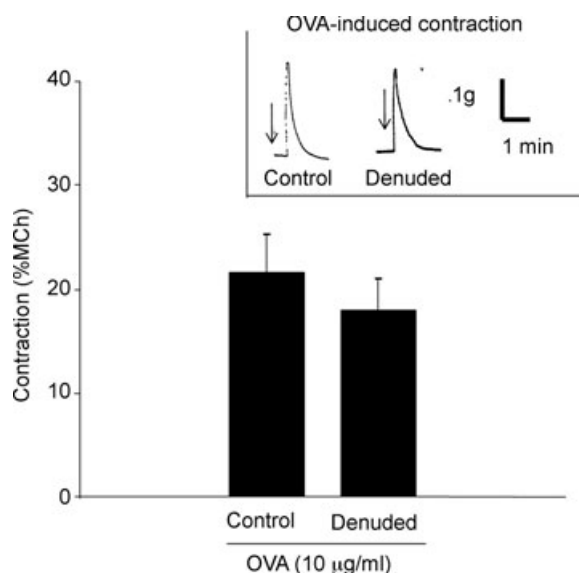


Figure 5. OVA ($10 \mu\text{g ml}^{-1}$)-induced contractions of trachea isolated from actively sensitized mice

The contractile responses were compared in trachea in which the epithelium was intact (control) vs. trachea in which the epithelium was mechanically removed (denuded). In each case histological assessment was obtained to showing the status of the epithelium. In denuded tissues approximately 80–100% of the basement membrane was free of any epithelial cells (not shown). The bar graphs represent the means $\pm$ S.E.M. of the peak contraction as a percentage of the maximum obtainable contraction induced by methacholine (Mch), $n = 7$ –8. Inset, representative trace of OVA-induced contractions in control and epithelium denuded trachea showing that the time course of the contraction is not influenced by the absence of epithelium. Arrows indicate the addition of OVA to the tissue bath.

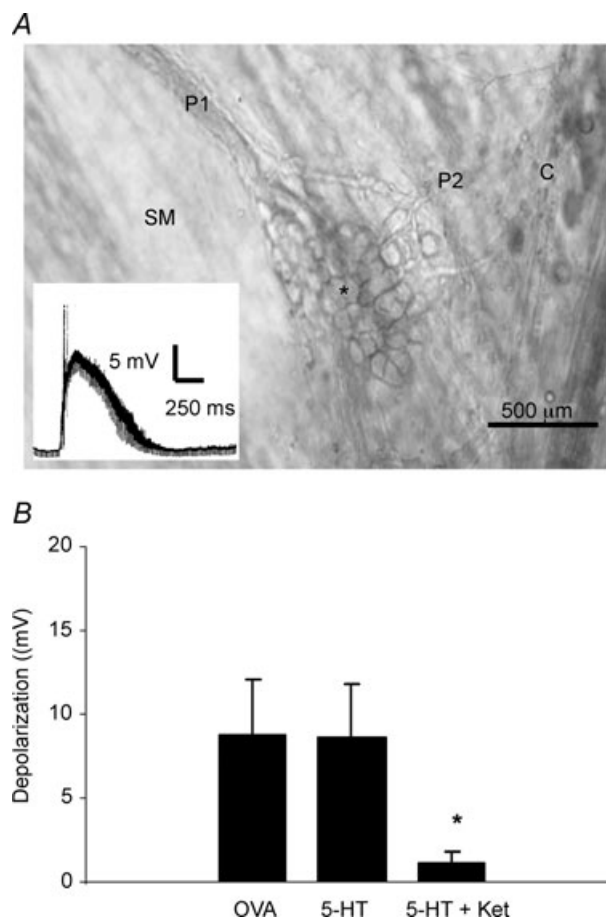


Figure 6. 5-HT and OVA cause membrane depolarization in airway parasympathetic neurons

A, photomicrograph of a parasympathetic ganglion within the trachea of a C57BL/6J mouse. An asterisk (*) indicates one neuron in the centre of one ganglion composed of approximately 40–60 neurons. This ganglion was located on the serosal aspect of the smooth muscle layer. P1 and P2 respectively are pre- and post-ganglionic nerve fibres; C indicates a capillary; and SM indicates smooth muscle fibres beneath the ganglion. Inset, example of membrane potential depolarization obtained with an intracellular recording of a neuron within a parasympathetic ganglion after exposure to the nicotinic agonist DMPP ($30 \mu\text{M}$ for 10 s); the depolarization reached action potential threshold as evidenced by 2 spikes (the action potentials are not readily resolved at this time scale but can be seen as a thin vertical line). B, mean membrane depolarization of airway neurons after exposure to OVA ($10 \mu\text{g ml}^{-1}$ for 1 min) and 5-HT ($10 \mu\text{M}$ for 1 min); mean resting membrane potential = -49 ± 4.5 mV in 4 neurons and -53 ± 2.4 mV in 9 neurons respectively. 5-HT-induced depolarization is sensitive to $1 \mu\text{M}$ -ketanserin (Ket). Data are means $\pm$ S.E.M., * $P < 0.01$, $n = 3$.

tracheal contractions were abolished by a 5-HT receptor antagonist, and inhibited $\sim 70\%$ by atropine. This indicates that the 5-HT evoked tracheal contractions depend mainly on a cholinergic mechanism with the additional involvement of a minor non-cholinergic component. The question that was further addressed here is the source of the 5-HT and acetylcholine in these responses.

In mice, 5-HT is released from mast cells upon activation by antigen-specific IgE (Kitamura, 1989). Our results support the hypothesis that mast cells are indeed obligatory for the antigen-induced contraction of mouse trachea. This is based on our observations that the mast cells (tryptase-positive cells) in the trachea also contained 5-HT and that the trachea from OVA-sensitized *sash*^{-/-} mice did not contain mast cells, and failed to respond to antigen with either mediator release or smooth muscle contraction. Additional proof of this hypothesis was sought by an attempt to selectively reconstitute the trachea of *sash*^{-/-} mice with syngeneic bone marrow-derived mast cells. Regrettably, while we were able to reconstitute mast cells into the lung parenchyma, the injected mast cells failed to repopulate the trachea; this is consistent with the findings of others (Grimbaldeston *et al.* 2005). Nevertheless, the observations that (1) the tracheal responses were due to 5-HT, (2) mast cells in the trachea contain 5-HT, and (3) the responses were absent in the mast cell deficient animals provide direct support for mast cells being the source of 5-HT in this system.

5-HT-mediated contraction of murine airway smooth muscle *in vitro* and *in vivo* is inhibited by atropine (Levitt & Mitzner, 1989; Eum *et al.* 1999; Moffatt *et al.* 2004; Kummer *et al.* 2006). In a precision-cut mouse lung slice preparation, a large concentration (100 μM) of atropine nearly abolished the 5-HT-induced airway narrowing even in muscarinic M2/M3 receptor double knock out animals, raising the caveat that at this dose atropine may inhibit some non-cholinergic mechanism involved in 5-HT responses (Kummer *et al.* 2006). In the trachea, however, low concentrations of atropine inhibited the 5-HT responses (present study; Moffatt *et al.* 2004); moreover blocking acetylcholinesterase enhances the 5-HT responses (Eum *et al.* 1999), leaving little doubt that the atropine-sensitive responses in the trachea are indeed cholinergic in nature. There is, however, some controversy over the source of the acetylcholine involved in 5-HT-induced tracheal contractions. It has generally been assumed that cholinergic contraction of airway smooth muscle involves the release of acetylcholine from post-ganglionic parasympathetic nerve terminals. More recently, however, several cell types in the airways other than neurons have been considered as potential sources of acetylcholine (Wessler & Kirkpatrick, 2001). Of particular relevance to the present discussion is the airway epithelium as a potential source of acetylcholine. Moffatt

et al. (2004) used Triton X-100 to remove the epithelium from the mouse trachea and found that this inhibited the subsequent 5-HT-induced cholinergic contractions. In addition these investigators provided several lines of pharmacological evidence that were inconsistent with the hypothesis that the 5-HT-induced cholinergic contractions required cholinergic nerve stimulation. By contrast, our data show that physically removing the epithelium did not influence either the rate or the magnitude of the 5-HT-dependent cholinergic response that was evoked by antigen challenge. On the other hand, these responses were significantly reduced by tetrodotoxin, implicating a dependency on action potential discharge in the response. These data favour the hypothesis that the source of acetylcholine is the postganglionic parasympathetic nerve terminal. Our electrophysiological findings that 5-HT can cause a ketanserin-sensitive depolarization of the cholinergic parasympathetic neurons in the trachea and that this is mimicked by OVA challenge provide additional direct evidence of a prominent role for parasympathetic nerves in the tracheal responses.

It is possible that exogenously applied 5-HT can evoke release of acetylcholine from tracheal epithelium and nerves, but when the source of the 5-HT is the tissue mast cell, the major effect is on the parasympathetic nerves. In this light it is noteworthy that the majority of mast cells in the mouse trachea were situated in the smooth muscle portion of the tissue well beneath the epithelium, a location that would have them juxtaposed to postganglionic parasympathetic nerves. That mast cells can release mediators that directly interact with airway parasympathetic neurons is consistent with our previous observations in guinea pigs (Myers *et al.* 1991; Kajejar *et al.* 2003).

The relevance of the present findings in the mouse to allergic airway disease in humans can be questioned. The antigen-induced contraction of human isolated bronchi (and guinea pig airways) is a sustained response that persists for over an hour. This is explained by a prominent role played by cys-leukotrienes (Adams & Lichtenstein, 1979). We found that the antigen-induced contraction of the mouse isolated airway is transient, likely to be due to the major role played by the pulsate release of acetylcholine, with apparently no role for leukotrienes. It should also be mentioned that histamine rather than 5-HT is the major biogenic amine in human mast cells. It may be relevant to point out, however, that 5-HT is stored in human mast cells and can be released upon antigen challenge (Kushnir-Sukhov *et al.* 2007). That mast cells are commonly associated with peripheral nerves in many tissues indicates that the mast cell-parasympathetic nerve interaction reported here may have relevance in other species as well as other tissues.

The results presented provide conclusive evidence that mast cells are required for antigen-induced contraction

in murine isolated trachea, and demonstrates immune modulation of parasympathetic nerve activity that adds new insight into the understanding the mechanisms involved in anaphylactic bronchoconstriction in the mouse.

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Author contributions

L.W. carried out all experiments, analysed data, and wrote the first draft of the manuscript. S.M. carried out experiments, analysed data, and edited the manuscript. A.M. designed and oversaw all studies on electrophysiology and immunohistochemistry, and edited the manuscript. B.U. oversaw the entire project, analysed data, and edited the manuscript.

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