Synergistic interactions between airway afferent nerve subtypes mediating reflex bronchospasm in guinea pigs

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Mazzone, Stuart B., and Brendan J. Canning. Synergistic interactions between airway afferent nerve subtypes mediating reflex bronchospasm in guinea pigs. Am J Physiol Regulatory Integrative Comp Physiol 283: R86–R98, 2002.—The hypothesis that airway afferent nerve subtypes act synergistically to initiate reflex bronchospasm in guinea pigs was addressed. Laryngeal mucosal application of capsaicin or bradykinin or the epithelial lipoxygenase metabolite 15(S)-hydroxyeicosatetraenoic acid evoked slowly developing but pronounced and sustained increases in tracheal cholinergic tone in situ. These reflexes were reversed by atropine and prevented by vagotomy, trimethaphan, or laryngeal denervation. Central nervous system-acting neurokinin receptor antagonists also abolished the reflexes without altering baseline cholinergic tone. Baseline tone was, however, reversed by disrupting pulmonary afferent innervation while preserving the innervation of the trachea and larynx. Surprisingly, selective pulmonary denervation also prevented the laryngeal capsaicin-induced tracheal reflexes, suggesting that laryngeal C-fibers act synergistically with continuously active intrapulmonary mechanoreceptors to initiate reflex bronchospasm. Indeed, reflex bronchospasm evoked by histamine was markedly potentiated by bradykinin, an effect mimicked by intracerebroventricular, but not intravenous, substance P. These data, as well as anatomic evidence for afferent nerve subtype convergence in the commissural nucleus of the solitary tract, suggest that airway nociceptors and mechanoreceptors may act synergistically to regulate airway tone.

Central sensitization; airway hyperreactivity; nucleus of the solitary tract; gastroesophageal reflux; 15(S)-hydroxyeicosatetraenoic acid

MULTIPLE AIRWAY AFFERENT NERVE subtypes have been identified on the basis of their neurochemistry, electrophysiological properties, and responsiveness to physical and chemical stimuli. Myelinated airway mechanoreceptors, of which there are at least two types, are sporadically active throughout the respiratory cycle (4, 36, 39). Mechanoreceptors respond to the dynamic and/or sustained physical effects of lung inflation and deflation and may also be activated indirectly by bronchoconstrictors such as histamine, cysteinyl leukotrienes, or acetylcholine (4, 7, 11, 38). The ongoing activity of these mechanoreceptors contributes to respiratory rhythm. A subset of these mechanoreceptors may also be a primary driving force behind the baseline parasympathetic tone measurable in the airways of all mammalian species (22, 23). When acutely activated, airway mechanoreceptors induce cough and parasympathetic reflexes such as bronchospasm, mucus secretion, and vasodilatation (42).

Unmyelinated afferent C-fibers also innervate the airways. Generally quiescent throughout the respiratory cycle but activated by inflammation and proinflammatory mediators such as bradykinin, these airway afferent nerves are physiologically similar to the nociceptors of the somatic nervous system. Activation of bronchopulmonary C-fibers can also precipitate cough and increased parasympathetic nerve activity and produce distinct effects on respiratory pattern and cardiovascular function (10, 28).

In the somatosensory nervous system, stimulation of nociceptors induces pain sensation, allodynia, and hyperalgesia by acting in synergy with mechanically sensitive sensory nerves in a process known as central sensitization (29, 45). This synergy is made possible by the convergent terminations of these sensory nerve subtypes on subsets of integrative relay neurons in the spinal cord. Neurons located in the integrative centers of the brain stem receive similar convergent inputs from visceral afferent nerves (20, 24, 34). Given the many physiological and morphological similarities of the somatosensory and visceral afferent nerves, we reasoned that synergistic interactions between airway afferent nerve subtypes might also precipitate airway parasympathetic reflexes.

METHODS

Surgery and animal preparation. All experiments were approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee. Male Hartley guinea pigs (300–400 g; n = 121; Hilltop, Scottsdale, PA) were anesthetized with urethane (1 g/kg ip) and placed supine on a heated pad. This dose of urethane provides deep, stable anesthesia for up to 9 h, although experiments rarely lasted for >4 h. The adequacy of the anesthesia was assessed throughout the course of the experiments by monitoring cardiovascular responses to a sharp pinch of the hindlimb.

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The caudalmost portion of the extrathoracic trachea was cannulated, and the animals were ventilated (60 breaths/min, 6 ml/kg, 2–3 cmH2O positive end-expiratory pressure) after induction of paralysis (suxamethonium chloride, 2 mg/kg sc). Reflex-mediated alterations in tracheal smooth muscle tone were monitored isometrically in the rostral extrathoracic trachea, as described previously (7, 22). Pulmonary insufflation pressure was monitored with a pressure transducer attached to a side port of the tracheal cannula. The abdominal aorta and vena cava were cannulated to monitor blood pressure and deliver intravenous drugs, respectively. Tracheal tone, pulmonary insufflation pressure, and arterial blood pressure were monitored and recorded on a Grass polygraph.

Krebs bicarbonate buffer was perfused through the tracheal lumen via a small incision in the caudal extrathoracic trachea. The buffer was recovered using a gentle suction catheter directed toward the caudal extrathoracic trachea. The buffer was recovered using a gentle suction catheter directed toward the caudal extrathoracic trachea. The buffer (composition in mM: 118 NaCl, 5.4 KCl, 1 NaH2PO4, 1.2 MgSO4, 1.9 CaCl2, 25 NaHCO3, 11.1 dextrose) contained 3 mM indoetharine, 2.5 mM propranolol, and 1 mM phenolamine. These drugs were used to block the local effects of prostaglandins and to block any effects of circulating and neurally released catecholamines on the tracheal segment studied. All animals were also pretreated with propranolol (1 mg/kg iv) at the beginning of each experiment.

At the end of each experiment, animals were killed using carbon dioxide delivered through the inspiratory port of the ventilator.

**Experimental design.** The anatomy of the extrinsic vagal pathways projecting to the guinea pig airways permits selective transections of the afferent nerve fibers innervating the intrapulmonary airways or larynx while leaving the preganglionic parasympathetic fibers innervating the trachea intact (Fig. 1) (9, 22). Moreover, the trachea provides a convenient window on the activity of parasympathetic nerves throughout the airways (7, 22, 30). In the present study, we exploited these attributes to study interactions between airway afferent nerve subtypes on airway smooth muscle tone.

**Reflex tracheal contractions evoked from the larynx.** First, we determined whether brief (1–2 min) application of capsaicin (30 μM, 0.2 ml; n = 7) to the larynx evoked reflex-mediated increases in tracheal smooth muscle tone. Capsaicin was applied selectively to the laryngeal mucosa by means of a laryngeal cannula (27 gauge) inserted through the cricoid membrane, such that the distal end of the cannula was positioned on the laryngeal mucosa. The selectivity of this laryngeal stimulation was confirmed in vagotomized animals by comparing responses to laryngeal capsaicin with responses evoked by addition of capsaicin directly to the tracheal perfusate. The reflex nature of any contractions evoked by laryngeal capsaicin was assessed by attempting to reverse the contraction with tracheal instillation of atropine (1 μM; n = 7) or by preventing contractions with cervical vagotomy (n = 3) or systemic administration of the ganglionic blocker trimethaphan (5 mg/kg iv; n = 3). We attempted to determine the afferent nerve subpopulations mediating the reflex responses evoked by laryngeal application of capsaicin by severing the recurrent and/or superior laryngeal nerves bilaterally, adjacent to their termination in the larynx (n = 3–4; Fig. 1).

In similar experiments, we assessed the ability of laryngeal application of bradykinin (3 μM; n = 7) and the putative (18) vanilloid receptor (VR1) agonist 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE, 3 μM; n = 4] to mimic the effects of capsaicin on tracheal cholinergic tone. The VR1-dependent nature of the effects evoked by 15(S)-HETE were assessed by attempting to reverse or prevent any tracheal contractions evoked by the lipoxygenase product with tracheal instillation of the selective VR1 antagonist capsazepine (30 μM; n = 3–4). The adequacy of the capsazepine concentration was determined by assessing the ability of capsazepine to prevent the direct contractile effects of capsaicin (10 μM) when administered to the trachea (n = 4).

**Role of neurokinins in reflex tracheal contractions evoked from the larynx.** The role and site of action of neurokinins in the reflex-mediated contractions evoked by capsaicin were determined first by studying the effects of neurokinin receptor antagonists on these responses. We employed potent and structurally unrelated antagonists throughout the studies (see below) to ensure the validity of experimental results. Neurokinin receptor antagonists were administered by one of three routes: 1) tracheal/laryngeal administration of SR-140333 [neurokinin type 1 (NK1) receptor], SR-48968 [neurokinin type 2 (NK2) receptor], and SB-223412 [neurokinin type 3 (NK3) receptor] at 0.3 μM each (n = 3), 2) intravenous administration of SR-140333 and SB-223412 at 1 mg/kg each (n = 7) or the nonselective neurokinin receptor antagonist ZD-6021 at 5 mg/kg (n = 5) (7, 41), or 3) intracerebroventricular infusion of ZD-6021 at 0.67–6.7 nmol/min (n = 4). For these latter studies, neurokinin receptor antagonists were
administered centrally (intracerebroventricularly) via a stainless steel cannula, which was stereotaxically cemented into the right lateral cerebral ventricle (2 mm caudal and 1.8 mm lateral to bregma and 4.8 mm below the surface of the skull). The cannula was attached to a Hamilton microsyringe via polyethylene tubing, and drugs were infused using a syringe pump (model A-99, Razel) at a speed of 40 µl/h. Injection sites were confirmed with Evans blue dye at the end of the experiments. For all experiments, control studies were performed in parallel in which the appropriate vehicle (see Drugs) was administered instead of the antagonist.

**Synergistic interactions between airway afferent subtypes.**

In previous studies, we presented evidence that baseline cholinergic tone in the trachea is absolutely dependent on the ongoing activation of intrathoracic airway mechanoreceptors (22). The very presence of this ongoing activity and the dependence of baseline tone on peripheral input suggest that reflex-mediated alterations in parasympathetic nerve actions might depend on alterations in the activity and/or synergistic interactions with these airway mechanoreceptors. To test the hypothesis that laryngeal (capsaicin-sensitive) afferent innervation of the trachea act synergistically with intrathoracic airway mechanoreceptors to evoke reflex bronchospasm, the effect of denervating the lower airways on the ability of laryngeal capsaicin to evoke reflex contractions of the trachea was assessed. In these studies, a bilateral (intrathoracic) vagotomy was performed in which the vagi were severed immediately caudal to the origin of the recurrent laryngeal nerves (n = 7; Fig. 1). In doing so, the intrapulmonary airways and lungs were denervated, thereby removing all central input from airway mechanoreceptors, while the afferent and efferent innervation of the trachea and larynx were left completely undisturbed (9, 22). The appropriateness and selectivity of all nerve cuts were confirmed at autopsy. Animals were excluded from subsequent statistical analyses if the transections were inappropriate or damage to the recurrent laryngeal nerves was evident.

Synergistic interactions between airway afferent nerve subtypes were further assessed by determining the ability of the nociceptor stimulant bradykinin to augment the reflex actions evoked by activating airway rapidly adapting receptors (RARs) with histamine (3, 7). Increasing concentrations of bradykinin (starting at 0.01 nmol/kg) were injected intravenously as a bolus until the threshold for evoking reflex contractions of the trachea was reached (average threshold dose of bradykinin was 0.08 ± 0.01 nmol/kg, range 0.04–0.1 nmol/kg, n = 8). Histamine (0.5–10 µg/kg) was subsequently dissolved in the threshold dose of bradykinin and injected intravenously into the same animals (n = 8). In control animals, histamine was administered in the presence of vehicle (isotonic saline), rather than bradykinin. To determine the role of the parasympathetic nervous system in the synergistic responses evoked by simultaneous injections of bradykinin and histamine, we compared responses in control animals with those obtained in vagotomized animals (n = 5) or animals pretreated with the muscarinic receptor antagonist atropine (1 mg/kg iv; n = 5). In all studies employing intravenous histamine and bradykinin, the histamine H1 receptor antagonist pyrilamine (1 µM) and the bradykinin B2 receptor antagonist FR-173657 (0.3 µM) were added to the tracheal perfusate to prevent any direct effects (via the vasculature) of the autacoids on the tracheal segment under study (7, 30).

We then attempted to determine the role of neurokinins in the synergistic responses evoked by combined histamine and bradykinin injections. Animals were pretreated with ZD-6021 (5 mg/kg iv (n = 5) or 6.7 nmol/min for up to 20 min intracerebroventricularly (icv; n = 5)) before the injection of the autacoids. To ensure that any observed effect of centrally (intracerebroventricularly) administered ZD-6021 was not due to a peripheral site of action, we compared tracheal tension and pulmonary insufflation responses to intravenously administered neurokinin A (NKA, 0.5 nmol/kg; n = 4–5) in control (vehicle-treated) animals and animals treated intravenously or centrally with ZD-6021. In parallel experiments, we also assessed the effect of a threshold dose of the neurokinin receptor agonist substance P (SP), rather than bradykinin, on histamine-evoked reflex alterations in airway smooth muscle tone. In these studies, histamine was dissolved in a threshold dose of SP (determined by injecting increasing doses of the neuropeptide, starting at 0.05 nmol/kg; n = 3) and injected intravenously. Neurokinin receptor antagonists (SR-140333, SR-48968, and SB-223412 at 0.3 µM each) were added to the tracheal perfusate to prevent the direct effects of SP on the tracheals.

To further support a role for neurokinins in the synergistic response, we then assessed the ability of SP (13 pmol/min; n = 4) to increase tracheal cholinergic tone when administered centrally (into the fourth ventricle). In these studies, a stainless steel cannula (22 gauge) was stereotaxically cemented into the fourth ventricle (midline and 18.2 mm caudal to bregma and 8.8 mm below the surface of the skull). SP was infused for 10 min using a Razel syringe pump, and injection sites were marked as described above. SR-140333, SR-48968, and SB-223412 at 0.1 µM each were included in the tracheal perfusate in these experiments to prevent any potential peripheral actions of SP on the tracheal segment under study. The appropriateness of the neurokinin receptor antagonist concentrations was determined by comparing atropine-insensitive tracheal contractions evoked by 10 µM capsaicin (added to the tracheal perfusate) in the absence and presence of the neurokinin antagonists. The selectivity of SP for central neurokinin receptors was further assessed by injection of ZD-6021 (25 nmol in 5 µl over 1–2 min; n = 3) into the lateral ventricle at the peak of the SP-evoked tracheal contraction.

At the end of each experiment, the level of baseline cholinergic tone was determined by adding 1 µM atropine to the tracheal perfusate and subsequently evoking a maximum contraction by adding 300 mM barium chloride to the perfusate. The amount of tone (in g) reversed by adding atropine to the tracheal perfusate divided by the grams of contraction subsequently evoked by barium chloride was the baseline cholinergic tone. Reflex-mediated effects are expressed as a percent increase in baseline cholinergic tone or as a percentage of the maximum contraction.

**Neuronal tracing and immunohistochemistry.** An anatomic basis for airway afferent nerve convergence in the brain stem was assessed by neuronal tracing with retrograde fluorescent tracers. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip) and mounted in a Kopf stereotaxic frame. The brain stem was exposed between the occipital bone and first cervical vertebra by careful dissection of the dorsal neck muscles and overlying atlantooccipital membrane and dura mata. Rhodamine-coated latex microspheres (200 nl; LumaFluor, Naples, FL) were injected unilaterally into the commissural subnucleus of the nucleus of the solitary tract (nTS, 0.5–1 mm caudal, 0.5 mm lateral to oex, 0.8 mm deep; n = 4). The incisions were sutured, and the animals were removed from the frame and positioned ventral side up on a heated pad. The extrathoracic trachea was then exposed, and a second tracer (15 µl of 2.5% fast blue) was injected into the tracheal lumen. These incisions were also
sutured, and the animals were allowed to recover under close supervision (sterile techniques were used for all surgeries).

Seven days after the injections, animals were deeply anesthetized with pentobarbital (100 mg/kg) and then transcardially perfused, first with 10 mM heparinized PBS containing 0.1% procaine and then with 4% paraformaldehyde in PBS. After perfusion, the brain stem, nodose ganglia, and jugular ganglia were removed, fixed (4% paraformaldehyde at 4°C for 2 h), and cryoprotected (18% sucrose at 4°C overnight) before they were frozen in OCT medium and cut.

Frozen sections (12 μm) were stained for SP and neurofilament immunoreactivity as described previously (39). Briefly, sections were placed in 0.01 M PBS and covered in blocking solution (10% goat serum, 1% BSA, 0.5% Tween 20) with a polyclonal rabbit anti-SP antibody (Oncogene Research Products, Boston, MA; diluted 1:200) or a monoclonal mouse antineurofilament antibody (160 kDa; clone NN18, Boehringer Mannheim, Indianapolis, IN; diluted 1:30), each diluted in PBS containing 0.3% Triton X-100 and then with 4% paraformaldehyde in PBS. After several PBS washes, labeled sections were incubated (1 h at room temperature) with fluorescein-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:40 dilution; Calbiochem, San Diego, CA) and rinsed in PBS, and coverslips were applied using a commercially available antifade kit (Slow Fade, Molecular Probes, Eugene, OR). Cells labeled with either tracer (fast blue and/or rhodamine) or stained for SP or neurofilament were visualized using an Olympus BX60 fluorescent microscope. The number of cells labeled with fast blue and/or rhodamine was manually counted per animal in a 1-mm² area using 6–10 representative sections of each nodose and jugular ganglion. The sum of these representative sections (from a single animal) was used to calculate the total number of labeled cells in each ganglion. The total number of labeled cells was subsequently used to generate the mean ± SE for the group.

Statistics. Values are means ± SE. Differences between group means were assessed using analysis of variance on Statview for Macintosh (Berkeley, CA). P < 0.05 was considered significant. When significant variation between groups was detected, treatment group means were compared using Scheffé’s F test for unplanned comparisons.

Occasionally (<10% of experiments), animals exhibited little (<10% of the maximum contraction) or no baseline cholinergic tone or baseline tone that approximated the maximum cholinergic tone (>50% of the maximum contraction) attainable in this preparation (22). Such preparations rarely exhibited any reflex effects in response to any stimulus and were thus excluded from subsequent statistical analyses.

Drugs. Atropine sulfate, barium chloride, capsaicin, heparin sulfate, indomethacin, histamine, urethane (ethyl carbamate), succinylcholine chloride, pentabarbital sodium, pyrilamine, phenotamine hydrochloride, and dl-propranolol hydrochloride were purchased from Sigma (St. Louis, MO). 15S-hydroxy-5Z,11Z,13E-eicosatetraenoic acid [15(S)-HETE] was purchased as a solution (100 μg/ml) in ethanol from Cayman Chemicals (Ann Arbor, MI). Capsazepine was purchased from Tocris (Ellisville, MO); SP and NKA from Peninsula Laboratories (Belmont, CA); trimethaphan camyslate from Roche Laboratories (Nutley, NJ); and Tissue-Tek OCT embedding medium from VWR (Bridgeport, NJ). Bradykinin, SR-140333, and ZD-6021 were kindly provided by AstraZeneca (Wilmington, DE), FR-173657 by Fujisawa (Osaka, Japan), and SR-48968 and SB-223412 by Schering Plough (Kenilworth, NJ) and Glaxo SmithKline (King of Prussia, PA), respectively. Stock solutions (10–20 mM) of all drugs added to the tracheal perfusate were made in distilled water, except indomethacin (30 mM) and capsaicin (0.1 M), which were dissolved in absolute ethanol, and SR-140333, SR-48968, SB-223412, and FR-173657 (1 mM), which were dissolved in dimethyl sulfoxide (DMSO). For laryngeal application, capsaicin was dissolved in absolute ethanol (0.1 M) and diluted (30 μM) using Krebs buffer. For 15(S)-HETE, the supplied solution in 100% ethanol was evaporated under a constant stream of nitrogen and resuspended with 2% ethanol in Krebs buffer. ZD-6021 (5 nmol/μl icv) was dissolved in DMSO (41). Drugs administered intravenously or subcutaneously (1–50 mg/ml) were dissolved in saline, except SR-48968 and SR-140333, which were dissolved in DMSO (10 mg/ml) and diluted (1 mg/ml) in saline; ZD-6021 (5 mg/ml), which was dissolved in saline containing 15% Tween 20; and SB-223412, which was dissolved in DMSO (10 mg/ml) and diluted (1 mg/ml) with acid in saline.

RESULTS

Effect of laryngeal application of capsaicin on tracheal cholinergic tone. Baseline cholinergic tone in all experiments (excluding those in which a manipulation was performed to abolish tone, e.g., vagotomy) averaged 32 ± 1% of the maximum attainable contraction of the trachealis (n = 89). Brief (1–2 min) laryngeal challenge with 30 μM capsaicin produced a biphasic effect on baseline tracheal cholinergic tone. Tone initially fell on application of capsaicin to the laryngeal mucosa (55 ± 18% decrease in cholinergic tone), a response that was consistently followed by a pronounced and sustained increase in tone (61 ± 14% increase in baseline cholinergic tone; Fig. 2). This latter effect developed slowly (10–30 min) but showed no signs of reversal for the duration of all control experiments, lasting long after the brief capsaicin challenge (up to 60 min). The effects of laryngeal application of capsaicin on tracheal smooth muscle tone were not accompanied by any detectable increases in insufflation pressure or any consistent effects on arterial blood pressure.

We confirmed the reflex and parasympathetic nature of the response to capsaicin by reversing the contraction with topical application of atropine (Fig. 2) and by abolishing the contraction after intravenous pretreatment with the ganglionic blocker trimethaphan (Fig. 3). Complete laryngeal denervation by sectioning the superior and recurrent laryngeal nerves also abolished the reflex without altering baseline cholinergic tone (Fig. 3). Sectioning the superior laryngeal nerves alone did not affect the reflex. Further evidence for the reflexive nature of the response to laryngeal capsaicin was provided by vagus nerve transection. After bilateral vagotomy (which reversed completely baseline cholinergic tone), capsaicin applied to the larynx failed to alter tracheal tone, while, as expected, 3 μM capsaicin subsequently applied directly to the tracheal mucosa evoked atropine-insensitive (neurokinin-mediated; see below) contractions of the trachealis (18 ± 8% of the maximum attainable contraction, n = 3, P < 0.05 vs. baseline). All these observations confirm that the effects of capsaicin, when applied to the laryngeal mucosa, were not due to a direct action in the trachea after diffusion from the larynx but, rather, occurred...
secondary to a central nervous system (CNS)-dependent parasympathetic reflex.

Role of laryngeal C-fibers in contractions of the trachealis evoked by laryngeal application of capsaicin. The sustained increases in tracheal cholinergic tone evoked by capsaicin were also evoked by other stimuliants of airway C-fibers. Bradykinin (3 μM) applied to the laryngeal mucosa evoked a comparable increase in tracheal tension (52 ± 19% of the peak increase in tracheal cholinergic tone, n = 7). As previously reported (23) and similar to the reflexes initiated by laryngeal capsaicin, these effects of bradykinin developed slowly (20–30 min) after an initial transient fall in tone and were reversed entirely by atropine. The epithelial lipoxigenase product and putative VR1 agonist 15(S)-HETE (3 μM) also evoked gradually developing (20–30 min) but sustained and pronounced increases in tracheal cholinergic tone, which generally followed an initial relaxant response (Fig. 4). We confirmed the role of VR1 in this response to 15(S)-HETE by blocking these reflexes with the VR1 antagonist capsazepine (30 μM; Fig. 4; P < 0.01). Interestingly, capsazepine was utterly ineffective at reversing the 15(S)-HETE-induced cholinergic reflexes, which were nevertheless reversed entirely by atropine (n = 4; Fig. 4). The VR1 antagonist did, however, nearly abolish the atropine-insensitive contractions evoked subsequently when 10 μM capsaicin was administered intratracheally (51 ± 4 and 8 ± 5% of the maximum contraction in the absence and presence of 30 μM capsazepine, respectively, n = 3–4, P < 0.01).

The data presented above and elsewhere are consistent with the hypothesis that the laryngeal capsaicin-induced reflexes may be initiated by C-fibers. We pre-
by 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE]. Values (means ± SE) represent increase in tracheal cholinergic tone over 50 min evoked by 15(S)-HETE in vehicle (control) animals (●, n = 4) and animals in which 30 μM capsaïnine (CPZ) was added to the tracheal perfusate 15 min before laryngeal stimulation (●; n = 4). Pretreatment with capsaïnine (CPZ) prevented (P < 0.01) entirely the increase in tracheal cholinergic tone evoked by 15(S)-HETE but did not significantly alter baseline cholinergic tone in the absence and presence of neurokinin receptor antagonists. When applied selectively to the laryngeal and tracheal mucosa, NK1 (SR-140333), NK2 (SB-48968), and NK3 (SB-223412) receptor-selective antagonists (0.3 μM each) had no effect on reflex-mediated contractions of the trachealis evoked by laryngeal capsaïcin challenge (Fig. 5). The mucosally applied antagonists did, however, abolish the atropine-insensitive tracheal contractions evoked by tracheal administration of 10 μM capsaïcin (51 ± 4 and 2 ± 1% of the maximum contraction in the absence and presence of the antagonists, respectively, n = 3–4, P < 0.01). It thus seems unlikely that peripherally released neurokinins mediate the laryngeal capsaïcin-induced parasympathetic-cholinergic reflexes. The capsaïcin-induced reflexes were, however, significantly reduced after systemic pretreatment with SR-140333 and SB-223412 (1 mg/kg iv each, P < 0.05) or by the potent but nonselective neurokinin receptor antagonist ZD-6021 (5 mg/kg iv, P < 0.05). ZD-6021 (but not vehicle) administered intracerebroventricularly also reversed increases in tracheal tension evoked by laryngeal capsaïcin (Fig. 5).

As reported previously (7), intratracheally, intracerebroventricularly, or intravenously administered neurokinin receptor antagonists on reflex tracheal contractions evoked by capsaïcin. A: reflex tracheal responses evoked by laryngeal application of 30 μM capsaïcin (●; n = 9) were inhibited by intravenous (systemic) pretreatment with 5 mg/kg ZD-6021 (●; n = 5, P < 0.05). B: intracerebroventricular (icv; central) infusion of ZD-6021 dose-dependently reversed effects of laryngeal capsaïcin on tracheal cholinergic tone (vehicle (●; n = 2), 0.67 nmol/min (●; n = 1), 3.3 nmol/min (●; n = 1), and 6.7 nmol/min (●; n = 2)). C and D: effects of neurokinin antagonists on baseline cholinergic tone and peak increase in cholinergic tone evoked by laryngeal capsaïcin, respectively. SR-140333, SR-48968, and SB-223412 were applied topically, directly to the tracheal and laryngeal mucosa (0.3 μM each; n = 3). ZD-6021 was administered systemically (5 mg/kg iv; n = 5) or centrally (0.67–6.7 nmol/min icv; n = 4). Vehicle animals (n = 10) were pooled, since neither baseline cholinergic tone nor reflex tracheal contractions evoked by capsaïcin differed among vehicle treatment groups. Systemic injections of the NK1 and NK2 receptor-selective antagonists SR-140333 and SB-223412, respectively (1 mg/kg iv each), also significantly attenuated reflex tracheal contractions evoked by laryngeal capsaïcin administration. These antagonists abolished responses to laryngeal capsaïcin in 3 of 7 guinea pigs and substantially reduced responses in the remaining 4 animals: 67 ± 19% (n = 7) and 29 ± 10% (n = 7) mean percent increase in tracheal cholinergic tone in vehicle- and antagonist-pretreated animals, respectively (P < 0.05). *P < 0.05 vs. vehicle.
neurokinin receptor antagonists did not alter baseline cholinergic tone in the trachea in situ (Fig. 5).

**Synergistic interactions between airway afferent nerve subtypes.** In the airways, RARs are sporadically active throughout the respiratory cycle, responding to the dynamic mechanical effects of lung inflation and deflation (4, 37). We have presented evidence that baseline cholinergic tone is absolutely dependent on the ongoing activity of intrapulmonary airway mechanoreceptors (most likely RARs) (22). We hypothesized that stimulation of capsaicin-sensitive laryngeal afferent nerves evokes reflex tracheal contractions at least in part by acting in synergy with the cyclically active airway mechanoreceptors. We addressed this hypothesis by studying the effects of laryngeal capsaicin challenge after bilateral transection of the vagi but still no damage to the recurrent nerves, baseline tone was unaffected (26 ± 3% of the maximum contraction), whereas the reflex contraction to capsaicin was still significantly reduced (peak increases averaged 3 ± 1% of the maximum contraction, P < 0.01). This would suggest that tracheal tone itself does not affect the laryngeal C-fiber reflex. It is also unlikely that intrathoracic vagotomy reduces tracheal tension, such that the bioassay is no longer optimal for measuring tracheal smooth muscle contractions, since passive tracheal tension is optimal in this preparation between 500 mg and 3 g (30), yet tracheal tension after vagotomy is typically −1.5 g. In addition, tracheal contractions evoked by electrical stimulation of the recurrent laryngeal nerves are unaltered after selective denervation of the lungs (22).

The data above suggest that airway afferent nerve subtypes may act synergistically to induce reflex bronchospasm. A prediction of this hypothetical interaction is that coactivation of neurokinin-containing airway C-fibers and airway mechanoreceptors would initiate reflex bronchospasm greater than the sum of activating the two afferent nerve subtypes alone. We tested this hypothesis by analyzing the effect of threshold doses of the C-fiber stimulant bradykinin on reflexes evoked by the airway mechanoreceptor stimulant histamine (3, 7). Injections of histamine (1–10 μg/kg iv, diluted in saline) evoked dose-dependent increases in tracheal tension and pulmonary insufflation pressure. Similar to our previous study (7), the doses of histamine required to evoke responses equivalent to 50% of the maxima were 4 ± 1 and 6 ± 1 μg/kg for tracheal tension and pulmonary insufflaction pressure, respectively. Bradykinin (0.04–0.1 nmol/kg iv) coadministered with histamine potentiated the duration of histamine-evoked increases in tracheal cholinergic tone and pulmonary insufflation pressure by as much as 500% (Fig. 7). The effects of histamine were potentiated by bradykinin at all doses studied, producing a five- to sevenfold leftward shift in the histamine concentration-response curves (not shown). These effects of bradykinin on responsiveness to histamine were prevented by atropine (1 mg/kg iv; n = 5) or bilateral cervical vagotomy (n = 5).

A dose of SP (0.1 nmol/kg iv) that evoked reflexes comparable to those evoked by threshold doses of bradykinin failed to potentiate responses to 2 μg/kg histamine (17 ± 8 and 27 ± 14% increase in cholinergic tone after histamine with vehicle and histamine with SP, respectively, n = 3–7). This suggests that bradykinin-induced hyperresponsiveness to histamine is not due to neurokinin-dependent peripheral sensitization. Nevertheless, the effects of bradykinin on responsiveness to histamine are probably mediated by neurokinins. ZD-

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**Fig. 6.** Effect of selectively denervating intrapulmonary airways and lungs on reflex-mediated contractions of the trachea evoked by laryngeal capsaicin challenge. Denervation of the afferent innervation to the intrapulmonary airways, while leaving the preganglionic input to the trachea intact (intrathoracic vagotomy), almost abolished baseline cholinergic tone (A) and tracheal contractions evoked by laryngeal capsaicin application (B). *P < 0.01 vs. control. Values are means ± SE of 5–7 experiments.
6021 (5 mg/kg iv or 6.7 nmol/min icv) prevented the bradykinin-induced potentiation of the histamine response (Table 1), leaving the reflex effects initiated by histamine in the presence of bradykinin identical to those initiated in the absence of bradykinin. As reported above, ZD-6021 had no effect on baseline cholinergic tone in these experiments. It seems unlikely that ZD-6021 reversed the bradykinin-induced hyperresponsiveness to histamine by acting in the periphery, inasmuch as ZD-6021 administered intracerebroventricularly was as effective as intravenous ZD-6021 at reversing the hyperresponsiveness, yet intracerebroventricular ZD-6021 failed to block the effects of peripherally administered NKA (which were blocked by

Table 1. Effects of ZD-6021 on histamine-, bradykinin-, and NKA-induced increases in tracheal tone and pulmonary insufflation pressure

<table>
<thead>
<tr>
<th></th>
<th>Histamine</th>
<th>Histamine + Bradykinin</th>
<th>NKA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>PT</td>
<td></td>
</tr>
<tr>
<td>Vehicle control ZD-6021</td>
<td>17 ± 8</td>
<td>18 ± 10</td>
<td>84 ± 19*</td>
</tr>
<tr>
<td>5 mg/kg iv</td>
<td>10 ± 3</td>
<td>25 ± 10</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>6.7 nmol/min icv</td>
<td>19 ± 7</td>
<td>30 ± 8</td>
<td>21 ± 7*</td>
</tr>
</tbody>
</table>

Values for histamine and histamine + bradykinin are means ± SE expressed as percent increase in tracheal tension (TT, percentage of baseline cholinergic tone) or pulmonary insufflation pressure (PT) evoked by histamine (2 µg/kg iv) in the absence or presence of threshold doses of bradykinin (0.08 ± 0.01 nmol/kg iv; n = 5–7). Bradykinin evoked increases in cholinergic tone (7 ± 5% increase) and pulmonary inspiratory pressure (4 ± 2% increase) in control experiments (n = 7) but was without effect on cholinergic tone or pulmonary insufflation pressure after intravenous or intracerebroventricular (icv) pretreatment with ZD-6021 (n = 5 each). Vehicle for ZD-6021 (iv or icv) had no effect on bradykinin-induced potentiation of histamine responses (n = 3 each; data not shown). See text for details of experimental design. Values for neurokinin A (NKA) are means ± SE expressed as increase in tracheal tension (percentage of BaCl2 contraction) or pulmonary insufflation pressure evoked by NKA (0.5 nmol/ kg iv) in 3–5 experiments. Atropine (1 µM) was added to tracheal perfusate before NKA challenge. *Significantly less than histamine + bradykinin in animals pretreated with vehicle for ZD-6021, P < 0.01. † Significantly different from corresponding control responses (histamine alone or NKA in the absence of ZD-6021), P < 0.05.
intravenous ZD-6021; Table 1). Rather, the site of action of ZD-6021 and, thus, the mechanism by which bradykinin potentiated reflex responses to histamine are likely in the CNS (Table 1). Indeed, fourth ventricular injection of SP at 13 pmol/min (the trachea was first pretreated with NK1, NK2, and NK3 receptor antagonists) evoked marked and persistent increases in tracheal cholinergic tone to levels approximating the maximum attainable cholinergic tone [57 ± 3% of the maximum contraction (a 92 ± 27% increase), n = 4]. The kinetics of this effect of centrally administered SP were similar to those produced by laryngeal stimulation: increasingly slowly over 10–20 min and sustained once equilibrium had been reached. ZD-6021 (25 nmol icv) significantly reversed this effect of fourth ventricular SP (89 ± 6% reversal of the peak response, n = 3, P < 0.05).

Anatomic evidence for central convergence of afferent nerve subtypes. A necessary prerequisite for central synergistic interactions between airway afferent nerve subtypes is convergence of these neural inputs at an integrative site along the parasympathetic-cholinergic reflex arc, most likely in the nTS (20, 24, 34). We tested this hypothesis by simultaneous retrograde neuronal tracing of afferent nerves after microinjections of fluorescent tracers into the trachea and brain stem.

Consistent with previous studies, neuronal tracing from the airways with fast blue revealed the bilateral origin of three subpopulations of airway vagal afferent neurons (39). Airway afferent neurons with cell bodies in the nodose ganglia had a relatively large somal diameter and stained positively for neurofilament (a marker for myelinated axons) but lacked immunoreactivity to SP (Fig. 8, a, c, and d). In jugular ganglia, airway afferent neurons with large and small somal diameters were also labeled when fast blue was injected into the airways. Similar to the nodose neurons projecting to the airways, airway jugular ganglia neurons with large somal diameters stained positively for neurofilament. Only the labeled jugular neurons with small soma expressed SP (Fig. 8, e, g, and h).

Unilateral brain stem microinjections [0.5–1 mm caudal, 0.5 mm lateral (right) to obex, 0.8 mm deep] of rhodamine-coated microspheres were confined to the region of the commissural nTS (diffusion of the beads in the dorsoventral plane from the site of injection was <300 μm). The microspheres retrogradely labeled
perikarya in the right and left nodose and jugular ganglia, suggesting considerable contralateral projection of vagal afferent neurons in the commissural nTS but also revealing that this portion of the brain stem is a primary site of vagal afferent nerve termination. Among the three vagal afferent nerve perikarya subtypes in the nodose and jugular ganglia retrogradely labeled by fast blue instilled into the airways, ~20% of each subtype (16–24%) were also retrogradely labeled by the microspheres injected into the commissural nTS (Fig. 8, a, b, e, and f, Table 2). About 40% of the neurons labeled with the microspheres were labeled with fast blue. Injecting the microspheres dorsal to the commissural nTS, in the region of the gracile nucleus, failed to colabel any identified airway afferent nerves in the nodose or jugular ganglia.

**DISCUSSION**

Airway smooth muscle possesses a baseline level of cholinergic contraction that is dependent on the activity of airway parasympathetic nerves. This cholinergic tone in the airways is subject to reflex adjustments in response to multiple afferent nerve inputs (6, 7, 10, 31, 42). We previously showed that disrupting the afferent innervation of the guinea pig lungs (while leaving the parasympathetic innervation of the trachea intact) abolishes baseline cholinergic tone in the trachealis (22, 23). Similar observations have been made in cats (19). We confirmed this observation in the present study and propose that cholinergic nerve activity in the airways is absolutely dependent on ongoing input from afferent (presumably mechanically sensitive) nerve fibers innervating the intrapulmonary airways and lungs. The presence of this baseline cholinergic tone and its likely dependence on ongoing activity of airway mechanoreceptors have important implications in the study of reflex-mediated alterations in airway caliber.

We have presented compelling evidence that laryngeal C-fiber-mediated parasympathetic reflexes are absolutely dependent on the ongoing afferent input arising from the intrapulmonary airways and lungs. No other interpretation is compatible with the observation that these reflexes are completely abolished by selective denervation of the larynx or the intrapulmonary airways and lungs. The implication of this observation is of potentially fundamental importance to understanding mechanisms of C-fiber-mediated reflexes and perhaps mechanisms of airway responsiveness. In effect, the stimulus for the parasympathetic reflexes evoked by laryngeal C-fiber activation is not only the capsaicin, bradykinin, or 15(S)-HETE applied to the mucosa, the stimulus is also the dynamic mechanical force delivered to the lung (and thus the pulmonary mechanoreceptors) during the respiratory cycle.

The laryngeal capsaicin-sensitive nerve-mediated parasympathetic reflexes described in the present study share many physiological attributes with inflammation-induced allodynia and hyperalgesia described in the somatic nervous system. Typically innocuous stimuli such as joint flexions or tidal lung stretches, which normally fail to evoke noxious sensation or defensive reflexes, produce pain in somatic tissues or reflex bronchospasm when initiated subsequent to or coincident with nociceptor stimulation (45). In animals, allodynia and hyperalgesia are initiated by neurokinins released in the CNS that sensitize central integrative neurons receiving convergent input from nociceptors and mechanoreceptors (17, 29). Central sensitization therefore reduces the threshold for reflex activation by subsequent mechanoreceptor input (36). A comparable role for neurokinins in mediating the reflexes initiated from the larynx in the present study is also apparent. We speculate, therefore, that laryngeal C-fibers evoke reflex bronchospasm by facilitating ongoing synaptic transmission through relay neurons of airway mechanoreceptors in the CNS, perhaps in the nTS.

The necessity of the laryngeal and the intrapulmonary afferent nerve inputs for mediating reflexes evoked from the larynx described here all but requires convergence of these separate reflex pathways at some level in the brain stem. Ultimately, this convergent input must lead to heightened activity in the preganglionic parasympathetic nerves regulating airway cholinergic tone. Although this convergence can occur at any number of brain stem locations along the reflex arc, we provide anatomic evidence that the interaction between airway nociceptors (jugular ganglia) and the mechanoreceptors (nodose ganglia) is likely to occur in the commissural nTS. Previous studies are consistent with this scenario (20, 24, 34).

The synergistic interactions between nociceptors and pulmonary mechanoreceptors might not be unique to laryngeal nociceptor-dependent reflexes. The potentiating effects of bradykinin on histamine-induced reflex bronchospasm suggest that pulmonary mechanoreceptors and nociceptors may also act synergistically to evoke reflex bronchospasm. In addition to the precedent set in our studies of the laryngeal reflexes as well as the anatomic evidence for afferent nerve conver-

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**Table 2. Dual retrograde labeling of afferent neuronal cell bodies in nodose and jugular ganglia of vagus nerves from airways (fast blue) and commissural nucleus of the solitary tract (rhodamine-coated microspheres)**

<table>
<thead>
<tr>
<th>%Ganglion Neurons Labeled</th>
<th>% FB Labeled Neurons Also Labeled by Rhodamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhodamine</td>
</tr>
<tr>
<td><strong>Nodose ganglia</strong></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Left</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td><strong>Jugular ganglia</strong></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>5.1 ± 1.1</td>
</tr>
<tr>
<td>Left</td>
<td>3.4 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 4 separate experiments expressed as percentage of total number of neuronal cell bodies visualized or as percentage of the number of fast blue (FB)-labeled (airway-specific) cell bodies. Rhodamine-coated microspheres were injected into the commissural nucleus of the solitary tract (0.5-1 mm caudal, 0.5 mm lateral (right) to obex, 0.8 mm deep). Diffusion of microspheres in the dorsoventral plane was minimal (<300 μm).

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gence, several additional lines of evidence are consistent with this notion. Thus bradykinin-induced hyperresponsiveness to histamine is reversed entirely by centrally acting neurokinin receptor antagonists and mimicked by centrally, but not peripherally, administered SP. The ability of intracerebroventricular ZD-6021 to prevent the bradykinin-induced hyperresponsiveness while having no effect on the histamine-induced reflexes seems to rule out the possibility that bradykinin sensitizes airway afferent nerve endings to histamine. Of course, the inclusion of pyrilamine and FR-173657 in the tracheal perfusate rules out entirely any modulatory effects of bradykinin or histamine on histamine. Of course, the inclusion of pyrilamine and ZD-6021 mimicked by centrally, but not peripherally, administered SP. The laryngeal mucosal afferent nerves mediated by centrally acting neurokinin receptor antagonists prevented or reversed the reflexes evoked by laryngeal stimulation with capsaicin, while the VR1 antagonist capsazepine prevented reflexes initiated by 15(S)-HETE. The laryngeal mucosal afferent nerves mediating the capsaicin-, bradykinin-, and 15(S)-HETE-induced reflexes are thus likely to be neurokinin-containing C-fibers carried primarily by the superior laryngeal nerves (8). This is in agreement with our previous studies showing that pulmonary C-fiber-evoked, but not mechanoreceptor-evoked, reflex bronchospasm is abolished by centrally administered neurokinin receptor antagonists (7).

Although we provide compelling evidence that the sustained increase in tracheal tone after laryngeal capsaicin application is mediated by C-fiber-evoked increases in cholinergic nerve activity, the nature of the initial relaxant response is less clear. The relaxant response may be a result of a coincidental noncholinergic relaxant reflex evoked by capsaicin. In support of this, we previously showed that laryngeal application of capsaicin evokes vagally mediated relaxations with kinetics comparable to those of the initial relaxant response observed in the present study (31). Alternatively, laryngeal capsaicin application in spontaneously breathing guinea pigs evokes an apnea followed by a period of rapid shallow breathing, suggesting that the relaxation observed in the trachea may also reflect a removal of cholinergic drive to the airways correlating with the apneic episode. Regardless of the underlying mechanism, the relaxant response was also observed after bradykinin and 15(S)-HETE application and was reduced by intravenous pretreatment with neurokinin receptor antagonists, suggesting that it is a common feature of laryngeal C-fiber activation.

The pulmonary afferent nerves regulating baseline cholinergic tone and the pulmonary afferent nerves also necessary for coregulating the laryngeal capsaicin-induced reflex are unknown. We speculate, however, that these intrapulmonary afferent nerves are one and the same and are probably RARs. RARs, much like baseline tone, are highly sensitive to changes in dynamic lung compliance and the rate and volume of lung inflation (4, 37). By contrast, sustained lung inflation or positive end-expiratory pressure, stimuli that activate slowly adapting receptors, inhibit reflex tracheal contractions evoked by histamine and reduce baseline cholinergic tone in the airways (6, 40). It is unlikely, therefore, that slowly adapting receptors are responsible for mediating baseline tone or the capsaicin reflex. Bronchopulmonary C-fibers are also unlikely to be the afferent nerves responsible for driving baseline tone or the laryngeal reflex. C-fiber-mediated airway reflexes are abolished by neurokinin receptor antagonists (1, 7), yet these antagonists have no effect on baseline cholinergic tone (7, 22; present study). C-fibers are also generally quiescent throughout the respiratory cycle.

Role of neurokinins in mediating reflex bronchospasm. It is interesting that centrally acting neurokinin receptor antagonists not only prevent the airway parasympathetic reflex evoked by laryngeal C-fiber stimulation, they also reverse the response when administered long after the challenge is terminated. The long-lasting effects of laryngeal C-fiber activation may thus not be due to an irreversible (agonist-independent) enhancement (or plasticity) of synaptic transmission in the brain stem but may be due to the persistent effects of the neurotransmitters. The duration of action of the neurokinins released in the brain stem may be exceedingly high. Hyperalgesia can be sustained by continuous activation of NK2 receptors, perhaps NK3 receptors localized to extrasynaptic sites (2, 17). Alternatively, once activated by capsaicin, bradykinin, or 15(S)-HETE, laryngeal C-fibers may remain active long after the brief (1–2 min) challenges. We observed, however, that although capsaizepine prevented the reflexes initiated by 15(S)-HETE, the VR1 antagonist failed to reverse the effects. In vitro studies of capsaicin- and bradykinin-induced activation of tracheal and laryngeal C-fibers are also inconsistent with a prolonged action of these afferent nerves (14, 21). These observations seem consistent with the notion of a long duration of action of the neurokinins in the brain stem once released, but alternative hypotheses are equally likely and await systematic evaluation (15).
the neurokinins in the present study. Rather, the data indicate that neurokinins are acting in the brain. The specific neurokinin receptors mediating these responses are unknown. Previous studies have shown that all three neurokinin receptors are present and functional in regions of the nTS likely containing vagal afferent nerve terminals (32, 33, 35). We observed that systemic pretreatment with a combination of NK1 and NK3 receptor antagonists was equally effective at reducing the laryngeal capsaicin-induced reflex and blockade of all neurokinin receptor subtypes. We thus speculate that NK1 and NK3 receptors in the brain stem mediate the C-fiber-induced parasympathetic reflexes described in the present study and elsewhere (7).

Physiological implications. The data presented in this study have important implications for the mechanisms of airway defensive reflexes. The ongoing activity of airway mechanoreceptors should always be considered a major factor influencing any reflexes initiated by C-fiber activation. The data may also have important implications for mechanisms of pulmonary disease. For example, diseases such as allergic rhinitis, gastroesophageal reflux disease, and upper respiratory tract infection can precipitate airway hyperresponsiveness and cough through vagal mechanisms (6, 12, 13, 16, 44). These inflammatory diseases may not directly affect the lower airways, indicating that they initiate pulmonary symptoms via extrapulmonary effects. Synergistic interactions between extrapulmonary afferent nerves and the spontaneously active airway mechanoreceptors might contribute to the pulmonary consequences of these extrapulmonary disorders. Finally, the results presented here have potentially important implications for therapeutic interventions targeted at visceral hyperreflexia, such as asthma and chronic obstructive pulmonary disease and perhaps gastroesophageal reflux disease and rhinitis. Drugs that selectively target the afferent pathways, by blocking the actions of their neurotransmitters in the CNS or by selectively preventing their activation peripherally, might prove superior to anticholinergics in the treatment of reactive airway diseases.

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