Endogenous neurokinins facilitate synaptic transmission in guinea pig airway parasympathetic ganglia

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The hypothesis that NK3 receptor activation enhances the ability of the parasympathetic nervous system to produce bronchospasm.

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ELECTROPHYSIOLOGICAL STUDIES of synaptic neurotransmission in airway parasympathetic ganglia reveal that the majority of nicotinic fast excitatory postsynaptic potentials (fEPSPs) evoked by activation of preganglionic parasympathetic nerves are subthreshold for action potential formation (5, 24, 33, 34, 41). Summation of as many as four fEPSPs may be required to produce membrane depolarizations sufficient to reach action potential threshold (33, 34). These data, along with other indirect measures of synaptic transmission in the airways (3, 51), highlight the important integrative role played by airway parasympathetic ganglia neurons.

The neurokinins substance P and neurokinin A are found in nerve fibers innervating autonomic ganglia throughout the viscer a of all mammalian species. These nerve terminals originate from a variety of neuronal subtypes, including preganglionic autonomic neurons (35, 45), intrinsic neurons (15, 16, 20, 48), and collaterals of visceral afferent nerves (27, 28, 49, 50). Activation of these nerves or exogenous application of tachykinins produces membrane depolarization and/or action potentials in many autonomic neurons, primarily through the activation of neurokinin type 1 (NK1) and/or type 3 (NK3) receptors (22, 23, 31, 39, 40, 50).

Although prejunctional effects of neurokinin receptor activation on airway cholinergic nerves have been studied (2, 38, 52), the effects of neurokinins on synaptic transmission in airway parasympathetic ganglia are unknown. Tachykinin-containing nerve fibers are, however, found in airway ganglia of several species, including humans (15, 16, 21, 30, 40, 47). In the guinea pig, these nerve fibers are probably collaterals of capsaicin-sensitive afferent nerves (28, 39). Our previous studies indicate that activation of these tachykinin-containing nerve endings evokes NK3 receptor-mediated depolarization of the airway ganglia neurons and a marked decrease in membrane resistance (39, 40). It is thus unclear what, if any, effect neurokinin receptor activation will have on synaptic neurotransmission in airway ganglia. Accordingly, in the present study, in vivo and in vitro techniques were utilized to address the hypothesis that NK3 receptor activation enhances synaptic efficacy in airway cholinergic ganglia, thereby enhancing the ability of the parasympathetic nervous system to produce bronchospasm.

METHODS

Vagally Mediated Contractions of the Isolated Guinea Pig Bronchus

Tissue preparation. All experiments were approved by The Johns Hopkins Medical Institutions Animal Care and Use Committee. Guinea pigs (male, 200–400 g, Hartley strain;
Hilltop, Scottsdale, PA) were asphyxiated in a vessel filled with 100% CO₂ and exsanguinated. The extrapulmonary airways with extrinsic innervation intact were removed in toto and placed in oxygenated (95% O₂-5% CO₂) Krebs bicarbonate buffer of the following composition (mM): 118 NaCl, 5.4 KCl, 1 NaH₂PO₄, 1.2 MgSO₄, 1.9 CaCl₂, 25 NaHCO₃, and 11.1 dextrose. The right and left bronchi, the intrathoracic trachea, and the right vagus nerve were dissected free of extraneous tissues and placed in a water-jacketed dissecting dish with a Sylgard-coated bottom. The dissecting dish was continuously perfused (20 ml/min) with warmed (37°C), oxygenated Krebs buffer. Tissues were pinned to the bottom of the dish, and the right bronchus was prepared for isometric tension measurements as described elsewhere (51). Baseline isometric tension was set (1 g) and continually readjusted during the 90-min equilibration period. After equilibration, the viability of the preparation and the reproducibility of the vagally mediated cholinergic contractions were assessed by three consecutive vagal stimulations using a suction electrode (World Precision Instruments, Sarasota, FL). Stimuli were delivered at a 0.1 M frequency producing contractions that were 20 and 50% of the maximum contraction evoked by vagus nerve stimulation were 50% of the maximum contraction of the airway smooth muscle. This contractile response was used as a means of assessing the relative contractility of each preparation within the various treatment groups and was used as a means of assessing nonspecific effects of the various treatments.

**Electrophysiological Studies of Synaptic Transmission in Airway Ganglia**

The right main stem bronchi of guinea pigs with the right vagus and peribronchial nerves attached were isolated, cut longitudinally along the ventral surface, and opened as a sheet. Bronchi were tightly pinned to the floor of a Sylgard-coated recording chamber (0.2 ml volume) with Z-shaped pins and were subsequently equilibrated in flowing (5–8 ml/min) Krebs bicarbonate buffer containing 0.1 µM atropine at 36–37°C for 1 h. Ganglia located near peribronchial nerves were visualized without staining and exposed by fine dissection as previously described (37, 39, 41). Micropipettes for intracellular recording were fabricated from thick-walled capillary stock (0.5-mm ID, 1.0-mm OD; World Precision Instruments) by a Brown-Flaming microelectrode puller (model P-87, Sutter Instrument, San Rafael, CA). Intracellular recordings were performed with the electrometer in a continuous current-clamp (3.0- to 4.0-kHz sampling rate) or active-bridge mode. A Macintosh computer equipped with a data translation interface digitized and stored data epochs (Axodata, Axon Instruments, Foster City, CA). Intracellular data (voltage and current) were displayed on-line with a dual-beam storage oscilloscope and a chart recorder and stored on digital audio tape.

Intracellular recordings of vagus nerve-stimulated fEPSPs were analyzed for afferent nerve- and/or drug-induced changes in mean amplitude. Vagus nerve-mediated fEPSPs were evoked by 1-Hz square pulses, ranging from 5 to 40 V and from 0.02- to 0.1-s duration (pulse duration and voltage adjusted to obtain subthreshold fEPSPs), delivered to the caudal cut end of the vagus nerve 10–30 mm from the ganglion. Vagus nerve-evoked slow excitatory postsynaptic potentials (sEPSPs) were induced with a 30-Hz train (5 s) of 5- to 40-V, 1.2-ms-duration square pulses. To study the effects of neurokinin receptor stimulation on fEPSPs, 50 consecutive vagus nerve-evoked fEPSPs were averaged for a control value. At 2 min after drug application (1 µM capsaicin or 0.1 µM senktide analog diverted from an in-line reservoir) or antidromic afferent nerve stimulation, the resting potential was current clamped to the predrug or prestimulus potential, and the amplitudes of 50 additional consecutive
fEPSPs after drug application/nerve stimulation were averaged. In time-control studies, fEPSP amplitudes did not vary significantly over the periods studied at this stimulation frequency. Antagonists were bath applied for \( \geq 10 \) min before agonist or capsaicin treatment. The effects of antidromic afferent nerve stimulation, capsaicin, or senktide analog were compared before and after pretreatment with the antagonists.

**Effects of Senktide Analog on Airway Smooth Muscle Tone In Vivo and In Situ**

Baseline cholinergic tone in the trachea was measured in situ using previously described methods (9, 25). Guinea pigs (300–400 g, male) were anesthetized with urethane (1 g/kg ip) and positioned ventral side up on a heated pad. This dose of anesthetic was sufficient to induce effective surgical anesthesia (which was repeatedly assessed throughout the course of these experiments by monitoring cardiovascular responses to a sharp pinch of the hindlimb) for the duration of the experimental period (typically 3–4 h). The caudalmost portion of the extrathoracic trachea was cannulated, and the animals were mechanically ventilated (60 breaths/min, 6 ml/kg, 2–3 cmH\(_2\)O positive end-expiratory pressure to preserve airway patency). Ventilation was initiated after induction of paralysis with an injection of succinylcholine chloride (2 mg/kg sc). (Paralysis was necessary to eliminate skeletal movements, which diminish our ability to measure tracheal smooth muscle tension.) Tracheal smooth muscle tension was measured in situ by placing stainless steel hooks between two cartilage rings (rings 6 and 7 caudal to the larynx) on either side of the trachea, rostral to the tracheal cannula. One hook was sutured to a fixed bar, and the other hook was sutured to an isometric force transducer (model FT03C, Grass Instruments, Quincy, MA). Optimal baseline tension was set (1.5–2 g) and maintained throughout the equilibration period. The lumen of the tracheal segment studied was perfused with warmed (37°C), oxygenated Krebs buffer (containing 3 mM indomethacin, 2 mM propranolol, and 1 mM phenolamine) delivered through a small slit made in the ventral trachea, caudal to the hooks. By perfusion of the lumen of the extrathoracic tracheal segment in a caudal-to-rostral direction, the buffer was removed from the rostralmost end of the trachea with gentle suction. Throughout all these manipulations, great care was taken to preserve the blood flow and extrinsic innervation of the trachea. After 20–30 min of equilibration, senktide analog (1 μM) was added to the tracheal perfusate, and the effects of the NK\(_3\) receptor agonist on baseline cholinergic tone were monitored. In some experiments, bilateral vagotomies were performed before administration of senktide analog, or, alternatively, SB-223412 (0.1 or 1 μM) was added to the perfusate \( \geq 20 \) min before addition of the NK\(_3\)-selective agonist. The effects of senktide analog were expressed as a percent increase in baseline cholinergic tone. Cholinergic tone was quantified by dividing the absolute amount (in mg) of isotropic tracheal tension lost on 1 μM atropine administration by the absolute amount of tension regained by addition of BaCl\(_2\) to the perfusate. In experiments where senktide analog produced an increase in tension, this increase was subtracted from the total relaxation produced by atropine to obtain baseline cholinergic tone.

In a separate set of experiments, the effects of senktide analog (10–100 nmol/kg iv) on pulmonary insufflation pressure were studied. Senktide analog was administered intravenously through a cannula placed in the abdominal segment of the vena cava. The effects of vagotomy, atropine (1 mg/kg iv), or SB-223412 (3 mg/kg iv) on the response to senktide analog were assessed in a nonpaired design. In these experiments, propranolol (1 mg/kg iv) was administered to counteract the effects of catecholamines on airway reactivity.

In all experiments described in this study, the abdominal aorta was cannulated (just rostral to the origin of the femoral arteries) to monitor blood pressure. Blood pressure, pulmonary insufflation pressure, and tracheal smooth muscle tension were recorded on a polygraph (model 79E, Grass Instruments). At the conclusion of each experiment, animals were killed by inhalation of 100% CO\(_2\), delivered through the inspiratory port of the ventilator.

**Immunofluorescent Staining for Substance P in Airway Ganglia**

Bronchi were incubated in oxygenated Krebs solution (37°C) with vehicle or with capsaicin (10 μM, 60 min) in an attempt to acutely deplete afferent nerve endings of neuropeptides (40, 51). The tissue was then fixed with 4% formaldehyde in PBS and rinsed with PBS. Tissues were cryoprotected overnight in 18% sucrose in PBS, covered with mounting medium, and frozen in liquid nitrogen. Sections (12 μm) of each tissue were cut on a cryostat, collected on silanecoated slides, and air dried. Sections were incubated with blocking solution containing 1% bovine serum albumin, 10% normal goat serum, and 0.1% Tween 20 in PBS for 1 h at room temperature and then incubated overnight (4°C) in a mixture of mouse monoclonal antiserum to the general neuronal marker protein gene product 9.5 (PGP 9.5, UltraClone, Cambridge, UK; diluted 1:400) to facilitate identification of bronchial ganglia neurons and a rabbit polyclonal antibody to substance P (Peninsula Laboratories, Belmont, CA; diluted 1:200). Slides were washed in PBS and incubated with a mixture of rhodamine-labeled anti-mouse antibody raised in goat (Alexa 594, Molecular Probes, Eugene, OR) and a fluorescein-labeled anti-rabbit antibody raised in goat (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Separate sections were processed similarly, but the primary antibody was excluded to evaluate nonspecific staining. Antifade glycerol (Molecular Probes) was used to apply coverslips to washed slides. The slides were evaluated and photographed with an epifluorescence microscope (Olympus BX60, Olympus America, Melville, NY) equipped with appropriate filter sets to allow separate visualization of rhodamine or fluorescein. Ganglia were photographed with Kodak TMY400 film, and the negatives were scanned and digitized (SprintScan 35, Polaroid, Cambridge, MA).

**Data Presentation and Statistical Analysis**

Unless otherwise stated, values are means ± SE of \( n \) experiments, where \( n \) is the number of preparations obtained from different animals. Depending on the experiment, the effects of the NK\(_3\) receptor antagonists on vagally mediated contractions and the effects of capsaicin-sensitive nerve stimulation on the magnitude of the fEPSPs in the airway ganglia neurons were assessed using paired or unpaired Student’s \( t \)-tests. The effects of senktide analog on baseline cholinergic tone in the guinea pig trachea in situ were assessed by one-way ANOVA. The effects of senktide analog on pulmonary insufflation pressure were also assessed by ANOVA. When statistically significant differences were detected by ANOVA, group means were compared using Scheffe’s \( F \) test for unplanned comparisons. \( P < 0.05 \) was considered significant.
Drugs and Reagents

Atropine, capsaicin, 4-acetyl-1,1-dimethylpiperazinium (DMPP), histamine, indomethacin, dl-propranolol, phen tolamine, and tetrodotoxin were purchased from Sigma (St. Louis, MO). Trimethaphan was obtained from Hoffmann-La Roche (Nutley, NJ). CP-99994 was a generous gift from Merck Frosst (Quebec, Canada). SB-223412, SR-142801, and SR-142806 were gifts from SmithKline Beecham (King of Prussia, PA). SR-48968 was a gift from Zeneca (Wilmington, DE). Senkide analog ([Asp<sub>6</sub>,Asp<sub>7</sub>,MePh<sub>e</sub>]<sub>6</sub>-SP(6–11)) was obtained from Peninsula Laboratories (Belmont, CA). Atropine (10 mM), DMPP (10 mM), hexamethonium (1 M), propranolol (10 mM), senkide analog (1 mM), and tetrodotoxin (1 mM) were dissolved in water. Trimethaphan (84 mM) was dissolved in 0.013% sodium acetate. CP-99994, SB-223412, SR-142801, SR-142806, and SR-48968 (1 mM each) were dissolved in DMSO. Indomethacin (30 mM) and capsaicin (10 mM) were dissolved in ethanol. For intravenous administration, histamine (1 mg/ml), propranolol (5 mg/ml), and senktide analog (100 nM–1 μM) were dissolved in isotonic saline, and SB-223412 (0.1 mM) was diluted in 20% DMSO and 0.05 N HCl in isotonic saline.

RESULTS

Immunohistochemical Studies of Substance P in Airway Ganglia

Parasympathetic ganglia containing 3–11 neurons (3.5 ± 0.2 neurons/ganglia, 196 ganglia from 17 guinea pigs) were readily localized to the serosal surface of guinea pig main stem bronchi and trachea after they were labeled with antisera to PGP 9.5. Substance P-immunoreactive nerve fibers could be localized to these bronchial ganglia, with virtually every ganglion neuronal cell body (>90%) adjacent to (≤20 μm) a substance P-containing axon. Consistent with previous studies (28, 40), the perikarya of the ganglia neurons did not contain substance P, providing further evidence for an extrinsic origin of the neuropeptide-containing nerves. Indeed, when the tissues were pretreated in vitro with 10 μM capsaicin, the number of substance P-immunoreactive fibers was greatly reduced or absent (Fig. 1).

Vaguely Mediated Cholinergic Contractions of the Right Bronchus

In the presence of NK<sub>1</sub> and NK<sub>2</sub> receptor antagonists, electrical stimulation (1-ms pulse duration, 24 Hz, 10-s train, 1–150 V) of the right vagus nerve evoked cholinergic contractions of the guinea pig right bronchus. These vagally mediated contractions became larger as the stimulation voltage increased from 2 to 25 V, reaching an asymptote in magnitude at 25–50 V. These contractions were substantially inhibited or abolished by the muscarinic antagonist atropine or the nicotinic antagonist trimethaphan, indicating that they were necessarily dependent on nicotinic synaptic neurotransmission through airway parasympathetic-cholinergic ganglia (Fig. 2).

The NK<sub>3</sub> receptor-selective antagonists SR-142801 (0.1 μM) and SB-223412 (0.1 μM) were without effect on vagally mediated contractions of isolated right bronchi produced at supramaximal stimulus intensities (150 V, 1 ms) over an entire range of stimulation frequencies. Similarly, the NK<sub>3</sub> antagonists were without effect on EFS-induced contractions of isolated tracheal strips. By contrast, SR-142801 and SB-223412 produced rightward shifts in the voltage-response curves produced using the right bronchus-right vagus nerve preparations. Thus, whereas 14 of 19 (74%) control preparations contracted when the vagi were stimulated at 5 V, only 7 of 19 (37%) preparations pretreated with SR-142801 or SB-223412 contracted when the vagi were stimulated at 5 V. SR-142806 (0.1 μM), the less-active enantiomer of SR-142801, was without effect on the vagally mediated contractions (Fig. 2, Table 1).

The contractions evoked by threshold vagal stimulation were also much smaller in preparations pretreated with the NK<sub>3</sub> receptor antagonists. At 5-V stimulation voltage, cholinergic contractions averaged 26 ± 6, 10 ± 5, and 4 ± 3% of the maximum response in tissues pretreated with vehicle, SR-142801, or SB-223412, respectively (n = 9–19, P < 0.05). In control preparations and preparations pretreated with SR-142806, 5-V nerve stimulation evoked contractions ≥50% of the maximum response frequency (6 of 23 preparations), while responses ≥20% of maximum in these two groups were typical (14 of 23 preparations). In preparations pretreated with SR-142801 or SB-223412, 12 of 19 preparations failed to contract with 5-V stimulation, while only 3 of 19 preparations pretreated with the antagonists contracted >20% (35, 31, and 29%) of the maximum with 5-V stimulation. As stimulation volt-
Fig. 2. Effects of neurokinin type 3 (NK₃) receptor-selective antagonists on vagally mediated cholinergic contractions of isolated guinea pig airways. A: representative traces of effects of vehicle or NK₃ receptor antagonist SB-223412 on vagally mediated contractions of guinea pig bronchus in vitro. Vagis were stimulated at 24 Hz for 1 ms with 10-s trains at 3- to 5-min intervals and at various voltages until maximal contractions were evoked. B: effects of ganglionic blocker trimethaphan, muscarinic antagonist atropine, or SB-223412 on vagally mediated contractions evoked at optimal stimulus intensities. C and D: voltage-response curves generated noncumulatively in preparations pretreated with vehicle (n = 8–10, control), SB-223412 (n = 10, C), or SR-142801 (n = 9, D). SB-223412 and SR-142801 produced rightward shifts in voltage-response curves without affecting maximal responses or frequency-response curves generated using optimal stimulus intensities (150 V, 1 ms; see Table 1). *Statistically significant difference from control (P < 0.05).

Vagally Mediated fEPSPs Recorded in Bronchial Ganglia Neurons

Intracellular recordings were made from the somal membrane of principal neurons located in bronchial parasympathetic ganglia. Consistent with previous studies of guinea pig and human bronchial ganglia neurons (24, 37, 39–41), membrane potential and resistance averaged 52 ± 4 mV and 46 ± 9 MΩ, respectively, and did not change considerably during the course of the experiments (n = 16). Vagus nerve-stimulated fEPSPs evoked at threshold stimulation intensities (4–40 V, 0.02- to 0.08-ms pulse duration) were highly reproducible when evoked at 1-s intervals (1 Hz for 1–2 min). The magnitude of the fEPSPs was ad-

Table 1. Effects of NK₃ receptor antagonists on cholinergic nerve-mediated contractions of guinea pig airway smooth muscle in vitro evoked by vagus nerve stimulation or EFS

<table>
<thead>
<tr>
<th>Vagus Nerve Stimulation</th>
<th>EFS</th>
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<tr>
<td>n</td>
<td>V₀, V</td>
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<tr>
<td>Vehicle control</td>
<td>5–9</td>
</tr>
<tr>
<td>SR-142801 (0.1 μM)</td>
<td>5–10</td>
</tr>
<tr>
<td>SR-142806 (0.1 μM)</td>
<td>3–4</td>
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<tr>
<td>Vehicle control</td>
<td>5–10</td>
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<tr>
<td>SB-223412 (0.1 μM)</td>
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Values are means ± SE. V₀ and V₀, estimated vagal stimulation voltages that will evoke 20 and 50% of maximum attainable nerve-mediated contraction, respectively; EF₀ is estimated nerve stimulation frequency (Hz) that will induce 50% of maximum contraction evoked by vagus nerve or electrical field stimulation (EFS). Neurokinin type 3 (NK₃) receptor antagonists SR-142801 and SB-223412 inhibited vagally mediated contractions evoked at threshold stimulation voltages (2–10 V), thereby increasing V₀, while having little, if any, effect on maximum responses or EF₀ (n = 9–10). SR-142806, the less-active isomer of SR-142801, was without effect on vagally mediated cholinergic contractions of isolated bronchi. Atropine (1 μM) or the neurotoxin TTX (2 μM) completely abolished contractions evoked by EFS at all stimulation frequencies studied (n = 5). By contrast, the NK₃ antagonists SR-142801 and SB-223412 were without effect on EFS-induced cholinergic contractions of guinea pig tracheal strips (n = 4–5). The NK₃ receptor agonist senktide analog (0.3 μM) was also without effect on EF₀ (14 ± 2 Hz) or maximum contractions (35 ± 2%) evoked by EFS (n = 5, P > 0.1). All preparations were pretreated with 3 μM indomethacin, 1 μM propranolol, 1 μM phentolamine, 0.1 μM SR-48968, and 0.1 μM CP-99994. Maximum (Max) contractions of airway preparations were induced with 300 mM BaCl₂. Neurokinin antagonists were without effect on contractions evoked by BaCl₂ (not shown; P > 0.1). *Statistically significant difference relative to matched vehicle controls (P < 0.05). NA, not assessed.

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justed (by altering stimulation intensity) to be subthreshold for action potential formation, averaging 6.3 ± 0.3 mV (n = 16). Subsequent tetanic stimulation of the vagus nerves at supramaximal stimulus intensities (30 Hz, 5 s, 40 V, 1.2-ms pulse duration) evoked sEPSPs. The fEPSPs evoked subsequent to sEPSP generation were potentiated in all preparations studied, and in two of six preparations the fEPSPs reached threshold for action potential generation. The NK3 receptor antagonist SB-223412 (1 μM) prevented the vagally mediated sEPSPs and the sEPSP-dependent potentiation of the fEPSPs (Fig. 3).

In subsequent experiments, vagus nerve-stimulated fEPSPs were evoked before and after capsaicin (1 μM) challenge. Consistent with previous studies, capsaicin evoked a long-lasting depolarization of the ganglia neurons, a depolarization that was accompanied by a marked decrease in membrane resistance. Perhaps because of the marked decrease in membrane resistance, capsaicin alone did not initiate action potential formation in the ganglia neurons, despite the marked membrane depolarization; at such depolarized potentials, sodium channels may also be inactivated, thus inhibiting action potential generation. As with electrically induced sEPSPs, however, capsaicin challenge did potentiate subsequently evoked fEPSPs in all preparations studied, and, again, in two of six preparations, the fEPSPs reached action potential threshold after capsaicin challenge. The amplitude of the capsaicin-induced depolarization was reduced and fEPSP amplitude was not potentiated in the presence of the NK3 receptor antagonist SB-223412 (1 μM; Fig. 4).

The potentiation of fEPSPs evoked by tetanic vagus nerve stimulation or capsaicin could be mimicked with the NK3 receptor-selective agonist senktide analog (0.1 μM). This effect of senktide analog on the fEPSPs, as well as the depolarization evoked by the agonist, could be inhibited by NK3-selective antagonists SR-142801 (1 μM) or SB-223412 (1 μM; Fig. 4). By contrast, senktide analog was without effect on depolarization of the ganglia neurons evoked by the nicotinic agonist DMPP (1–10 μM), which depolarized airway cholinergic ganglia neurons 7.5 ± 1 and 6.5 ± 1 mV in the absence and presence of 0.1 μM senktide analog, respectively (n = 4).

Effects of Senktide Analog on Baseline Cholinergic Tone Measured In Situ

Consistent with previous studies (9, 25), the parasympathetic-cholinergic nerves innervating the guinea pig trachea were spontaneously active during mechanical ventilation, producing contractions of the trachealis in situ that averaged 32 ± 4% of the maximum obtainable contraction (n = 12). Also consistent with previous observations, vagotomy essentially abolished these contractions (0 ± 0%, n = 5), providing further evidence that baseline cholinergic tone in the airways in vivo is dependent on ongoing preganglionic vagal input from the brain stem.

Adding senktide analog to the tracheal perfusate produced slowly developing contractions of the trachealis in situ, contractions that increased baseline cholinergic tone of the trachealis by >25% in five of six preparations studied and by an average of 34 ± 8% overall (range 0–56% increase). These contractions were completely reversed by atropine and were prevented entirely by vagotomy (Fig. 5).

Pretreating the tracheal segment with SB-223412 was without effect on baseline cholinergic tone in the trachealis. The effects of subsequent administration of senktide analog were, however, attenuated by 0.1 μM

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**Fig. 3.** Effects of antidromic electrical stimulation of neurokinin-containing vagal afferent nerves on bronchial ganglia neurons. A and B: subthreshold fast excitatory postsynaptic potentials (fEPSPs) were reproducibly evoked at 1-Hz stimulation frequency (4–40 V, 0.02- to 0.8-ms pulse duration). Vagus nerve was then stimulated at 30 Hz for 5 s at a higher stimulation intensity (40 V, 1.2-ms pulse duration) to evoke slow excitatory postsynaptic potentials (sEPSPs). As reported previously (39, 40), sEPSPs evoked in this manner have wide-ranging durations (10–30 s) and amplitudes (1–15 mV) and are accompanied initially by a marked decrease in membrane resistance that is occasionally followed by a sustained increase in resistance. A: after 30-Hz train, fEPSP amplitudes were increased coincident with sEPSP and thereafter. In 2 of 6 preparations, fEPSPs were increased in amplitude sufficient to reach action potential threshold (not shown). B: NK3 receptor antagonist SB-223412 (1 μM) inhibited sEPSPs and prevented potentiation of fEPSPs evoked by 30-s train. C: mean data showing effects of evoking sEPSPs on fEPSP amplitude in the presence and absence of 1 μM SB-223412. Values are means ± SE of 6 experiments. *Statistically significant potentiation of fEPSP amplitude relative to control (P < 0.05).
Increases in pulmonary insufficiency also produced dose-dependent effects of senktide analog (0.1 μM capsaicin and senktide analog was inhibitory by NK3 receptor antagonist SB-223412 (1 μM; n = 6). Similar inhibitory effects on senktide analog-induced fEPSP amplitude were observed using 1 μM SR-142801 (n = 2; not shown). Statistically significant potentiation of fEPSP amplitude relative to control (P < 0.05).

SB-223412 and abolished by pretreatment with 1 μM SB-223412 (Fig. 5).

Intravenous administration of the NK3 receptor agonist senktide analog also produced dose-dependent increases in pulmonary insufflation pressure in paralyzed, mechanically ventilated guinea pigs. Prior vagotomy or pretreatment with atropine (1 mg/kg iv) or SB-223412 (3 mg/kg iv) markedly attenuated the effects of senktide analog on pulmonary insufflation pressure (Fig. 5).

The effects of senktide analog on pulmonary insufflation pressure were rapid in onset and reversal and rapidly desensitized, with responses to repeated administration producing progressively smaller responses. This apparent desensitization did not seem to occur in the cardiovascular system, inasmuch as 10 nmol/kg senktide analog produced a marked decrease in blood pressure and heart rate that persisted for the duration of the experiment. Similar to previous reports (11, 53), the cardiovascular effects of senktide analog were nearly abolished by SB-223412 and reduced in animals pretreated with atropine but not markedly affected by vagotomy.

DISCUSSION

In the airways of most species studied, neurokinin-containing nerve fibers innervate parasympathetic ganglia (15, 16, 21, 30, 40, 47). The primary (if not exclusive) origin of these nerves in guinea pigs is primary vagal afferent neurons with cell bodies in the jugular (superior vagal) ganglia (28, 39, 40). Peripheral activation of these nerve endings by antidromic electrical stimulation or capsaicin administration induces depolarization of the airway cholinergic ganglia neurons (39, 40). These depolarizations are mediated by activation of NK3 receptors, inasmuch as they are abolished by potent and selective NK3 receptor antagonists (present study), unaffected by NK1 and NK2 receptor antagonists, and mimicked by NK3 receptor-selective agonists (39). The morphological and electrophysiological data presented in this study provide further evidence for this hypothesis. Similar effects of capsaicin and NK3 receptor agonists have been noted in studies of human bronchial ganglia (36).

NK3 receptor activation does not evoke contractions, atropine sensitive or otherwise, of isolated airway preparations obtained from humans or other species (18) and has no effect on airway tone in vivo after vagotomy (present study). Accordingly, it seems likely that neurokinins will have a neuromodulatory role in airway ganglia and not the role of a primary mediator of synaptic neurotransmission. Consistent with this assertion, we present evidence that endogenously released neurokinins facilitate synaptic neurotransmission in airway parasympathetic-cholinergic ganglia through activation of NK3 receptors. The effects of NK3 receptor activation on airway cholinergic nerve activity were striking: in 4 of 12 experiments, activation of the tachykinin-containing nerves electrically or by capsaicin rendered subthreshold fEPSPs threshold for action potential formation. In all preparations, fEPSP magnitude was increased after NK3 receptor activation. This effect on fEPSPs suggests that neurokinins acting at the level of the ganglia might potentiate cholinergic nerve-dependent contractions and, thus, bronchospasm simply by modulating synaptic neurotransmission. Indeed, the NK3 receptor agonist senktide analog applied selectively to the trachea increased baseline cholinergic tone of the trachealis in situ by >30%, without any apparent benefit of systemic or central nervous system effects, to levels nearing the maximum attainable cholinergic contraction of the trachea (10, 25). When administered intravenously, senktide analog also increases atropine-sensitive bronchoconstriction. These effects of senktide analog were dependent on preganglionic input, inasmuch as the NK3 receptor agonist was without effect in vagotomized animals and had no effect on EFS-induced contractions of the trachealis. Similarly, in vitro, NK3 receptor antagonists...
markedly reduced vagally mediated contractions evoked at threshold stimulation voltages. Again, this effect seemed dependent on the preganglionic parasympathetic input, inasmuch as the antagonists were without effect on baseline cholinergic tone in situ or on EFS-induced contractions in vitro. These data highlight the profound influence of airway ganglia integration on parasympathetic cholinergic tone and the potentially important influence neurokinins may have on the airways through effects on ganglionic integration of preganglionic input.

It is not readily apparent how neuropeptides facilitate synaptic transmission in airway ganglia. We have reported that NK3 receptor activation of airflow cholinergic ganglia neurons evokes marked but transient membrane depolarization and decreased membrane resistance, perhaps by opening nonspecific cation channels (39). Comparable mechanisms of NK3 receptor-mediated depolarization have been noted in studies of guinea pig cardiac and gallbladder ganglia neurons (22, 31). It seems unlikely that a similar mechanism accounts for the effects on synaptic transmission, however, inasmuch as in the present study the effects of NK3 receptor activation on synaptic transmission persist long after resolution of evoked sEPSPs. Moreover, the decrease in membrane resistance associated with the sEPSPs would likely render the ganglia neurons less excitable to subsequent or coincident excitatory stimuli. Zhang and colleagues (53) reported that low doses of substance P potentiate responses of most guinea pig cardiac ganglia neurons to exogenous acetylcholine, suggesting a postsynaptic effect on synaptic transmission, but higher doses of substance P (1–10 μM) evoked marked depolarization of the neurons and
often attenuated responses to acetylcholine. This effect of high-dose substance P was mimicked by voltage clamping the neurons at depolarized membrane potentials. We observed that senktide analog was without effect on nicotine agonist-induced depolarization of the ganglia neurons. It seems possible, then, that presynaptic mechanisms of NK3 receptor activation may be important in amplifying fEPSPs.

The results of the present study in no way conflict with previous studies demonstrating NK1 and NK2 receptor-mediated potentiation of cholinergic contractions of the airways via postganglionic mechanisms (2, 38, 52). The experiments presented here were designed to limit the influence of these postganglionic effects on the cholinergic nerves and on the airway smooth muscle, our bioassay for cholinergic nerve activity. Consequently, it was necessary to include NK1 and NK2 receptor antagonists in our buffers for all the in vitro functional assays of cholinergic nerve activity. Similarly, the data in no way discount the important role of NK1 and NK2 receptors on the airway smooth muscle (17). Rather, the data presented here merely highlight the important role of NK3 receptors in regulating synaptic transmission in the airway ganglia. It should also be noted that substance P and neurokinin A released from guinea pig sensory nerve terminals can readily activate NK3 receptors (22, 31, 39, 40). Neurokinin B, the endogenous ligand with highest affinity for NK3 receptors, is not expressed in airway nerves (28).

It is possible that NK1 receptors might also play a role in modulating synaptic transmission in airway ganglia. We reported previously that sEPSPs are occasionally followed by an increase in membrane resistance, an effect mimicked by NK1 receptor agonists (39, 40). We have also reported NK1 receptor-dependent activation of airway noncholinergic parasympathetic nerves in guinea pigs (8). Perhaps the ability of low-dose substance P to potentiate acetylcholine-induced effects in the cardiac ganglia can be attributed to NK1 receptor activation, whereas the inhibitory effects associated with higher doses are NK3 receptor dependent (53). These issues await a more systematic electrophysiological analysis, inasmuch as the functional assays used in the present study are not suitable for testing this hypothesis because of the effects of NK1 receptor activation of the airway smooth muscle. In any event, it seems likely that the mechanism of the NK3 receptor agonist activation of airway cholinergic ganglia neurons differs from that of noncholinergic ganglia innervating the guinea pig airways and neurons in the spinal cord involved in nociception (8, 29). In the spinal cord, NK3 receptor-mediated effects are nitric oxide (NO) dependent, whereas NK3 receptor activation produces action potentials in the noncholinergic parasympathetic ganglia neurons (which contain NO synthase) innervating guinea pig airways. Airway cholinergic neurons [all neurons intrinsic to the guinea pig airways are cholinergic (6)] do not express NO synthase (6, 46) and rarely form action potentials in response to NK3 receptor activation (39, 40).

**Physiological Significance**

Tachykinin-containing afferent nerves innervate the airways of all mammalian species. Tachykinin-containing nerve fibers are particularly prevalent in airway parasympathetic ganglia of human and animal airways (15, 16, 21, 30, 40, 47). Parasympathetic ganglia might thus be a primary site of action of tachykinins in the airways. Indeed, in guinea pigs, NK3 receptor antagonists (including the NK2-selective but NK3-active SR-48968) prevent hyperresponsiveness and other airway responses evoked by citric acid, antigen, and interleukin-5 (1, 4, 13, 26, 34, 42). Substance P challenge mimics the effects of these proinflammatory challenges on airway reactivity in guinea pigs, an effect that is prevented by the NK3 receptor antagonist SR-142801 (14). Anticholinergic agents such as atropine and ipratropium bromide also reverse allergen-induced airway hyperresponsiveness in guinea pigs (43) and markedly reduce airway reactivity in asthmatic patients to a wide variety of stimuli (7). NK3 receptor antagonists may also decrease pathological cough and elevated parasympathetic nerve activity (cholinergic and noncholinergic) in the airways through central and peripheral mechanisms (1, 7–9, 13; present study). The present study demonstrates that NK3 receptor antagonists have the potential ability to decrease parasympathetic nerve activity in the airways by regulation of ganglionic synaptic transmission.

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