Inhibition of 5-Lipoxygenase Diminishes Neurally Evoked Tachykinergic Contraction of Guinea Pig Isolated Airway

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ABSTRACT
The role of endogenous 5-lipoxygenase products in modulating tachykinergic neurotransmission in guinea pig isolated trachea was investigated. Tachykinin-containing afferent nerve fibers were stimulated with either electrical field stimulation or antidromic stimulation of the right vagus nerve. This resulted in contractions of the stimulated caudal trachea and bronchus that could be blocked with either tetrodotoxin or a combination of neurokinin-1 and neurokinin-2 receptor antagonists. The 5-lipoxygenase inhibitor ZD 2138 (1 μM) significantly inhibited these neurally mediated tachykinergic contractions, by approximately 50%, yet had no effect on the contractions evoked by stimulating tachykinergic fibers in an action potential-independent fashion with capsaicin or by exogenously applied neurokinin A. The effect of ZD 2138 on action potential-driven tachykinergic contractions was mimicked by pobilukast, pranolukast, montelukast and zafirlukast, four structurally unrelated antagonists of the cysteinyl leukotriene 1 receptor subtype. Pobilukast had no effect on the tachykinergic contraction in tissues pretreated with ZD 2138. Likewise, ZD 2138 had no effect on the tachykinergic contractions in tissues pretreated with pobilukast. Intracellular electrophysiological recording of the membrane properties of jugular ganglion neurons, the source of tachykinins in the guinea pig trachea/bronchus, demonstrated that leukotriene D₄ caused a membrane depolarization of vagal afferent C-fiber neurons and an increase in input impedance, both of which were abolished by zafirlukast. Taken together, these data indicate that in the resting guinea pig isolated trachea/bronchus, endogenous 5-lipoxygenase activity leads to the production of cysteinyl leukotrienes that amplify action potential-dependent release of tachykinins from airway afferent nerve fibers.

Stimulation of pulmonary afferent fibers causes central reflexes (Coleridge and Coleridge, 1984) as well as local release of the bioactive peptides known as tachykinins, through an axon reflex (Lundberg and Saria, 1982a). In the guinea pig, this local release of neuropeptides causes a number of proinflammatory processes, including vasodilation, plasma extravasation and airway smooth muscle contraction. This noncholinergic contraction is well characterized; nerve stimulation releases substance P and neurokinin A, which contract the smooth muscle via NK-1 and NK-2 receptor activation (Renzi et al., 1992). Thus, the guinea pig isolated airway is a convenient model to study neuroregulation of tachykinergic transmission.

We have previously demonstrated that the cys-LT1 receptor antagonist pobilukast (previously referred to as SKF 104353) is an effective inhibitor of tachykinergic contractions elicited by afferent nerve stimulation in the guinea pig isolated trachea (Ellis and Undem, 1991). Moreover, this compound inhibited the extent of plasma extravasation evoked by antidromic stimulation of tachykinin-containing afferent fibers in vivo (Ellis and Undem, 1991). This led to the hypothesis that there is 5-LO activity in the resting guinea pig airway capable of producing enough cys-LTs to amplify tachykinergic neurotransmission. Alternatively, the cys-LT receptor antagonists may have been acting as inverse agonists in this preparation. The presumption of the inverse agonist theory is that the cys-LT receptors on the afferent nerve fibers are spontaneously active and that this activity is inhibited by binding of the antagonist (Schutz and Freissmuth, 1992). A 5-LO inhibitor that effectively inhibits leukotriene production in guinea pig airways could be used to investigate these alternative hypotheses. Our current results suggest that the 5-LO inhibitor ZD 2138 is highly effective at inhibiting leukotriene production in the guinea pig isolated airway, so we have employed this compound to learn more about the regulatory influence of 5-LO activity on tachykinergic neurotransmission in the guinea pig isolated trachea and bronchus.

Our results indicate that 5-LO activity in the guinea pig isolated airways produces enough cys-LTs to amplify electrically evoked tachykinergic neurotransmission. Furthermore,
the data are consistent with the contention that this occurs via the interaction of cys-LTs with receptors on the afferent nerve fibers, leading to a selective amplification of action potential-dependent tachykinin release.

Materials and Methods
Reagents. Chromotrope 2R, atropine sulfate, propranolol hydrochloride, indomethacin, LNNa and pyrilamine maleate were purchased from Sigma Chemical Co. (St. Louis, MO). Each was dissolved in distilled water at a concentration of 10 mM. ZD 2138, SKF 104353, zafirlukast, montelukast, and pranlukast were generous gifts from SmithKline Beecham Pharmaceuticals (Philadelphia, PA). Each compound was dissolved in DMSO at a concentration of 10 mM before dilution to the appropriate concentration in buffer solution.

Isolation of tissue. Male Hartley guinea pigs (100–400 g) were killed by asphyxiation in a CO 2 chamber and exsanguination. After exsanguination, the thorax was opened, and the trachea, bronchi, lungs and vagus nerves with intact nodose and jugular ganglia were removed and placed into a dissection dish containing a modified oxygenated Krebs' buffer solution (composition in mM: 118 NaCl, 5.4 KCl, 1 NaH 2 PO 4 , 1.9 CaCl 2 , 25 NaHCO 3 , and 11.1 glucose).

Effect of ZD 2138 against antigen. The effectiveness of ZD 2138 in preventing antigen-induced contraction of the isolated guinea pig trachea was examined as previously described (Adams and Lichtenstein, 1979). Briefly, guinea pigs were passively sensitized by an i.p. injection of serum from an animal that had been actively sensitized to ovalbumin. The animals were sacrificed 48 hr after the injection, at which time they had been actively sensitized to ovalbumin. The trachea was examined as previously described (Adams and Lichtenstein 1979), as well as 1 μM atropine, 1 μM propranolol, and 3 μM indomethacin. Each tissue ring received either vehicle (DMSO) or ZD 2138 (10 nM, 100 nM, or 1 μM). After a 30-min incubation with either drug or vehicle, the tracheal rings were challenged with 10 μg/ml of ovalbumin. After the antigen-induced contraction had reached equilibrium, maximum contractile responses were obtained by the addition of 30 mM BaCl 2 to the bathing solution.

Field stimulation. After transfer of the airways to the dissection dish, either bronchial rings (three or four cartilage rings in width) or caudal tracheal strips (two cartilage rings in width) were isolated and then placed in a 10-ml bath containing the same Krebs' solution (composition in mM: 118 NaCl, 5.4 KCl, 1 NaH 2 PO 4 , 1.9 CaCl 2 , 25 NaHCO 3 , and 11.1 glucose).

Vagal stimulation. After removal of the airways, the trachea and bronchi with intact extrinsic innervation were placed in a water-jacketed dissection dish filled with buffer solution containing 1 μM propranolol and 3 μM indomethacin, gassed with 95% O 2 /5% O 2 , and maintained at 37°C. Connective tissue was cleaned away from the bronchi and caudal trachea. The vagi were transected caudal to the nodose ganglia. The free end of the right vagus was then placed in a suction electrode (World Precision Instruments, Sarasota, FL). Two longitudinal sections were made in the right bronchus, approxi- mately three to four cartilage rings apart, and then the bronchus was tied with nonsterile surgical suture to a Grass model FT03C force-displacement transducer for recording of base-line tension and opened with a transverse cut. The tissue was perfused for an hour after the application of 1.5 g of base-line tension. After the equilibration period, the vagus was stimulated at 5 Hz, 1 ms, 150 V for 15 s. After the initial stimulation, the bath was switched from a perfusion system to a 50-ml static bath, and 1 μM atropine and 30 μM LNNa were added. The vagus was subsequently stimulated at 15-min intervals until two consistent control responses were elicited. ZD 2138 (1 μM) was then added for 30 min, after which the vagus was stimulated again. After the experiment, the tissue's maximum contraction was obtained by the addition of 30 mM BaCl 2.

Intracellular recording. For recording direct effects of LTD 4 on C-type sensory neurons, left jugular ganglia with the vagus and superior laryngeal nerves attached were removed along with the airways (as above) and transferred to Krebs’ buffer. The jugular ganglia were trimmed of adhering connective tissue and the neurons exposed by fine dissection. The preparations were pinned in a recording chamber (100 μl in volume) and superfused with Krebs' solution (36–37°C, 8–10 ml/min) throughout the experiments. Microperipettes for recording conducted action potentials and membrane properties were fabricated from thick-walled capillary stock (0.5-mm I.D., 1.0-mm O.D., World Precision Instruments Co., Inc., Sarasota, FL) by a Brown-Flaming microelectrode puller (Model P-87, Sutter Instr. Co., San Rafael, CA). Electrodes were filled with 3M KCl (pH 7.4), and the electrolyte in the micropipette was connected by a Ag-AgCl wire in an electrode holder (Axon Instruments, Foster City, CA) to a headstage to an electrometer (Axoclamp 2A, Axon Instruments). The electrode DC resistance in Krebs’ solution ranged between 50 and 70 MΩ. A Ag-AgCl pellet in the bath was connected to headstage ground. Impalement of the neurons was aided by a 20-mV overcompensation (i.e., buzz) of the capacitance neutralization circuit of the Axoclamp amplifier. Intracellular recordings were performed with the electrometer in either discontinuous current clamp (3.0–4.0-kHz sampling rate) or active bridge mode. For estimating the conduction velocity of the action potential in the axon of the impaled neuron, both vagus and superior laryngeal nerves were pulled into a suction (stimulating) electrode and stimulated; the distance between the stimulating and recording electrodes was divided by the time between the shock artifact and the somal action potential recorded with the intracellular electrode. Only neurons with conduction velocities less than 1 m/s were used in these studies (i.e., C-type neurons). Voltage stimuli through the suction electrode originated in a Grass (Astra-Med, Inc., West Warwick, RI) stimulator; the stimulus amplitudes ranged from 70 V to 100 V, and the
pulse durations ranged from 1.0 to 1.2 ms, parameters optimal for eliciting sensory nerve-evoked sEPSPs in bronchial parasympathetic neurons (Myers et al., 1996) or NANC contractions of bronchial smooth muscle (Undem et al., 1990). The response of the jugular ganglion neurons to LTD₄ was examined by superfusing the ganglion with 0.1 μM LTD₄ for 3 to 5 min (24–50 ml). The effect on membrane properties was obtained by comparing the control resting potential, before drug application, to the peak of the drug-induced change in membrane potential. Net changes in input resistance, calculated using voltage transients elicited by 100-pa hyperpolarizing current steps, were also noted at these times. In a separate series of experiments, we determined the ability of zafirlukast to block the LTD₄-induced depolarization by using the same protocol, but with 1 μM zafirlukast introduced into the superfusion solution at least 30 min before impalement of the neuron.

**Statistical analysis.** Data were compared using Student’s t test, and P values below .05 were considered statistically significant. When multiple means were involved, an ANOVA was first performed.

**Results**

To evaluate the effectiveness of ZD 2138 at inhibiting the production of cys-LTs in the guinea pig isolated airway preparation, we examined antigen-induced contraction in the presence of the histamine H-1 receptor antagonist pyrilamine. These contractions are dependent on leukotriene formation and can be blocked by cys-LT1 receptor antagonists (Adams and Lichtenstein, 1979). At a concentration of 1 μM, ZD 2138 effectively inhibited the leukotriene-dependent component of the antigen-induced contractions (fig. 1), so we chose this concentration in our studies on neuromodulation. Parenthetically, ZD 2138 (1 μM) did not appear to act as a leukotriene receptor antagonist, inasmuch as pretreatment of tracheal strips for 30 min produced no difference in the concentration-response curve to LTC₄ \[\text{EC}_{50} \text{ of } 8.14 \pm 0.26 \text{ vs. } 8.08 \pm 0.21 \text{ for control; } n = 4 \]. In the presence of inhibitors of the peptidase enzyme that metabolizes LTC₄ to LTD₄, LTC₄ contracts the trachea via both cys-LT1 and non-cys-LT1 receptor mechanisms (Snyder and Krell, 1984).

**Vagus nerve stimulation and EFS experiments.** Treatment of tissues with 1 μM ZD 2138 reduced the peak magnitude of tachykinergic contraction elicited by EFS from 30.7 ± 2.7% to 15.5 ± 1.9% \((n = 22; P < .001; \text{fig. 2A})\). Vagus nerve stimulation was also employed to elicit tachykinergic smooth muscle contractions. These experiments were performed to avoid the potential release of autacoids from non-neural cells after EFS of the tissue (Fernandes et al., 1994). The peak magnitude of contraction in vagal stimulations was also significantly reduced by 1 μM ZD 2138, from 21.2 ± 2.9% to 13.9 ± 3.1% \((n = 9; P < .001; \text{fig. 2B})\). There was no effect of time alone on the magnitude of the nerve stimulation-induced contractions. In four time control experiments, the magnitude of last nerve-evoked response was 101 ± 4% of the initial response. In 3 out of 3 experiments, contractions were completely abolished by crushing the vagus. There was no statistically significant difference in the peak magnitude of inhibition by ZD 2138 in the tissues subjected to EFS and the vagus nerve-stimulated tissues \((P > .1)\).

We previously reported that pobilukast (also known as SKF 104353) inhibits the tachykinergic contraction to EFS of the guinea pig isolated trachea (Ellis and Undem, 1991). In the present study, we found that the magnitude of the inhibition of tachykinergic contractions caused by ZD 2138 was mimicked by the four cys-LT1 receptor antagonists zafirlukast, pobilukast, montelukast, and pranlukast, each at a concentration of 3 μM (fig. 3). As with ZD 2138, the magnitude of the inhibition of the tachykinergic contractions by the cys-LT1 receptor antagonists was independent of whether the nerves were stimulated by vagus nerve stimulation or EFS. For example, zafirlukast had the same effect on tachykinergic contractions evoked by bipolar (see “Materials and Methods”) pulses of EFS (46.6 ± 9.9% inhibition, \(n = 5\)) and vagus nerve stimulation (58.3 ± 6.6% inhibition, \(n = 4\)). This was also previously noted with pobilukast (Ellis and Undem, 1991).

In a set of four experiments in which 1 μM ZD 2138 was added to the preparation after the application of 3 μM pobilukast, no further inhibition of the EFS-induced tachykinergic contraction was observed \((7.2 ± 2.0 \text{ to } 6.5 ± 2.2\% \text{ of maximum, } P > .05)\). Likewise, pobilukast (3 μM) produced no additional inhibition in tissues pretreated with ZD 2138 (7.1 ± 1.1 to 6.2 ± 2.0% of maximum, \(P > .05, n = 5\)). ZD 2138 did not alter smooth muscle contractions elicited by addition of capsaicin in a cumulative manner; \(-\log (M) \text{EC}_{50} \text{ was } 6.96 ± 0.20 \text{ in the presence of ZD 2138 and } 7.05 ± 0.15 \text{ in its absence (n = 6; fig. 4A).} \text{ZD 2138 also did not alter tracheal smooth muscle contractions induced by NKA. In four experiments, } -\log (M) \text{EC}_{50} \text{ was } 7.63 ± 0.05 \text{ in the presence of ZD 2138 and } 7.66 ± 0.15 \text{ in its absence (n = 4; fig. 4B).} \text{Electrophysiology.} \text{The tachykinin-containing afferent C-fibers that innervate the guinea pig trachea and bronchus arise from cell bodies located in the jugular ganglia situated on the vagus nerves (Kummer et al., 1992; Riccio et al., 1996). We investigated the effect of LTD₄ on the resting membrane potential of jugular ganglion C-fiber neurons. Superfusion of C-fiber cells within the isolated jugular ganglion with buffer
solution containing 100 nM LTD₄ resulted in a consistent depolarization of the resting membrane potential (table 1). The onset of the depolarization was approximately 15 s, and the depolarization peaked within 3 min. During the time of peak depolarization, the input impedance was increased by 23% (table 1). In 6 of 8 experiments, the membrane potential returned to its original value after application of LTD₄. Both the depolarization and the increase in input impedance were abolished by inclusion of 1 μM zafirlukast in the superfusion solution (table 1). Zafirlukast itself had no effect on the resting potential or input impedance of the neurons studied (table 1).

**Discussion**

We have previously reported that cys-LT1 receptor antagonists inhibit the tachykinergic contractions of guinea pig isolated airways evoked by electrical stimulation of the afferent nerve fibers (Ellis and Undem, 1991). This led to the suggestion that either there was 5-LO activity in the resting airways, resulting in sufficient leukotriene production to amplify the nerve response, or the leukotriene receptors were spontaneously active and the receptor antagonists were acting as inverse agonists in this preparation. The present study supports the former hypothesis—that endogenous 5-LO activity in the resting guinea pig airway results in amplification of the action potential-dependent release of tachykinins from airway sensory nerve fibers. The hypothesis that endogenous 5-LO activity is sufficient to modulate tachykinin release in this system is supported by the fact that a selective 5-LO inhibitor, ZD 2138, significantly inhibited tachykinin-mediated contractions of airway smooth muscle induced by either EFS or vagus nerve stimulation. The results further indicate that the 5-LO products involved in this neuromodulatory effect are entirely cys-LTs. Thus four structurally unrelated cys-LT1 receptor antagonists inhibited tachykinergic neurotransmission in this model to the same extent as 5-LO blockade. Moreover, as predicted from the hypothesis, after treatment with a cys-LT receptor antagonist, the 5-LO inhibitor produced no further inhibition of the response.

In vagus nerve-stimulated or field-stimulated tissues, inhibiting 5-LO enzymes with ZD 2138 reduced the magnitude of tachykinin-mediated airway smooth muscle contraction by approximately 50%. This could be through a decreased amount of tachykinergic neurotransmission, increased inhibitory NANC neurotransmission or a nonselective effect of the drug. It seems highly unlikely that ZD 2138 was acting nonselectively, because its effect on the tachykinin-evoked contractions was mimicked by four structurally distinct compounds. Also supporting the conclusion that ZD 2138 was indeed acting by blocking 5-LO activity is the observation that its effect was not additive with the cys-LT1 receptor antagonists. The possibility that the inhibitory NANC system is being amplified, causing a functional antagonism of the tachykinergic response, is also unlikely, because the 5-LO inhibitor inhibited the response to both EFS and vagus nerve stimulation. We have previously reported that the preganglionic fibers responsible for NANC relaxation of guinea pig isolated airways interact with ganglia within the esophagus. If the esophagus is removed, vagus nerve stimu-
cation does not stimulate the NANC relaxant innervation to the airway smooth muscle (Canning and Undem, 1993). Therefore, it seems most likely that ZD 2138 is inhibiting basal production of cys-LTs that amplify tachykinergic neurotransmission.

Although the precise mechanisms by which endogenous 5-LO products amplify neuronally evoked tachykinergic contractions was not determined, the data are consistent with a prejunctional site of action. This is based on the observation that the concentration-response curve to exogenously applied NKA was unaffected by ZD 2138. We have previously noted that the concentration-response curve for percentage of maximum tension evoked by NKA in the isolated caudal trachea in the presence (A) and absence of (B) 1 μM ZD 2138 (n = 6). The values represented are the mean ± S.E.M. and are normalized as a percentage of the maximum tension generated by the addition of 30 mM BaCl2 to the bath after the experiment.

**TABLE 1**

Effect of 100 nM LTD4 on membrane properties of guinea pig jugular C-fiber neurons

<table>
<thead>
<tr>
<th></th>
<th>No Antagonist</th>
<th>1 μM Zafirlukast</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>100 nM LTD4</td>
</tr>
<tr>
<td>Resting potential (mV)</td>
<td>-57.4 ± 3.7</td>
<td>-52.3 ± 3.8**</td>
</tr>
<tr>
<td>Input resistance (MR)</td>
<td>33.8 ± 5.9</td>
<td>43.8 ± 6.8*</td>
</tr>
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Values represent mean ± S.E.M. of eight experiments in the absence of zafirlukast and six experiments in its presence. Asterisks indicate significant differences between LTD4-treated and control groups (**P < .01; *P < .05).

The observation that LTD4 and zafirlukast inhibit electrically evoked tachykinergic contractions but not capsaicin-induced responses not only supports a prejunctional site of action but also demonstrates that the mechanism of potentiation by the cys-LTs is selective for action potential-driven tachykinin release.

It is not technically feasible to study the electrophysiologic properties of the afferent nerve terminals in the airway. The cell bodies of these fibers are, however, accessible to electrophysiologic recordings. The tachykinins in the guinea pig trachea/bronchus are located in C-fibers derived from cell bodies located in the jugular sensory ganglia (Riccio et al., 1996; Kummer et al., 1992). The observation that LTD4 caused a consistent depolarization and boosted input impedance in jugular C-fiber neurons supports the speculation that cys-LTs may act directly on the afferent nerve fibers to enhance the action potential-dependent release of tachykinins.

We have previously reported that the 5-LO inhibitor AA 861 did not significantly reduce the EFS-induced tachykinergic contractions in this model. This compound, however, was found to be less effective in inhibiting the production of leukotrienes in our model, as assessed by its ability to inhibit antigen-induced contractions in tissues pretreated with a histamine H-1 receptor antagonist (Ellis and Undem, 1991). These contractions are blocked by cys-LT receptor antagonists and are thought to be due to the production of leukotrienes by airway mast cells (Adams and Lichtenstein, 1979).
Whereas ZD 2138 nearly abolished the antigen-induced contractions, AA 861 inhibited the contractile response only by approximately 50%. Therefore, the discrepancy in these findings can probably be attributed to the relative effectiveness of the two inhibitors at blocking 5-LO activity in the guinea pig isolated airway.

An issue that remains unsolved is the source of leukotrienes in this preparation. Although we cannot rule out the possibility that the nerve fibers release cys-LTs, which then have an autocrine effect, a more likely source is mast cells within the guinea pig airway. Mast cells are known to produce cys-LTs (Peters et al., 1985) and are frequently found close to nerve fibers (Undem and Weinreich, 1989). Another potential candidate is the eosinophil. Eosinophils also have 5-LO enzymes, produce cys-LTs and are routinely found in the airway wall of naive guinea pigs. In fact, in the present study, tissue sections of bronchi from six different naive guinea pigs were stained for eosinophils with chromotrope 2R and were found to contain 438.7 ± 168.9 eosinophils per square millimeter. That ZD 2138 inhibits the neuronal response but did not relax the airway smooth muscle suggests that the source of the endogenous leukotriene may be closer to the nerve fiber than the smooth muscle. Alternatively, cys-LTs may be more potent at potentiating neuronal responses than causing smooth muscle contraction. This latter suggestion is supported by the observation that exogenously applied LTD4 potentiated tachykinergic contractions at concentrations that were subthreshold for causing smooth muscle contraction (Ellis and Undem, 1991). It should also be noted that the potentiation of electrically evoked tachykinergic transmission by endogenous leukotrienes is not limited to the guinea pig airway. Goldhill et al. (1995) recently noted that the cys-LT1 receptor antagonist inhibited EFS-induced noncholinergic contractions of the mouse intestine by 80% while having no effect on the response to exogenously applied neurokinin A.

In conclusion, these data suggest the involvement of endogenous 5-LO products, most likely cys-LTs, in tachykinergic contractions of airway smooth muscle induced by either EFS or vagus nerve stimulation. The 5-LO products appear to act on the nerve terminal of tachykinin-containing afferent fibers that innervate the airways to potentiate action potential-induced release of transmitter. Inhibition of neuronal responses should therefore be considered in the airway pharmacology of 5-LO inhibitors and cys-LT1 receptor antagonists.

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References


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