Absence of Tachykinin Involvement in Leukotriene D₄ and Antigen-Induced Contraction of Guinea Pig Isolated Bronchus

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Accepted for publication August 15, 1997

ABSTRACT
In guinea pig airways, contractions induced by leukotriene D₄ or antigen are thought to be mediated primarily by an action of the agonist or of released mast cell-derived mediators directly on the airway smooth muscle cell. An indirect contractile action mediated by endogenous tachykinins also has been described for both of these stimuli. The present study evaluated the contribution of endogenous tachykinins to ovalbumin- and leukotriene D₄-induced contractions in the guinea pig bronchus by modulating the concentrations of tachykinins within the tissues and by using neurokinin receptor antagonists. Acute depletion of tachykinins with capsaicin had no effect on responses elicited by either stimulus. Similarly, tetrodotoxin treatment failed to influence leukotriene D₄-induced contractions. Inhibitors of neutral endopeptidase (thiorphan) and angiotensin-converting enzyme (lisinopril) enhanced neurally mediated tachykininergic responses and potentiated leukotriene D₄. The latter effect persisted in the presence of tetrodotoxin or the neurokinin antagonists CP99994 and SR48968 and in tissues treated acutely with capsaicin. The potentiation was absent, however, from bronchi incubated with L-cysteine. Ovalbumin-induced contractions were unaltered by inhibition of neutral endopeptidase and angiotensin-converting enzyme. These observations suggest that tachykinins are not involved in mediation of leukotriene D₄- or antigen-induced contractions of the guinea pig bronchus. The ability of protease inhibitors to potentiate leukotriene D₄ but not antigen-induced responses is therefore ascribed to inhibition of bioinactivation of leukotriene D₄ to leukotriene E₄.

Guinea pig central airways are well supplied with tachykinin-containing nerves (Lundberg et al., 1984; Manzini et al., 1989). A variety of pharmacological agents have been shown to stimulate the release of tachykinins from guinea pig airways. These include muscarinic and nicotinic cholinergic agonists (Kizawa and Takayanagi, 1985; Saria et al., 1988; Martins et al., 1991), bradykinin (Saria et al., 1988), histamine (Saria et al., 1988; Martins et al., 1991), platelet-activating factor (Martins et al., 1991) and leukotriene D₄ (Bloomquist and Kream, 1990; Martins et al., 1991). Given that the tachykinins, substance P and neurokinin A, are potent contractile agonists on airway smooth muscle (Saria et al., 1988), it is conceivable that contractions of airway smooth muscle by the aforementioned pharmacological agonists could be supplemented or mediated indirectly by tachykinins released from intrinsic airway nerves. Several studies support this contention. First-generation tachykinin antagonists abolish nicotine-induced contraction of the guinea pig bronchus (Kizawa and Takayanagi, 1985) and attenuate contractions of the guinea pig trachea caused by leukotriene D₄ (Bloomquist and Kream, 1990). Depletion of endogenous tachykinins by acute capsaicin treatment prevents the contractile activity of nicotine in the guinea pig bronchus (Kizawa and Takayanagi, 1985). Furthermore, inhibition of tachykinin metabolism enhances increases in guinea pig airway opening pressure induced by histamine and methacholine (Martins et al., 1991) and augments antigen- and leukotriene C₄-induced contractions of the guinea pig bronchus (Kohrogi et al., 1991). Employing pharmacological tools used by previous investigators and newer, more selective neurokinin antagonists, the present study re-evaluates the contribution of an indirect tachykinin-dependent mechanism to the mediation of contractions induced by leukotriene D₄, an agonist for which there is the greatest functional evidence of tachykinin involvement. Given the contribution of leukotrienes to antigen-induced responses, we also assessed the role of tachykinins in ovalbumin-induced contractions in airway tissue obtained from sensitized guinea pigs.

Materials and Methods
Animal sensitization. Male guinea pigs (200–250 g) were sensitized to ovalbumin using the method of Andersson (1980). In summary, ovalbumin was adsorbed to aluminum hydroxide (10 mg/ml) and administered as a single i.p. dose (40 μg/kg). Fourteen to 21 days later, animals were used in experiments in which antigen-induced effects were examined.

ABBREVIATIONS: KHS, Krebs-Henseleit solution; NEP, neutral endopeptidase; NK, neurokinin.
In experiments on the effects of leukotrienes, male Hartley guinea pigs that weighed 250 to 450 g, were anesthetized using sodium pentobarbital (100 mg/kg i.p.). Upon attainment of surgical anesthesia, the trachea and lungs were removed en bloc and placed in KHS of the following composition (mM): NaCl, 118.2; KCl, 4.74; CaCl₂, 2.54; KH₂PO₄, 1.19; MgSO₄, 1.19; NaHCO₃, 26.2; (d-+)-glucose, 11.1. Indomethacin (2.8 μM) was included in the KHS to abolish the influence of cyclooxygenase products on tissue responses. Right and left main stem bronchial ring preparations, obtained immediately distal to the tracheal bifurcation, were isolated from each animal. Tissue segments were cleaned of extraneous connective tissue and mounted on stainless steel tissue hooks in 15-ml glass-jacketed organ baths containing KHS maintained at 37°C and gassed with 5% CO₂ in O₂. An initial load of 6 g was placed on bronchial segments. Tissues were allowed to equilibrate for 60 min, during which time the bathing solution was exchanged at 10-min intervals. Upon attainment of a stable base-line tone, a maximal contractile concentration of ACh (1 mM) was administered to all tissues. After the ACh response had stabilized, the tissues underwent a 60-min “washout period” during which the bathing solution was exchanged at 10-min intervals. Upon reattainment of a steady base-line tone, tissues were subjected to various treatments as noted below.

(1) Depletion of endogenous tachykinins. Endogenous tachykinins were depleted using acute capsaicin treatment (Kizawa and Takayanagi, 1985; Thompson et al., 1987). One of the pair of airway preparations was exposed to capsaicin (10 μM) for 60 min, the other to capsaicin vehicle (15 μl of ethanol) for 60 min. Tissues then underwent a 60-min “washout period” during which the bathing solution was exchanged at 10-min intervals.

(2) Protease inhibition. One of the pair of airway preparations was exposed to a neutral endopeptidase inhibitor, thiorphan (10 μM), to an angiotensin-converting enzyme inhibitor, lisinopril (10 μM), or to their combination, and the other of the pair was exposed to an equivalent volume of inhibitor vehicle. An incubation period of 30 min was then allowed before leukotriene D₄ or ovalbumin was applied to the tissues.

(3) Inhibition of action potential conduction. One of the pair of airway preparations was exposed to tetrodotoxin (10 μM), the other to an equivalent volume of inhibitor vehicle. An incubation period of 30 min was then allowed before leukotriene D₄ or ovalbumin was applied to the tissues.

(4) Inhibition of neurokinin receptors. One of the pair of airway preparations was exposed to NK₁ antagonist, CP99994 (1 μM) (McKee et al., 1999), and to NK₂ antagonist, SR48968 (10 μM) (Advenir et al., 1992), and the other of the pair was exposed to an equivalent volume of inhibitor vehicle. An incubation period of 30 min was then allowed before leukotriene D₄ or ovalbumin was applied to the tissues.

(5) Inhibition of conversion of leukotriene D₄ to leukotriene E₄. Tissues were treated with L-cysteine (3 mM) 30 min before commencement of the leukotriene D₄ concentration-response curve to prevent metabolism of leukotriene D₄ to leukotriene E₄ by tissue aminopeptidases (Snyder et al., 1984; Snyder and Krell, 1984).

After the appropriate treatment regimen, a concentration-response curve to ovalbumin (antigen) or leukotriene D₄ was elicited by the cumulative addition of agonist to the bathing solution. Experimental protocols were designed on a paired basis such that one of each pair of tissues from each animal (e.g., left or right hilar bronchus) acted as a control (vehicle or no treatment) while the other of the pair was subjected to treatment. Experimental protocols were designed such that equivalent numbers of each airway segment were assigned to control and test groups. No differences in responsiveness of left and right hilar bronchi were evident. Neurally mediated tachykininergic responses were induced in bronchial preparations treated with atropine (1 μM), guanethidine (10 μM), propranolol (1 μM) (to abolish cholinergic and adrenergic neural influences) and thiorphan (10 μM) and lisinopril (10 μM) (to inhibit tachykinin degradation). Pseudo-cumulative frequency-response curves for electrical field stimulation (0.1–80 Hz, pulse duration 0.3 ms, pulse amplitude 10 V, 5 s stimulation duration/60 s) were applied to preparations using rectangular wave pulses generated by a Grass S-88 stimulator in series with a signal conditioner (Stimu-Splitter, Med. Lab. Instruments, Fort Collins, CO). All responses were measured isometrically using force-displacement transducers (Grass FT.03c) and were displayed on a MacLab8 (ADI Instruments, Sydney, Australia)/Macintosh (Apple Computer Corp., Cupertino, CA) computer system.

Materials. All reagents were of analytical grade. Drugs were obtained from the following sources: ACh perchlorate, capsaicin, L-cysteine, leukotriene D₄, leukotriene E₄, ovalbumin (grade V), tetrodotoxin, thiorphan (Sigma Chemical Co., St. Louis, MO); CP99994, lisinopril, SR48968 (Merck Frosst, Kirkland, Québec, Canada). Indomethacin was initially dissolved in 0.1 M Na₂CO₃ and diluted 10,000-fold in KHS. Capsaicin, CP99994 and SR48968 were dissolved in ethanol and diluted in KHS. All other drugs were dissolved in distilled water and diluted in KHS.

Statistical analysis. Data are presented as means and associated S.E.M. The negative logarithmic values of the concentrations of contractile agonist that produced a 40% maximal ACh response are denoted as log EC₄₀ ACh. This concentration was selected (rather than log EC₅₀ or pD₉) because the maximal effect of leukotriene D₄ was usually not obtained in experiments. Nevertheless, in the presence of L-otine (where a maximum did appear to be achieved), the maximal effect of leukotriene D₄ was ~50% ACh maximum. Accordingly, the leukotriene D₄ log EC₄₀ ACh, approximates the leukotriene D₄ log EC₅₀. Log EC₄₀ ACh values were determined by regression analysis and interpolation of the linear portions of individual concentration-response curves. Statistical comparisons of concentration-response curves were conducted using two-way analysis of variance (ANOVA). Post-hoc Student’s unpaired t tests were used to compare responses at specific agonist concentrations. P < .05 was considered significant.

Results

Initial studies were conducted to verify the efficacy of treatments. A combination of the protease inhibitors thiorphan and lisinopril enhanced neurally mediated tachykininergic responses in the guinea pig bronchus (fig. 1). These inhibitor-augmented responses were absent from tissues treated with the neurokinin receptor antagonists CP99994 and SR48968 (fig. 1). This same neurokinin antagonist combination induced a 57-fold rightward shift of the substance P concentration-response curve (control, −7.97 ± 0.17; treated, −6.22 ± 0.08, mean log EC₅₀ ± S.E.M.). Acute capsaicin treatment also abolished neurally mediated tachykininergic responses (data not shown).

Leukotriene D₄ induced concentration-dependent contraction of the guinea pig bronchus, threshold responses becoming manifest at about 1 nM. In bronchi from sensitized animals, ovalbumin evoked concentration-dependent contractions with a threshold of 0.1 ng/ml. Acute capsaicin exposure had no influence on either the leukotriene D₄ (P = .18, ANOVA) or the ovalbumin (P = .17, ANOVA) concentration-response relationships in the bronchus (fig. 2). Similarly, neither contractile responses induced by leukotriene D₄ (P = .25, ANOVA) nor those induced by ovalbumin (P = .50, ANOVA) were affected by CP99994 and SR48968 (fig. 3) or by tetrodotoxin (P = .66, ANOVA for leukotriene D₄) (data not shown). By contrast, treatment of tissues with the combination of thiorphan and lisinopril resulted in marked potentiation of leukotriene D₄ at all concentrations except the maximal concentration tested, 0.1 μM (fig. 4). Neither inhibitor alone, however, altered the leukotriene D₄ concentration-
response curve (P = .71 for lisinopril; P = .91 for thiorphan, ANOVA) (fig. 5). The combination of thiorphan and lisinopril failed to influence ovalbumin (P = .88, ANOVA)- or ACh (P = .73, ANOVA)-induced contractions (fig. 6).

The mechanism of the potentiation of leukotriene D4 by the combination of thiorphan and lisinopril was examined further in additional experiments. The potentiation persisted in tissues treated with tetrodotoxin (fig. 7) or with the neurokinin antagonists CP99994 and SR48968 (fig. 7) and in those tissues that had undergone acute capsaicin treatment (fig. 8). Conducting experiments in the presence of L-cysteine resulted in abolition of the potentiation (P = .85, ANOVA) (fig. 9). It should be noted, however, that L-cysteine treatment alone served to enhance the potency of leukotriene D4 independently of the presence of thiorphan and lisinopril (control log EC40 ACh = −7.78 ± 0.08 vs. L-cysteine control log EC40 ACh = −8.24 ± 0.25). Contractions induced by leukotriene E4 were unaltered by thiorphan and lisinopril (P = .53, ANOVA) (fig. 10).

Discussion

Leukotriene D4 is a potent constrictor of airway smooth muscle. In the present study, leukotriene D4 induced concentration-dependent contraction in the guinea pig bronchus in the range 1 to 100 nM. The contractile actions of leukotriene D4 on the airway smooth muscle were thought to be mediated directly via a specific leukotriene receptor (Mong et al., 1985). However, a more recent study has implicated an additional indirect mechanism that involves the release of endogenous tachykinins, the contractile activity of which reinforces or supplements leukotriene D4 receptor-mediated contraction of the guinea pig trachea (Bloomquist and Kream, 1990). The tachykinins, substance P and neurokinin A, are localized in unmyelinated sensory nerves, afferent C-fibers, innervating the airways (Lundberg et al., 1984). A variety of stimuli are known to activate C-fibers in the airways in vivo (Coleridge and Coleridge, 1984), and many of these, including bradykinin, capsaicin and histamine, elicit tachykinin release from guinea pig airways in vitro (Saria et al., 1988; Martins et al., 1991). Given that tachykinins, such as substance P and neurokinin A, exert contractile effects on the guinea pig airway smooth muscle (Saria et al., 1988; Buckner et al., 1991), it is reasonable to expect that tachykinins released by contractile agonists contribute to the overall contractile response. The possible contribution of endogenous tachykinins to the manifestation of contractions elicited by leukotriene D4 was investigated because there is evidence linking this agonist to tachykinins or tachykinin-containing nerves (Stewart et al., 1984; Bloomquist and Kream, 1990; Martins et al., 1991). In the present study, we evaluated the role of tachykinins by pharmacologically modulating the concentrations of endogenous tachykinins within guinea pig bronchus or by antago-
nizing neurokinin receptors that mediate tachykinin effects. The guinea pig bronchus was chosen because it possesses a denser tachykininergic nervous innervation than the guinea pig trachea (Manzini et al., 1989). The role of tachykinins in antigen-induced responses was also assessed, because contractions in the guinea pig airways are mediated primarily by leukotrienes and histamine (Undem et al., 1989), two mediators that have been shown to elicit the release of tachykinins (Saria et al., 1988; Bloomquist and Kream, 1990; Martins et al., 1991).

Capsaicin administered acutely to guinea pig airway tissue results in the abolition of tachykinin-mediated nonadrenergic noncholinergic excitatory responses induced by electrical field stimulation (Kizawa and Takayanagi, 1985; Thompson et al., 1987). This treatment had no effect on the concentration-response relationships for antigen (ovalbumin) or leukotriene D₄, which suggests that endogenous tachykinins of C-fiber origin (or other agents subject to capsaicin-induced depletion) do not contribute to the contractile responses elicited by these agents. To evaluate more directly the contribution of tachykinins to ovalbumin- and leukotriene D₄-induced contractions, we examined the inhibitory effects of newer, more selective antagonists of NK₁ (i.e., CP99994) and NK₂ (i.e., SR48968) receptors. In these studies, concentrations of CP99994 and SR48968 that abolished neurally mediated tachykinergic responses failed to influence contractions elicited by either ovalbumin or leukotriene D₄. These results contrast with those obtained by Bloomquist and Kream (1990), wherein the substance P antagonist [d-Pro⁴, d-Trp⁷,⁹]-substance P 4-11 was shown to inhibit leukotriene D₄-induced contractile responses in the guinea pig trachea. These variant results may reflect a tissue-dependent difference in the role of tachykinins in leukotriene D₄-induced contraction; Bloomquist and Kream utilized the trachea, whereas the present study was conducted in the bronchus. However, the possibility that [d-Pro⁴, d-Trp⁷,⁹]-substance P 4-11 acted as a leukotriene receptor antagonist was not excluded by Bloomquist and Kream (1990). The lack of inhibitory activity of CP99994 and SR48968 on leukotriene D₄ responses excluded such a possibility in the present study.

The airway contractile actions of the tachykinins are modulated by tissue enzymes, including NEP and ACE, such that the potency of the endogenously released or exogenously applied tachykinins is enhanced in preparations in which these enzymes are inhibited (Djokic et al., 1988; Thompson et al., 1989; Warner et al., 1990). Alterations in tissue expression of NEP or ACE also appear to regulate the biological activity of the tachykinins. For example, viral or cigarette smoke exposure enhances the airway smooth muscle contractile activity of endogenous and exogenous tachykinins by down-regulating NEP expression (Dusser et al., 1989a,b). To enhance the potential for tachykinin involvement in contractions, experiments were repeated in the presence of thiorphan and lisinopril, inhibitors of NEP and ACE, respectively. Initial studies verified that neurally mediated tachykinergic responses in the guinea pig bronchus were enhanced by such treatment, as were contractile responses induced by substance P and neurokinin A (Thompson et al., 1989). In the presence of both of these inhibitors, leukotriene D₄ was po-
tentiated, which suggests that tachykinins released by leukotriene D₄ contribute to the contractile response but normally do not do so as a result of rapid degradation by tissue proteases. It is notable that leukotriene D₄-induced responses were not affected by the individual application of thiorphan or lisinopril. A similar requirement for the combination of both inhibitors for enhancement of neurally mediated tachykininergic responses in the guinea pig bronchus has been noted previously (Thompson et al., 1989).

The potentiation of leukotriene D₄ by thiorphan and lisinopril provided circumstantial evidence in favor of a role for tachykinins. However, it is possible that other substances, such as calcitonin gene-related peptide, were protected from proteolysis by these inhibitors and contributed to the response (Martling et al., 1988; Tschirhart et al., 1990; Katayama et al., 1991). Accordingly, we further examined the role of neurally released tachykinins using other pharmacological tools shown in initial experiments to abolish neurally mediated tachykininergic responses. The potentiation of leukotriene D₄ was not prevented by either acute capsaicin desensitization or the selective neurokinin receptor antagonists, a result that argues strongly against tachykinins contributing to the enhancement of leukotriene D₄-induced responses by thiorphan and lisinopril. Clearly, had tachykinins been implicated in the potentiation, these treatments would have prevented it, particularly when the concentrations used were sufficient to prevent neurally mediated tachykininergic responses and/or elicit a 57-fold rightward shift of the substance P concentration-response curve. It should be noted that prevention by acute capsaicin desensitization of NEP inhibitor-induced enhancement of antigen-induced responses has been used as evidence of tachykinin involvement in the guinea pig bronchus (Kohrogi et al., 1991).

The enhancing action of thiorphan and lisinopril appeared specific for leukotriene D₄ because contractions induced by ACh or antigen were unaffected by these inhibitors. In guinea pig airways, leukotriene D₄ can be metabolized to leukotriene E₄ by an aminopeptidase (Snyder et al., 1984; Aharony et al., 1985). This represents a bioinactivation step insofar as the contractile potency of leukotriene D₄ in guinea pig airways is enhanced when conversion to leukotriene E₄ is prevented by L-cysteine, an inhibitor of the aminopeptidase (Snyder et al., 1984; Snyder and Krell, 1984). Inhibition of leukotriene D₄ metabolism by thiorphan and lisinopril could...
therefore underlie the observed potentiation. The present study reproduced the results of previous studies in that L-cysteine potentiated leukotriene D₄ in the bronchus. Under these conditions, however, thiorphan and lisinopril failed to affect leukotriene D₄-induced contractions. L-Cysteine did not prevent the combination of thiorphan and lisinopril from enhancing substance P-induced contractions of the bronchus (data not shown), and this excludes the possibility that L-cysteine was interfering with thiorphan or lisinopril in a physicochemical manner. Finally, leukotriene E₄-induced contractions were not altered by thiorphan and lisinopril, which makes it unlikely that the observed potentiation of leukotriene D₄ was due to protease inhibitor-induced potentiation of its metabolite, leukotriene E₄. Taken together, these results provide evidence that potentiation of leukotriene D₄ in the guinea pig bronchus involves inhibition of the conversion of leukotriene D₄ to leukotriene E₄.

Leukotrienes mediate, at least in part, contractions elicited by antigen in sensitized guinea pig airways (Undem et al., 1989; Ro et al., 1991). Given the potentiating action of lisinopril and thiorphan on leukotriene D₄, a similar effect might have been anticipated on antigen-induced responses. Indeed, NEP inhibitors have been shown previously to enhance contractions induced by a single ovalbumin concentration (10 μg/ml) in sensitized guinea pig bronchi (Kohrogi et al., 1991). In their studies, the NEP inhibitors, thiorphan and phosphoramidon, did not affect the peak of the antigen-induced contraction but rather augmented the waning phase of the antigen response, i.e., after attainment of the peak contraction. In the present study, thiorphan and lisinopril had no
Fig. 10. Protease inhibition has no effect on leukotriene E4-induced contractions. Leukotriene E4 was administered in a cumulative manner (closed circles). Responses were expressed as a percentage of the response to ACh (1 mM). Data represent the mean with one S.E.M. from six experiments.

**Table**: Protease inhibition has no effect on leukotriene E4-induced contractions. Leukotriene E4 was administered in a cumulative manner (closed circles). Responses were expressed as a percentage of the response to ACh (1 mM). Data represent the mean with one S.E.M. from six experiments.

**References**


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