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Effects of the cysteinyl leukotriene receptor antagonists pranlukast and zafirlukast on tracheal mucus secretion in ovalbumin-sensitized guinea-pigs *in vitro*

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1 We investigated the inhibitory effects of the cysteinyl leukotriene ($CysLT_1$) receptor antagonists, pranlukast and zafirlukast, on $^{35}SO_4$ labelled mucus output, *in vitro*, in guinea-pig trachea, induced by leukotriene D_4 (LTD_4) or by antigen challenge of sensitized animals. Agonists and antagonists were administered mucosally, except in selected comparative experiments where drugs were administered both mucosally and serosally to assess the influence of the epithelium on evoked-secretion.

2 LTD_4 increased $^{35}SO_4$ output in a concentration-related manner with a maximal increase of 23 fold above controls at 100 μM and an approximate EC_{50} of 2 μM. Combined mucosal and serosal addition of LTD_4 did not significantly affect the secretory response compared with mucosal addition alone. Neither LTC_4 nor LTE_4 (10 μM each) affected $^{35}SO_4$ output. Pranlukast or zafirlukast significantly inhibited 10 μM LTD_4-evoked $^{35}SO_4$ output in a concentration-dependent fashion, with maximal inhibitions of 83% at 10 μM pranlukast and 78% at 10 μM zafirlukast, and IC_{50} values of 0.3 μM for pranlukast and 0.6 μM for zafirlukast. Combined mucosal and serosal administration of the antagonists (5 μM each) gave degrees of inhibition of mucosal-serosal 10 μM LTD_4-evoked $^{35}SO_4$ output similar to those of the drugs given mucosally. Pranlukast (0.5 μM) caused a parallel rightward shift of the LTD_4 concentration-response curve with a pK_B of 7. Pranlukast did not inhibit ATP-induced $^{35}SO_4$ output.

3 Ovalbumin (10–500 μg ml^{-1}) challenge of tracheae from guinea-pigs actively sensitized with ovalbumin caused a concentration-related increase in $^{35}SO_4$ output with a maximal increase of 20 fold above vehicle controls at 200 μg ml^{-1}. The combination of the antihistamines pyrilamine and cimetidine (0.1 mM each) did not inhibit ovalbumin-induced $^{35}SO_4$ output in sensitized guinea-pigs. Neither mucosal (10 μM or 100 μM) nor mucosal-serosal (100 μM) histamine had any significant effect on $^{35}SO_4$ output.

4 Pranlukast or zafirlukast (5 μM each) significantly suppressed ovalbumin-induced secretion in tracheae from sensitized guinea-pigs by 70% and 65%, respectively.

5 We conclude that LTD_4 or ovalbumin challenge of sensitized animals provokes mucus secretion from guinea-pig trachea *in vitro* and this effect is inhibited by the $CysLT_1$ receptor antagonists pranlukast and zafirlukast. These antagonists may be beneficial in the treatment of allergic airway diseases in which mucus hypersecretion is a clinical symptom, for example asthma and allergic rhinitis.

Keywords: Adenosine 5′-triphosphate (ATP); cysteinyl leukotrienes; $CysLT_1$ receptors; mucus; mucus secretion; ovalbumin sensitization; percutaneous anaphylactic reaction (PCA); pranlukast; zafirlukast

Introduction

Secretion of high molecular weight mucous glycoproteins (mucus) onto the internal surface of the airways is an acute protective mechanism in response to inhaled irritants and noxious chemicals. In contrast, mucus hypersecretion is one of the features of airway inflammatory disorders such as asthma (Liu *et al*., 1998) and contributes to morbidity and mortality. Thus, modulating mucus secretion may be an important therapeutic strategy in chronic inflammatory airway disorders with airway obstruction and mucus hyperproduction.

Several lines of evidence suggest that cysteinyl leukotrienes (CysLTs) are putative mediators in asthma (see Hay *et al*., 1995). Of relevance to the present study is the demonstration that CysLTs are secretagogues in human, guinea-pig and cat airways *in vitro* and *in vivo* (Peatfield *et al*., 1982; Shelhamer *et al*., 1982; Nakata *et al*., 1997). Agents that block the action of CysLTs at their receptors or inhibit their formation are beneficial in the amelioration of asthma attacks (Chung, 1995). Recently, a $CysLT_1$ receptor antagonist, zafirlukast, has been shown to be effective in relieving symptoms, including rhinorrhea, in acute seasonal allergic rhinitis (Donnelly *et al*., 1995).

Two broad subgroups of CysLT receptors are currently recognized (Alexander & Peters, 1997). $CysLT_1$ receptors are characterized by a relative order of potency of $LTD_4 = LTC_4 > LTE_4$, whereas $CysLT_2$ receptors are characterized by an order of potency of $LTC_4 > LTD_4 \gg LTE_4$. A number of $CysLT_1$ receptor antagonists are now available, including pranlukast (ONO-1078) (Nakai *et al*., 1988) and zafirlukast (ICI 204,219) (Krell *et al*., 1990). $CysLT_2$ receptor antagonists are also being developed, for example Bay u9773 (Back *et al*., 1996).

The aim of the present study was to investigate the inhibitory effect of selective $CysLT_1$ receptor antagonists, pranlukast and zafirlukast, on *in vitro* tracheal mucus output induced by CysLTs in unsensitized guinea-pigs and by ovalbumin in sensitized guinea-pigs. We used the guinea-pig trachea to explore control of mucus secretion because: (1) guinea-pigs have well defined secretory apparatus (Jeffery, 1983) and (2) guinea-pigs are sensitive to ovalbumin, which is an established model for the study of allergic mucus secretion (Andersson, 1980; Nakata *et al*., 1997). We used $^{35}SO_4$ as a mucus marker

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(Ramnarine *et al*., 1994). We also investigated whether or not the epithelium acted as a barrier to drug diffusion.

Methods

Tissue preparation

Male Dunkin-Hartley guinea-pigs (Harlan, Bicester, Oxon.) weighing 400–600 g were kept 5–6 to a cage and allowed one week to recuperate after delivery. Animals had free access to food (standard guinea-pig diet) and water. They were terminally anaesthetized with pentobarbitone sodium (Sagatal: 60 mg kg^{-1}, i.p.) and the tracheae were removed, cleared of adventitia and bathed in aerated (95% O_2 : 5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_2$ 25.5 and glucose 5.05 (pH 7.4 after aeration). Tracheae were cut longitudinally through the dorsal membrane, opened flat and cut transversely to give four segments. Each segment was pinned and clamped across the aperture separating the two halves of perspex Ussing-type chambers so that the tissue divided the chambers into mucosal ('luminal': i.e. mucus-producing) and 'submucosal' (serosal) sides. The exposed surface area of each segment was 0.28 cm^2. Each side of the tissue was bathed with 4 ml warmed (37°C) Krebs-Henseleit solution which was aerated (95% O_2 : 5% CO_2) and circulated using gas-lift pumps.

At time 0 h, $Na_2{}^{35}SO_4$ (0.1 mCi; Amersham International plc, Aylesbury, Bucks.) was added to the submucosal half-chambers, to label newly-synthesized intracellular mucus, where it remained throughout the experiment. At unit time intervals, the fluid in the luminal side of the chamber (containing secretions) was collected and the half chamber was replenished with 4 ml fresh Krebs-Henseleit solution (Meini *et al*., 1993; Ramnarine *et al*., 1994).

Measurement of $^{35}SO_4$-labelled macromolecule output

The collected luminal fluid, ~4 ml and comprising secretions in Krebs-Henseleit solution, was drained into tubes containing 5.4 g guanidine hydrochloride, to dissolve the mucus; the final concentration of guanidine hydrochloride in the fluid was 6 M. Following this, each sample was dialyzed exhaustively against distilled water containing excess Na_2SO_4 and sodium azide (10 mg l^{-1}), to remove unbound $^{35}SO_4$, using cellulose tubing (Medicell International Ltd., London) which allowed molecules of 12–14 KDa or less to pass through. Sodium azide was used to prevent bacterial degradation of the mucus. The samples were recovered after at least six changes of distilled water when the radioactive count of the dialysis water was the same after dialysis as before dialysis (~15 disintegrations per minute (d.p.m.)). The recovered samples were weighed and the radioactivity in 1 ml duplicates of each sample mixed with 2 ml scintillant (Ultima Gold XR, Canberra Packard Ltd., Pangbourne, Berks.) was determined by scintillation spectrometry (model 1900CA Spectrophotometer, Canberra Packard Ltd., Pangbourne, Berks.). The total radioactivity of each sample was determined by multiplying the radioactivity present in a 1 ml aliquot of that sample by the total weight of the sample (assuming a 1 ml sample weighs 1 g).

Ovalbumin sensitization and challenge

Male Dunkin-Hartley guinea-pigs weighing 400–450 g were actively sensitized by a single intraperitoneal injection of 0.7 ml of normal saline containing 30 μg hen ovalbumin (Grade III) and 150 mg aluminium hydroxide (Andersson, 1980). Control (sham-sensitized) animals were treated with a single intraperitoneal injection of 0.7 ml normal saline containing 150 mg aluminium hydroxide. Both control and sensitized animals were kept under the same conditions for 3 weeks before use.

For ovalbumin challenge, animals were terminally anaesthetized and the tracheal tissue prepared as above. The tissue was challenged at 2.5 h by adding ovalbumin 10–500 μg ml^{-1} to the luminal half chambers with samples collected every 15 min thereafter.

Passive cutaneous anaphylaxis reaction

Passive cutaneous anaphylactic reactions (PCA) were performed according to the method of Watanabe & Ovary (1977). Blood (~5 ml per animal) was obtained by cardiac puncture from normal, non-sensitized guinea-pigs, the sham-sensitized group and the ovalbumin-sensitized group. Serum was collected 24 h after clot retraction, and was centrifuged (400 *g* for 5 min) and pooled for each group. Aliquots of serum were stored frozen at −20°C before use. To differentiate IgE (heat labile) from IgG (heat resistant) antibodies, a sample of serum from the ovalbumin-sensitized group was maintained at 56°C for 4 h in a water bath (Perini & Mota, 1972). Non-sensitized guinea-pigs weighing 400–450 g were shaved with an electric hair clipper on both flanks and 1 h later marked skin sites were injected intradermally with 0.1 ml undiluted non-sensitized, sham-sensitized or pre-heated ovalbumin-sensitized serum. Unheated ovalbumin-sensitized serum was diluted accordingly and 0.1 ml injected intradermally in the non-sensitized animals. On day 8, these animals were challenged by an intravenous injection, via a pedal vein, of 0.4 ml normal saline containing 2 mg ovalbumin together with 10 mg kg^{-1} Evans blue dye. Day 8 represents a latent period of greater than 72 h which will differentiate IgE from IgG (Perini & Mota, 1972). Twenty to 30 min later, the animals were terminally anaesthetized, the skin was removed and inverted, and the lesion diameter, dyed with Evans blue, was measured on the inner surface using a transparent ruler. If the diameter of reaction area was equal to or greater than 5 mm, a positive PCA response was indicated. IgE antibody titre was estimated by determining the highest dilution of anti-serum causing a positive PCA reaction.

Protocols for secretory studies in non-sensitized guinea-pigs

To define baseline secretion and establish optimal conditions under which collections were taken after the addition of drugs and control solutions, at 0 h, $Na_2{}^{35}SO_4$ 0.1 mCi was added to the submucosal half-chambers, where it remained throughout the experiment. The fluid in the luminal side of the chamber (containing secretions) was collected and replaced with fresh Krebs-Henseleit solution every 30 min during the first 2 h, followed by every 15 min during the next 4 h (6 h in total). All test drugs were added to the luminal half-chamber unless stated otherwise. To determine secretory potential and tissue viability, adenosine 5′-triphosphate (ATP) 1 mM (Kim *et al*., 1993) was administered to the luminal side at 2.5 h and again at 5.75 h (i.e. 15 min before the end of the experiment).

To determine the optimal LTD_4 concentration for investigation of the inhibitory effect of the CysLT receptor antagonists on mucus secretion, LTD_4 (0.1–100 μM) or vehicle (distilled water) was added to the luminal side at 2.5 h. To

determine whether or not the epithelium was a barrier to drug diffusion between the mucosal and serosal halves of the Ussing chambers, the effect on secretion of mucosal administration of LTD_4 was compared with combined mucosal and serosal administration.

To study the effects of CysLT receptor antagonists on stimulated secretion induced by LTD_4 and to determine the optimal concentration of antagonist for the ovalbumin challenge study in ovalbumin-sensitized guinea-pigs, pranlukast, zafirlukast (0.1–10 μM each) or vehicle (DMSO) were added to the luminal chamber 30 min before the administration of a submaximal concentration of LTD_4 (10 μM). To determine whether or not the epithelium acted as a barrier to antagonist diffusion, mucosal administration was compared with mucosal-serosal administration (at 5 μM for each drug).

To investigate the potency of pranlukast, tissues were pretreated with pranlukast 0.5 μM for 30 min before the addition of LTD_4 (0.1–100 μM). To elucidate whether pranlukast affected non-LTD_4-induced mucus secretion, pranlukast (5 μM) was administered 30 min before the addition of ATP (1 mM).

We also examined the effect of LTC_4 or LTE_4 (10 μM each) on secretion; studies with LTC_4 were conducted with or without borate-serine complex (boric acid and L-serine, 50 mM each), an inhibitor of γ-glutamyl transpeptidase, the enzyme involved in the conversion of LTC_4 to LTD_4 (Buckner *et al*., 1990).

Protocols for secretory studies in sensitized guinea-pigs

To determine the concentration of ovalbumin and the collection time points optimal for ovalbumin-induced mucus secretion, ovalbumin (10–500 μg ml^{-1}) or vehicle (normal saline) was added to the luminal side at 2.5 h and luminal fluid ($\sim$4 ml) was collected and replenished with fresh Krebs-Henseleit solution containing ovalbumin every 15 min for 1 h.

To study the effects of the CysLT receptor antagonists on ovalbumin-induced mucus secretion in sham- and ovalbumin-sensitized guinea-pigs, tissue was pretreated with pranlukast, zafirlukast (5 μM for both) or vehicle 30 min before the administration of ovalbumin (200 μg ml^{-1}).

To elucidate the influence of endogenous histamine in evoking mucus secretion in ovalbumin-sensitized guinea-pigs, the effects of the antihistamines cimetidine and pyrilamine (0.1 mM for both) in combination against ovalbumin-induced secretion, or effect of exogenous histamine (10–100 μM) alone, were examined; the antagonists or histamine were administered to the luminal half chamber at 2.5 h and samples collected 15 min later. The effect on secretion of combined mucosal-serosal administration of histamine (100 μM) was also investigated.

Drugs

The following drugs were used: ATP, boric acid, L-serine, LTE_4, chicken egg albumin (ovalbumin, grade III), aluminium hydroxide, dimethyl sulphoxide (DMSO), histamine, pyrilamine (Sigma Chemical Company Ltd., Poole, Dorset); pentobarbitone sodium B.P. (Sagatal; RMB Animal Health Ltd., Dagenham, Essex); cimetidine (Tagamet; SmithKline Beecham Pharmaceuticals, Welwyn Garden City, Hertfordshire). Pranlukast, zafirlukast, LTC_4 and LTD_4 were kind gifts from SmithKline Beecham (King of Prussia, Pennsylvania, U.S.A.). Pranlukast, also known as ONO-1078 (Hay, 1997), has the chemical formula 4-oxo-8-[4-(4-phenylbutoxy)benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran-8-yl]-4-(4-phenylbutoxy) benzamine (Nakai *et al*., 1988). The chemical formula of zafirlukast is (4-5(-cyclopentyloxycarbonylamino-1-methylindol-3-ylmethyl)-3-methoxy-*N*-*o*-toly-sulfonylbenzamide) (Krell *et al*., 1990).

Solutions of ATP, boric acid, L-serine, pyrilamine, histamine, ovalbumin and aluminium hydroxide, dissolved in distilled water or normal saline, were made fresh on each day, $\sim$1 h before experimentation. LTE_4 stock solution (0.11 mM) was kept at -70°C and its vehicle was methanol : water (70 : 30) solution containing 17 mM acetate buffer adjusted to pH 5.4 with NH_4OH. Stock solutions of pranlukast and zafirlukast were both 1 mM and their vehicle was DMSO, divided into aliquots and stored at -20°C. The stock solutions of LTC_4 and LTD_4 were both approximately 6.4 mM, dissolved in distilled water, and kept at 4°C on ice before use. Exact concentrations of LTC_4 and LTD_4 were calibrated by ultraviolet spectroscopy at 282 nm before experimentation.

Data analysis

Data in Results are presented as the arithmetic mean and one standard error of the mean (s.e.mean), with n values being the number of animals. Because of the variability in baseline output of $^{35}SO_4$ between tracheal segments, secretory responses obtained from individual segments were calculated as percentage changes in radiolabel output for the difference between response to drug or vehicle and the preceding collection. Statistical comparisons for concentration-response effects were determined using the Kruskal-Wallis test followed by Dunn's multiple comparison test if $P<0.05$, and non-parametric two-way analysis of variance for comparison between experimental groups. The null hypothesis was rejected at $P<0.05$ for all tests. The concentrations of agonist or antagonist causing 50% stimulation (EC_{50}) or inhibition (IC_{50}) were calculated by non-linear regression using GraphPad Prism software (San Diego, CA, U.S.A.).

Results

Median baseline radioactivity in the following series of experiments was of the order of 300 d.p.m. (range 130–498 d.p.m., depending upon the specific experiment). There were no significant differences between treatment groups. In the following, drug administrations are to the luminal half-chamber (mucosal), unless stated otherwise.

Baseline secretion and the effect of ATP

In tissues incubated for 5.75 h, baseline $^{35}SO_4$ output increased by 173% over the first 2.25 h, after which it remained stable for the remaining 3.5 h (Figure 1). ATP (1 mM), added to the luminal side to test secretory potential and tissue viability, increased $^{35}SO_4$ output above baseline by 65% at 2.5 h and 76% at 5.75 h, values which were not significantly different from each other (Figure 1).

Effect of CysLTs on secretion

LTD_4 increased $^{35}SO_4$ output in a concentration-dependent manner (Figure 2). There was no significant difference between mucosal administration of LTD_4 and combined mucosal-serosal administration: 23 fold and 26 fold increases above controls at 100 μM LTD_4 with approximate EC_{50}s of 2.0 μM and 4.5 μM after mucosal administration and mucosal-serosal administration, respectively. We chose 10 μM LTD_4 as a

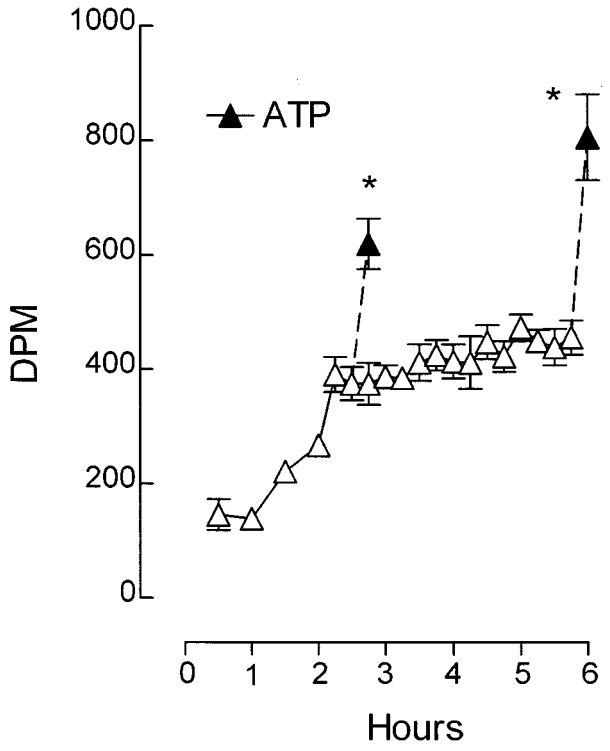

Figure 1 Time course of baseline mucus secretion in guinea-pig trachea *in vitro*, and the effect of ATP (1 mM). Data are mean disintegrations per minute (d.p.m.) for $^{35}SO_4$ output (a marker for mucus). Vertical lines show s.e.mean ($n=5$ for all groups). *$P<0.05$ compared with baseline $^{35}SO_4$ output immediately before the addition of ATP.

submaximal concentration for investigation of the inhibitory effects of pranlukast and zifirlukast on LTD_4-induced secretion.

LTC_4 (10 μM), either alone or after pretreatment with borate-serine complex (50 mM), did not significantly increase $^{35}SO_4$ output (2 and 1 fold increases above control, respectively, $n=8$ for both). Similarly, LTE_4 10 μM did not significantly increase $^{35}SO_4$ output (2 fold increase, $n=7$, which was indistinguishable from vehicle controls).

Effect of pranlukast and zafirlukast on LTD_4-induced secretion

Both pranlukast and zafirlukast significantly inhibited 10 μM LTD_4-induced $^{35}SO_4$ output in a concentration-dependent fashion, with maximal inhibitions of 83% at 10 μM pranlukast and 78% at 10 μM zafirlukast, and IC_{50} values of 0.3 μM for pranlukast and 0.6 μM for zafirlukast (Figure 3, left panel). DMSO (vehicle for antagonists) had no significant effect on LTD_4-induced $^{35}SO_4$ output (compare a and b in Figure 2). The degree of inhibition of mucosal 10 μM LTD_4-induced $^{35}SO_4$ output by 5 μM pranlukast (IC_{85}) or zafirlukast (IC_{75}) was not significantly different from the degree of inhibition of mucosal-serosal LTD_4-induced secretion afforded by this same concentration of antagonist administered mucosally-serosally (Figure 3, right panel). We chose 5 μM pranlukast and zafirlukast as the concentration for examination of their effects on ovalbumin-induced mucus secretion in sensitized guinea-pig tracheae. We chose 0.5 μM pranlukast ($\sim IC_{50}$) as the concentraiton for the investigation of LTD_4 *versus* pranlukast competition curves.

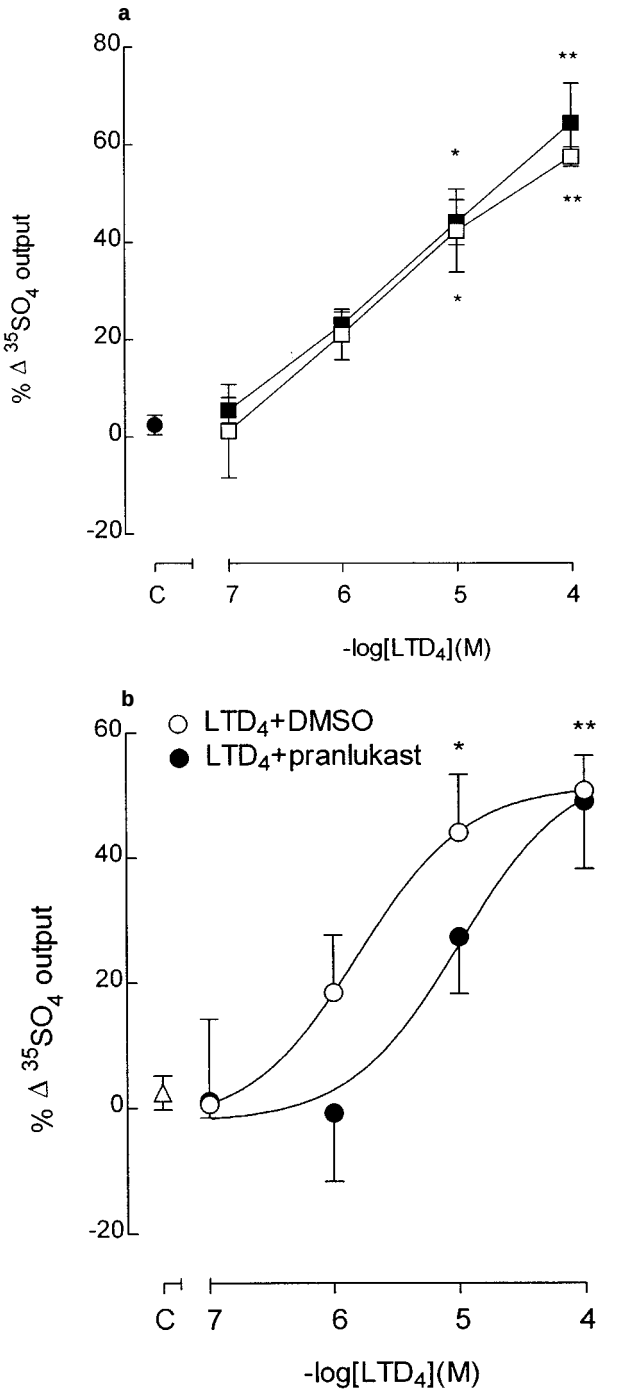

Figure 2 Effect of leukotriene D_4 (LTD_4) on mucus secretion in guinea-pig trachea *in vitro* and inhibition by the cysteinyl LT receptor antagonist, pranlukast. (a) Comparison of administration of LTD_4 mucosally (open squares; $n=5-6$) or mucosally-serosally combined (solid squares; $n=3-4$). C, control ($n=4$). (b) Effect of pranlukast (0.5 μM) on LTD_4-induced secretion ($n=5-6$). Dimethyl sulphoxide (DMSO) was the vehicle for pranlukast. Data are mean % change in $^{35}SO_4$ output (a marker for mucus). Vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$ compared with distilled water (vehicle for LTD_4).

LTD_4 versus *pranlukast competition curves*

Pranlukast (0.5 μM) caused a parallel rightward shift of the LTD_4 concentration-response curve with a pK_B of 7.0 (Figure 2b).

Effect of ATP on secretion

ATP (1 mM) significantly increased $^{35}SO_4$ output by 22 fold above control (Figure 4). Pre-incubation with pranlukast for 30 min did not significantly effect ATP-induced $^{35}SO_4$ output (21 fold increase above control) (Figure 4).

Concentration-dependent ovalbumin-induced secretion in sensitized guinea-pigs

Ovalbumin challenge of tracheae from sensitized guinea-pigs caused a concentration-related increase in $^{35}SO_4$ output with a maximal increase of 8 fold above vehicle controls at 200 μg ml^{-1}, measured at 30 min, an effect which was significantly higher than that in sham-sensitized guinea-pigs (Figure 5a). In sham-sensitized animals, the response to ovalbumin challenge was indistinguishable from vehicle controls (Figure 5a). The concentration of 200 μg ml^{-1} ovalbumin was chosen for the time course experiments.

Time course of ovalbumin-induced secretion

In tracheal segments from sensitized guinea pigs, ovalbumin (200 μg ml^{-1}) increased $^{35}SO_4$ output over the first 45 min after challenge (Figure 5b) compared with the sham-sensitized animals, with a maximal increase in $^{35}SO_4$ output of 20 fold above sham-treated animals. At 60 min after challenge, $^{35}SO_4$ output was reduced to approximately sham control values (Figure 5b).

Effects of pranlukast and zafirlukast on ovalbumin-induced secretion

Ovalbumin 200 μg ml^{-1} challenge significantly increased $^{35}SO_4$ output in sensitized tracheae compared with that in sham-sensitized tracheae, with a maximal increase of 20 fold above sham tracheae at 45 min (Figure 5c). The response to ovalbumin in sensitized tracheae was significantly suppressed by pranlukast or zafirlukast (5 μM for each) by 70% and 65%, respectively, at 45 min; there was no statistical difference between the effects of the two drugs (Figure 5c).

Effects of antihistamines and histamine in ovalbumin-sensitized guinea-pigs

The combination of cimetidine and pyrilamine (0.1 mM for each) did not influence ovalbumin (200 μg ml^{-1})-induced $^{35}SO_4$ output in sensitized guinea-pigs compared with vehicle controls (% Δ in $^{35}SO_4$ output: 2.3±5.4% vs 6.5±3.2% at 15 min, 21.3±13.8% vs 24.0±8.6% at 30 min, and 36.8±0.5% vs 32.3±8.4% at 45 min; n=4–8). Histamine had no significant effect on secretion, either when administered mucosally (% Δ in $^{35}SO_4$ output: controls 5.2±3.9%; histamine 10 μM, 8.6±4.9%; histamine 100 μM, 11.4±5.3%, n=6 each) or mucosally-serosally (histamine 100 μM, 9.7±5.5%, n=4).

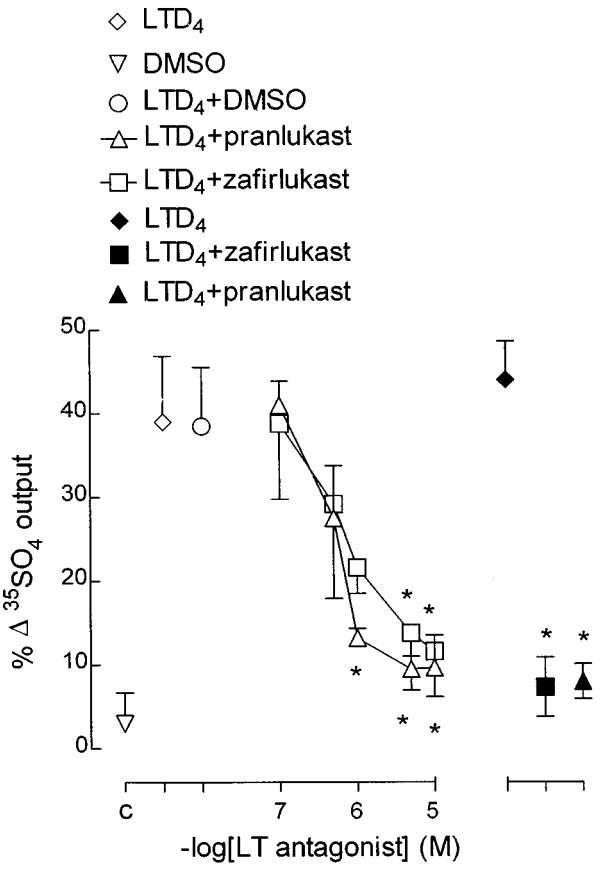

Figure 3 Effects of the cysteinyl leukotriene receptor antagonists, pranlukast or zafirlukast, on mucus secretion induced by leukotriene D_4 (LTD_4 10 μM) in guinea-pig trachea *in vitro*. Left panel (open symbols): mucosal administration of drugs (n=6 for all groups). Right panel (solid symbols): combined mucosal-serosal administration of drugs (n=4–5); pranlukast and zafirlukast, 5 μM each. Data are mean % change in $^{35}SO_4$ output (a marker for mucus). Vertical lines show s.e.mean. *P<0.05 compared with LTD_4+DMSO group (dimethyl sulphoxide, vehicle for pranlukast and zafirlukast).

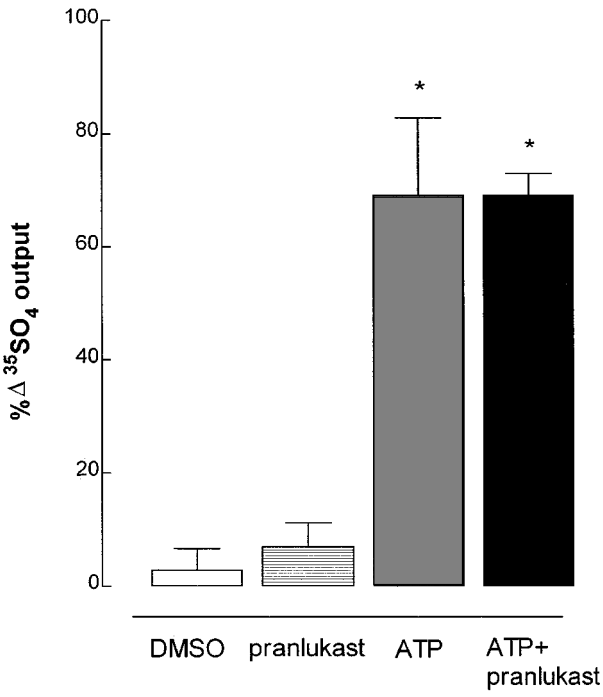

Figure 4 Effect of a cysteinyl leukotriene receptor antagonist, pranlukast (5 μM), on mucus secretion induced by ATP (1 mM) in guinea-pig trachea *in vitro*. Data are mean % change in $^{35}SO_4$ output (a marker for mucus). Vertical lines show s.e.mean (n=5–6 for all groups). Dimethyl sulphoxide (DMSO) was the vehicle for pranlukast. *P<0.05 compared with distilled water group (vehicle for ATP).

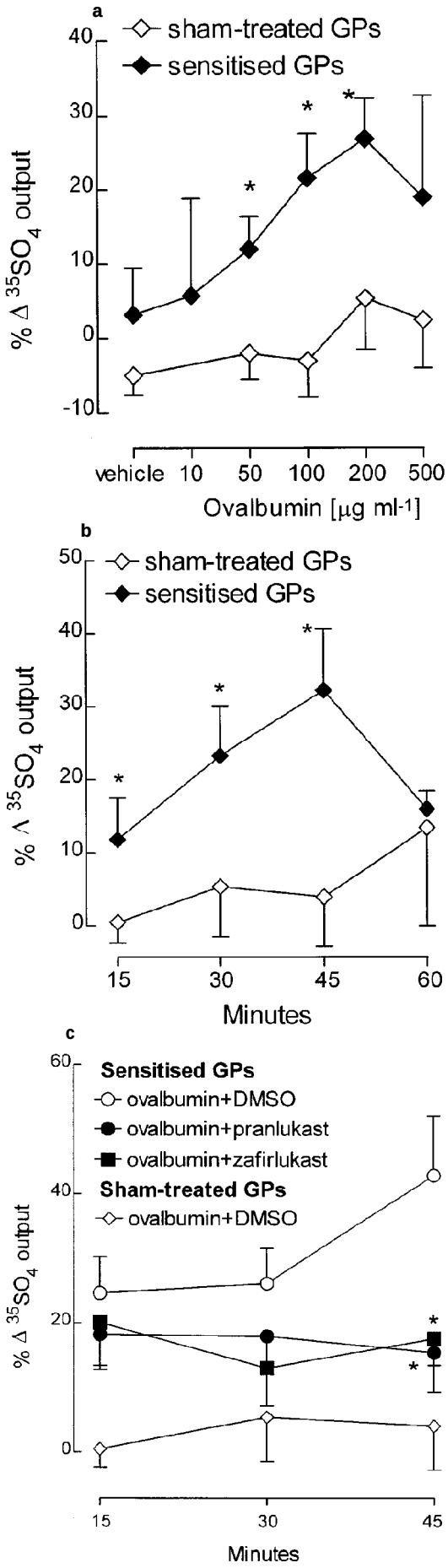

Figure 5 Ovalbumin-induced mucus secretion *in vitro* in tracheae from ovalbumin-sensitized guinea-pigs and the effect of cysteinyl leukotriene (CysLT) receptor antagonists. Data are mean % change in $^{35}SO_4$ output (a marker for mucus). Vertical lines show s.e.mean. (a) Concentration-response curve for effect of ovalbumin challenge in sensitized and sham-sensitized tracheae at 30 min after challenge ($n=5-6$ for all groups), $^*P<0.05$ compared with equivalent concentration of ovalbumin in sham-sensitized group. (b) Time course of secretion induced by ovalbumin (200 μg ml^{-1}) challenge in sensitized and sham-sensitized tracheae ($n=6-8$ for all groups). $^*P<0.05$ compared with equivalent time point in sham-sensitized group. (c) Inhibitory effect of the CysLT receptor antagonists pranlukast or zafirlukast (5 μM each) on secretion induced by ovalbumin (200 μg ml^{-1}) challenge in sensitized and sham-sensitized tracheae ($n=7-13$ for all groups). Dimethyl sulphoxide (DMSO) was the vehicle for the antagonists. $^*P<0.05$ compared with ovalbumin + DMSO at equivalent time time point.

PCA reaction

The pooled, undiluted non-sensitized ($n=6$), sham-sensitized ($n=6$) or pre-heated ovalbumin-sensitized ($n=8$) serum elicited no reaction in non-sensitized guinea-pig skin ($n=3$). The diluted serum (both 1 : 2 and 1 : 8) provoked a reaction in which the Evans blue diameter was greater than 5 mm in different sites in three different guinea-pigs.

Discussion

In the present study, stimulation by LTD_4 and by ovalbumin challenge in guinea-pig tracheae *in vitro* significantly increased $^{35}SO_4$ output and this response was inhibited by the $CysLT_1$ receptor antagonists pranlukast and zafirlukast. The secretory apparatus in guinea-pig trachea is predominantly goblet cells (Jeffery, 1983) with some submucosal glands. The $^{35}SO_4$ is localized to the goblet cells in cat, goose and ferret trachea (Lamb, 1975; Phipps *et al*., 1977; Gashi *et al*., 1987) and the submucosal gland cells in cat and ferret trachea (Gashi *et al*., 1987; Davies *et al*., 1990), and is released upon stimulation (Gashi *et al*., 1987). Thus, in the present study, the increase in $^{35}SO_4$ output is consistent with an increase in mucus secretion by the goblet cells and, to a lesser extent, submucosal glands in the guinea-pig tracheal segments.

In the present study, over the 6 h incubation with $^{35}SO_4$ we observed a rapid increase in baseline mucus secretion during the first 2.25 h followed by stable secretion over the remaining 3.5 h. This is similar to previous data showing that over a period of $\sim$5 h incubation with $^{35}SO_4$ in ferret trachea, basal secretion increased markedly during the first 2 h followed by a smaller rate of increase over the remaining 3 h (Borson *et al*., 1984; Meini *et al*., 1993). We found herein that ATP increased mucus secretion when given at the beginning of stabilization of baseline and gave a reproducible increase in secretion when given at the end of baseline incubation period. ATP is an established secretagogue in inducing mucus secretion from goblet cells in primary airway epithelial cell culture of humans and hamsters (Lethem *et al*., 1993; Kim *et al*., 1993), and from isolated submucosal glands of cats (Shimura *et al*., 1994). The stable baseline of $^{35}SO_4$ output and reproducible secretion potential during the latter 3.5 h demonstrate that a balance had been reached between labelling and spontaneous release of $^{35}SO_4$ over this period, and this period is optimal for the investigation of drug effects in this *in vitro* preparation of guinea-pig trachea.

We found that LTD_4 increased $^{35}SO_4$ output in a concentration-dependent manner with an increase of $\sim$40% above baseline at 10 μM (EC_{50} of $\sim$2.0 μM). This compara-

tively weak effect of LTD_4 is consistent with previous *in vitro* studies where mucus output was increased by ~40% by 20 ng ml^{-1} LTD_4 in human bronchial explants (Marom *et al*., 1982) and by 24% by 30 μM LTD_4 in hamster tracheal epithelial cells (Kim *et al*., 1989). In the latter two studies in culture, the low potency of the LTD_4 is unlikely to be due to restricted access of drug to the secretory cells because both mucosal and serosal surfaces were bathed in drug solution. In contrast, in the present study using Ussing chambers, LTD_4 was administered to the mucosal surface only, which may have reduced its potency. Guinea-pig tracheal epithelium is a barrier to smooth muscle contractile agents, including cholinomimetic drugs, histamine and hypertonic KCl (Munakata *et al*., 1989; Fedan & Frazer, 1992). In the present study, we found that there was no significant difference in mucus output between the LTD_4 given mucosally or mucosally-serosally. This is similar to the observation *in vivo* in guinea-pig trachea that there is no difference in granule fractional volume, a morphometric measure of goblet cell mucus secretion, between aerosol and intravenous administration of LTD_4 (Hoffstein *et al*., 1990). Thus, in terms of guinea-pig tracheal mucus secretion, the latter observation and our present data indicate either, (1) that the epithelium is not a barrier to LTD_4, (2) that the principal site of action of the drug is the surface epithelial goblet cells rather than the submucosal glands, (3) that the principal source of mucus is the goblet cells rather than the comparatively sparse submucosal glands, or (4) a combination of these options. Whatever the explanation, the studies above and our present data indicate that LTD_4 is not a particularly potent secretagogue in the airways. Similarly, in the present study, LTC_4 (10 μM), either alone or in the presence of borate-serine complex, elicited minimal mucus secretion. This is consistent with its lack of effect in inducing mucus output in cat trachea *in vitro* (Peatfield *et al*., 1982), and its low potency in inducing secretion in human cultured airway explants (Shelhamer *et al*., 1982) or cat trachea *in vivo* (Peatfield *et al*., 1982). In the present study, LTE_4 (10 μM) also caused minimal mucus release. We know of no other study of the effect of this LT on mucus output. The lack of effect of LTE_4 is compatible with the relative order of potencies of the CysLTs for $CysLT_1$ receptors (i.e. $LTD_4 = LTC_4 > LTE_4$) (Alexander & Peters, 1997). In contrast to the weak effects of CysLTs on mucus secretion, LTD_4, C_4 and E_4 are considerably more potent in inducing guinea-pig tracheal smooth muscle contraction (Buckner *et al*., 1990). The reason for the discrepancy in potency between secretion and smooth muscle contraction is unclear, but may be due to differential receptor number and affinity between the two tissues. In an autoradiographic study of guinea-pig airways, LTC_4 had a high concentration of binding sites in the tracheal epithelium and smooth muscle, whereas there was scanty LTD_4 binding (Carstairs *et al*., 1988), despite the observations that LTD_4 is a potent bronchoconstrictor and inducer of airway hypersensitivity (Buckner *et al*., 1990; Kurosawa *et al*., 1994). High radio-density might be an over-estimate of receptor number, because LTC_4 not only binds to its receptors but also binds to intracellular isoenzymes of glutathione-*S*-transferase and to mitochondrial membranes (Sun *et al*., 1986; Chau *et al*., 1986).

In our present study, LTD_4-induced mucus secretion was significantly inhibited by pranlukast and zafirlukast with IC_{50} values of 0.3 and 0.6 μM, respectively. Furthermore, pranlukast inhibited the LTD_4 concentration-response curve with a pK_B of 7. The pK_B values for pranlukast are ~10 for LTD_4-induced tracheal contraction and ~8 for LTD_4-induced lung strip contraction in guinea-pigs (Obata *et al*., 1992). The pK_B for zafirlukast in attenuating LTD_4-induced airway contraction is ~9.5 in guinea-pigs (Krell *et al*., 1990) and ~8.5 in human intralobar airways (Buckner *et al*., 1990). The reason for the difference in potency of the two antagonists between inhibition of mucus secretion and smooth muscle contraction is unclear, but is unlikely to be due to an epithelial barrier effect (see above) because mucosal and mucosal-serosal administration of 5 μM of either drug gave similar degrees of inhibition of LTD_4-induced secretion. Pranlukast produced a parallel rightward shift of the LTD_4 concentration-response curve which is consistent with pranlukast being a selective $CysLT_1$ receptor antagonist.

Sensitization of guinea-pigs to ovalbumin is established as an animal model of allergy. In the present study, we used a protocol of sensitization with low amounts of ovalbumin together with alum adjuvant which produces both IgG_1 and IgE antibodies (Andersson, 1980). We used PCA to assess whether or not our animals were sensitized. Depending upon the sensitization protocol, and the timing of PCA assay, IgG_1 and/or IgE antibodies can mediate the PCA reaction (Ovary *et al*., 1963; Undem *et al*., 1985). In our chosen sensitization protocol, IgE antibodies are considered to play an important role in the anaphylactic reaction (Andersson, 1980). Thus, we timed our PCA assay to detect IgE antibodies (Perini & Mota, 1972). Our PCA results demonstrated no detectable reaction in the controls, with sensitization only in ovalbumin-treated animals. The heat lability of the sera indicated the presence of IgE antibody.

In terms of mucus secretion, we found that as the concentration of ovalbumin elevated, the output of mucus increased accordingly, with a maximal increase of 40% above control animals at 200 μg ml^{-1} at 45 min. This is consistent with the observation that supernatant fluids from sensitized peripheral human lung significantly increased output of radiolabelled mucus markers from human airways by 25–40% above control, and ragweed pollen increased secretion in IgE-sensitized human airways by 36% (Shelhamer *et al*., 1980). Furthermore, intravenous challenge by ovalbumin increased output of fluorescent fucose residues (a mucus marker) in a concentration-dependent fashion in sensitized guinea-pigs *in vivo* with a maximal increase of 120% above control at 30–45 min (Yeadon *et al*., 1995). Ovalbumin challenge also evoked mucus secretion, estimated by a morphometric method, in guinea-pig airways in a concentration-dependent manner with a maximal increase of 6 fold in secretion (Nakata *et al*., 1997). Studies using both radiolabelling and chemical detection methods in parallel revealed that the size and quantity of the mature mucins could be under-estimated using radioactivity alone (Sheehan *et al*., 1995; 1996). Thus, the difference in mucus output induced by allergen among the above studies might be due to species variation, individual study designs and different methods to evaluate mucus release.

In our present study, the peak effect of mucus release after allergen challenge was at 45 min. The time course of CysLT release in response to allergen challenge in human lung parenchymal fragments *in vitro* peaked for LTC_4 at 5 min and at 15 min for both LTD_4 and LTE_4 (Salari *et al*., 1985). The present time-course of secretory response after allergen exposure is consistent with the generation of LTD_4 and its subsequent effect on mucus output.

Allergen (200 μg ml^{-1})-induced mucus secretion was not attenuated by the combination of pyrilamine and cimetidine, at appropriate concentrations (Shelhamer *et al*., 1980), which is consistent with the lack of inhibitory effect of antihistamines on allergen-induced bronchoconstriction in sensitized guinea-pigs (Ashida *et al*., 1987). In the latter study, a 5-lipoxygenase inhibitor attenuated the antigen-induced bronchoconstriction.

Consistent with the lack of effect of the antihistamines herein, we found minimal mucus output in response to histamine administration, whether administered mucosally or mucosally-serosally, which is consistent with the lack of effect of an aerosol of histamine in inducing goblet cell discharge in guinea-pig trachea (Hoffstein *et al*., 1990). Thus, it would appear that histamine is not a principal mediator of certain antigen-induced airway responses in guinea-pigs. In contrast, histamine did cause mucus secretion from human airways via a mechanism which was inhibited by cimetidine (Shelhamer *et al*., 1980), which suggests that there are species differences in the influence of histamine on mucus secretion in the airways.

In the present study, pranlukast or zafirlukast significantly suppressed ovalbumin-challenged mucus secretion in sensitized guinea-pigs. A 5-,15-lipoxygenase inhibitor, BW B70C, has been shown to inhibit glycoconjugate release substantially from sensitized guinea-pig trachea, whereas a 5-lipoxygenase inhibitor, ZD-2138, did not (Yeadon *et al*., 1995). Several studies, including the present one, indicate that the products of 5-lipoxygenase, CysLTs and prostanoids are important secretagogues in human, cat, dog and guinea-pig airways (Peatfield *et al*., 1982; Shelhamer *et al*., 1982; Hoffstein *et al*., 1990; Nakata *et al*., 1997).

In summary, we have shown that LTD_4 and ovalbumin provoke mucus secretion from guinea-pig trachea *in vitro*, via a mechanism which is inhibited by the $CysLT_1$ receptor antagonists, pranlukast and zafirlukast. In addition to asthma and allergic rhinitis (Busse, 1996), CysLT concentrations are raised in sputum from subjects with other hypersecretory disorders, including chronic bronchitis, bronchiectasis and cystic fibrosis (Zakrzewski *et al*., 1987a,b). Initial clinical trials have shown that both pranlukast and zafirlukast are beneficial in ameliorating asthma attacks induced by various challenges, including allergen, aspirin or exercise, and in improving lung function in asthmatics (Spector, 1995; Barnes *et al*., 1997; Okudaira, 1997). It is possible that the therapeutic effects of $CysLT_1$ receptor antagonists in asthma may be due in part to inhibition of airway mucus secretion; formal investigation of this hypothesis would be an important area of research.

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