Interactions among Three Classes of Mediators Explain Antigen-Induced Bronchoconstriction in the Isolated Perfused and Ventilated Guinea Pig Lung

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ABSTRACT
Intravascular challenge of isolated perfused and ventilated guinea pig lung (IPL) from actively sensitized guinea pigs, with cumulatively increasing (10−10,000 μg) doses of ovalbumin (OVA), resulted in dose-dependent and reproducible reductions in lung conductance. The antihistamines mepyramine (1 M) and metiamide (1 M), the leukotriene antagonist zafirlukast (0.1 μM), or the cyclooxygenase enzyme (COX) inhibitor diclofenac (10 μM) each caused a parallel and rightward shift in the dose-response relation for OVA, providing evidence for contributions of histamine, cysteinyl-leukotrienes, and COX products to the OVA-induced bronchoconstriction in the IPL. Moreover, when all three drugs were combined there was a complete abolishment of the response to OVA. When two antagonists or inhibitors were combined, the results, however, were more complex. The 5-lipoxygenase inhibitor BAY U1005 (30 μM) and the thromboxane (TP) receptor antagonist BAY u3405 (1 μM) given as single treatment did not inhibit the response to OVA. However, combinations of different antagonists/inhibitors, including BAY x1005 and BAY u3405, caused pronounced inhibitions of the antigen responses, suggesting synergism in action. On the basis of these data it was concluded that although histamine and cysteinyl-leukotrienes mediate the major part of the bronchoconstriction, one or several prostanooids other than thromboxane contribute to the bronchoconstriction evoked by OVA. Moreover, the effect of diclofenac involved a dual action because it also made the IPL less sensitive to histamine and LTD4. The findings resemble and extend recent observations in clinical studies of patients with asthma and support the usefulness of this particular model in airway pharmacology.

In airway pharmacology there is a great need to have experimental in vitro models that are relevant with regard to the central components of allergen-induced airway obstruction in people with asthma. Such models are important for characterization of the actions of new or existing pharmacological agents, and for the detailed exploration of novel mechanisms or drugs before going into humans. A fundamental shortcoming of most currently used models using tissues from small animals is that the mediators of airway inflammation and bronchoconstriction are distinctly different from those in humans. In particular, mouse and rat airways do not respond with significant bronchoconstriction to challenge with histamine or leukotrienes (Hedqvist et al., 1980; Piper and Samhoun, 1981; Martin et al., 1988; Uhlig and Wendel, 1995; Held et al., 1999), whereas these two classes of mediators account for the major part of allergen-induced airway obstruction in humans with asthma (Roquet et al., 1997) and IgE-dependent contraction of isolated human bronchi (Dahlén et al., 1983a; Björck and Dahlén, 1993). We have previously reported that the Schultz-Dale contraction evoked by antigen challenge of isolated guinea pig lung parenchyma preparations in tissue baths is predominantly mediated by histamine and leukotrienes (Wikström Jonsson and Dahlén, 1994), thus making it a relevant model in asthma pharmacology.

In this study, we therefore decided to determine the mediators of the Schultz-Dale response in the isolated perfused and ventilated guinea pig lung preparation (IPL). The novelty of this approach may at first seem questionable, as the perfused guinea pig lung has been used extensively in pharmacology to discover release of biologically active principles and in particular arachidonic acid-derived metabolites (eicosanoids) (Piper and Vane, 1969; Svensson et al., 1975) or to follow metabolism of endogenous compounds (Vane, 1964) or drugs (Ryrfeldt et al., 1988; Kröll et al., 1990). Surprisingly
enough, however, a literature review disclosed that the mechanisms involved in the actions of antigen or individual mediators on airway reactivity in the perfused guinea pig lung itself has not received the same attention. One previous study of vascular and tracheal responses to antigen challenge in perfused guinea pig lungs provided evidence for a major role of histamine and weak indications of participation of leukotrienes in the response (Selig et al., 1993). In the same study, the cyclooxygenase inhibitor indomethacin paradoxically potentiated the antigen-induced bronchoconstriction but inhibited release of the bronchoconstrictor thromboxane A2 (TXA2).

Therefore, it was decided to perform a comprehensive study of the effects of intervention with three different mediator classes, histamine, leukotrienes, and cyclooxygenase products such as prostaglandins and TXA2, by the use of specific antagonists or inhibitors. In addition to characterizing the effects of interventions with each pathway alone, we investigated the effects of combining antagonists/inhibitors of two or all three classes of mediators. This has previously not been done, although it is documented in asthma patients that interactions occur between mediators (Curzen et al., 1987; Roquet et al., 1997; O'Sullivan et al., 1998).

The only previous investigation of mediators in perfused guinea pig lung used challenges with single bolus doses of antigen (Selig et al., 1993). We hypothesized that challenge with cumulatively increasing doses of antigen would increase the repeatability of the antigen-induced bronchoconstriction and increase the resolution of the model by allowing studies of shifts in the dose-response relations. This assumption is supported by our previous observations of antigen-induced contractions in the isolated guinea pig lung parenchyma (Wikström Jonsson and Dahlén, 1994).

Furthermore, for the complete characterization of the model and to establish the effectiveness of the pharmacological tools used, the reactivity of the preparation to histamine, cysteinyl-leukotrienes, and TXA2 was characterized initially, the latter with the aid of the stable TP agonist U-46619. That part of the study was initially conducted in the lungs from nonsensitized animals, which in addition provided us with the opportunity to test whether or not the active sensitization changed airway reactivity as assessed by cumulative challenge with LTD4. Increased airway reactivity to bronchoconstrictors is a typical feature of asthma (Hargreave et al., 1981).

### Materials and Methods

**Materials.** The lungs were perfused with Krebs-Ringer-bicarbonate buffer (118.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 24.9 mM NaHCO3, 1.2 mM KH2PO4), with the addition of 5.5 mM glucose and 12.6 mM HEPES. NaCl, KCl, CaCl2, MgSO4, NaHCO3, KH2PO4, and glucose were obtained from a local supplier. Bovine serum albumin, fraction V, and HEPES were purchased from Roche Diagnostics (Mannheim, Germany). Salbutamol hemisulfate, histamine dihydrochloride, pyrilamine maleate (mepramine), metiamide, and diclofenac sodium were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved as follows: salbutamol in redistilled water, histamine and metiamine in 0.9% saline, and diclofenac sodium and metiamide in 95% ethanol. Leukotrienes C4, D4, and E4 from Cascade Biochemicals Ltd. (Reading, UK) were dissolved in 10% ethanol. The concentration and purity of LTE4, LTD4, and LTE4 stock solutions were checked by UV spectrophotometry. Zafirlukast (AstraZeneca Pharmaceuticals, Alderley, UK) was dissolved in DMSO. Ovalbumin (chicken egg, grade II) from Sigma-Aldrich was dissolved in 0.9% saline. BAY u3405 and BAY x1005 were obtained from Bayer AG (Wuppertal, Germany); BAY u3405 was dissolved in 95% ethanol, BAY x1005 in DMSO. U-46619 from Cayman Chemical (Ann Arbor, MI) was dissolved in 10% ethanol.

All used receptor antagonists and enzyme inhibitors were freshly made from the stock and added to the perfusion buffer shortly before the start of the pretreatment periods. Pretreatment periods and concentrations (i.e., final molar concentration in perfusate reservoir) of the receptor antagonists and enzyme inhibitors used were 5 min for 1 μM mepyramine, 1 μM metiamide, 0.1 μM zafirlukast, and 1 μM BAY u3405; 20 min for 10 μM diclofenac; and 30 min for 30 μM BAY x1005. Thereafter, the drugs were constantly present in the perfusate during the remainder of the experiments.

**Ethics.** The study was approved by the local Ethical Review Board (N317/98 and N14/02).

**Isolated Perfused and Ventilated Lung Model.** Male Dunkin-Hartley guinea pigs (n = 251) weighing 400 to 600 g were used in this study. The IPL was prepared essentially as described in Kröll et al., 1986, with following modifications: 1) positive pressure ventilation was not used during preparation; 2) the buffer with addition of 2% bovine serum albumin, fraction V, was used only during the surgical procedure and the initial part of a stabilization period; 3) the preparation was stabilized with 33 nM salbutamol (Atzori et al., 1992).

Only lungs with stable baseline values for lung conductance, dynamic compliance, and perfusion flow were used. The baseline values for lung conductance, dynamic compliance, and perfusion flow were 86 ± 18 ml/s/kPa, 9 ± 2 ml/kPa, and 29 ± 5 ml/min (mean ± S.D., n = 25) in that order. The lungs were allowed to equilibrate for 10 min in the medium without albumin and with single pass perfusion before administration of the test compounds, which were injected in a constant volume of vehicle (0.1 ml) into the pulmonary artery.

Lung conductance and dynamic compliance were evaluated as percentage of baseline values. Since changes in compliance followed a pattern similar to the changes in conductance throughout all experiments, we have chosen to show the values of conductance in our presentation, except for Figs. 1 and 2.

**Animal Sensitization.** The guinea pigs (n = 141) were sensitized to chicken egg albumin (ovalbumin, OVA) at least 4 weeks before experiments by one i.p. and one s.c. injection of 10 mg of OVA dissolved in 0.4 ml of saline-aluminum hydrogel (1:1).

**Statistical Analysis.** Data in the figures are given as mean ± S.D. The obtained experimental data were statistically evaluated by Student’s t test for paired and unpaired observations; P < 0.05 was generally considered significant except for Figs. 3 and 4, where several comparisons were done and the P value was set to 0.02 to minimize the alpha error.

### Results

**Characterization of the Responses to Histamine, the Cysteinyl-Leukotrienes, and the Thromboxane Mimetic U-46619 in Nonsensitized Animals.** Challenge of nonsensitized guinea pig lungs with escalating doses of LTC4, LTD4, LTE4, and the thromboxane mimetic U-46619 (Fig. 5) or histamine (Fig. 6) resulted in distinct and dose-dependent bronchoconstriction. Repeated administration of U-46619 and LTD4 was not associated with tachyphylaxis (Table 1). Dose-response relations for the responses to LTC4, LTD4, and LTE4 revealed that LTD4 was the most potent bronchoconstrictor, followed by LTC4, LTE4, and U-46619 (Fig. 5). This is documented for D50 values (the agonist dose causing 50% reduction in conductance) (Table 2). Dose-response relations for the effects of the challenge with histamine and LTD4 were parallel and showed that LTD4 on a
molar basis was about 1500 times as potent as histamine (Fig. 6 and Table 2). The response to histamine was prompt and reached maximal amplitude within 16 ± 0.5 s (mean ± S.D., n = 7) after provocation with doses causing 50% decrease in conductance, whereas the peak response to equioactive doses of LTD₄ was reached within 42 ± 5 s (mean ± S.D., n = 7; P < 0.001 compared with histamine) (Fig. 1, A and B; Fig. 2A). In addition, the effect of LTD₄ was more persistent and several rounds of hyperinflation were required for return to baseline conductance. This was not the case with histamine.

Effects of Receptor Antagonists and Enzyme Inhibitors on the Responses to Histamine, LTD₄, and the Thromboxane Mimetic U-46619 in Nonsensitized Animals. The histamine H₁ receptor antagonist mepyramine (1 μM) substantially inhibited the response to histamine (Fig. 7A), whereas pretreatment with the histamine H₂ receptor antagonist metiamide (1 μM) enhanced the response to histamine (Fig. 7B). The selective cysLT₁ receptor antagonist zafirlukast (0.1 μM) abolished the response to LTD₄ (Fig. 7C). The selective TP receptor antagonist BAY u3405 (1 μM) abolished the response to the thromboxane mimetic U-46619 (Fig. 7D).

The response to histamine or LTD₄ was significantly reduced in the presence of 10 μM diclofenac (Fig. 8A). The effect of 10 μM diclofenac was, however, surmountable by higher doses of LTD₄ and a parallel shift to the right of the dose-response curve for LTD₄ was obtained in the presence of 10 μM diclofenac (Fig.
6 and Table 2). Maximal reduction in lung function was obtained by about 30-fold higher doses, i.e., by 300 pmol of LTD₄ after diclofenac compared with 10 pmol of LTD₄ in control. Moreover, the peak of the response to LTD₄ in the presence of diclofenac was much delayed and was reached within 112/11006 ± 34 s (mean ± S.D., n = 4; P < 0.001 compared with LTD₄ alone) (see Fig. 1D). The diclofenac-resistant component of the response to 300 pmol of LTD₄ was, however, subsequently abolished by 0.1 μM zafirlukast (Fig. 8B).

Responses in Sensitized Animals

Basal Characteristics of Antigen and Agonist Responses. First, lungs from sensitized animals showed a dose-dependent bronchoconstriction in response to the OVA-challenge (Figs. 3 and 4). The onset of the response to OVA (Fig. 2C) was slower than that obtained after challenge with the agonists histamine (Fig. 2A), U-46619 (Fig. 2B), or LTD₄ (not shown). The peak effect occurred after about 1 min and the peak amplitude of response to OVA producing more than 50% decrease in conductance was reached within 210 ± 88 s (mean ± S.D., n = 11).

Second, OVA up to 1000 μg did not elicit bronchoconstriction when administered to the lungs from nonsensitized animals (n = 4), whereas histamine (10 nmol) evoked the expected bronchoconstrictory response in these control lungs (maximal drop in conductance was 73 ± 21%; mean ± S.D., n = 4) (Fig. 7A).

Third, there was no difference in the responsiveness to LTD₄ between the lungs from OVA-sensitized and control animals (Fig. 9).

Inhibition of One Class of Mediators at a Time. Pretreatment with 1 μM mepyramine caused a significant shift to the right of the dose-response relationship for the reaction to OVA (Fig. 3A). Pretreatment with the combination of 1 μM mepyramine and 1 μM metiamide (Figs. 3A and 4C) did not show a significantly different effect on the response to OVA compared with that of mepyramine alone (Table 3). A significant shift to the right of the dose-response relationship for the reaction to OVA was also observed after pretreatment with 0.1 μM zafirlukast (Fig. 3B) and 10 μM diclofenac (Fig. 3C). Pretreatment with the

**TABLE 1**

Effect of repeated administration of LTD₄ and U-46619

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Decrease in Conductance (%)</th>
<th>1st</th>
<th>2nd</th>
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<tr>
<td></td>
<td></td>
<td>mean ± S.D.</td>
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<tr>
<td>LTD₄ (3 pmol) (n = 6)</td>
<td>29 ± 18</td>
<td>38 ± 31 ns</td>
<td></td>
</tr>
<tr>
<td>U-46619 (100 pmol) (n = 3)</td>
<td>57 ± 16</td>
<td>65 ± 24 ns</td>
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ns, P > 0.05.

**TABLE 2**

Potency of test agonists measured as D₅₀ values

<table>
<thead>
<tr>
<th>Agonist</th>
<th>D₅₀, Mean ± S.D. (n)</th>
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<tbody>
<tr>
<td>Histamine</td>
<td>4.7 ± 2.4 nmol (5)</td>
</tr>
<tr>
<td>LTC₄</td>
<td>10 ± 4 pmol (7) vs. LTD₄**</td>
</tr>
<tr>
<td>LTD₄</td>
<td>3 ± 0.2 pmol (5) vs. Histamine**</td>
</tr>
<tr>
<td>LTE₄</td>
<td>24 ± 15 pmol (4) vs. LTD₄*</td>
</tr>
<tr>
<td>LTD₄ after 10 μM diclofenac</td>
<td>96 ± 43 pmol (5) vs. LTD₄**</td>
</tr>
<tr>
<td>U-46619</td>
<td>43 ± 15 pmol (4) vs. LTD₄***</td>
</tr>
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n, number of subjects; *P < 0.05, **P < 0.01, ***P < 0.001.

D₅₀, the agonist dose causing 50% decrease in conductance.

Fig. 2. Time courses of the effects on dynamic compliance following consecutive challenges with agonists and antigen in one lung preparation from an OVA-sensitized animal. A, 5 nmol of histamine; B, 100 pmol of U-46619; C, 10 μg of OVA.
TP receptor antagonist BAY u3405 (1 μM) had no effect on the response to OVA (Fig. 4B), neither had pretreatment with the 5-lipoxygenase inhibitor BAY x1005 (30 μM) (Fig. 4C).

Inhibition of Two Classes of Mediators at a Time. The combination of 0.1 μM zafirlukast (Figs. 3B and 4B) or 10 μM diclofenac (Fig. 3C) with 1 μM mepyramine caused even more pronounced and highly significant inhibition of the response to OVA compared with the effects of either drug alone (Table 3). Compared with control, a significant shift to the right of the dose-response relationship to OVA was also achieved by the combination of 0.1 μM zafirlukast and 10 μM mepyramine (Fig. 3D). The effect of this combination of drugs was, however, not greater than the effects of either drug alone. The combination of BAY x1005 (30 μM) with antihistamines (1 μM each of mepyramine and metiamide) caused a major inhibition of the response to OVA (Fig. 4C). The effect of this combination of drugs was much more pronounced...
compared with that achieved with the antihistamines alone (Fig. 4C and Table 3).

**Inhibition of All Three Classes of Mediators.** The combination of $\frac{1}{1000\text{M}}$ mepyramine, $\frac{0.1}{1000\text{M}}$ zafirlukast, and $10\frac{1}{1000\text{M}}$ diclofenac abolished the response to OVA (Fig. 4A). This drug combination, however, did not change reactivity of the preparation for methacholine (3 nmol; not shown).

The combination of $\frac{1}{1000\text{M}}$ mepyramine, $\frac{0.1}{1000\text{M}}$ zafirlukast, and $10\frac{1}{1000\text{M}}$ BAY u3405 caused a major inhibition of the response to OVA (Fig. 4B). The effect of this combination of drugs was more pronounced compared with that achieved by the combination of $\frac{1}{1000\text{M}}$ mepyramine and $\frac{0.1}{1000\text{M}}$ zafirlukast (Fig. 4B and Table 3).

**Discussion**

This study documented that the guinea pig IPL provides a model where a dose-dependent, reproducible, and specific response is obtained with intravascular challenge with antigen (OVA) in lungs of animals previously actively sensitized to OVA. Moreover, the pharmacologic interventions support that the bronchoconstrictor response to OVA is mediated by the concerted action of histamine, cysteinyl-leukotrienes, and one or several COX-derived prostanoids. Thus, the pharmacology of mepyramine, $\bullet$, and combined leukotriene CysLT1 receptor antagonism, histamine H1 receptor antagonism, and TP receptor antagonism ($\frac{0.1}{1000\text{M}}$ mepyramine, $\frac{1}{1000\text{M}}$ zafirlukast, $\frac{10}{1000\text{M}}$ diclofenac, and $\frac{1}{1000\text{M}}$ mepyramine, $\bullet$, $n = 4$) compared with controls ($\bigcirc$, $n = 19$). B, TP receptor antagonism ($\frac{1}{1000\text{M}}$ BAY u3405, $\blacksquare$, $n = 5$); leukotriene CysLT1 receptor antagonism combined with histamine H1 receptor antagonism ($\frac{0.1}{1000\text{M}}$ zafirlukast and $\frac{1}{1000\text{M}}$ mepyramine, $\bullet$, $n = 5$); and combined leukotriene CysLT1 receptor antagonism, histamine H1 receptor antagonism, and TP receptor antagonism ($\frac{0.1}{1000\text{M}}$ mepyramine, $\frac{1}{1000\text{M}}$ mepyramine, and $\frac{1}{1000\text{M}}$ BAY u3405, $\bigcirc$, $n = 5$), compared with controls ($\bigcirc$, $n = 5$). C, 5-lipoxygenase inhibition ($30\frac{1}{1000\text{M}}$ BAY x1005, $\blacksquare$, $n = 6$); histamine H1 and H2 receptor antagonism ($\frac{1}{1000\text{M}}$ mepyramine and $\frac{1}{1000\text{M}}$ metiamide, $\downarrow$, $n = 6$); and combined 5-lipoxygenase inhibition and histamine antagonism ($30\frac{1}{1000\text{M}}$ BAY x1005, $\frac{1}{1000\text{M}}$ mepyramine, and $\frac{1}{1000\text{M}}$ metiamide, $\downarrow$, $n = 6$) compared with controls ($\bigcirc$, $n = 6$). Level of significance compared with controls: $**$, $P < 0.02$; $***$, $P < 0.01$. Levels of significance for the comparisons between different treatments are presented in Table 3.

**Fig. 4.** Effects of pharmacological interventions on dose-response relationships obtained by cumulative challenge with antigen (OVA) of the lungs from actively sensitized guinea pigs. Results are presented as peak effect on conductance expressed as percentage decrease related to the baseline values, mean ± S.D. A, combined leukotriene CysLT1 receptor antagonism, cyclooxygenase inhibition, and histamine H1 receptor antagonism ($0.1\frac{1}{1000\text{M}}$ zafirlukast, $10\frac{1}{1000\text{M}}$ diclofenac, and $\frac{1}{1000\text{M}}$ mepyramine, $\bullet$, $n = 4$) compared with controls ($\bigcirc$, $n = 19$). B, TP receptor antagonism ($\frac{1}{1000\text{M}}$ BAY u3405, $\blacksquare$, $n = 5$); leukotriene CysLT1 receptor antagonism combined with histamine H1 receptor antagonism ($0.1\frac{1}{1000\text{M}}$ zafirlukast and $1\frac{1}{1000\text{M}}$ mepyramine, $\bullet$, $n = 5$); and combined leukotriene CysLT1 receptor antagonism, histamine H1 receptor antagonism, and TP receptor antagonism ($0.1\frac{1}{1000\text{M}}$ mepyramine, $1\frac{1}{1000\text{M}}$ mepyramine, and $1\frac{1}{1000\text{M}}$ BAY u3405, $\bigcirc$, $n = 5$), compared with controls ($\bigcirc$, $n = 5$). C, 5-lipoxygenase inhibition ($30\frac{1}{1000\text{M}}$ BAY x1005, $\blacksquare$, $n = 6$); histamine H1 and H2 receptor antagonism ($\frac{1}{1000\text{M}}$ mepyramine and $\frac{1}{1000\text{M}}$ metiamide, $\downarrow$, $n = 6$); and combined 5-lipoxygenase inhibition and histamine antagonism ($30\frac{1}{1000\text{M}}$ BAY x1005, $1\frac{1}{1000\text{M}}$ mepyramine, and $1\frac{1}{1000\text{M}}$ metiamide, $\downarrow$, $n = 6$) compared with controls ($\bigcirc$, $n = 6$). Level of significance compared with controls: $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$. Levels of significance for the comparisons between different treatments are presented in Table 3.

**Fig. 5.** Dose-response relations for the peak effect on conductance, obtained in nonsensitized lungs with escalating doses of LTC4 ($\downarrow$, $n = 8$), LTD4 ($\bigcirc$, $n = 6$), LTE4 ($\bullet$, $n = 4$), and U-46619 (□, $n = 6$). Data are expressed as percentage decrease related to the baseline values, mean ± S.D.
cology of this particular preparation is closely similar to that of allergen-induced bronchoconstriction in subjects with asthma (Curzen et al., 1987; Roquet et al., 1997). Furthermore, the sensitization protocol we used has previously been shown to produce reproducible contraction responses to OVA in isolated guinea pig lung parenchyma with no decay in responsiveness over 4 months after the sensitization (Wikström Jonsson and Dahlén, 1994). Taken together, all these aspects should make the guinea pig IPL OVA model useful in airway pharmacology. Observed drug actions in this particular model would seem to have a great probability of being predictive of corresponding effects in humans.

As it may be speculated that the sensitized state by itself changes airway reactivity to the mediators of bronchoconstriction, much as patients with asthma suffer from bronchial hyper-responsiveness to histamine (Hargrave et al., 1981) and LTD₄ (Smith et al., 1985), it was furthermore assessed whether the sensitization procedure changed the reactivity to LTD₄. This assessment was also considered important in the current project, as marked changes in effector cell reactivity could have implications for the dose level of antagonists to be used. However, it was documented that the sensitivity to LTD₄ in the IPL was unaltered by the OVA sensitization. Lungs from nonsensitized but weight-matched littermates had dose-response relations for LTD₄ that were superimposable on those from the sensitized animals (Fig. 9). We have in fact been unable to find other publications that have made this direct comparison of mediator reactivity between sensitized and nonsensitized animals. The finding indicates that it is not the passive state of being sensitized with an allergen per se that changes airway reactivity. Airway hyper-responsiveness in allergic individuals is rather the result of repeated allergen stimulation that occurs naturally by exposure to specific allergen (Ihre and Zetterström, 1993). The animals in this study were not exposed to OVA in the interval between sensitization and the challenge with OVA in our experiments.

To clarify the sometimes complex effects of the antagonists and inhibitors used on the responses to OVA in the sensitized animals, and also to introduce the rationale behind some of the protocols in the OVA challenges, it is necessary to first consider the influence of the pharmacologic interventions on responses obtained by injecting the mediators themselves in lungs from nonsensitized animals. First, we discovered that there was an apparently relaxant H₂ receptor involved in the response to histamine, as the effect of histamine was potentiated after H₂ antagonism by metiamide (Fig. 7B). Relaxant H₂ receptors have previously been described in isolated human and guinea pig airways (Black et al., 1972; Advenier et al., 1987), but not in the IPL.

Second, the predominant part of the response to cysteinyl-leukotrienes in human airways is mediated by activation of the CysLT₁ receptor (Brink et al., 2003), whereas in certain guinea pig airway preparations there is a significant contribution by CysLT₂ receptors. However, it was found that the potent and clinically used selective CysLT₁ antagonist zafirlukast effectively antagonized LTD₄ (Fig. 7C). It was also found that LTD₄ was more potent than LTC₄ and LTE₄ in this particular model (Fig. 5), which incidentally fits with agonist data for CysLT₁ receptors in binding assays (Brink et al., 2003), and justifies our use of LTD₄ as the probe for the cysteinyl-leukotrienes in this particular study.

Third, similar to guinea pig airways in vitro (Weichman et al., 1982; Dahlén et al., 1983b) or anesthetized animals (Omini et al., 1981; Weichman et al., 1982; Dahlén, 1983), it was established that the response to LTD₄ had two components. Thus, after pretreatment with the COX inhibitor diclofenac, the actions of low doses of LTD₄ were abolished (Fig. 8A). However, the effect of diclofenac was surmountable and a complete dose-effect curve for LTD₄ could be obtained also in the presence of diclofenac (Fig. 6). Therefore, it is concluded that the effect of LTD₄ in the presence of diclofenac represents the direct action of LTD₄ on the airway smooth muscle, whereas the effect of LTD₄ in the absence of COX inhibition is a combination of the direct and indirect actions.

Release of the potent bronchoconstrictor thromboxane A₂ (Hamberg et al., 1975) by cysteinyl-leukotrienes has in the guinea pig been shown to occur in isolated airways (Piper and Samhoun, 1982; Weichman et al., 1982; Dahlén et al., 1983b), perfused whole lungs (Folco et al., 1981; Piper and Samhoun, 1981), or in anesthetized whole animals (Omini et al., 1981; Dahlén, 1983). It has previously been shown that diclofenac in the concentration used in this study completely inhibited the release of TXB₂ in the guinea pig lung parenchyma preparation in vitro (Wikström Jonsson and Dahlén, 1994). Thus, the findings support the hypothesis that the COX-mediated part of the response to LTD₄ in the IPL could be due to release of TXA₂. However, there are reports suggesting that other contractile prostanoids are involved as well, inasmuch that potent TP receptor antagonists show less complete antagonism of leukotriene responses than COX inhibitors (Fitzpatrick and Lawson, 1988).

Although release of TXA₂ by histamine has been shown in isolated perfused guinea pig lungs (Berti et al., 1979) and in vivo (Rossoni et al., 1980), the role of TXA₂ in histamine-
induced bronchoconstriction has previously not been examined in the IPL. We discovered that the bronchoconstrictor response to histamine was significantly reduced after the administration of diclofenac (Fig. 8A). This suggests that the release of TXA2 presumably accounts for part of the bronchoconstriction evoked by histamine in this particular model, although experiments with selective receptor antagonists are required to define whether or not other contractile COX products are involved as well.

We conclude from the initial agonist studies with the individual mediators that both histamine and cysteinyl-leukotrienes cause bronchoconstriction via two mechanisms, direct smooth muscle activation and indirectly via release of contractile COX products, possibly predominantly TXA2. In fact, we therefore propose that release of contractile COX products is a mechanism common for all bronchoconstrictors in the IPL, although future studies will be required to test the general applicability of our hypothesis and to define which COX products are involved.

With regard to the experiments in OVA-sensitized animals, it should be appreciated that antagonism of histamine and cysteinyl-leukotrienes by an antihistamine and by a CysLT1 antagonist, respectively, will therefore have a dual action on the responses. First, the antagonists will remove the effect caused by the endogenously released mediator itself, but also by blocking the receptor-triggered release of...
COX products, the antagonist will remove a synergistic mechanism. Accordingly, there was a 30-fold decrease in sensitivity for LTD4 in preparations treated with diclofenac (Fig. 6).

Here, the objection may be raised that the identification of this distinct COX product-mediated amplification mechanism makes the guinea pig model dissimilar to the human airway. However, this contention must be dismissed, as the data with regard to the effects of COX inhibitors on human airways in vitro and in vivo are very conflicting (reviewed by Brink, 1988). Today, also in view of the emerging knowledge of the complex regulation of COX isoenzymes, it is likely that seemingly different results may be due to a lack of understanding of mechanisms in humans rather than caused by real species differences. In fact, we currently plan to reassess the role of COX products in some validated bronchoprovocation models in humans.

Turning to the interventions with the response to OVA challenge, the inhibition of single classes of mediators initially produced straightforward results. Thus, three different selective interventions produced about the same 1 to 1.5 log dose shifts in the dose-response relations for antigen (Fig. 3). Accordingly, there was a histamine component and the H1 receptor was mediating the response. Combined H1 and H2 antagonism had the same effect as H1 antagonism alone, in line with previous observations in anesthetized animals (Advenier et al., 1979). Furthermore, another component of the response to OVA was sensitive to CysLT1 antagonism by zafirlukast, supporting the involvement of the cysteinyl-leukotrienes. The third class of mediators in the response was apparently COX products, as diclofenac also had a distinct overall inhibitory effect on the response. However, in this particular case it should be recalled that the effect of diclofenac could relate to at least two different actions, namely 1) inhibition of the formation of a contractile prostanoid released primarily in response to the antigen-antibody reaction on mast cells, or 2) inhibition of the formation of contractile prostanoids released from the tissue by histamine and LTD4 (vide supra!). Again, the likely candidate for the latter compound would be TXA2, whereas release of mast cell-derived prostaglandin D2 (PGD2) (O’Sullivan et al., 1998) would be expected to occur as a result of the antigen-antibody reaction.

Next, when zafirlukast and mepyramine were combined, the preparation tolerated considerably higher doses of OVA and there was substantial reduction of the response to the highest dose of OVA (Fig. 3B), suggesting greater effects on the response by inhibiting two classes of mediators (histamine and cysteinyl-leukotrienes). In line with that assumption, the combination of mepyramine, zafirlukast, and di-
clobenac, to target all three classes of mediators, produced an overwhelming and unquestionable abolishment of the response to OVA (Fig. 4A). This remarkable inhibition, where the preparation tolerated 4 log orders of increase in the OVA dose without any response to the antigen, was not caused by unspecific depression of the reactivity of the preparation because the response to standard doses of methacholine was normal in the presence of the same drugs. Therefore, we interpret the results as strong evidence that by blocking histamine and cysteinyl-leukotrienes in the presence of a potent COX inhibitor there are no other important mediators of bronchoconstriction to consider in this model. It should in this context be recognized that about 75% of the allergen-induced bronchoconstriction in patients with asthma is mediated by histamine and cysteinyl-leukotrienes (Roquet et al., 1997). The combination of these antagonists with COX inhibitors has not yet been done in humans, but there are studies implicating that the small residual component of allergen-induced early reactions may be due to release of PGD2 or TXA2 (Curzen et al., 1987; Manning et al., 1991).

The findings in our study indicate that there also are complex interactions that need further experimentation. For example, the contributions of the individual COX products are difficult to assess, as diclofenac will remove both contractile and relaxant prostanooids. Therefore, combining selective receptor antagonists for different prostanooid receptors and direct measurement of release of COX products from the IPL will be required to address some of the unexpected and unexplained findings in this study. For example, when diclofenac was combined with zafirlukast, the expected additive effect was not observed (Fig. 3D). However, COX products, and in particular PGE2, may also regulate release of histamine and leukotrienes by feedback inhibition on mast cells (Raud et al., 1988). It is therefore conceivable that under conditions when the major effect of COX inhibition is to enhance release of histamine and leukotrienes due to removal of such an inhibitory effect, the decreased release of contractile COX products may have little overall effect on the antigen response. In fact, the observations by Selig et al. (1993) that indomethacin potentiated the antigen response in the perfused lung may be in line with this proposal.

In fact, the failure to achieve additive inhibition of the OVA response by combining diclofenac and zafirlukast (Fig. 3D) may theoretically be explained by enhanced histamine release after addition of diclofenac. The removal of PGE2 inhibition may be a relatively more prominent effect of diclofenac under these conditions, when zafirlukast has blocked the activation of the CysLT1 receptor by cysteinyl-leukotrienes and consequently has blocked the component of the OVA response that is due to LTD4-mediated release of contractile COX products. This speculation receives some support from the unexpectedly large inhibition of the OVA response that is observed when the antihistamine mepyramine is added to preparations given diclofenac (Fig. 3C).

Furthermore, as an indication of the necessity to dissect the COX pathway in greater detail, the findings with the selective TP antagonist BAY u3405 provide circumstantial support for the presence of contractile COX products that act on additional receptors. Thus, when given alone, BAY u3405 had no effect on the antigen response, whereas there was a significant additive effect when BAY u3405 was given to preparations already treated with mepyramine and zafirlukast (Fig. 4B). This supports the theory that TP receptors are involved, but interestingly enough the effect of combining the TP antagonist with mepyramine and zafirlukast (Fig. 4B) appeared less pronounced than the effect of adding diclofenac to the same antagonists (Fig. 4A). This would also support the view that the overall effect of COX inhibition in the IPL cannot be explained solely in terms of removal of TXA2 acting at TP receptors. It may be speculated that PGF2α acting on FP receptors or PGD2 acting on any of the two described DP receptors may be involved as well.

It may conceptually be easier to understand the failure of BAY x1005, a potent inhibitor of leukotriene biosynthesis, to inhibit the response to OVA when given alone (Fig. 4C). Thus, in contrast to the selective antagonism of cysteinyl-leukotrienes by zafirlukast, BAY x1005 causes an overall inhibition of the 5-lipoxygenase, with consequent inhibition of also the formation of leukotriene B4 and lipoxygenase interaction products, such as lipoxins. The latter compounds are known, for example, to have many important anti-inflammatory effects in other models of inflammation (Brink et al., 2003) and their removal could therefore be expected to produce an exaggerated response, similar to inhibition of PGE2 formation. The influence of LTD4 on the IPL has not been established, but it is generally supposed to be a proinflammatory mediator (Brink et al., 2003). Irrespective of the final explanation for the lack of effect of BAY x1005 alone, the combination of BAY x1005 with the antihistamines mepyramine and metiamide again provided striking inhibition of the antigen response (Fig. 4C). This lends strong support to the concept that cysteinyl-leukotrienes and histamine mediate a major part of the Schultz-Dale response in the IPL, and again indicates the similarity between this particular model and the airways of atopic asthmatic subjects.

In conclusion, the IPL is an experimental model that pro-

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**TABLE 3**

Statistical evaluation of different treatments

<table>
<thead>
<tr>
<th>Graph</th>
<th>Treatment</th>
<th>Dose OVA (µg), log</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A</td>
<td>Mepyramine + metiamide vs. mepyramine</td>
<td>ns ns ns ns</td>
</tr>
<tr>
<td>5B</td>
<td>Zafirlukast + mepyramine vs. zafirlukast</td>
<td>ns ns --- ***</td>
</tr>
<tr>
<td>5C</td>
<td>Diclofenac + mepyramine vs. diclofenac</td>
<td>ns ns --- ***</td>
</tr>
<tr>
<td>6B</td>
<td>Mepyramine + Zafirlukast vs. BAY u3405</td>
<td>ns ns ns --- ***</td>
</tr>
<tr>
<td>6B</td>
<td>Mepyramine + zafirlukast + BAY u3405 vs. BAY u3405</td>
<td>ns --- --- ***</td>
</tr>
<tr>
<td>6B</td>
<td>Mepyramine + zafirlukast + BAY u3405 vs. mepyramine + zafirlukast</td>
<td>ns ns ns --- ***</td>
</tr>
<tr>
<td>6C</td>
<td>Mepyramine + metiamide vs. BAY x1005</td>
<td>ns ns ns ns</td>
</tr>
<tr>
<td>6C</td>
<td>BAY x1005 + mepyramine + metiamide vs. BAY x1005</td>
<td>ns --- --- ***</td>
</tr>
<tr>
<td>6C</td>
<td>BAY x1005 + mepyramine + metiamide vs. mepyramine + metiamide</td>
<td>ns ns ns --- ***</td>
</tr>
</tbody>
</table>

ns, P > 0.02, *P < 0.02, **P < 0.01, ***P < 0.001.
vides a possibility for mechanistic studies with relevance to human asthma. The model allows very controlled studies of mediator mechanisms using animal lungs but yet avoiding the complication of interference from anesthesia that remains one of the major weaknesses with mechanistic in vivo investigations in animals. On the basis of our findings in this comprehensive pharmacological study we believe that further work must take into account several levels of interactions that may be overlooked when intervention with single classes of antagonists or inhibitors are administered. First of all, as illustrated by the interactions between COX products and histamine or LTD4, the mediators may synergize on downstream mechanisms at the effector cell level. In addition, there may be direct synergistic effects between released histamine and the cysteinyl-leukotrienes on the smooth muscle, as suggested by some studies (Lee et al., 1984). Interestingly, Wohlsen et al. (2003) recently described interactions between TP receptors and CysLT1 receptors in the anaphylactic contraction of human lung parenchyma. Finally, prostanoids and other mediators and modulators may also exert feedback control on the release of bronchoconstrictive mediators from the inflammatory cells. Considered together, the evaluation of drug candidates and experimental interventions need to take all these levels of actions and interactions into account, and to define the relative importance of each mechanism for a particular intervention.

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