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Interactions between three classes of mediators explain antigen-induced bronchoconstriction in the isolated perfused and ventilated guinea pig lung

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Running title: Schultz-Dale reaction in guinea pig lung

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Abbreviations: LT, leukotriene; TXA_2, thromboxane A_2; PGD_2, prostaglandin D_2; OVA, ovalbumin (chicken egg albumin); COX, cyclooxygenase; IPL, isolated perfused and ventilated guinea pig lung; G_{aw}, pulmonary conductance; C_{dyn}, dynamic compliance; ip, intraperitoneal; sc, subcutaneous; Mep, mepyramine; Met, metiamide; Zaf, zafirlukast; Diclo, diclofenac; DMSO, dimethylsulfoxide; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid.

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Abstract

Intravascular challenge of isolated perfused and ventilated guinea pig lung (IPL) from actively sensitised guinea pigs, with cumulatively increasing (10-10000 µg) doses of ovalbumin (OVA), resulted in dose-dependent and reproducible reductions in lung conductance. The antihistamines mepyramine (1 µM) and metiamide (1 µM), 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 were however more complex. The 5-lipoxygenase inhibitor BAY x1005 (30 µM) and the 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 this data it was concluded that although histamine and cysteinyl-leukotrienes mediate the major part of the bronchoconstriction, one or several prostanoids other than thromboxane contribute to the bronchoconstriction evoked by OVA. Moreover, the effect of diclofenac involved a dual action as it also made the IPL less sensitive to histamine and LTD_4. The findings resemble and extend recent observations in clinical studies of asthmatics, and support the usefulness of this particular model in airway pharmacology.

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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 asthmatics. Such models are important for characterisation 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 employing tissues from small animals is however 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 asthmatics (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

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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 bronchoconstrictory thromboxane A_2 (TXA_2).

Therefore, it was decided to perform a comprehensive study of the effects of intervention with three different mediator classes, histamine, leukotrienes and cyclo-oxygenase products such as prostaglandins and TXA_2, by the use of specific antagonists or inhibitors of the leukotriene pathway that now are available. In addition to characterising 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 asthmatics 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 hypothesised that challenge with cumulatively increasing doses of antigen would increase the repeatability of the antigen-induced bronchoconstriction and in addition 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 characterisation of the model and to establish the effectiveness of the pharmacological tools used, the reactivity of the preparation to histamine, cysteinyl-leukotrienes and TXA_2 was characterised 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

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non-sensitised animals, which in addition provided us with the opportunity to test whether or not the active sensitisation changed airway reactivity as assessed by cumulative challenge with LTD_4. Increased airway reactivity to bronchoconstrictors is a typical feature of asthma (Hargreave et al., 1981).

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Methods

Materials

The lungs were perfused with Krebs-Ringer bicarbonate buffer (composition in mM: NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2) with addition of Glucose, 5.5 mM and HEPES, 12.6 mM. NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4 and Glucose were obtained from a local supplier. Bovine serum albumin, fraction V and HEPES were from Boehringer Mannheim GmbH, Germany. Salbutamol hemisulfate, histamine dihydrochloride, pyrilamine maleate (mepyramine), metiamide and diclofenac sodium were purchased from Sigma Chemicals Co., St. Louis, MO, USA, and dissolved as follows: salbutamol in redistilled water, histamine and mepyramine in 0.9 % saline, diclofenac sodium and metiamide in 95 % ethanol. Leukotrienes C_4, D_4 and E_4 from Cascade Biochemicals LtD, Reading, UK, were dissolved in 10 % ethanol. The concentration and purity of LTC_4, LTD_4 and LTE_4 stock solutions was checked by UV spectrofotometry. Zafirlukast from AstraZeneca Pharmaceuticals, Alderly, UK, was dissolved in DMSO. Ovalbumin (chicken egg, grade II) from Sigma Chemicals Co., St. Louis, MO, USA, was dissolved in 0.9 % saline. BAY u3405 and BAY x1005 was from Bayer AG Leverkusen, Germany and dissolved as follows: BAY u3405 in 95 % ethanol, BAY x1005 in DMSO. U-46619 from Cayman Chemical Company, Ann Arbor, MI, USA, 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 pre-treatment periods. Pre-treatment periods and concentrations (i.e. final molar concentration in perfusate reservoir) of the utilised receptor antagonists and enzyme inhibitors were: 5 minutes for mepyramine (1μM), metiamide (1μM), zafirlukast (0,1 μM) and BAY u3405 (1 μM), 20 minutes for diclofenac (10 μM) and 30 minutes for BAY x1005 (30 μM). Thereafter, the drugs were constantly present in the perfusate during the reminder of the experiments.

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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 400-600 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 employed 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 stabilisation period. 3). The preparation was stabilised with salbutamol (33 nM) (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 ± SD, n = 251), respectively. The lungs were allowed to equilibrate for 10 minutes 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 per cent of baseline values. Since changes in compliance followed similar pattern as the changes in conductance throughout all experiments, we have chosen to normally show the values of conductance in our presentation, except of *Fig. 8* and *Fig. 9*.

Animal sensitisation

The guinea pigs (n = 141) were sensitised at least four weeks prior to experiments to chicken egg albumin (ovalbumin, OVA) by one ip and one sc injection of 10 mg OVA dissolved in 0.4 ml saline-aluminium hydrogel (1:1).

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Statistical analysis.

Data in figures are given as mean ± SD. 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 of *Fig. 5* and *Fig. 6*, where several comparisons were done and P value was set to 0.02 to minimize the alpha error.

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Results

Characterisation of the responses to histamine, the cysteinyl-leukotrienes and the thromboxane mimetic U-46619 in non-sensitised animals

Challenge of non-sensitised guinea pig lungs with escalating doses of LTC_4, LTD_4 , LTE_4 and the thromboxane mimetic U-46619 *(Fig. 1)* or histamine *(Fig. 2)*, resulted in distinct and dose-dependent bronchoconstriction. Repeated administration of U-46619 and LTD_4 was not associated with tachyphylaxis *(Table 1)*. Dose-response relations for the responses to LTC_4, LTD_4 and LTE_4 revealed that LTD_4 was the most potent bronchoconstrictor, followed by LTC_4, LTE_4 and U-46619 *(Fig. 1)*. This is documented for D_{50} values (the agonist dose causing 50 % reduction in conductance) *(Table 2)*. Dose-response relations for the effects of the challenge with histamine and LTD_4 were parallel and showed that LTD_4 on a molar basis was about 1500 times as potent as histamine *(Fig. 2 and Table 2)*. The response to histamine was prompt and reached maximal amplitude within 16 ± 0.5 seconds (mean ± SD, n = 7) after provocation with doses causing 50 % decrease in conductance, whereas the peak response to equiactive doses of LTD_4 was reached within 42 ± 5 seconds (mean ± SD, n = 7; $P < 0.001$ compared with histamine) *(Fig. 8A, 8B and Fig. 9A)*. In addition, the effect of LTD_4 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, LTD_4 and the thromboxane mimetic U-46619 in non-sensitised animals

The histamine H_1 receptor antagonist mepyramine (1μM) substantially inhibited the response to histamine *(Fig. 3A)* whereas pre-treatment with the histamine H_2 receptor antagonist metiamide (1μM) enhanced the response to histamine *(Fig. 3B)*. The selective cysLT_1 receptor antagonist zafirlukast (0.1 μM) abolished the response to LTD_4 *(Fig. 3C)*.

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The selective TP receptor antagonist BAY u3405 (1 μM) abolished the response to the thromboxane mimetic U-46619 *(Fig. 3D)*.

The response to histamine or LTD_4 was significantly reduced in the presence of diclofenac (10 μM) *(Fig. 4A)*. The effect of diclofenac (10 μM) was, however, surmountable by higher doses of LTD_4 and a parallel shift to the right of the dose-response curve for LTD_4 was obtained in the presence of diclofenac (10 μM) *(Fig. 2 and Table 2)*. Maximal reduction in lung function was obtained by about 30-fold higher doses, i.e. by 300 pmol of LTD_4 after diclofenac, compared with 10 pmol of LTD_4 in control. Moreover, the peak of the response to LTD_4 in the presence of diclofenac was much delayed and was reached within 112 ± 34 seconds (mean ± SD, n = 4; $P < 0.001$ compared with LTD_4 alone) *(Fig. 8D)*. The diclofenac-resistant component of the response to 300 pmol of LTD_4 was however subsequently abolished by zafirlukast (0.1 μM) *(Fig. 4B)*.

Responses in sensitised animals

Basal characteristics of antigen and agonist responses

First, lungs from sensitised animals showed a dose-dependent bronchoconstriction in response to the OVA-challenge *(Fig. 5 and Fig. 6)*. The onset of the response to OVA *(Fig. 9C)* was slower than that obtained after challenge with the agonists histamine *(Fig. 9A)*, U-46619 *(Fig. 9B)* or LTD_4 *(not shown)*. The peak effect occurred after about 1 minute and the peak amplitude of response to OVA producing more than 50 % decrease in conductance was reached within 210 ± 88 seconds (mean ± SD, n = 11).

Second, OVA up to 1000 μg did not elicit bronchoconstriction when administered to the lungs from non-sensitised animals (n = 4), whereas histamine (10 nmol) evoked the expected bronchoconstrictory response in these control lungs (maximal drop in conductance was 73 ± 21%; mean ± SD, n = 4) *(cf. Fig. 3A)*.

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Third, there was no difference in the responsiveness to LTD_4 between the lungs from OVA-sensitised and control animals *(Fig. 7)*.

Inhibition of one class of mediators at the time

Pre-treatment with mepyramine (1 μM) caused a significant shift to the right of the dose-response relationship for the reaction to OVA *(Fig. 5A)*. Pre-treatment with the combination of mepyramine (1 μM) and metiamide (1 μM) *(Fig. 5A and Fig. 6C)* did not show a significantly different effect on the response to OVA compared to that of mepyramine alone *(Table 3)*. Significant shift to the right of the dose-response relationship for the reaction to OVA was observed also after pre-treatment with zafirlukast (0.1 μM) *(Fig.5B)* and diclofenac (10 μM) *(Fig.5C)*. Pre-treatment with TP receptor antagonist BAY u3405 (1 μM) had no effect on the response to OVA *(Fig. 6B)*, neither had pre-treatment with the 5-lipoxygenase inhibitor BAY x1005 (30 μM) *(Fig. 6C)*.

Inhibition of two classes of mediators at the time

The combination of zafirlukast (0.1 μM) *(Fig. 5B and Fig. 6B)* or diclofenac (10 μM) *(Fig. 5C)* with mepyramine (1 μM) caused even more pronounced and highly significant inhibition of the response to OVA compared with the effects of either drug alone *(Table 3)*. Compared to control, significant shift to the right of the dose-response relationship to OVA was also achieved by the combination of zafirlukast (0.1 μM) and diclofenac (10 μM) *(Fig. 5D)*. The effect of this combination of drugs was however not greater than the effects of either drug alone. The combination of BAY x1005 (30μM) with antihistamines (mepyramine 1μM and metiamide 1 μM) caused a major inhibition of the response to OVA *(Fig. 6C)*. The effect of this combination of drugs was much more pronounced compared with that achieved with the antihistamines alone *(Fig. 6C and Table 3)*.

Inhibition of all three classes of mediators

The combination of mepyramine (1 μM), zafirlukast (0.1 μM) and diclofenac (10 μM)

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abolished the response to OVA *(Fig. 6A)*. This drug combination however did not change reactivity of the preparation for methacholine (3 nmol; *not shown*).

The combination of mepyramine (1 µM), zafirlukast (0.1 µM) and BAY u3405 (10 µM) caused a potent inhibition of the response to OVA *(Fig. 6B).* The effect of this combination of drugs was more pronounced compared with that achieved by the combination of mepyramine (1 µM) and zafirlukast (0.1 µM) *(Fig. 6B and Table 3).*

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Discussion

The 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 sensitised 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 this particular preparation is closely similar to that of allergen-induced bronchoconstrition in subjects with asthma (Curzen et al., 1987; Roquet et al., 1997). Furthermore, the sensitisation 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 four months after the sensitisation (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 sensitised state by itself changes airway reactivity to the mediators of bronchoconstriction, much as asthmatics suffer from bronchial hyper-responsiveness to histamine (Hargreave et al., 1981) and LTD_4 (Smith et al., 1985) it was furthermore assessed whether the sensitisation procedure changed the reactivity to LTD_4. 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 LTD_4 in the IPL was unaltered by the OVA sensitisation. Lungs from non-sensitised but weight-matched littermates had dose-response relations for LTD_4 that were superimposable on those from the sensitised animals *(Fig. 7).* We have in fact been unable to find other publications that have made this direct

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comparison of mediator reactivity between sensitised and non-sensitised animals. The finding indicates that it is not the passive state of being sensitised 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 sensitisation and the challenge with OVA in our experiments.

In order to clarify the sometimes complex effects of the employed antagonists and inhibitors on the responses to OVA in the sensitised 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 non-sensitised animals. First, we discovered that there was an apparently relaxant H_2 receptor involved in the response to histamine, as the effect of histamine was potentiated after H_2 antagonism by metiamide *(Fig. 3B)*. Relaxant H_2 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 $CysLT_1$ receptor (Brink et al., 2003), whereas in certain guinea pig airway preparations there is a significant contribution by $CysLT_2$ receptors. However, it was found that the potent and clinically used selective $CysLT_1$ antagonist zafirlukast effectively antagonised LTD_4 *(Fig. 3C)*. It was also found that LTD_4 was more potent than either of LTC_4 and LTE_4 in this particular model *(Fig. 1)*, which incidentally fits with antagonist data for $CysLT_1$ receptors in binding assays (Brink et al., 2003), and justifies our use of LTD_4 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 anaesthetised animals (Omini et al., 1981; Weichman et al., 1982; Dahlén, 1983c), it

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was established that the response to LTD_4 had two components. Thus, after pre-treatment with the COX inhibitor diclofenac, the actions of low doses of LTD_4 were abolished *(Fig. 4A)*. However, the effect of diclofenac was surmountable and a complete dose-effect curve for LTD_4 could be obtained also in the presence of diclofenac *(Fig. 2)*. Therefore, it is concluded that the effect of LTD_4 in the presence of diclofenac represents the direct action of LTD_4 on the airway smooth muscle, whereas the effects of LTD_4 in the absence of COX inhibition is a combination of the direct and indirect actions.

Release of the potent bronchoconstrictor thromboxane A_2 (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 anaesthetised whole animals (Omini et al., 1981; Dahlén et al., 1983c). It has previously been shown that diclofenac in the concentration used in this study, completely inhibited the release of TXB_2 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 LTD_4 in the IPL could be due to release of TXA_2. However, there are reports suggesting that other contractile prostanoids are involved as well, inasmuch that potent TP receptor antagonist show less complete antagonism of leukotriene responses than COX inhibitors (Fitzpatrick and Lawson, 1988).

Although release of TXA_2 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 TXA_2 in histamine-induced bronchoconstriction has previously not been examined in the IPL. We discovered that the bronchoconstrictor response to histamine was significantly reduced after diclofenac *(Fig. 4A)*. This suggests that the release of TXA_2 presumably accounts for part of the bronchoconstriction evoked by histamine in this particular model, although experiments with

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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 TXA_2. 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 that are involved.

With regard to the experiments in OVA-sensitised animals, it should be appreciated that antagonism of histamine and cysteinyl-leuktorienes by an antihistamine and by a $CysLT_1$ 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 about 30-fold decrease in sensitivity for LTD_4 in preparations treated with diclofenac *(Fig. 2)*.

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* in fact 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 re-assess the role of COX products in some validated bronchoprovocation models in humans.

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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 1-1.5 log dose shifts in the dose-response relations for antigen *(Fig. 5)*. Accordingly, there was a histamine component, and the H_1 receptor was mediating the response. Combined $H_1$and H_2 antagonism had the same effect as H_1 antagonism alone, in line with previous observations in anaesthetised animals (Advenier et al., 1979). Furthermore, another component of the response to OVA was sensitive to $CysLT_1$ 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, (a) inhibition of the formation of a contractile prostanoid released primarily in response to the antigen-antibody reaction on mast cells, or (b) inhibition of the formation of contractile prostanoids released from the tissue by histamine and LTD_4 *(vide supra!)*. Again, the likely candidate for the latter compound would be TXA_2, whereas release of mast cell-derived prostaglandin D_2 (PGD_2) (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. 5B)*, 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 diclofenac, in order to target all three classes of mediators, produced an overwhelming and unquestionable abolishment of the response to OVA *(Fig. 6A)*. This remarkable inhibition, where the preparation tolerated four log orders of increase in the OVA dose without any response to the antigen, was not caused by unspecific

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depression of the reactivity of the preparation as the response to standard doses of methacholine were 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 recognised that about 75% of the allergen-induced bronchoconstriction in asthmatics is mediated by histamine and cysteinyl-leukotrienes (Roquet et al., 1997). The combination of these antagonists with COX inhibitors have 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 PGD_2 or TXA_2 (Curzen et al., 1987; Manning et al., 1991).

The findings in our study indicate that there also are complex interactions that need further experimentations. For example, the contributions of the individual COX products are difficult to assess, as diclofenac will remove both contractile and relaxant prostanoids. Therefore, combining selective receptor antagonists for different prostanoid receptors as well as direct measurements 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. 5D)*. However, COX products, and in particular PGE_2, may also regulate release of histamine, and leukotrienes, by feedback inhibition on mast cells (Raud et al., 1987). 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. (Selig et al., 1993) that indomethacin potentiated the antigen response in the perfused lung may be in line with this proposal.

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In fact, the failure to achieve additive inhibition of the OVA response by combining diclofenac and zafirlukast *(Fig. 5D)* may theoretically be explained by enhanced histamine release after addition of diclofenac. The removal of PGE_2 inhibition may be a relatively more prominent effect of diclofenac under these conditions, when zafirlukast has blocked the activation of the $CysLT_1$ receptor by cysteinyl-leukotrienes and consequently has blocked the component of the OVA response that is due to LTD_4-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. 5C)*.

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. 6B)*. This supports that TP receptors are involved, but, interestingly enough, the effect of combining the TP antagonist with mepyramine and zafirlukast *(Fig. 6B)* appeared less pronounced than the effect of adding diclofenac to the same antagonists *(Fig. 6A)*. 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 TXA_2 acting at TP receptors. It may be speculated that $PGF_{2\alpha}$ acting on FP receptors or PGD_2 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. 6C)*. 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

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formation of leukotriene B_4 as well as lipoxygenase interaction products such as lipoxins. The latter compounds are for example known 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 PGE_2 formation. The influence of LTB_4 on the IPL has not been established, but it is generally supposed to be a pro-inflammatory 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. 6C)*. 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 provides 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 anaesthesia that remains one of the major weaknesses with mechanistic *in vivo* investigations in animals. On the basis of our findings in this comprehensive pharmacologic 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 LTD_4, the mediators may synergise on downstream mechanisms at the effector cell level. In addition, there may be direct synergistic effects between released histamine and the cysteinyl-leuktorienes on the smooth muscle, as suggested by some studies (Lee et al., 1984). Interestingly, Wohlsen et al. (Wohlsen et al., 2003) recently described interactions between TP receptors and $CysLT_1$ receptors in the anaphylactic contraction of human lung parenchyma. Finally, prostanoids as well as other mediators and modulators may

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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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Footnotes

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Fig. 1. Dose-response relations for the peak effect on conductance, obtained in non-sensitised lungs with escalating doses of LTC_4 (▼, n = 8), LTD_4 (◆, n = 6), LTE_4 (●, n = 4) and U-46619 (□, n = 6). Data are expressed as per cent decrease related to the baseline values, mean ± SD.

Fig. 2. Dose-response relations for the peak effect on conductance, obtained in non-sensitised lungs with escalating doses of LTD_4 (◆, n = 6), LTD_4 in the presence of cyclooxygenase inhibitor diclofenac (10 μM) (◇, n = 6) and histamine (■, n = 6). Data are expressed as per cent decrease related to the baseline values, mean ± SD.

Fig. 3. Drug effects on response to agonists in non-sensitised lungs; □, control; ■, effects of agonists after pre-treatment with drugs. **A**. Effect of pre-treatment with histamine H_1 receptor antagonist mepyramine (1μM) on the maximal drop in conductance following the challenge with histamine: 3 nmol (n = 4, the left bars) and 10 nmol (n = 5, the right bars). **B**. Effect of pre-treatment with histamine H_2 receptor antagonist metiamide (1μM) on the maximal drop in conductance following the challenge with histamine: 5 nmol (n = 5, the left bars) and 10 nmol (n = 5, the right bars). **C**. Effect of pre-treatment with leukotriene $CysLT_1$ receptor antagonist zafirlukast (0.1μM) on the maximal drop in conductance following the challenge with 3 pmol of LTD_4; (n = 6). **D**. Effect of pre-treatment with TP receptor antagonist BAY u3405 (1 μM) on the maximal drop in conductance following the challenge with thromboxane analogue U-46619: 100 pmol (the filled bar) and 300 pmol (the striped bar), compared with the effect of 100 pmol of U-46619 as control; (n = 3). Data are expressed as per cent decrease related to the baseline, mean ± S.D.; ns $P > 0.05$; *$P < 0.05$; ***$P < 0.001$.

Fig. 4. Effect of cyclooxygenase inhibition on response to agonists in non-sensitised lungs.

A. Effect of pre-treatment with cyclooxygenase inhibitor diclofenac (10μM) on the maximal drop in conductance following the challenge with 5 nmol of histamine (n = 4, the left bars)

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and 3 pmol of LTD_4 (n = 5, the right bars); □, control; ■, effects of agonists after pre-treatment with diclofenac. **B**. Effect of pre-treatment with leukotriene $CysLT_1$ receptor antagonist zafirlukast (0.1μM, ■) on the maximal drop in conductance following the challenge with 300 pmol of LTD_4 in the presence of cyclooxygenase inhibitor diclofenac (10μM, □); (n = 4). Data are expressed as per cent decrease related to the baseline, mean ± S.D.; *P < 0.05.

Fig. 5. Effects of pharmacological interventions on dose-response relationships obtained by cumulative challenge with antigen (OVA) of the lungs from actively sensitised guinea pigs. Results are presented as peak effect on conductance, expressed as per cent decrease related to the baseline (mean ± SD). Controls, ○, n = 19. **A**. Histamine H_1 receptor antagonism (mepyramine 1μM, ■, n = 13) and combined histamine H_1 and H_2 receptor antagonism (mepyramine 1 μM and metiamide 1 μM, ▲, n = 5). **B**. Leukotriene $CysLT_1$ receptor antagonism (zafirlukast 0.1 μM, ■, n = 9) and combined leukotriene $CysLT_1$ receptor antagonism and histamine H_1 receptor antagonism (zafirlukast 0.1 μM and mepyramine 1 μM, ▲, n = 4). **C**. Cyclooxygenase inhibition (diclofenac 10 μM, ■, n = 8) and combined cyclooxygenase inhibition and histamine H_1 receptor antagonism (diclofenac 10 μM and mepyramine 1 μM, ▲, n = 5). **D**. Combined leukotriene $CysLT_1$ receptor antagonism and cyclooxygenase inhibition (zafirlukast 0.1 μM and diclofenac 10 μM, ▲, n = 5). * Level of significance compared with controls: * P < 0.02, ** P < 0.01, *** P < 0.001. Levels of significance for the comparisons between different treatments are presented in *Table 3*.

Fig. 6. Effects of pharmacological interventions on dose-response relationships obtained by cumulative challenge with antigen (OVA) of the lungs from actively sensitised guinea pigs. Results are presented as peak effect on conductance, expressed as per cent decrease related to

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the baseline (mean ± SD). **A**. Combined leukotriene $CysLT_1$ receptor antagonism, cyclooxygenase inhibition and histamine H_1 receptor antagonism (zafirlukast 0.1 μM, diclofenac 10 μM and mepyramine 1μM, ▲, n = 4), compared with controls (○, n = 19). **B**. TP receptor antagonism (BAY u3405 1 μM, ■, n = 5), leukotriene $CysLT_1$ receptor antagonism combined with histamine H_1 receptor antagonism (zafirlukast 0.1 μM and mepyramine 1 μM, ▲, n = 5) and combined leukotriene $CysLT_1$ receptor antagonism, histamine H_1 receptor antagonism and TP receptor antagonism (zafirlukast 0.1 μM, mepyramine 1 μM and BAY u3405 1 μM, ▼, n = 5), compared with controls (○, n = 5). **C**. 5-lipoxygenase inhibition (BAY x1005 30 μM, ■, n = 6), histamine H_1 and H_2 receptor antagonism (mepyramine 1 μM and metiamide 1 μM, ▲, n = 6) and combined 5-lipoxygenase inhibition and histamine antagonism (BAY x1005 30 μM, mepyramine 1 μM and metiamide 1 μM, ▼, n = 6), compared with controls (○, n = 6). * Level of significance compared with controls: * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$. Levels of significance for the comparisons between different treatments are presented in *Table 3*.

Fig. 7. Dose-response relations for the peak effect on conductance obtained with escalating doses of LTD_4 in the lungs from non-sensitised guinea pigs (○, n = 4) and from weight matched littermates that were previously actively sensitised to OVA (●, n = 4). Data are expressed as per cent decrease related to the baseline, mean ± SD.

Fig. 8. Time courses of the effects on dynamic compliance following consecutive challenges with agonists performed in one lung preparation from a non-sensitised animal **A.** Histamine (10 nmol), **B.** LTD_4 (10 pmol), **C.** LTD_4 (10 pmol) after pre-treatment with diclofenac (10 μM), **D.** LTD_4 (50 pmol) after pre-treatment with diclofenac (10 μM).

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Fig. 9. Time courses of the effects on dynamic compliance following consecutive challenges with agonists and antigen in one lung preparation from an OVA-sensitised animal. **A.** Histamine (5 nmol), **B.** U-46619 (100 pmol), **C.** OVA (10 µg).

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Fig. 1.

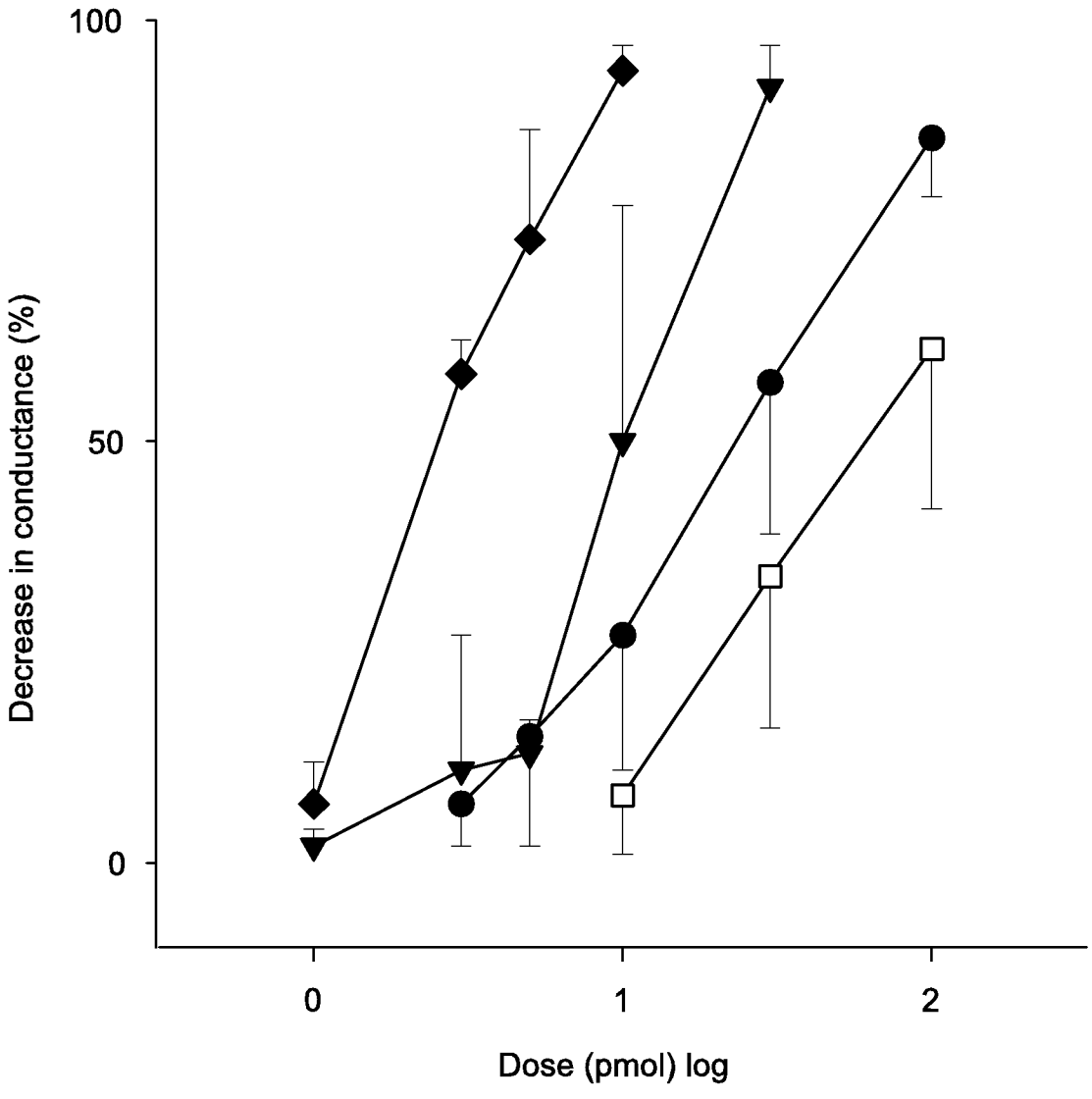

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Fig. 2.

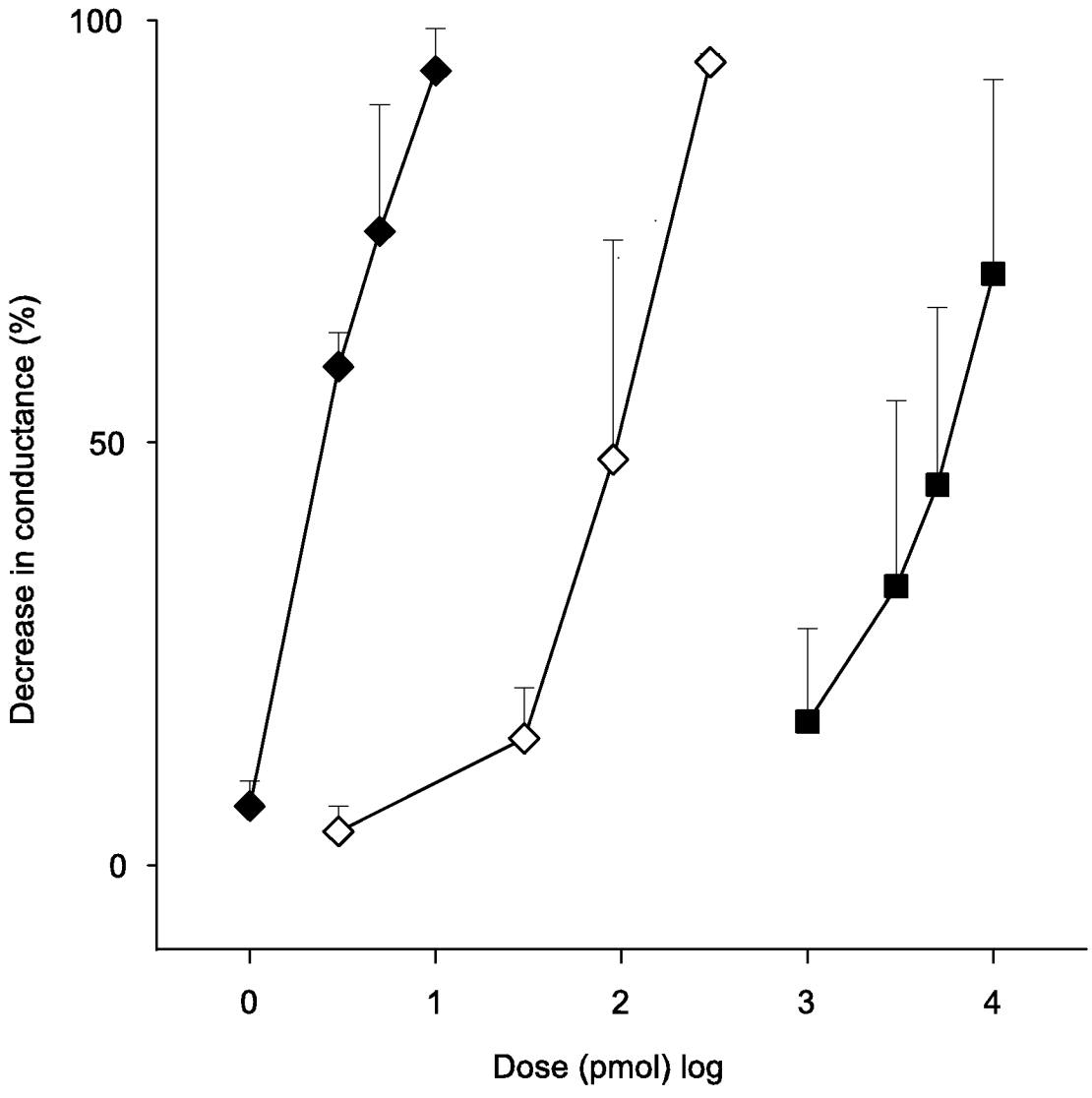

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Fig. 3.

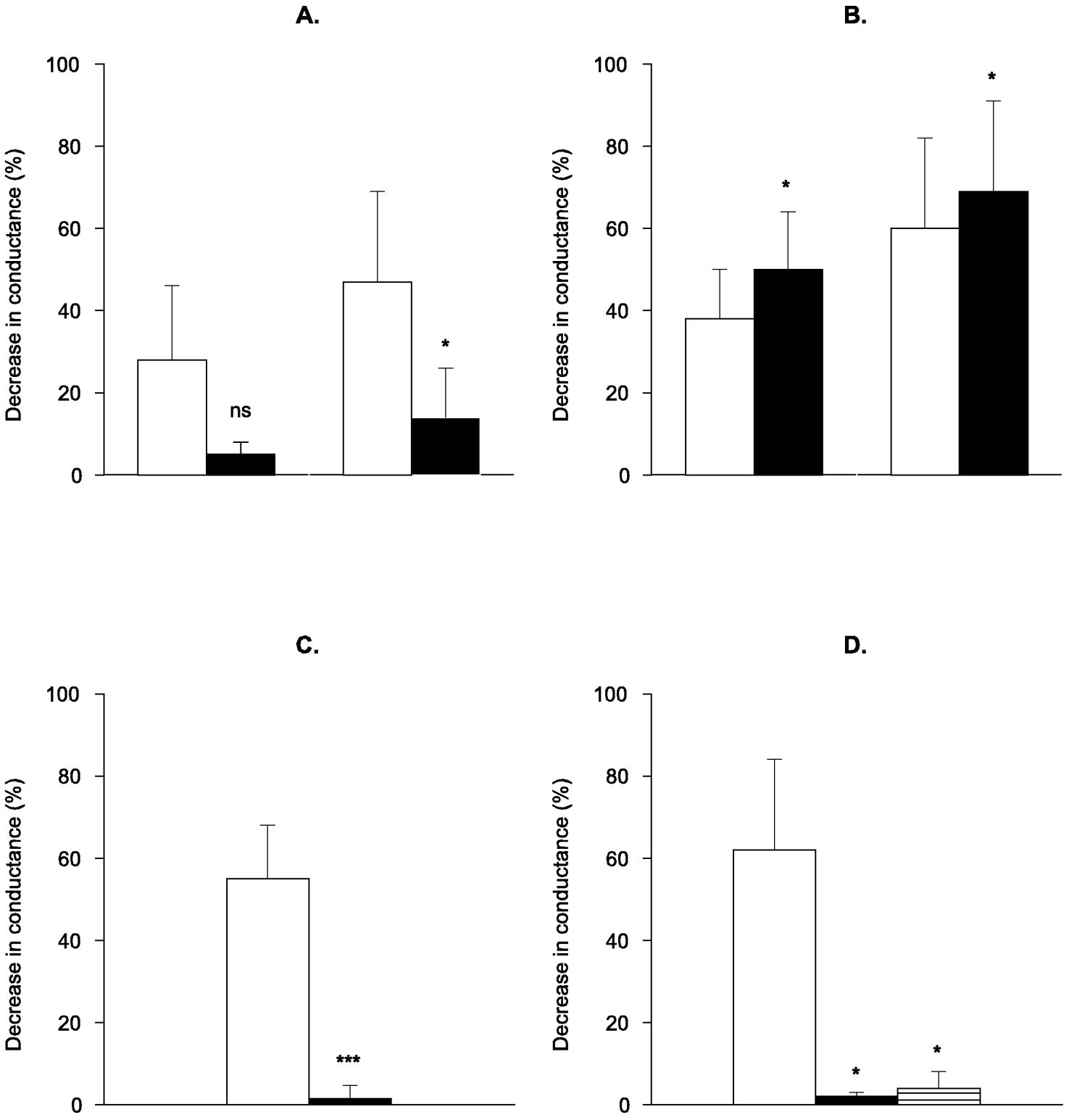

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Fig. 4.

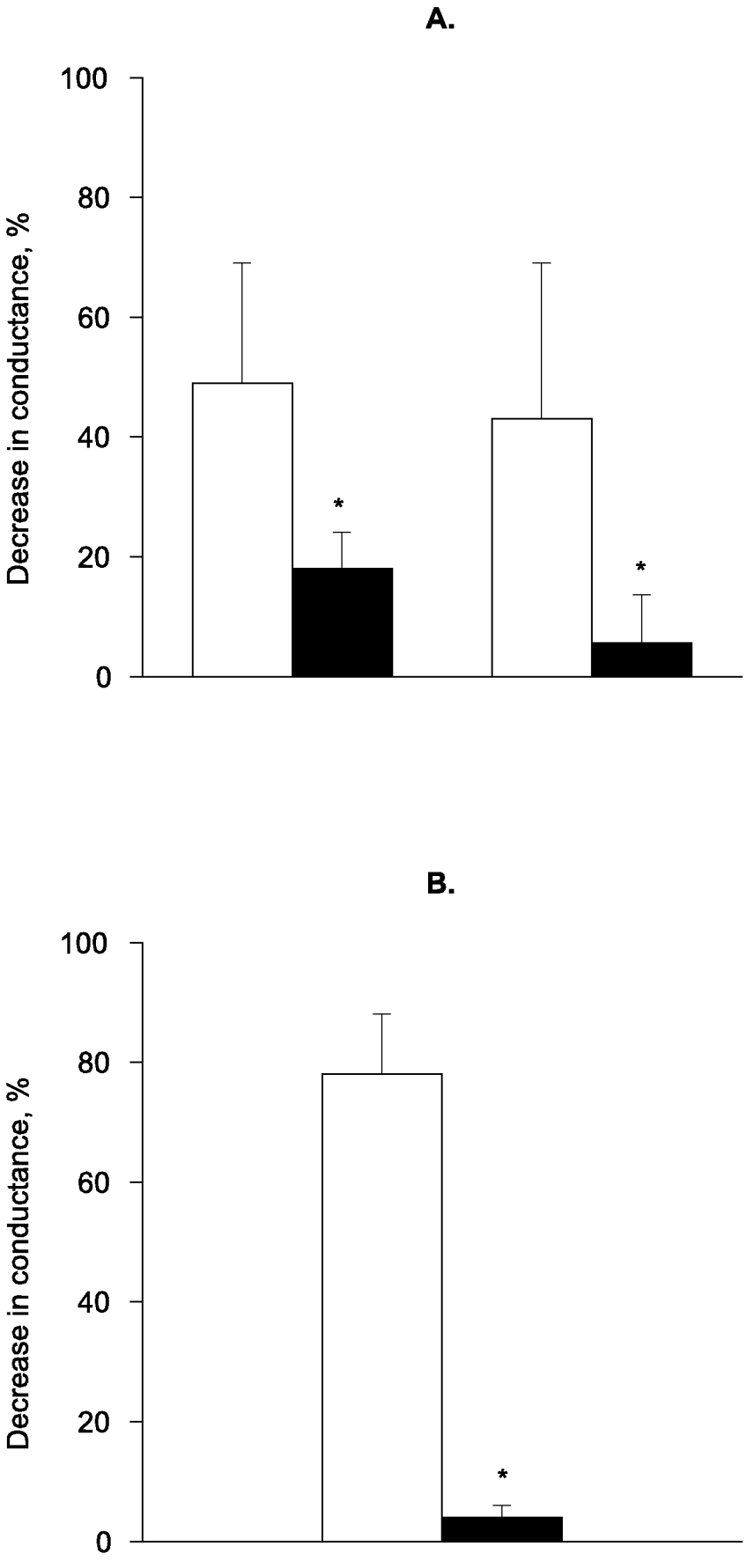

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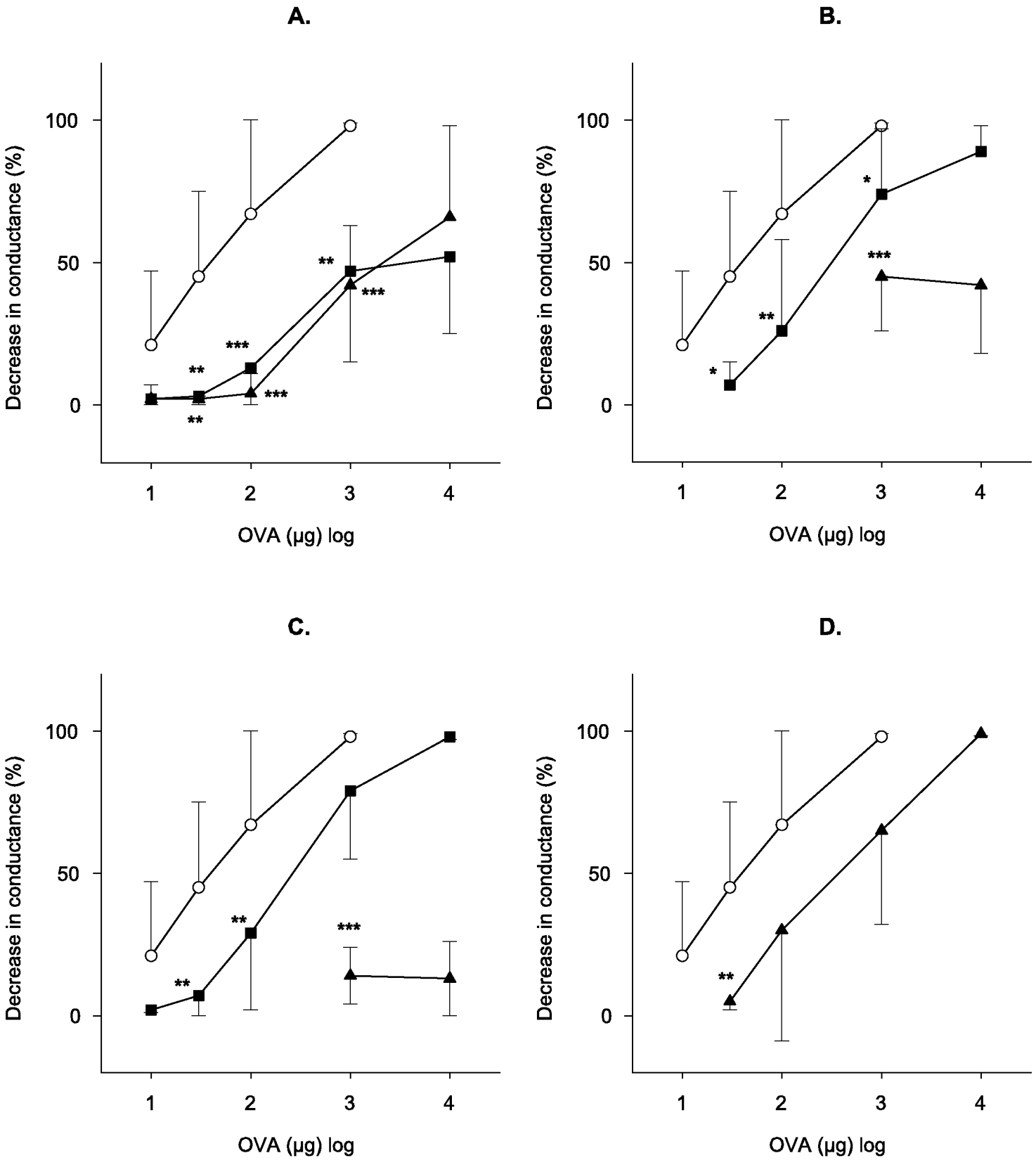
Fig. 5.



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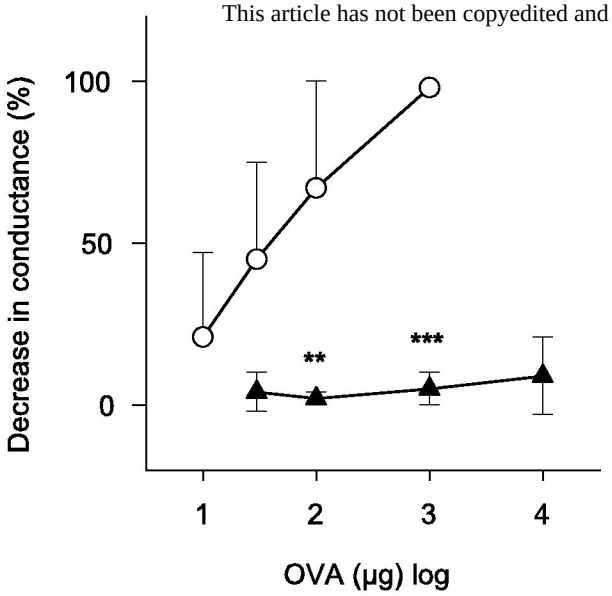

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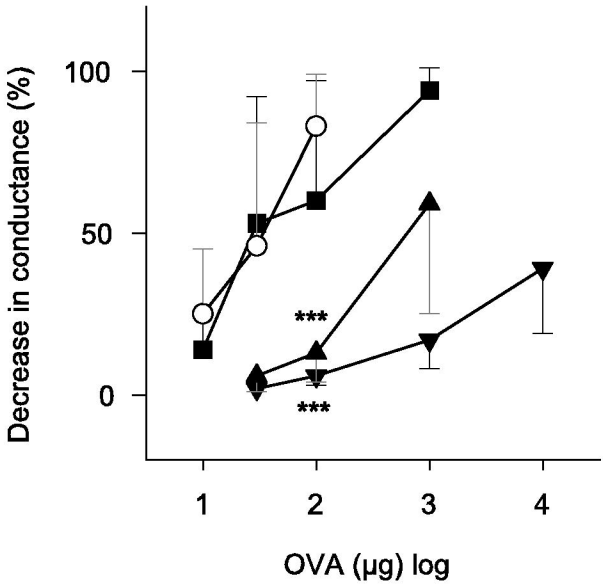

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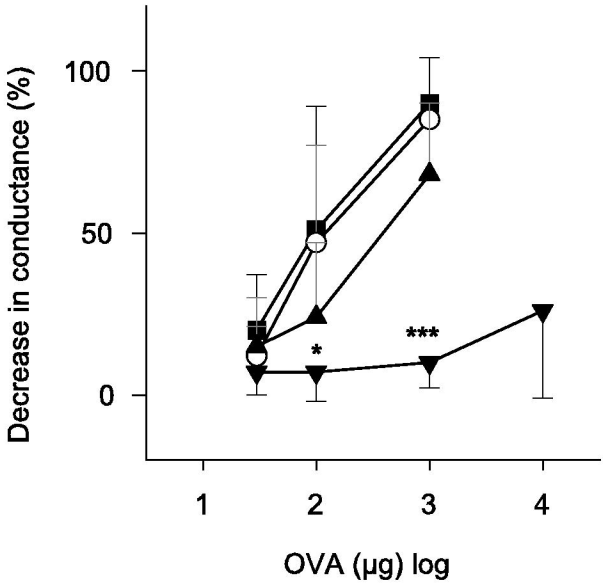

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Fig. 7.

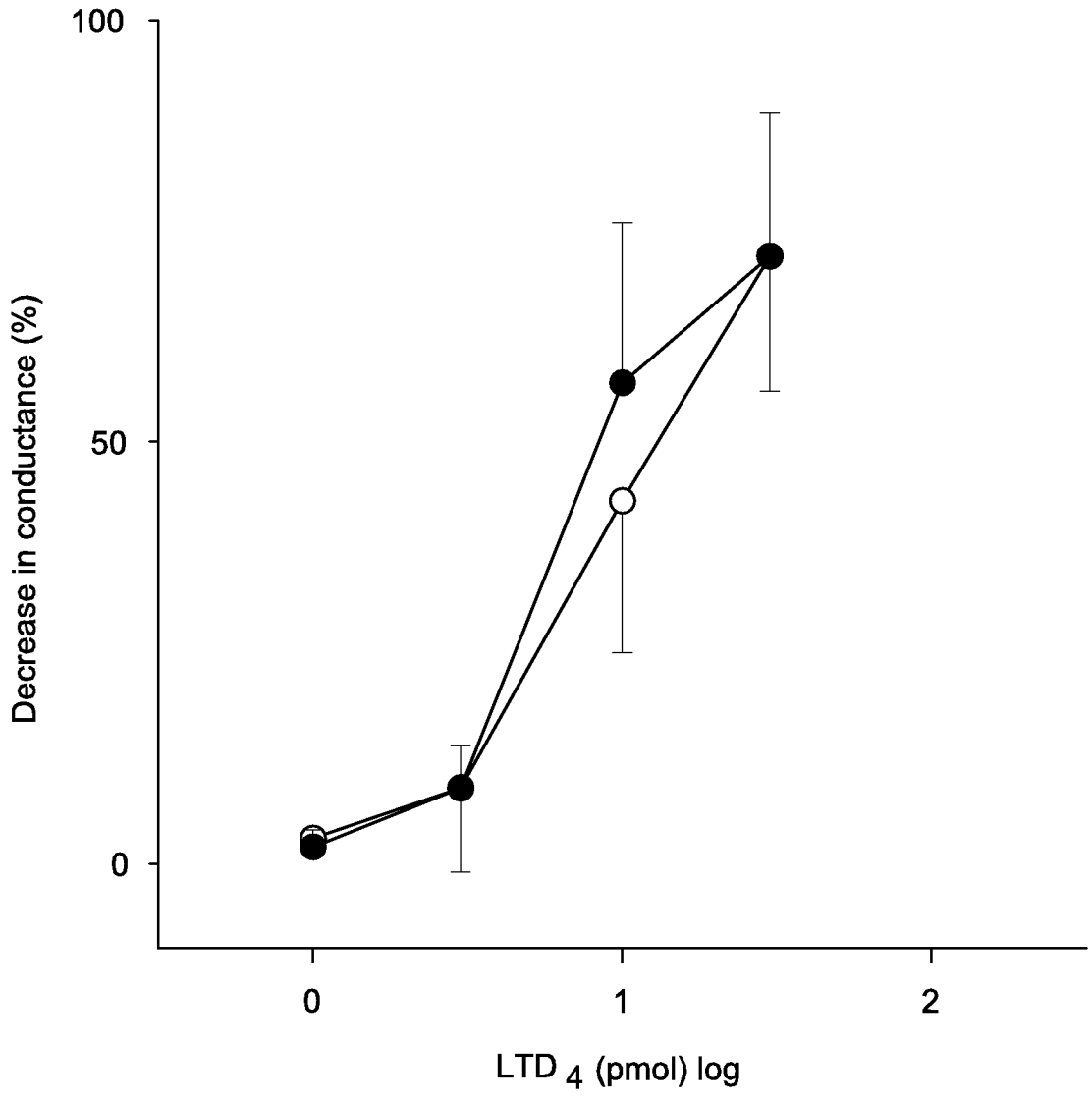

Fig. 8.

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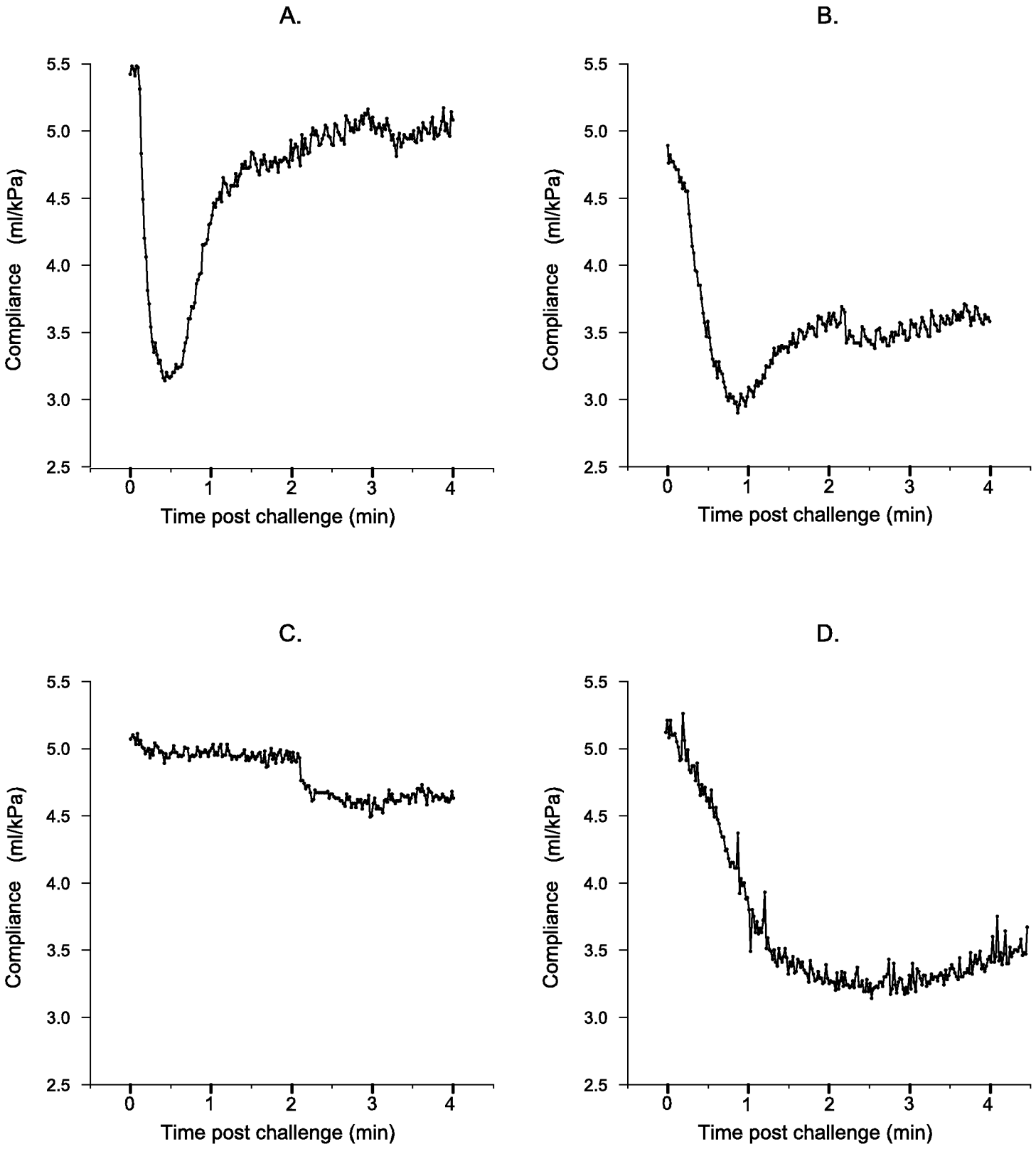

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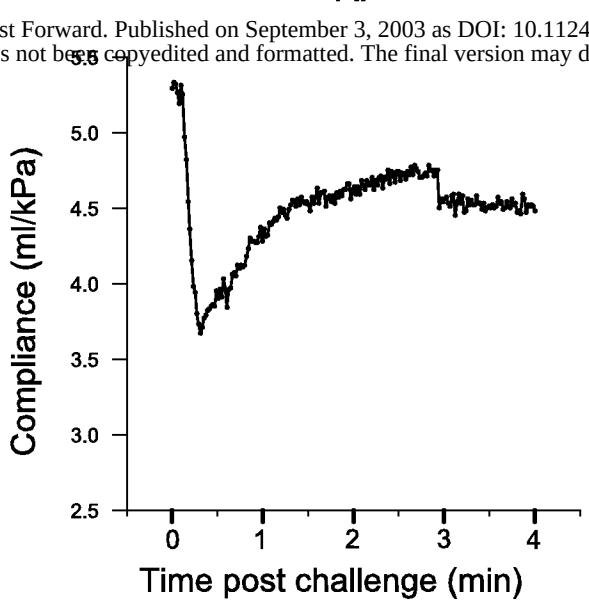

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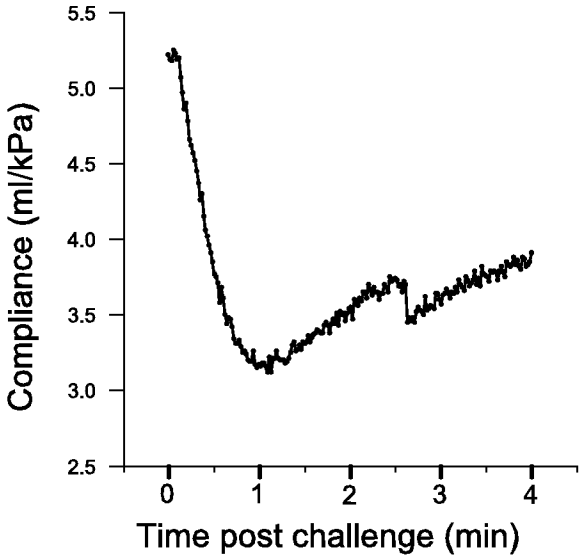

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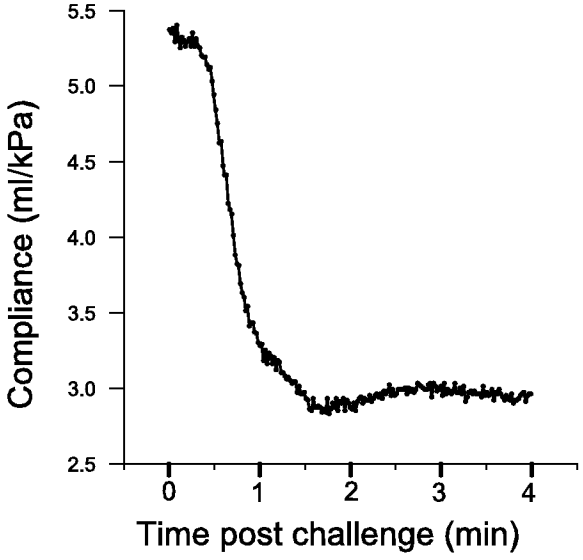

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TABLE 1.

Effect of repeated administration of LTD_4 and U-46619.

Time between 1st and 2nd administration of agonists was 10 minutes.
ns P > 0.05

Agonist	Decrease in conductance (%), mean ± SD		
	1st	2nd	
LTD_4 (3 pmol) (n = 6)	29 ± 18	38 ± 31	ns
U-46619 (100 pmol) (n = 3)	57 ± 16	65 ± 24	ns

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TABLE 2.
Potency of test agonists measured as D_{50}[a] values.
*P < 0.05, **P < 0.01, ***P < 0.001

Agonist	D_{50}, mean ± SD (n)	
Histamine	4.7 ± 2.4 nmol (5)	
LTC_4	10 ± 4 pmol (7)	** vs. LTD_4
LTD_4	3 ± 0.2 pmol (5)	** vs. Histamine
LTE_4	24 ± 15 pmol (4)	* vs. LTD_4
LTD_4 after diclofenac (10 μM)	96 ± 43 pmol (5)	** vs. LTD_4
U-46619	43 ± 15 pmol (4)	*** vs. LTD_4

[a]D_{50} : the agonist dose causing 50 % decrease in conductance.

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TABLE 3.
Statistical evaluation of different treatments.
Mep: mepyramine, Met: metiamide, Zaf: zafirlukast, Diclo: diclofenac
ns P > 0.02, *P < 0.02, **P < 0.01, ***P < 0.001

		Dose OVA (µg), log			
Graph	Treatment	1.5	2	3	4
5A	Mep + Met vs. Mep	ns	ns	ns	ns
5B	Zaf + Mep vs. Zaf			ns	**
5C	Diclo + Mep vs. Diclo			***	***
6B	Mep + Zaf vs. BAY u3405	ns	ns	ns	
6B	Mep + Zaf + BAY u3405 vs. BAY u3405	*	*	***	
6B	Mep + Zaf + BAY u3405 vs. Mep + Zaf	ns	ns	*	
6C	Mep + Met vs. BAY x1005	ns	ns	ns	
6C	BAY x1005 + Mep + Met vs. BAY x1005	ns	*	***	
6C	BAY x1005 + Mep + Met vs. Mep + Met	ns	ns	***	

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