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Modulation of *in vitro* anaphylaxis of guinea-pig isolated tracheal segments by azelastine, inhibitors of arachidonic acid metabolism and selected antiallergic drugs

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1 The ability of azelastine to influence antigen-induced contractile responses (Schultz-Dale phenomenon) in isolated tracheal segments of the guinea-pig was investigated and compared with selected antiallergic drugs and inhibitors of arachidonic acid metabolism.

2 Indomethacin produced a significant leftward shift of the antigen concentration-effect curve.

3 The inhibitory activity of azelastine on anaphylactic responses in guinea-pig trachea was dependent on the duration of exposure (preincubation period).

4 The relative order of potency (antianaphylactic activity) at calculated IC_{50} level was as follows: FPL 55712 (a leukotriene receptor antagonist) > nordihydroguaiaretic acid (a lipoxygenase inhibitor) > *p*-bromophenacyl bromide (a phospholipase A_2 inhibitor) > BW 755c (a dual inhibitor of lipoxygenase and cyclo-oxygenase) > theophylline (a phosphodiesterase inhibitor) > azelastine > diphenhydramine (H_1 histamine-receptor antagonist) > ketotifen > disodium cromoglycate. FPL 55712 (added 5 min before antigen challenge) was about 12 times as potent as azelastine (added 2 h before antigen challenge).

5 The incubation of tracheal segments with azelastine and BW 755c for a period of 30 min was found to inhibit indomethacin-augmented anaphylactic responses. These observations seem to suggest that azelastine and BW 755c interfere with the synthesis/release of the products of lipoxygenase/leukotriene synthetase pathway (e.g., leukotrienes) in the mediation of allergic responses in airway smooth muscles.

Introduction

Azelastine, 4-(*p*-chlorobenzyl)-2- (hexahydro-1-methyl-1H- azepine-4yl) -1- (2H)-phthalazinone hydrochloride, is a new, orally effective and long-acting antiallergic agent. Azelastine inhibits passive cutaneous anaphylaxis and allergic bronchoconstriction (Zechel *et al.*, 1981; Katayama *et al.*, 1981; Mandi *et al.*, 1981; Diamantis *et al.*, 1984a,b; Perhach *et al.*, 1984; Chand *et al.*, 1984, 1985a; Atkins *et al.*, 1985; Storms *et al.*, 1985). It inhibits allergic and nonallergic release of histamine (Chand *et al.*, 1983b; 1985b,c; Fields *et al.*, 1984). Azelastine has also been shown to inhibit the allergic release of slow reacting substance of anaphylaxis (SRS-A, leukotrienes) during passive peritoneal anaphylaxis in rats as well as calcium ionophore A23187-stimulated release of SRS (leukotrienes) from rat isolated mixed peritoneal cells (Diamantis *et al.*, 1982). It also affords protection against antihistamine-resistant, leukotriene-mediated allergic bronchospasm in guinea-pigs (Chand *et al.*, 1983a). These data seem to suggest that azelastine may be acting by interfering with the leukotriene synthetase pathway of arachidonic acid metabolism.

The antigen-induced *in vitro* contraction (Schultz-Dale phenomenon) of guinea-pig isolated tracheal smooth muscle has been used as a model for evaluating antiasthmatic activity of drugs and exploring their mode of action (Chand & Eyre, 1978). Indomethacin, a potent cyclo-oxygenase inhibitor, has been reported to augment allergic responses in guinea-pig isolated trachea (Hand & Buckner, 1979; Burka & Paterson, 1980) and human bronchus (Adam & Lichtenstein,

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1985). In this study the effects of azelastine on the indomethacin-augmented component of allergic responses in guinea-pig isolated tracheal segments were investigated and compared with selected antiallergic drugs and agents that are known to interfere with arachidonic acid metabolism.

Methods

Sensitization

Adult male Hartley strain guinea-pigs (Charles River Breeding Laboratories, N. Wilmington, MA) weighing 300–500 g were sensitized by intraperitoneal injections of 1 mg ovalbumin (OA) and 5×10^9 killed *Bordetella pertussis* organisms (Ritchie *et al.*, 1981).

Preparation of tracheal segments and recording of isometric responses

On days 12 to 18 of sensitization, guinea-pigs were killed by cervical dislocation. The thoracic segment of the trachea was dissected and cut into four segments, each 3–4 mm wide. Tracheal segments were randomly attached to force-displacement transducers (FT.03C) under an initial load of about 2 g in 10 ml isolated tissue baths containing Krebs-Henseleit solution. The composition of Krebs-Henseleit solution (mM) was as follows: NaCl 118.4, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0 (pH 7.4 at 37°C).

During the period of equilibration for 1 to 2 h, tracheal segments spontaneously and gradually developed an additional tone of 2–6 g. After the equilibration period and depending on the nature of the experiment, indomethacin (14 μM) was added to tissue baths. Indomethacin induced a slowly developing relaxation (reduction in spontaneous tone = 1470 ± 77 mg, $n = 150$) over a period of 30 to 60 min. After 60 min of indomethacin treatment, loading tensions were readjusted to 4 to 4.5 g. Then each segment was contracted with acetylcholine (100 μM) to determine acetylcholine maximum response (ACh*max*).

Effect of inhibitors on Schultz-Dale anaphylactic response

Thirty to sixty minutes later each potential inhibitor drug was allowed to remain in contact with the tissues for a period of 120 min, except disodium cromoglycate (DSCG) and FPL 55712. One or two tracheal segments served as an untreated 'no antagonist' control in each experiment. Five to 10 min before antigen ovalbumin (OA) challenge the loading tensions were readjusted to 4 to 4.5 g. DSCG was added immediately before antigen challenge. Compound FPL 55712 (Chand, 1979a) was added 5 min before OA challenge. The OA-induced responses were measured in the absence and presence of drugs and expressed as percentage of ACh*max*. The OA-induced responses, expressed as a percentage of ACh*max*, recorded on 5 to 9 tracheal segments of different guinea-pigs per week for a period of several months were subjected to analysis of variance. The mean ± s.e.mean of these observations did not vary significantly ($P > 0.05$) from week to week.

Time course of development of anaphylactic response

In an additional set of experiments, the effects of 30 min exposure of guinea-pig isolated tracheal segments to azelastine (50 and 100 μM) and BW 755c (10 and 20 μM; a dual inhibitor of cyclo-oxygenase and lipoxygenase) on the time course of the development of the indomethacin-augmented anaphylactic response were also studied. The parameters used in this part of the study were: (a) *the duration of the onset of contraction* (latent period in minutes), i.e., the time required for the initiation of the anaphylactic responses after the addition of OA to the tissues; (b) *time to peak* (in min), i.e., the time required for the establishment of plateau (maximum) contractile responses after the addition of antigen. The anaphylactic responses were expressed as percentage of ACh*max* at peak effect (initial phase) and also at 30, 45, 60 and 90 min intervals during the course of the secondary phase (maintenance and recovery phases) of the anaphylactic responses. These parameters were compared by Student's *t* test between (a) 'control' (no indomethacin) and indomethacin-treated tissues, i.e., to

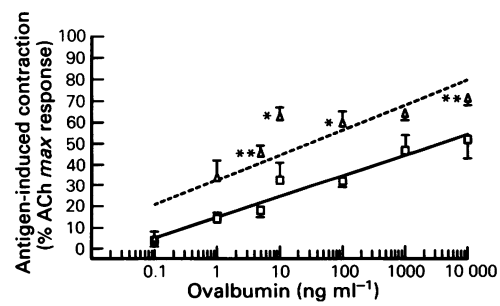

Figure 1 Augmentation of ovalbumin-induced contractile responses (Schultz-Dale phenomenon) by indomethacin (14 μM) in guinea-pig isolated tracheal segments. Only one concentration of ovalbumin was tested on each segment. Values are mean of ($n = 4$ to 6 segments; vertical lines show s.e.mean). The augmentation of anaphylactic responses by indomethacin (△) was significant ($P < 0.05$) at 5, 10, 100 and 10,000 ng ml^{-1} of ovalbumin as compared to untreated tracheal segments (□).

measure the magnitude of the augmentation of anaphylactic responses by indomethacin (14 μM, 60 min preincubation before antigen challenge); and (b) between indomethacin (14 μM, 60 min) alone versus combination of indomethacin (14 μM, 60 min) plus drug (azelastine or BW 755c, 30 min exposure before antigen challenge).

The significance of the inhibitory activity of the drugs on allergic airway responses was determined by comparing the OA-responses (% of ACh*max*) in the absence and presence of drugs by Student's *t* test. The significance of the augmentation of antigen- and acetylcholine-induced contractile responses by indomethacin was also determined by Student's *t* test.

The IC_{50} values, i.e., the concentration of drugs inhibiting antigen-induced contractile responses of guinea-pig tracheal segments by 50%, were calculated from the line of best fit of the concentration-effect curve of each drug and the 95% confidence limits were also calculated.

Drugs

Sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxy propoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate (FPL 55712), disodium cromoglycate (DSCG, Fisons, Bedford, MA), ketotifen (Sandoz Pharmaceuticals, E. Hanover, NJ), theophylline (Ganes Chemicals, NY), diphenhydramine (Parke-Davis, Morris Plains, NJ) and 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride (BW 755c) (Wellcome Research Laboratories, Beckenham, Kent, U.K.) were dissolved in double glass distilled water immediately before use. Azelastine (Wallace laboratories, Cranbury, NJ) (100 μM) dissolved in dimethylsulphoxide (DMSO, final bath concentration, 0.1%) and distilled water, respectively, produced 97.4 ± 2.6% and 98 ± 3% inhibition of anaphylactic responses. Therefore, the data from all these experiments were pooled. Nordihydroguaiaretic acid (NDGA), and *p*-bromophenacylbromide (PBPB, Sigma Chemical Co., MO) were dissolved in propylene-glycol. Propylene-glycol in a final bath concentration (0.005 to 0.1%) was found to have no significant ($P > 0.05$) inhibitory influence on allergic tracheal contractile responses. Indomethacin (Sigma Chemical Co., MO) was dissolved in warm phosphate buffer (1 mg ml^{-1}).

Results

Schultz-Dale response

In an initial series of experiments, the concentration effect curve for OA (0.1–10,000 ng ml^{-1}) was established in the absence and presence of indomethacin (14 μM). The antigen (OA)-induced contractile responses were expressed as percentage of ACh*max*. As shown by the data presented in Figure 1, indomethacin augmented allergic airway responses, i.e., significantly shifted the OA-response curve to the left. The acetylcholine (100 μM)-induced contractile responses of isolated tracheal segments in the absence and presence of indomethacin were 2985 ± 276 mg ($n = 31$) and 5912 ± 458 mg ($n = 34$), respectively ($P < 0.001$).

From the concentration-effect curve of ovalbumin, a suboptimal concentration of OA (5 ng ml^{-1}) producing 46 ± 3% of ACh*max* responses in the presence of indomethacin (14 μM) was selected for examination of the effects of azelastine and other drugs. Indomethacin was used: (i) to exclude the possible modulatory role of the products of cyclo-oxygenase pathway of arachidonic acid (AA) metabolism, e.g., prostaglandin E_2; (ii) to enhance the allergic responses, perhaps by shunting AA metabolism via 5-lipoxygenase/leukotriene synthetase pathways, (s) leading to increased production of leukotrienes.

Effect of duration of preincubation

In preliminary experiments, azelastine (100 μM) was found to exert 40% and 89% inhibition of OA (5 ng ml^{-})-induced contractile responses following 30 and 120 min exposure, respectively (Table 1). Subsequently, the antianaphylactic activities of azelastine and other drugs were evaluated and compared with a 120 min exposure period of the tissues.

Pharmacological modulation of Schultz-Dale responses in guinea-pig isolated tracheal segments

The effects of azelastine, inhibitors of arachidonic acid metabolism, compound FPL 55712, and selected antiallergic drugs on antigen (ovalbumin: 5 ng ml^{-1})-induced contractile responses are summarized in Table 2. Following 2 h contact with the tissues, the IC_{50} values (μM) were as follows: compound FPL 55712 = 4.2; NDGA = 10.7; PBPB = 21.4; theophylline = 29; BW 755c = 29.6; azelastine = 49.9; diphenhydramine = 96.2; and ketotifen = 198.3. DSCG in a concentration range of 10–1000 μM failed to exert any significant ($P > 0.05$) anti-anaphylactic effect (Table 2). Following 2 h contact period, 100 μM concentrations of ketotifen and diphenhydramine also did not produce any significant ($P > 0.05$) inhibition of anaphylactic responses.

Time course of the anaphylactic response in guinea-pig isolated tracheal segments

In the absence of indomethacin, OA (5 ng ml^{-1}) induced contractile responses (35.7 ± 5.3% of ACh*max*) with a delay in onset of 54 ± 6 s after antigen

Table 1 Influence of the duration of preincubation of azelastine on antigen (ovalbumin, 5 ng ml^{-1})-induced contractile responses in guinea-pig isolated tracheal segments in the presence of indomethacin (14 μ)

Azelastine conc (μM)	n	*Incubation time* (min)	*anaphylactic contractile response* (% ACh*max response*)	*% inhibition*
0	10		59.2 ± 5.4	
(Control)		30		51.7 ± 7.7
100	14		28.1 ± 4.9	
0	8		60 ± 5.2	
(Control)		120		86.8 ± 2.4*
100	14		8 ± 1.5*	

Values are means ± s.e.mean.
*$P < 0.05$ (30 min vs 120 min preincubations).

challenge of the tracheal segments and achieved a peak effect in 16.5 ± 2.2 min. The second phase of allergic response was characterized by gradual recovery over a period of > 90 min. Indomethacin (14 μM) added to the tissues 1 h before antigen challenge did not change the time to peak but significantly augmented the magnitude of the initial phase (peak effect) as well as the secondary phase of the anaphylactic response at 30 min but not at 45, 60 or 90 min time intervals (Figure 2). The treatment of the tissues with azelastine (50 μM) for a period of 30 min significantly delayed the duration of the onset and peak effect and slightly, but not significantly, attenuated the magnitude of the indomethacin-augmented component of anaphylactic responses. At a higher concentration (100 μM), azelastine prolonged ($P < 0.05$) the duration of onset but not the peak effect of the allergic responses. At this concentration azelastine also inhibited ($P < 0.05$) the indomethacin-augmented component of initial and secondary phases of anaphylactic responses of guinea-pig isolated tracheal segments. BW 755c (10 μM) only prolonged ($P < 0.05$) the duration of onset of the allergic response with little or no effect on the duration of peak effect and the magnitude of anaphylactic

Table 2 Summary of the inhibitory activity of compound FPL 55712, inhibitors of arachidonic acid metabolism and antiallergic drugs on allergic contractile responses of guinea-pig tracheal segments (ovalbumin 5 ng ml^{-1} in the presence of indomethacin 14 μM).

Drug	*IC_{50}*	*95% confidence limits*	*Slope*	r
Leukotriene receptor antagonist:				
FPL 55712	4.2	0.9– 18.9	22.70	0.89
Lipoxygenase inhibitor:				
Nordihydroguaiaretic acid	10.7	6.6– 17.3	74.72	0.99
Phospholipase A_2 inhibitor:				
p-Bromophenacyl bromide	21.4	4.9– 92.8	38.95	0.93
Dual inhibitor of cycloxygenase and lipoxygenase:				
BW 755c	29.6	19.9– 44.2	75.07	0.88
Antiallergic drugs:				
Theophylline	29.0	14.9– 56.6	51.41	0.68
Azelastine	49.9	37.0– 68.0	80.86	0.67
Diphenhydramine	96.2	16.0–580.0	25.59	0.70
Ketotifen	198.3	177.0–222.1	123.25	1.00
Disodium cromoglycate	> 1000		6.54	0.23

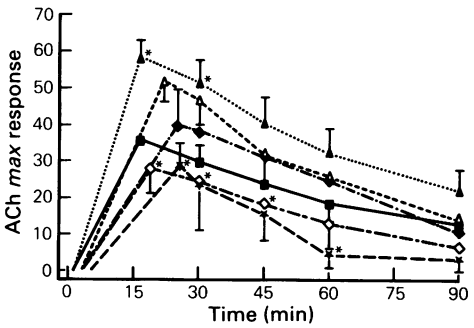

Figure 2 Pharmacological modulation of the time course of the *in vitro* anaphylactic responses (control: ■, $n = 14$) by indomethacin 14 μM, 60 min preincubation before ovalbumin challenge: ▲, $n = 13$), BW 755c (10 μM: △, $n = 6$; 20 μM: ★, $n = 5$) and azelastine (50 μM: ◆, $n = 5$; 100 μM: ◇, $n = 9$). Effects of BW 755c and azelastine (30 min preincubation) were evaluated in the presence of indomethacin. Each point represents mean with s.e.mean shown by vertical line. Asterisk (*) indicates significance ($P < 0.05$; control vs indomethacin; indomethacin alone vs BW 755c or azelastine in the presence of indomethacin, unpaired Student's t test). S.e.mean at certain points is not shown to avoid overlapping.

responses. Compound BW 755c (20 μM) not only prolonged ($P < 0.05$) the duration of onset and peak effect but also inhibited the indomethacin-augmented component of anaphylactic responses, i.e., initial phase, peak effect and secondary phase at 60 min ($P < 0.05$) (Figure 2).

Discussion

The data obtained in this study showed that allergic contractile responses of guinea-pig isolated tracheal segments in the presence of indomethacin are susceptible to compound FPL 55712 (a leukotriene receptor antagonist), nordihydroguaiaretic acid (NDGA, a 5-lipoxygenase inhibitor), BW 755c (a dual inhibitor of cyclo-oxygenase and lipoxygenase) and *p*-bromophenacyl bromide (a phospholipase A_2 inhibitor). These observations and reports from other investigators suggest that the activation of phospholipase A_2 and 5-lipoxygenase/leukotriene synthetase could play an important role in the mediation of allergic airway responses (this study; Chand, 1979, a,b; Hand & Buckner, 1979; Burka & Paterson, 1980; Chand & Altura, 1981; Samuelsson, 1983; Adams & Lichtenstein, 1985).

The blockade of the indomethacin-augmented airway responses (the time course of the development of anaphylactic responses) by BW 755c and azelastine (50–100 μM) also suggests the involvement of the products of leukotriene synthetase pathway of arachidonic acid metabolism in the mediation of allergic tracheal responses. Further direct biochemical evidence to demonstrate the inhibitory effects of BW 755c and azelastine on leukotriene synthesis in the lungs (airways) and leukocytes is in progress.

Ketotifen, diphenhydramine and DSCG at 100 μM concentrations failed to exert any significant inhibition of anaphylactic responses, whereas azelastine, theophylline and BW 755c (100 μM) after 2 h of incubation with the tissues produced virtually complete inhibition of the allergic responses. The failure of diphenhydramine (a classical H_1-histamine receptor antagonist) and ketotifen (a new, orally acting antihistamine-antiallergic agent) to influence these allergic airway responses suggests a minor role of histamine in the mediation of these responses.

The inhibition of allergic tracheal responses by azelastine was dependent on the duration of preincubation, suggesting a slow onset of action. On the contrary, azelastine inhibits antigen-, A23187- and concanavalin A-stimulated histamine secretion from mast cells even when added simultaneously or immediately prior to the addition of the secretagogues to the cell suspensions with an IC_{50} of 7 to 8.8 μM (Chand *et al.*, 1983b; 1985b,c; Fields *et al.*, 1984). Aerosolized azelastine administered immediately or 15 min before antigen challenge effectively inhibits lung anaphylactic responses, i.e., decline in dynamic lung compliance and increase in airway resistance (Chand *et al.*, 1985a). These observations suggest that azelastine exerts rapid and selective inhibitory effects on the synthesis/release of chemical mediators from mast cells (basophils, leukocytes), perhaps by interfering with a Ca^{2+}-dependent step in target cells of allergic inflammation (Chand *et al.*, 1983b).

It has been suggested that the products of 5-lipoxygenase/leukotriene synthetase pathway, e.g., 5-HPETE, 5-HETE and leukotrienes, play an important role in the pathophysiology of airway hyper-reactivity in asthmatics and could be responsible for aspirin-induced asthma (Chand & Altura, 1981; Copas *et al.*, 1982; Samuelsson, 1983). Earlier, azelastine has been shown to interfere with the synthesis/release of SRS-A (SRS-leukotrienes) *in vivo* as well as in *in vitro* model systems (Diamantis *et al*, 1982; Chand *et al.*, 1983a). Recently, azelastine was found to inhibit calcium ionophore A23187 (0.2 μM)-stimulated leukotriene C_4 (LTC_4) formation in rat mixed peritoneal cells by use of a radioimmunoassay technique, with an IC_{50} of 22.8 μM (unpublished observations). Therefore in addition to bronchodilatation in asthmatics (Storms *et al.*, 1985), i.e. antagonism of chemical mediators such as histamine, Ca^{2+}, and leukotrienes (Chand *et al.*, 1984), azelastine may also act by inhibiting histamine secretion and the synthesis of leukotrienes and sub-

sequent allergic airway responses (this study; Diamantis *et al.*, 1982; 1984a,b; Chand *et al.*, 1983a,b; 1984; 1985a,b,c).

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