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Phospholipase A_2 induced airway hyperreactivity to cooling and acetylcholine in rat trachea: pharmacological modulation

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1 Rat isolated tracheal smooth muscle preparations respond to phospholipase A_2 (PLA_2) and phospholipase C (PLC) with contractile responses of highly variable magnitudes. Rat tracheae exposed to PLA_2 or PLC for a period of 10–30 min, exhibit airway hyperreactivity (AH) to cooling (10°C), i.e., respond with strong contractile responses. Phospholipase D neither contracted rat tracheae nor induced AH to cooling.

2 PLA_2-induced AH to cooling was dependent on the presence of extracellular Ca^{2+} in the physiological solution.

3 Verapamil, azelastine, diltiazem and TMB-8 (each 10 μM) significantly attenuated PLA_2-induced AH. This effect was not shared by nifedipine (10 μM).

4 Bepridil (10 μM), a Ca^{2+} and calmodulin antagonist, also significantly attenuated AH induced by PLA_2.

5 Indomethacin (a cyclo-oxygenase inhibitor), AA-861 (a selective 5-lipoxygenase inhibitor), FPL 55712 (a leukotriene receptor antagonist), methysergide (a 5-hydroxytryptamine D-receptor antagonist) and pyrilamine (a histamine H_1-receptor antagonist) exerted little or no effect on PLA_2-induced AH to cooling.

6 Atropine significantly attenuated PLA_2-induced AH suggesting the participation of acetylcholine.

7 Nordihydroguaiaretic acid (an antioxidant; 5-lipoxygenase inhibitor) and BW 755C (an antioxidant; a dual inhibitor of cyclo-oxygenase and 5-lipoxygenase) significantly attenuated PLA_2-induced AH to cooling.

8 In conclusion, these data show that PLA_2 (an enzyme involved in the synthesis of Paf-acether, prostaglandins, thromboxanes, leukotrienes, diacylglycerol, superoxide free radicals and lipid peroxides, etc.) induces AH to cooling and acetylcholine in rat trachea. The induction of AH to cooling is dependent on the presence of extracellular Ca^{2+} and is significantly attenuated by verapamil, diltiazem, bepridil, atropine and azelastine (an antiallergic/antiasthmatic drug).

Introduction

Non-specific airway hyperreactivity (AH) to a variety of pharmacological and physical stimuli such as histamine, methacholine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and exercise is a hallmark of asthma (Bleeker, 1986). There are at least two hypotheses (cooling and/or drying of the airways and transient increase in the osmolarity/osmolality of tracheal mucosal fluid) which have been put forth to explain the underlying cause of exercise-induced asthma (Anderson, 1985). We have recently developed an *in vitro* model of AH to cooling in rat trachea, in which cooling (10°C) itself causes weak (5–10% acetylcholine maximum) contractile responses. However, tracheal segments exposed to threshold concentrations of chemical mediators such as platelet-activating factor (Paf-acether), acetylcholine and adenosine, or following recovery from allergic responses, exhibit AH to cooling (Chand *et al.*, 1986; 1987a,b). In the present study we now demonstrate that phospholipase A_2 (PLA_2) induces AH to cooling and acetylcholine in rat trachea. The pharmacological modulation of PLA_2-induced AH to cooling by a variety of drugs is also described.

Methods

The methodology used in this study was identical to that described previously (Chand *et al.*, 1986; 1987a). In brief, adult male Sprague-Dawley rats (Charles River Breeding Laboratories, N. Wilmington, MA) weighing 250–450 g were killed by exposure to CO_2. An 8–10 mm long segment of the thoracic trachea was dissected from each rat and placed in warm Krebs-Henseleit solution (37°C). Each tracheal tube was cut into two equal segments. These segments were set up in two isolated tissue baths containing Krebs-Henseleit solution which was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. The composition of the Krebs-Henseleit solution was (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.9, glucose 10.0 (pH 7.4). Tracheal segments were attached to force-displacement transducers (Grass FT.03C) under 1 g loading tension; this was determined to be the optimum applied load. Tissues were allowed to equilibrate for 1 to 3 h and were washed at 30–60 min intervals with fresh physiological salt solution. The loading force was maintained at 1 g during the stabilization period.

After an equilibration period the effects of PLA_2 (0.1 μM), PLC (0.05 μM) or PLD (0.7 μM) on the cooling (10°C) responses of rat tracheae were evaluated.

The effect of the threshold concentration of PLA_2 (0.1–1.0 μM, 10–30 min) on the responsiveness of rat tracheal segments to acetylcholine (ACh) was also examined. Cumulative-concentration effect curves for ACh were constructed and significance was determined at each ACh concentration by a paired t test.

Pharmacological modification of phospholipase A_2-induced airway hyperreactivity to cooling

To exclude the possible involvement of prostaglandins, indomethacin (14 μM) was added to all the tracheal segments and left in contact with the tissues for the entire length of the experiments, except in those experiments where the effect of BW 755C or indomethacin alone was evaluated.

One segment of each pair (the test tissue) was exposed to the appropriate concentration of a modifying agent (verapamil, diltiazem, TMB-8, nifedipine, bepridil, azelastine, pyrilamine, atropine, methysergide, indomethacin, BW 755C, AA 861, nordihydroguaiaretic acid, L-652,731 or Ca^{2+} free Krebs) for a period of 2 h before the addition of threshold concentrations of PLA_2 (0.1–1.0 μM). When FPL 55712 was used as a modifying agent, it was added 5 min before PLA_2 challenge. In one set of experiments, L-652,731 (100 μM) was added one minute before the addition of threshold concentrations of PLA_2 (0.1–1.0 μM). The second segment of each pair of tissues was treated identically to the test tissue but was not exposed to the modifying agent. It served as a vehicle-treated control.

Following recovery from PLA_2 contractile responses, cold (10°C) water was circulated in the outer jacket of the tissue bath. Cold-induced contractile responses were recorded for an additional 15–30 min or until a plateau was established. Warm water (37°C) was then circulated in the outer jacket of the tissue bath, which caused immediate reversal (relaxation) of cold-induced contractile responses. Fifteen to 30 min later, tissues were exposed to acetylcholine (ACh: 1 mM) to determine the ACh maximal response. When atropine (1 μM) was used, contractility was measured by adding acetylcholine (>30 mM).

The results are given as the mean ± s.e.mean and are expressed as % ACh. max. and mg tension. Statistical comparison between vehicle- and drug-treated segments was performed by use of paired t test; statistical significance was implied when the P value was <0.05.

Drugs

Phospholipase A_2 (from *N. naja* venom), phospholipase C (from *C. welchii*) and phospholipase D (from cabbage) (obtained from Sigma Chemical Co., St. Louis, MO) were dissolved in Krebs-Henseleit solution and stored at −70°C. Sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate (FPL 55712, Fisons, Bedford, MA), azelastine hydrochloride (Wallace Laboratories, Cranbury, NJ), acetylcholine chloride (ICN Nutritional Biochemicals, Cleveland, OH), pyrilamine maleate and atro-pine sulphate (Sigma), 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride (BW 755C, Wellcome Research Laboratories, Beckenham, Kent, U.K.), verapamil hydrochloride (Knoll Pharmaceutical Co., Whippany, NJ), diltiazem hydrochloride (Marion Laboratories, Kansas City, MO), 8-(N,N-diethylamino-octyl-3,4,5-trimethoxybenzoate (TMB-8, Aldrich Chemical Co., Milwaukee, WI) and methysergide hydrogen maleate (Sandoz Ltd., Basle, Switzerland) were dissolved in double glass distilled water immediately before use. Bepridil (Wallace Laboratories, Cranbury, NJ) and 2,3,4,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA-861, Takeda Chemical Industries, Ltd., Osaka, Japan) were dissolved in 10% ethanol (10 mM). (±)-2,5-*bis*(3,4,5-trimethoxyphenyl)tetrahydrofuran (L-652,731, Merck Sharp & Dohme, Rahway, NJ) was dissolved in DMSO (10 mM). Nordihydroguaiaretic acid (NDGA, Sigma) was dissolved in propylene glycol. Indomethacin

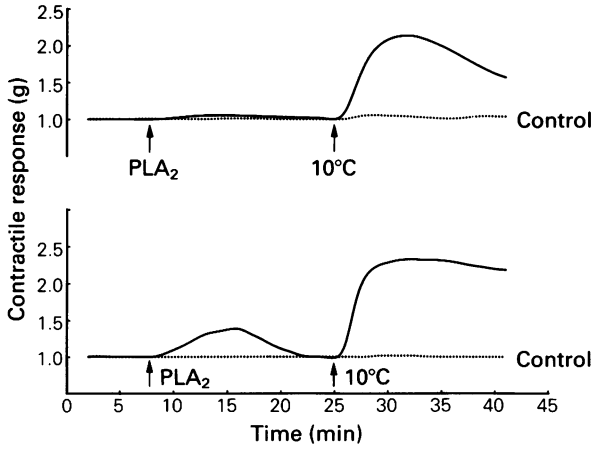

Figure 1 Typical tracings showing the induction of airway hyperreactivity (AH) to cooling (10°C) by a threshold concentration of phospholipase A_2 (PLA_2; 0.1 μM: solid line) in rat tracheal segments. Second segment of the pair served as vehicle treated control (dotted line).

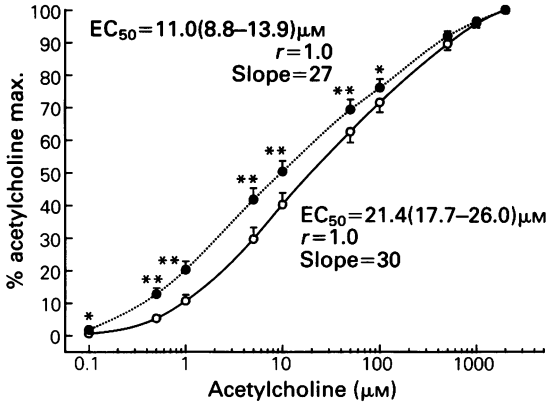

Figure 3 The cumulative log concentration-effect curves of acetylcholine in the absence (○) and presence (●) of phospholipase A_2 (PLA_2; 0.1–1.0 μM, 10–30 min). $^*P < 0.05$, $^{**}P < 0.01$; significance difference, determined by paired t test, between vehicle and PLA_2 exposed tracheal segments. Each point represents the mean ($n = 7$) and vertical lines indicate s.e.mean.

(Sigma) was dissolved (1 mg ml^{-1}) in warm phosphate buffered saline (0.15 M Na_2HPO_4, 0.15 M KH_2PO_4, 0.9% NaCl).

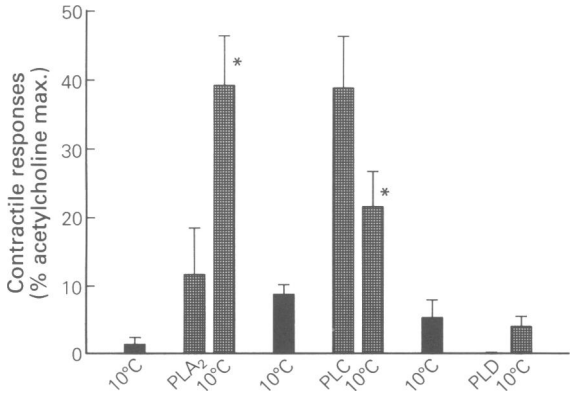

Figure 2 The effect of phospholipase A_2 (PLA_2; 0.1 μM), PLC (0.5 μM) and PLD (0.7 μM) (hatched columns) on the subsequent contractile responses to cooling (10°C) in rat isolated tracheal segments. Note: PLD neither contracted nor induced airway hyperreactivity (AH) to cooling. Asterisks indicate the significance ($P < 0.05$) of the induction of AH to cooling by PLA_2 and PLC as compared to the vehicle-treated tissues (solid columns) (determined by paired t test). Each column represents the mean ($n = 4$) and vertical lines show s.e.mean.

Results

Induction of airway hyperreactivity to cold provocation by phospholipase A_2 and C

Rat isolated tracheal segments responded to PLA_2 (0.1 μM) with contractile responses of variable magnitudes (362 ± 211 mg; 12 ± 7% ACh max) (Figures 1 and 2). PLC (0.05 μM) exerted stronger contractile responses (833 ± 239 mg; 39 ± 7% ACh max (Figure 2).

The tracheal segments which were exposed to PLA_2 or PLC for a period of 10–30 min (during this period tissues usually returned to their pre-contraction, resting loading tensions) responded with strong contractile responses to cooling (10°C) as compared to control (in the absence of PLA_2 or PLC) segments (Figures 1 and 2). PLD neither contracted rat tracheae nor induced AH to cooling ($n = 4$) (Figure 2).

Induction of airway hyperreactivity to acetylcholine by phospholipase A_2

The cumulative-concentration effect curve of ACh was significantly shifted to the left in the tracheal segments exposed to threshold concentrations of PLA_2 (0.1–1.0 μM) as compared to control segments (Figure 3).

Table 1 Effect of Ca^{2+}-channel blockers, azelastine, pyrilamine, atropine and methysergide on phospholipase A_2-induced airway hyperreactivity to cooling in rat isolated tracheae

Drugs (2 h)	*Bath conc.* (μM)	n	*% inhibition of cold provocation (10°C)-induced contractile responses* (mean ± s.e.mean)
Ca^{2+}-channel blockers			
Verapamil	1	5	16 ± 10
	5	5	11 ± 8
	10	6	64 ± 10***
Diltiazem	10	4	32 ± 9*
TMB-8	10	6	16 ± 5*
Nifedipine	10	7	24 ± 9
Ca^{2+} and calmodulin antagonist			
Bepridil	10	6	29 ± 8*
Antiasthmatic			
Azelastine	1	4	27 ± 11
	5	5	66 ± 10*
	10	6	66 ± 11**
H_1-histamine receptor antagonist			
Pyrilamine	10	4	28 ± 13
Cholinoceptor antagonist			
Atropine	0.1	4	64 ± 12***
	1.0	8	53 ± 11**
5-Hydroxytryptamine-receptor blocker			
Methysergide	1.0	4	17 ± 8

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significance of drug effect was determined by comparing contractile responses (% ACh max) to cooling in PLA_2 (0.1–1.0 μM) exposed tissues in the presence and absence of modifying drugs by paired t test. All the tissues were pre-exposed to indomethacin (14 μM, 3 h).

Pharmacological modulation of PLA_2-induced airway hyperreactivity

The addition of indomethacin (14 μM) to rat tracheae exerted quick and moderate to strong contractile responses (> 200–1450 mg, 4.9–47.9% ACh max) in 10% of the tracheal segments ($n = 46$), while, in other segments, this response was either poor or non-existent (0–200 mg, 0.0–12.1% ACh max, $n = 361$).

The effects of the pharmacological agents on PLA_2 (0.1–1 μM)-induced AH have been summarized in Tables 1 and 2. Atropine (0.1 and 1 μM), azelastine (5 and 10 μM), NDGA (10 μM) and BW 755C (20 μM) exerted significant attenuation of PLA_2-induced AH. Pyrilamine (10 μM), methysergide (1 μM), AA-861 (10 μM), FPL 55712 (10 μM), indomethacin (14 μM), and L-652,731 (1–100 μM, 2 h; 100 μM, 1 min) failed to exert any significant inhibition of PLA_2-induced AH to cold provocation (Tables 1 and 2).

Obligatory role of Ca^{2+} in the induction of airway hyperreactivity to cold provocation by phospholipase A_2

The incubation of one segment of each tissue pair in Ca^{2+}-free Krebs-Henseleit solution for a period of 2 h resulted in a loss of responsiveness to PLA_2 (0.1 μM) as well as in an abrogation of its ability to produce AH to cold provocation (Figure 4).

With the exception of nifedipine, the exposure of rat tracheal segments to calcium channel blockers (each at 10 μM) for a period of 2 h also attenuated PLA_2-induced airway hyperreactivity to cooling (Figure 5, Table 1).

Discussion

The data obtained in this study show that (i) phospholipase A_2 causes airway hyperreactivity (AH) to cold provocation and acetylcholine; (ii) the development of long lasting (> 30 to 60 min) PLA_2-induced AH to cooling is dependent on the presence of extracellular Ca^{2+} in the physiological solution, and is attenuated by some voltage-dependent Ca^{2+}-channel blockers (excluding nifedipine), atropine, azelastine, NDGA and BW 755C.

The failure of indomethacin, AA-861, FPL 55712, pyrilamine, methysergide and L-652,731 (a Paf-acether antagonist) to influence PLA_2-induced AH to cold provocation in rat tracheae suggests that prostaglandins, thromboxanes, the products of

Table 2 Effects of drugs which interfere with arachidonic acid metabolism on phospholipase A_2-induced airway hyperreactivity to cooling in rat tracheae

Drugs (2 h)	*Bath conc.* (μM)	n	*% inhibition of cold provocation (10°C)-induced contractile responses* (mean ± s.e.mean)
Dual inhibitor of cyclo-oxygenase and lipoxygenase			
BW 755C	20	5	45 ± 15*
Cyclo-oxygenase inhibitor			
Indomethacin	14	6	13 ± 11
Lipoxygenase inhibitors			
AA-861	10	4	18 ± 11
NDGA	5	4	9 ± 9
	10	7	40 ± 11*
Leukotriene receptor antagonist			
FPL 55712†	10	4	8 ± 6
Paf-acether antagonist			
L-652,731	1	4	10 ± 10
	10	6	34 ± 10
	100	6	30 ± 11
	100††	8	26 ± 10

* $P < 0.05$, significance of drug effect was determined by comparing contractile responses (% ACh max) to cooling in PLA_2 (0.1–1.0 μM) exposed tissues in the presence and absence of modifying drugs by paired t test. All the tissues were exposed to indomethacin (14 μM, 3 h) except when the effect of indomethacin or BW 755C was studied.
† added 5 min before PLA_2 addition.
†† added 1 min before PLA_2 addition.

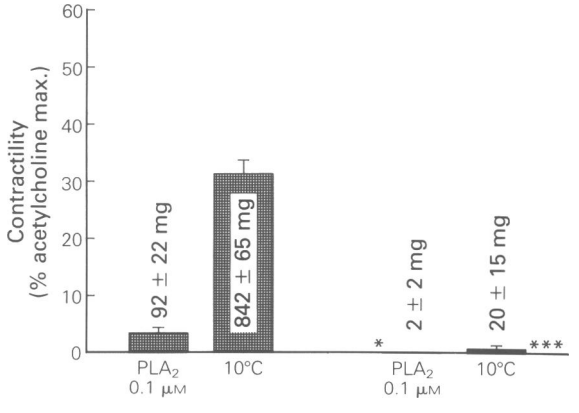

Figure 4 Abolition of phospholipase A_2 (PLA_2; 0.1 μM)-induced contractile responses and subsequent development of airway hyperreactivity to cooling in rat trachea by Ca^{2+} removal from the Krebs-Henseleit solution (solid columns). Hatched columns represent responses in normal Krebs solution. Each column represents the mean ($n = 5$) and vertical lines indicate s.e.mean. *$P < 0.05$, ***$P < 0.001$, determined by comparing contractile responses to PLA_2 and cold provocation in normal Ca^{2+}-containing Krebs with those in Ca^{2+}-free Krebs by paired t test. All the tissues were pre-exposed to indomethacin (14 μM, 3 h).

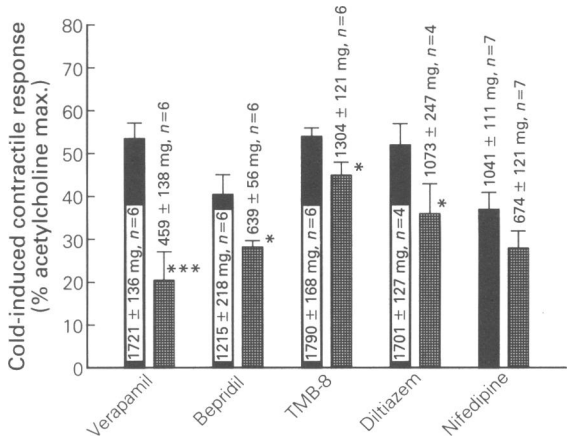

Figure 5 Effect of Ca^{2+} channel blockers on phospholipase A_2 (PLA_2; 0.1–1.0 μM)-induced airway hyperreactivity to cooling (10°C) in rat isolated trachea. All the tissues were pre-exposed to indomethacin (14 μM) before the addition of Ca^{2+}-channel blockers and PLA_2. Solid columns represent control responses and hatched columns responses in the presence of the drugs (each at 10 μM). *$P < 0.05$, ***$P < 0.001$, determined by comparing PLA_2-induced airway hyperreactivity to cooling (% ACh max) in drug-treated and vehicle-treated segments by paired t test. Each column represents the mean and vertical lines indicate s.e.mean.

lipoxygenase pathway, histamine, Paf-acether and 5-hydroxytryptamine play no role in the induction of AH in response to PLA_2.

NDGA (10 μM, 2h) and BW 755C (20 μM, 2h) attenuated PLA_2-induced AH (this study). This may be attributed to anti-PLA_2 or antioxidative activities of these compounds. Interestingly, NDGA is ineffective against allergen-induced AH to cold, whereas BW 755C potentiated allergic-induced AH (Chand *et al.*, 1987b). The generation of superoxide free radicals ($\cdot O_2^-$) may play an important role in the induction of AH (Calhoun *et al.*, 1987; Postma *et al.*, 1987). Azelastine has been shown to inhibit the generation of $\cdot O_2^-$ in guinea-pig polymorphonuclear cells with an IC_{50} of 4–18 μM (Taniguchi & Takanaka, 1984) and also inhibits the influx of Ca^{2+} in mast cells and airway smooth muscles (Chand *et al.*, 1983; 1984). A similar mechanism may explain its inhibitory activity against PLA_2- and antigen-induced AH (this study, Chand *et al.*, 1987b) and exercise-induced asthma (Motojima *et al.*, 1985).

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