

Mediators of arachidonic acid-induced contractions of indomethacin-treated guinea-pig airways: leukotrienes C₄ and D₄

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- 1 Arachidonic acid (AA) (66 μM) induced contractions of indomethacin-treated (8.4 μM) guinea-pig tracheal and lung parenchymal preparations. Mepacrine (210 μM) treatment did not affect the magnitude of contraction induced by AA.
- 2 Normal and ovalbumin-sensitized tissues responded identically to AA, and released equivalent amounts of the contractile mediators.
- 3 Nordihydroguaiaretic acid (100 μM) markedly reduced release of the contractile mediators and reduced AA-induced contractions of the airways.
- 4 The mediators of AA-induced, calcium ionophore A23187-induced, and antigen-induced contraction of the trachea and lung parenchyma were purified and identified by reverse-phase high performance liquid chromatography to be leukotrienes C₄ and D₄, being present in an approximate ratio of 20:1.
- 5 Mepacrine-treated trachea exhibited a smaller contractile response to stimuli (A23187 for normal tissues and ovalbumin for sensitized tissues). Addition of exogenous AA (66 μM) increased the magnitude of contraction, although not to the level observed on tissues not treated with mepacrine. There was no observable effect of AA on the response of mepacrine-treated parenchyma to the ionophore or antigen.
- 6 It was concluded that (a) immunological sensitization does not alter AA metabolism via the lipoxygenase pathway in guinea-pig airways and (b) the mediators of AA-induced contraction are leukotrienes C₄ and D₄.

Introduction

Arachidonic acid (AA) is metabolized via the cyclo-oxygenase and lipoxygenase pathways to yield prostaglandins and leukotrienes, respectively. The sulphidopeptide leukotrienes, LTC₄ and LTD₄, the major components of slow reacting substance of anaphylaxis (SRS-A) (Samuelsson *et al.*, 1980), are potent bronchoconstrictors important in the pathology of airways disease (Hanna *et al.*, 1981; Holroyde *et al.*, 1981; Jones *et al.*, 1981).

We have recently demonstrated that one of the mediators of calcium ionophore A23187-induced contraction of normal guinea-pig trachea and of antigen-induced contraction of sensitized trachea is LTC₄ (Saad *et al.*, 1983a,b). AA induces weak relaxation of trachea and contraction of lung parenchymal strip. However after treatment with indomethacin, a cyclo-oxygenase inhibitor, AA induces contraction of tracheal spirals and exerts lesser contractile effects

on lung parenchymal strips (Burka & Paterson, 1980; Mitchell & Denborough, 1980; Mitchell, 1982). Since the main AA metabolite in trachea is prostaglandin E₂ (PGE₂, Burka *et al.*, 1981) inhibition of its synthesis would remove its relaxant effect and allow the effects of contractile mediators, possibly synthesized via the lipoxygenase pathway, to appear (Burka & Paterson, 1980). Various contractile cyclo-oxygenase metabolites are produced from AA in the lung parenchyma (Hamberg & Samuelsson, 1974; Mathe *et al.*, 1977). These include thromboxane A₂ (TXA₂) and prostaglandins. The lung also has the capacity to synthesize leukotrienes in response to appropriate stimulation, as has been demonstrated for guinea-pig (Morris *et al.*, 1980) monkey (Weichman *et al.*, 1982) and human lung (MacGlashan *et al.*, 1982).

It was not known whether addition of substrate was

a sufficient stimulus for leukotriene synthesis. Thus it was necessary to examine whether AA-induced contractions of trachea and lung parenchymal strips after indomethacin-treatment would lead to leukotriene synthesis. This hypothesis was supported by earlier reports that lipoxygenase inhibitors can markedly reduce the contractile response to AA, as can FPL55712 (sodium 7-[3(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate), a selective leukotriene antagonist (Yen, 1981; Mitchell, 1982).

It has been assumed that leukotriene biosynthesis is dependent on membrane events which activate phospholipase A₂ such that free AA is made available to the metabolic enzymes (Burka & Flower, 1979). The initial membrane events such as calcium mobilization also appear to be necessary for activation of the lipoxygenase pathway (Borgeat & Samuelsson, 1979; Jakschik & Lee, 1980). The present study, however, presents evidence that availability of free substrate (exogenous AA) may be sufficient to activate the lipoxygenase pathway to induce leukotriene synthesis, release, and consequent effects.

Methods

Male English short-hair guinea-pigs (200–250 g) (Connaught Laboratories, Toronto, Ontario) were sensitized with ovalbumin (OA: Sigma, grade II), 100 mg subcutaneously and 100 mg intraperitoneally and used 3 weeks later. Normal animals were untreated. Animals were killed by stunning and exsanguination. The trachea was removed, placed in Krebs-Henseleit solution, spirally cut (Constantine, 1965), and divided into four equal segments. The lung was removed and parenchymal strips prepared from the distal edges of each lobe (Lulich *et al.*, 1976). The composition (mM) of the Krebs-Henseleit solution was: NaCl 118, KCl 4.7, MgSO₄·7H₂O 1.2, CaCl₂ 2.2, KH₂PO₄ 1.2, NaHCO₃ 24.9 and (+)-glucose 11.1. The tissues were placed in

10 ml jacketed organ baths in Krebs solution maintained at 37°C and bubbled with 95% O₂ and 5% CO₂. The tissues were attached by silk threads to force displacement transducers (Grass FTO3C) at a resting tension of 1 g and the responses displayed on Grass polygraphs (Model 7D). Following equilibration (2 h) the tissues were challenged with histamine (100 µM) until responses became constant. All tissues were then treated with indomethacin (8.4 µM), and this concentration was maintained in the bath for the remainder of the experiment. Indomethacin treatment reduced the tone of the trachea, but did not affect the tone of lung parenchymal strips. AA-induced contractions of the indomethacin-treated trachea were not significantly different when AA was added to trachea with reduced tone or to trachea for which the tone was mechanically readjusted to the initial 1 g tension. Drug treatments and stimuli applied are indicated in Table 1. The calcium ionophore A23187 was used on normal tissues whereas ovalbumin (OA) was used on sensitized tissues. Modulatory agents were added to the bath 30 min before challenge and maintained in the bath during the challenge period. Contractions were recorded for 60 min after which leukotrienes were extracted from the bath fluid for purification and analysis.

Extraction and initial purification of leukotrienes

The bath fluid was acidified to pH 3 with 1 N HCl and applied to a primed SEP-PAK C₁₈ cartridge (Waters Associates). Priming was carried out by washing the cartridge with 5 ml methanol followed by 40 ml water. The cartridge was successively washed with 5 ml water, 5 ml water/methanol (65:35 vol:vol) and 5 ml methanol.

The 100% methanol fraction was collected, and the methanol blown off under nitrogen at 50°C. Earlier experiments with [³H]-LTC₄ indicated that this fraction contains about 80% of the initial LTC₄ in the sample (Saad *et al.*, 1983a). The residue was dissolved in 200 µl of water and used for high performance liquid chromatography (h.p.l.c.) and bioassay on the guinea-pig ileum longitudinal strip treated

Table 1 Experimental protocol

<i>Experiment</i>	<i>Modulatory agent</i>	<i>Stimulus</i>
1	None	A23187 (5.7 µM) or OA (3 µg ml ⁻¹)
2	None	AA (66 µM)
3	Mepacrine (210 µM)	AA (66 µM)
4	Mepacrine (210 µM)	A23187 (5.7 µM) or OA (3 µg ml ⁻¹) and AA (66 µM)
5	Mepacrine (210 µM)	A23187 (5.7 µM) or OA (3 µg ml ⁻¹)
6	NDGA (100 µM)	AA (66 µM)
7	NDGA (100 µM)	A23187 (5.7 µM) or OA (3 µg ml ⁻¹) and AA (66 µM)

with atropine ($1\ \mu\text{M}$), mepyramine ($1\ \mu\text{M}$), and indomethacin ($8.4\ \mu\text{M}$). Synthetic LTC_4 was used as a standard.

High performance liquid chromatography

H.p.l.c. was carried out using a Radial-Pak C_{18} column (Waters Associates). The solvent system consisted of methanol/water/acetic acid (65:35:0.1, vol/vol) (pH 5.5, adjusted with ammonium hydroxide). Flow rate was $1\ \text{ml}\ \text{min}^{-1}$, and column back pressure 1000 psi.

Eluants were monitored continuously at 280 nm. Synthetic LTC_4 and LTD_4 were injected as standards. Peaks corresponding to these in retention time were collected, rechromatographed, recollected and bioassayed on guinea-pig ileum and lung parenchymal strip. FPL55712 ($0.17\text{--}8.7\ \mu\text{M}$) was used to reverse the characteristically slow contractions induced by the leukotrienes.

Statistics

Student's *t* test for paired and unpaired data was used to assess the significance of the difference between groups. Differences were considered to be statistically significant when $P < 0.05$.

Materials

Histamine dihydrochloride, nordihydroguaiaretic acid (NDGA), arachidonic acid, ovalbumin (grade II for sensitization and grade V for challenge), and calcium ionophore A23187 were purchased from Sigma (St. Louis, Mo.). We are grateful to Drs J. Rokach and Wm. D. Dorian, Merck Frosst Laboratories (Pointe Claire-Dorval, P.Q.) for the gifts of LTC_4 and LTD_4 and indomethacin respectively.

Indomethacin was dissolved ($10\ \text{mg}\ \text{ml}^{-1}$) in 1M Tris buffer (pH 8.4) and diluted with water. NDGA

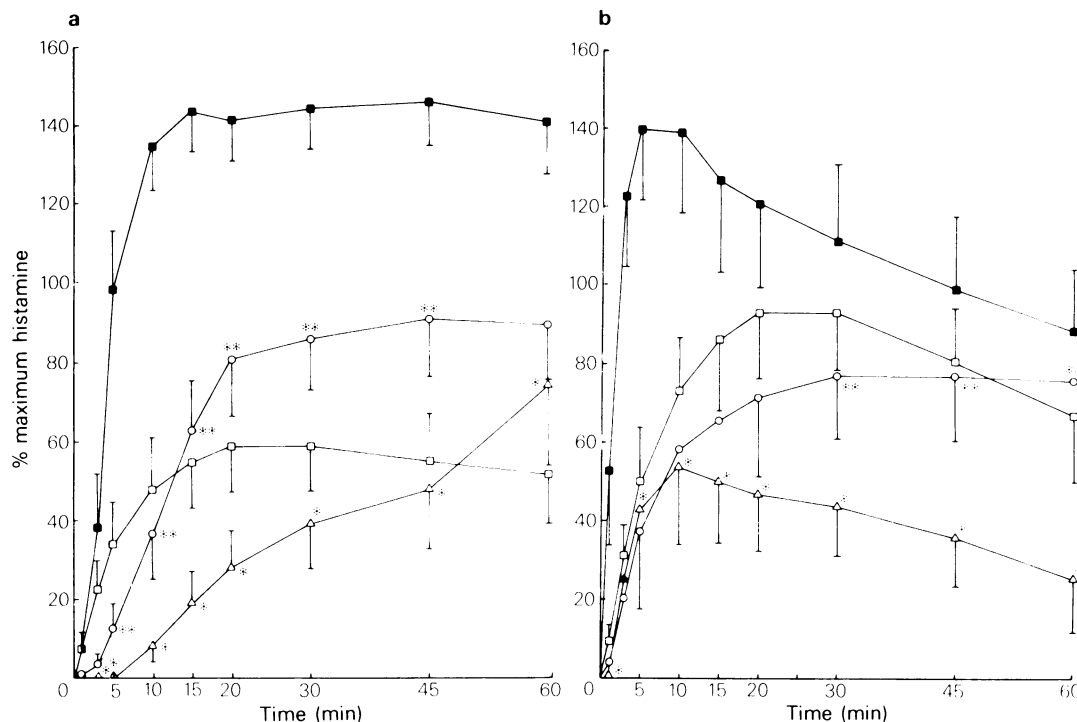


Figure 1 The contractile responses of indomethacin-treated tracheal spirals to arachidonic acid (AA), A23187 or ovalbumin: (a) normal trachea plus A23187; (b) sensitized trachea plus ovalbumin. Results are expressed as a % of the maximum histamine response and are the mean of at least 5 experiments; vertical lines show s.e. mean. Symbols indicate the following: (\square) AA alone; (\blacksquare) A23187 or ovalbumin alone; (\triangle) mepacrine and A23187 or mepacrine and ovalbumin; and (\circ) mepacrine AA and A23187 or mepacrine, AA and ovalbumin. *Significantly different ($P < 0.05$) from contractions to A23187 (a) or ovalbumin alone (b) indicating that mepacrine partly inhibits contractions induced by A23187 or ovalbumin; **significantly different ($P < 0.05$) from contractions to mepacrine-treated tissues stimulated with A23187 (a) or ovalbumin alone (b), indicating that addition of exogenous substrate (i.e. AA) enhanced contractions previously inhibited by mepacrine.

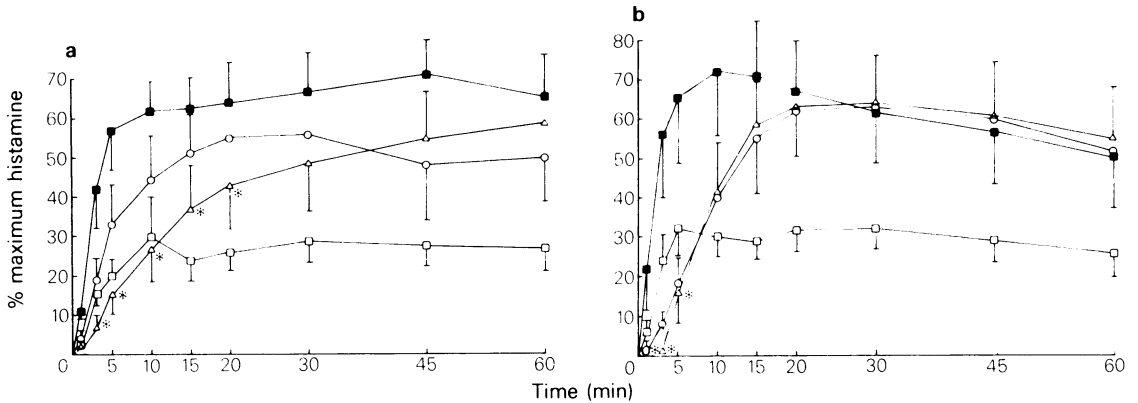


Figure 2 The contractile responses of indomethacin-treated lung parenchymal strips to arachidonic acid (AA), A23187 or ovalbumin; (a) normal parenchyma plus A23187, (b) sensitized parenchyma plus ovalbumin. Results are expressed as a % of the maximum histamine response, and are the mean of at least 5 experiments; vertical lines show s.e.mean. Symbols indicate the following: (□) AA alone; (■) A23187 or ovalbumin alone; (Δ) mepacrine and A23187 or mepacrine and ovalbumin; and (○) mepacrine, AA and A23187 or mepacrine, AA and ovalbumin. *Significantly different ($P < 0.05$) from contractions to A23187 or ovalbumin alone, indicating that mepacrine partly inhibits contractions induced by A23187 or ovalbumin.

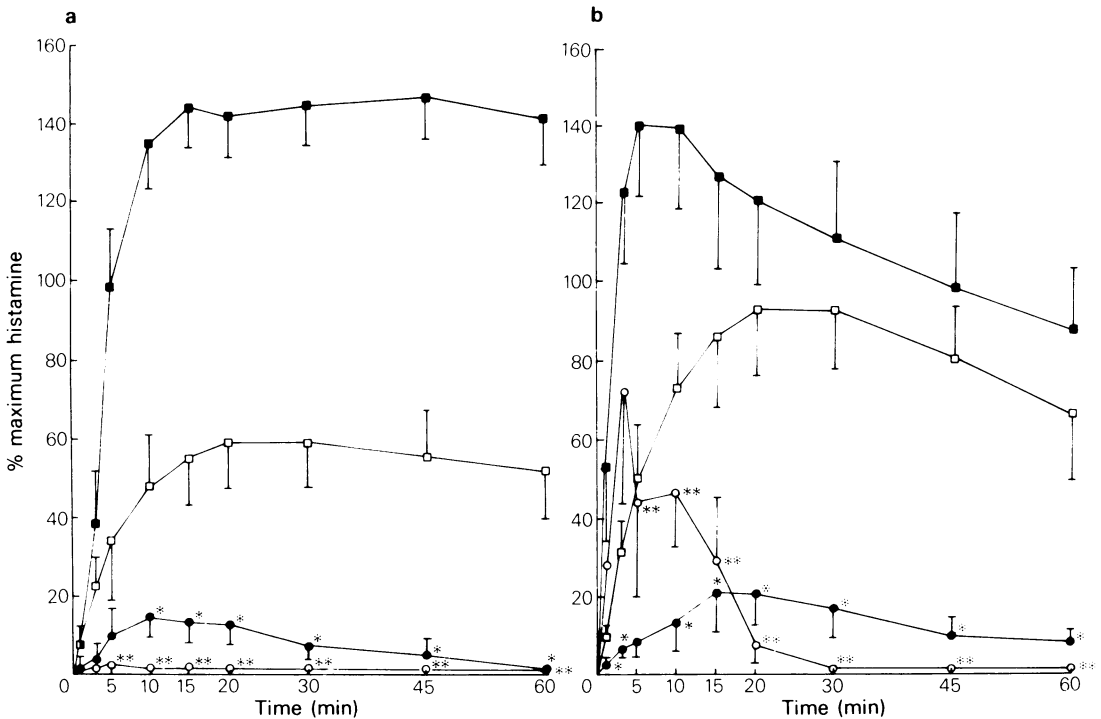


Figure 3 The contractile responses of indomethacin-treated tracheal spirals to arachidonic acid (AA), A23187 or ovalbumin: (a) normal trachea plus A23187; (b) sensitized trachea plus ovalbumin. Results are expressed as a % of the maximum histamine response and are mean results with s.e.mean shown by vertical lines. Symbols indicate the following: (□) AA alone ($n = 8$); (■) A23187 or ovalbumin alone ($n = 5$); (●) nordihydroguaiaretic acid (NDGA) and AA ($n = 5$); and (○) NDGA and A23187 and AA or ovalbumin and AA ($n = 3$). *Significantly different ($P < 0.05$) from contractions to AA alone and **significantly different ($P < 0.05$) from contractions to A23187 or ovalbumin alone.

(100 mM) was dissolved in water containing 1.5% NaOH (1N) to make a salt. A23187 was dissolved in ethanol (1 mg ml^{-1}). AA was dissolved (10 mg ml^{-1}) in 1M Tris buffer (pH 8.4), diluted in water and kept frozen at -70°C in the dark until used. Leukotrienes were dissolved in water, diluted, and stored as above.

Results

Arachidonic acid-induced contraction

AA relaxed tracheal spirals and contracted lung parenchymal strips prior to indomethacin treatment. The extent of contraction to AA ($66 \mu\text{M}$) in the presence of indomethacin is shown for normal and sensitized trachea (Figure 1) and for normal and sensitized lung parenchyma (Figure 2). The trachea ($93.3 \pm 20.1\%$ histamine maximum) was more responsive to AA than the lung strip ($32.5 \pm 5.1\%$ histamine maximum), contracting to a greater extent with respect to the maximal contraction obtained with histamine. NDGA ($100 \mu\text{M}$), a lipoxygenase inhibitor, significantly ($P < 0.05$) reduced the magnitude of the contraction at most time points studied (Figures 3 and 4). Phenidone ($185 \mu\text{M}$), another lipoxygenase inhibitor, exhibited similar effects (2 experiments). Mepacrine ($210 \mu\text{M}$) pretreatment of airway tissues did not significantly affect the magnitude of contraction to exogenous AA (results not shown). However, A23187-induced contractions (normal trachea) and OA-induced contractions (sensitized trachea) were significantly reduced ($P < 0.05$) after mepacrine pretreatment (Figure 1). Addition of exogenous AA to mepacrine-pretreated trachea increased the magnitude of the contraction to A23187 or OA, but not to the level observed in the absence of mepacrine (Figure 1). Mepacrine pretreatment significantly reduced the extent of contraction of lung parenchyma to A23187 or OA in the initial period (0–10 min) after stimulus addition. Exogenous AA did not further increase the magnitude of the contraction (Figure 2).

NDGA ($100 \mu\text{M}$) pretreatment of airway tissues markedly reduced the extent of contraction to AA and A23187 or AA and OA (normal or sensitized tissues) (Figures 3 and 4).

High performance liquid chromatography

Analysis of the partially purified SEP-PAK extract by h.p.l.c. indicated the presence of peaks co-chromatographing with synthetic LTC_4 and LTD_4 (Figure 5). The retention times for these substances were 5.27 and 11.50 min respectively. The peaks had an ultraviolet absorption maximum of (278–280 nm) and exhibited spasmogenic activity on guinea-pig

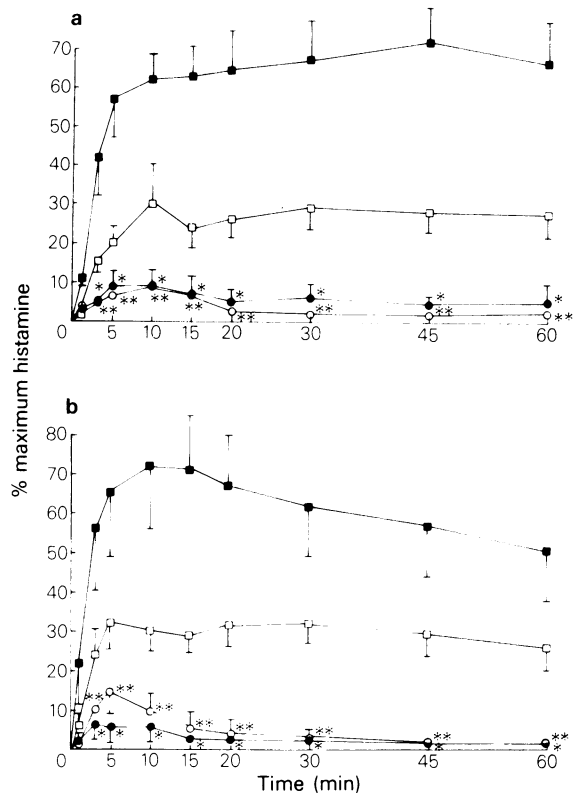


Figure 4 The contractile responses of indomethacin-treated lung parenchymal strips to arachidonic acid (AA), A23187 or ovalbumin: (a) normal parenchyma plus A23187; (b) sensitized parenchyma plus ovalbumin. Results are expressed as a % of the maximum histamine response and are mean results with s.e.mean shown by vertical lines. Symbols indicate the following: (□) AA alone ($n = 6$); (■) A23187 or ovalbumin alone ($n = 5$); (●) nordihydroguaiaretic acid (NDGA) and AA ($n = 5$); and (○) NDGA and A23187 and AA or ovalbumin and AA ($n = 3$). *Significantly different ($P < 0.05$) from contractions to AA alone and **significantly different ($P < 0.05$) from contractions to A23187 or ovalbumin alone.

ileum and guinea-pig parenchymal strips. Both contractile activities were reversed by the leukotriene antagonist FPL55712 (Figure 6). Furthermore the spasmogenic activity was partially destroyed by heating to 60°C at pH 3.

Bioassay of mediators released

LTC_4 was the predominant mediator released from the airways ($\text{LTC}_4:\text{LTD}_4$; 20:1 ratio) using AA and stimulus. This is based on % composition of samples determined by an integrator as a function of

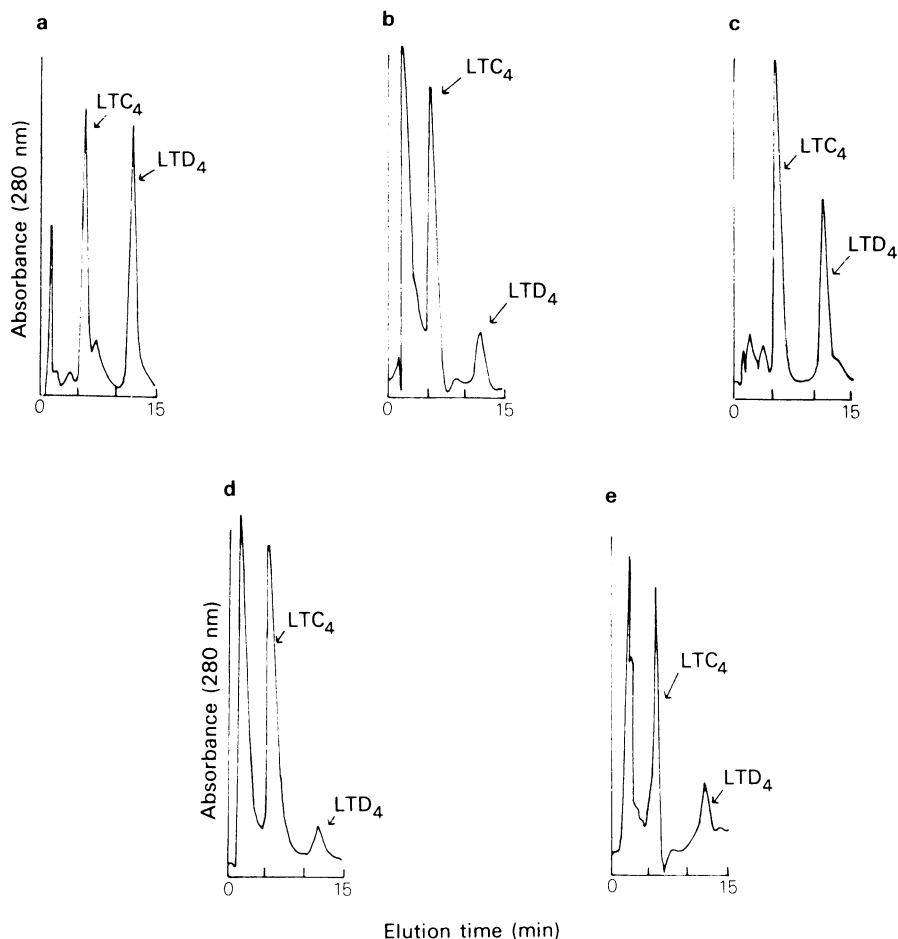


Figure 5 Typical h.p.l.c. chromatograms obtained after analysis of the medium surrounding airway tissues after appropriate stimulation. Synthetic standards leukotriene C₄ (LTC₄) and LTD₄ are shown in (a), normal trachea after stimulation with arachidonic acid (AA) in (b), normal parenchyma after AA stimulation in (c), sensitized trachea after mepacrine treatment and stimulation with AA and ovalbumin in (d), and normal parenchyma after stimulation with A23187 in (e). All absorbances were measured at 280 nm.

Table 2 Release of leukotrienes from guinea pig airway tissue (ng mg⁻¹ tissue [dry weight] in the first hour)

	<i>Normal trachea</i>	<i>Sensitized trachea</i>
Arachidonic acid (66 μM)	21.6 ± 8.8 ^a (13) ^b	33.9 ± 12.9 (9)
Arachidonic acid after NDGA (100 μM)	(1.8 – 124) ^c 3.1 ± 2.2 (9) (0 – 20.4)	(4.4 – 129) 1.1 ± 0.7 (8) (0 – 5.7)
	<i>Normal parenchyma</i>	<i>Sensitized parenchyma</i>
Arachidonic acid (66 μM)	11.3 ± 3.5 (8)	12.7 ± 3.5 (5)
Arachidonic acid after NDGA (100 μM)	(0.8 – 34) 4.1 ± 1.2 (7) (0 – 8.9)	(1.4 – 21) 0.2 ± 0.1 (6) (0 – 0.6)

^aMean ± s.e.mean; ^bnumber of animals; ^crange. NDGA, nordihydroguaiaretic acid.

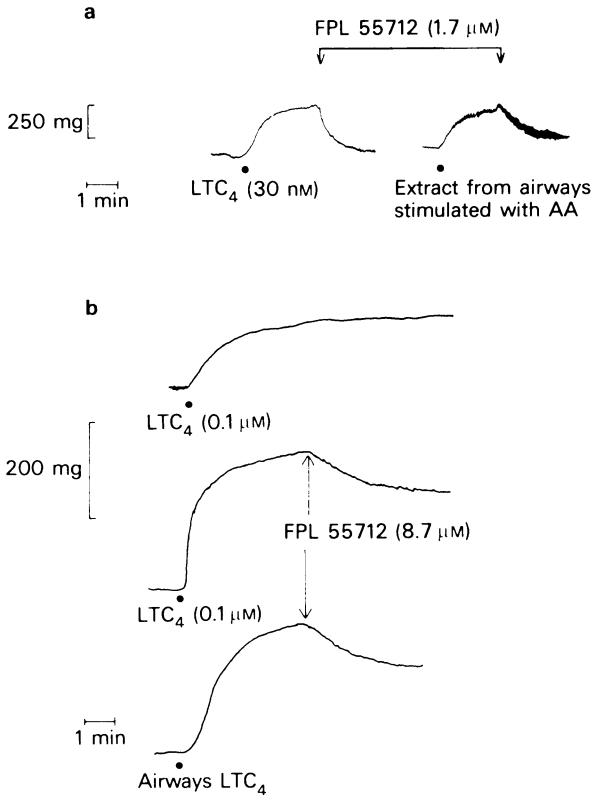


Figure 6 Bioassay of released leukotriene C₄ (LTC₄) on guinea-pig ileum (a) and lung parenchymal strips (b). Synthetic LTC₄ was used as a standard. FPL55712 (1.7 μM) was used to reverse contractions on the ileum. A higher concentration of FPL55712 was necessary to partially reverse contractions on the lung strip. The bioassay was carried out in the presence of atropine (1 μM) and mepyramine (1 μM).

area under the h.p.l.c. peaks. We used synthetic LTC₄ as the standard for bioassay on longitudinal strips of guinea pig ileum. Similar amounts of LTs were released from normal and sensitized tissues under the same conditions. NDGA pretreatment completely abolished or markedly reduced release of LT-like activity (Table 2). Samples obtained from mepacrine-treated tissues could not be bioassayed because the mepacrine residue after initial sample purification relaxed the ileum. Hence the data were considered unreliable.

Discussion

AA is metabolised by lung tissue into contractile cyclo-oxygenase products (Hamberg & Samuelsson,

1974; Mitchell & Denborough, 1980). Similarly, the guinea-pig trachea has been shown to release prostaglandins and thromboxane A₂ after challenge with OA, A23187 and AA (Burka *et al.*, 1981). AA can also be metabolised to lipoxygenase products by guinea-pig airways (Piper *et al.*, 1979; Mitchell & Denborough, 1980; Yen, 1981). In this work we have demonstrated that AA is metabolised by indomethacin-treated guinea-pig airway tissues to LTC₄ and LTD₄. This establishes the inherent capacity of the airways to produce these potent bronchoconstrictors. It is not essential for a stimulus such as A23187 or OA to be present for AA to be metabolized to the above products.

The magnitude of AA-induced contraction was greater on trachea than on lung parenchyma (Figures 1 and 2). This is significant especially because the small airways are particularly sensitive to leukotrienes (Drazen *et al.*, 1980; Saad & Burka, 1983). Lung parenchymal strips in the absence of indomethacin, contracted to a level equivalent to that observed on the trachea. Subsequent addition of indomethacin partially inhibited the contraction (results not shown). Furthermore the leukotrienes exert both direct and indirect contractile effects on the lung strip, the latter action being mediated by thromboxane A₂ (Piper & Samhoun, 1981). In our experiments this latter component was abolished by indomethacin. Our results are in agreement with those of Mitchell & Denborough (1980) and Yen (1981).

The effects of mepacrine, a phospholipase A₂ inhibitor, support the evidence that exogenous AA is metabolized to leukotrienes, since the magnitude of antigen- and ionophore-induced contractions was reduced whereas exogenous AA was unaffected. In the experiments conducted on mepacrine-treated tissues, AA probably had an additive effect with antigen or ionophore on the trachea. An additive effect of exogenous AA on the lung strip was not observed even though mepacrine appeared to inhibit phospholipase A₂, based on the criteria that the magnitude of antigen- and ionophore-induced contractions was significantly reduced.

The two inhibitors of lipoxygenase, NDGA and phenidone, both markedly reduced the effects and inhibited release by all three stimuli we used. This supported the observations that contractions induced by AA, OA, and A23187 are a result of leukotriene synthesis (this paper; Saad *et al.*, 1983a,b).

The presence of the lipoxygenase enzyme in lung tissue has been confirmed for both guinea-pig lung (Hamberg & Samuelsson, 1974) and for human foetal lung (Saeed & Mitchell, 1982). It has also been observed that the basal levels of glutathione-S-transferase, the enzyme that converts LTA₄ to LTC₄, are higher in normal lung than in sensitized lung (Morris *et al.*, 1982). However, no significant differ-

ence in leukotriene production between normal and sensitized tissues, when activated with AA, was observed in the present experiments. This is in contrast to the results of Piper & Seale (1979) who observed a greater production of SRS-A from sensitized lung than from normal lung. The use of fragments stimulated with A23187 by these authors may account for the difference from our results using parenchymal strips stimulated with AA whilst under tension in organ baths. Furthermore, our methods of purification of the released mediators differed.

Finally, we have shown that in indomethacin-treated guinea-pig airways, addition of substrate (AA) induces synthesis of similar lipoxygenase metabolites to those produced upon activation with ionophore or antigen. Similarly, rabbit platelets release SRS upon activation with either AA, thrombin

or platelet activating factor (Mencia-Huerta *et al.*, 1981). In some cells, such as human blood polymorphonuclear leukocytes, the AA products formed are dependent on the stimulus. AA stimulation leads to very little lipoxygenase product formation, whereas combination of AA with A23187 leads to the production of significant amounts of 5-hydroeicosatetraenoic acid and LTB₄ (Borgeat & Samuelsson, 1979). This does not appear to be the situation in the indomethacin-treated airways. Mere addition of substrate is an adequate stimulus for activation.

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