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ARACHIDONIC ACID METABOLISM AND MODULATION OF *in vitro* ANAPHYLAXIS BY 5,8,11,14-EICOSATETRAYNOIC ACID AND 9a,12a-OCTADECADIYNOIC ACID

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1 5,8,11,14-Eicosatetraynoic acid (ETYA) inhibited the antigen-induced contractions of tracheal spirals obtained from actively sensitized guinea-pigs. Consistent data were obtained only when the spirals were treated with indomethacin.

2 ETYA did not affect histamine-induced contractions of indomethacin-treated tracheal spirals.

3 9a, 12a-Octadecadiynoic acid (Ro-3-1314) a potential inhibitor of linoleic acid metabolism, stimulated the antigen-induced contraction of guinea-pig tracheal spirals and the immunological release of slow reacting substance of anaphylaxis (SRS-A) from actively sensitized guinea-pig lung fragments.

4 Both ETYA and Ro-3-1314 inhibited the immunological release of malondialdehyde from guinea-pig lung fragments.

5 The data indicate that the effects of ETYA were due to inhibition of lipoxygenase and the effects of Ro-3-1314 were due to inhibition of cyclo-oxygenase.

6 The results suggest that products of lipoxygenase contribute to the antigen-induced contraction of guinea-pig lung, particularly when cyclo-oxygenase is inhibited. Under these conditions there may be redirection of the metabolism of arachidonic acid to favour production of constrictor products of lipoxygenase such as SRS-A.

Introduction

Challenge of isolated actively sensitized guinea-pig trachea with antigen results in a contractile response. The tension developed can be enhanced by drugs which inhibit cyclo-oxygenase (Hitchcock 1980). This may be due to redirection of the metabolism of arachidonic acid to favour increased production of bronchoconstrictor products of lipoxygenase, such as slow reacting substance of anaphylaxis (SRS-A) which is now thought to be predominently leukotriene D (Morris, Taylor, Piper & Tippins 1980; Watanabe-Kohno & Parker, 1980). In support of this hypothesis, low concentrations of FPL 55712, an SRS-A antagonist, inhibited the antigen-induced contraction of human bronchus (Adams & Lichtenstein, 1979) and guinea-pig trachea (Hitchcock, 1980). Thus drugs which are known to inhibit lipoxygenase should inhibit the response of sensitized airway smooth muscle to antigen. In the present study, the effects of 5,8,11,14-eicosatetraynoic acid (ETYA), a selective inhibitor of lipoxygenase (Hamburg 1976), and 9a,-12a-octadecadiynoic acid (Ro-3-1314) a potential inhibitor of plant lipoxygenase (Nugteren, 1975) have been investigated on the antigen-induced contraction of guinea-pig airway smooth muscle (trachea) and the release of SRS-A and malondialdehyde from chopped lung fragments. Some of these results were presented at the first International Congress on Immunopharmacology (Hitchcock & Kokolis, 1980).

Methods

Paired tracheal spiral strips

Male albino guinea-pigs (Hartley strain, 250 g) were actively sensitized with a single injection (i.p.) of 10 mg egg albumin in 1 ml 0.9% w/v NaCl solution. Twenty-eight days later the trachea and lungs were removed and placed in Tyrode solution (pH 7.4), the composition of which was as follows (mM): NaCl 136.7, KCl 2.7, $MgCl_2$, 0.49, $NaHCO_3$ 11.9, $CaCl_2$ 1.8 NaH_2PO_4 0.36 and glucose 5.8. The trachea was spirally cut and separated into two halves of equal length. Each half spiral was suspended in a separate organ bath (capacity 10 ml) in Tyrode solution at 37°C, gassed with 5% CO_2 in 95% O_2. An initial

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tension of 6 g was applied and the spiral was permitted to equilibrate for 90 min. At the end of the equilibration period the resting tension was between 3.5 and 4.5 g. Isometric contractions were recorded as changes in tension on a Heathkit recorder with a Harvard force displacement transducer (Model 373). Throughout the experiment, contractions produced by identical concentrations of either histamine or antigen (egg albumin) were recorded from each tracheal preparation simultaneously. The volume of agonist or drug added to the organ bath never exceeded 100 μl. In all experiments, a dose-response curve to histamine (bath concentration 0.11 to 5.55 μg base/ml) was obtained for each of the paired tracheal spirals. When the response had reached a plateau, the preparation was washed several times with Tyrode solution and then allowed to return passively to its resting tension. One of the spirals was then incubated with one of the fatty acids for 20 min (concentration given in the text) and the responses of both spirals to antigen or histamine recorded. Dose-response curves to antigen were obtained from cumulative bath concentrations of 1, 5 and 10 ng/ml. Responses to antigen were calculated as a percentage of the maximum response to histamine determined before treatment with fatty acid. Enhancement or inhibition was calculated according to the formula

$$\frac{A_{ge}}{A_{gc}} \times 100$$

where A_{ge} and A_{gc} are the responses of respectively the fatty acid treated and control spirals to antigen. In some experiments, both of the paired spirals were treated with indomethacin (5 μg/ml for 20 min). One of the spirals was then treated with fatty acid and responses to antigen or histamine obtained as described above.

Determination of mediator release from chopped lung

The lung tissue was prepared, incubated, and mediator release determined by published techniques (Hitchcock, 1978). Briefly, weighed aliquots (100 or 200 mg) of chopped lung fragments were incubated in Tyrode solution at 37°C in a final volume of 5 ml. Following a 10 min equilibration period during which time the drugs under examination were present, the sensitized lung was challenged with egg albumin (20 μg/ml final concentration). After 15 min the incubates were filtered and the amount of SRS-A determined in the filtrate by bioassay on the guinea-pig ileum in the presence of atropine sulphate (3.4 μg/ml), pyrilamine maleate (0.34 μg/ml) and methysergide bimaleate (0.1 μg/ml). FPL 55712 (0.1 μg/ml) was used to block the contractions attributed to SRS-A. The contractions were compared with a laboratory standard of crude SRS-A and are expressed in arbitary units. The standard was obtained by incubating sensitized chopped lung (5 g) with egg albumin (5 mg) in a final volume of 25 ml at 37°C for 30 min. The incubate was filtered and aliquots of the filtrate were frozen at −70°C. One unit of SRS-A was contained in 50 μl of filtrate. The same standard was used in all of the experiments to be described. Malondialdehyde concentration was determined in 2 ml of filtrate (Flower, Cheung & Cushman, 1973). The data are expressed as fmol malondialdehyde equivalents formed per 200 mg chopped lung.

Determination of prostaglandins

The concentrations of prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) released by the trachea were determined directly on duplicate samples of organ bath fluid by radioimmunoassay. At 50% binding, the anti $PGF_{2\alpha}$ serum cross-reacted as follows: PGE_1 and PGE_2, 0.02%; PGA_1 and PGA_2, less than 0.01%; 13,14 dihydro-15 keto-prostaglandin $F_{2\alpha}$, 0.33%; and $PGF_{1\alpha}$, 10%. Assay of the PGE content was performed by measuring the amount of PGB produced by alkaline treatment (Levine, Gutierrez-Cernosak & Van Vunakis, 1971). The antibody used did not distinguish between PGB_1 and PGB_2, therefore the data for PGE_2 content are expressed in terms of total PGE. At 50% binding, the anti-PGB serum cross-reacted as follows: PGA_1 and PGA_2, 1%; PGE_1, 0.2%; PGE_2, 0.1%.

Statistics

Student's paired *t* test was used to determine statistical significance. *P* values of less than 0.05 were considered statistically significant.

Drugs

The following chemicals and drugs were used: histamine dihydrochloride (Sigma Chemical Co., St. Louis Mo.) and egg albumin 5 times crystallized (Nutritional Biochemicals Cleveland, Ohio), 5,8,11,14-eicosatetraynoic acid (ETYA), 9a,12a-octadecadiynoic acid (Ro-3-1314) (Hoffmann-La Roche Inc., Nutley, N.J.), indomethacin (Merck, Sharpe & Dohme, West Point, Pa) and sodium 7-[3-(4-acetyl-3-hydroxy-2-propyl phenoxy)-2-hydroxy propoxy]-4 oxo-8-propyl-4H-1-benzopyran-2-carboxylate (FPL 55712, Fisons, Ltd., Loughborough, Leics.) were kindly supplied by the manufacturers.

Stock solutions of all chemicals were freshly prepared each day. Indomethacin (20 mg), ETYA (10 mg) and Ro-3-1314 (5 mg) were dissolved in ethanol (1 ml) and diluted to the required concentration with Tyrode solution. FPL 55712 was prepared in distilled water (1 mg/ml) and diluted with Tyrode solution. All other chemicals and drugs were dissolved in Tyrode solution.

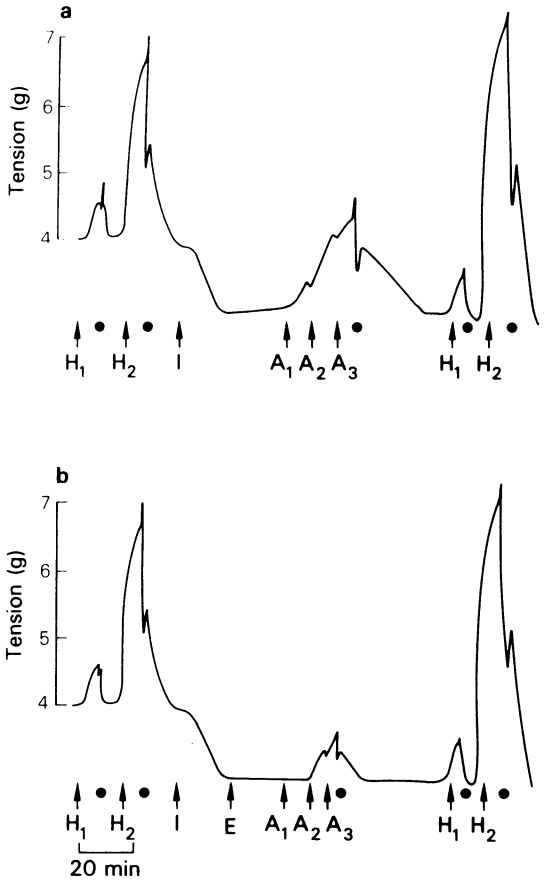

Figure 1 Inhibition by ETYA of the guinea-pig tracheal response to antigen in the presence of indomethacin. Contractions of spirally cut halves of indomethacin treated trachea (I = 5 μg indomethacin per ml bath) to histamine (H_1 = 0.11, H_2 = 5.55 μg histamine base per ml bath) and antigen (A_1 = 1.0, A_2 = 5.0, A_3 = 10.0 ng per ml cumulative concentration in bath); (a) in the absence of ETYA; (b) response of the paired half in the presence of ETYA (E = 1.0 μg per ml bath). Dots indicate points at which the preparation was washed.

Results

Experiments with paired tracheal spirals

Treatment of an actively sensitized tracheal spiral strip with cumulative doses of 1, 5 and 10 ng/ml antigen resulted in a concentration-dependent increase in tension (Figure 1). This concentration range produced complete cumulative dose-response curves in all tracheal spirals tested (n = 38). In the absence of drug treatment, the average responses to 1, 5 and 10 ng/ml antigen were respectively 9.95 ± 3.50, 30.51 ± 5.25 and 41.77 ± 5.15% of the maximum response to histamine (n = 19). In the presence of indomethacin (5 μg/ml), the average responses to 1, 5 and 10 ng/ml antigen were respectively 15.35 ± 8.34, 48.52 ± 8.46 and 53.11 ± 8.29% of the maximum response to histamine (n = 14). The responses to cumulative doses of antigen were less than those observed when equivalent single doses of antigen were studied (Hitchcock, 1980).

Treatment of one of the paired spirals with ETYA or Ro-3-1314 for 20 min resulted in a concentration-dependent relaxation of basal tone. ETYA 0.1 and 1 μg/ml produced relaxation of 0.12 ± 0.02 and 0.24 ± 0.05 g respectively; Ro-3-1314 0.001, 0.01 and 0.025 μg/ml produced relaxation of 0.12 ± 0.02, 0.14 ± 0.02 and 0.26 ± 0.02 g respectively. ETYA (0.1 to 1 μg/ml) inhibited the response to the lowest concentration of antigen used (1 ng/ml) but had inconsistent effects at higher concentrations (Table 1). In some instances, stimulation of the responses to 5 and 10 ng/ml antigen were observed particularly when 1.0 μg/ml ETYA was used. In the presence of indomethacin (5 μg/ml), ETYA inhibited the response of the tracheal spiral to all concentrations of antigen (Table 1). The effect of ETYA was concentration-related in that the inhibition was greater at 1.0 μg/ml than at 0.1 μg/ml. The degree of inhibition was inversely proportional to the concentration of antigen used at both concentrations of ETYA tested (Table 1) indicating that the inhibition was competitive. In contrast, Ro-3-1314 (0.001 to 0.025 μg/ml) enhanced in a dose-related manner, the response of the tracheal spiral to all concentrations of antigen. The threshold concentration for this effect was 0.001 μg/ml (3.6×10^{-9} M), with maximum enhancement occurring at 0.025 μg/ml (9.06×10^{-8} M). Figure 2 shows the results of experiments with 0.025 μg/ml which enhanced the responses to 1,5 and 10 ng/ml antigen by 96, 78 and 58% respectively. For comparative purposes, results of similar experiments with an optimal concentration of indomethacin (5 μg/ml, 1.35×10^{-5} M) are also shown in Figure 2.

In the absence of drug, contractions to antigen (1 ng/ml) started after an average delay of 3.52 ± 0.45 min and reached a maximum (antigen = 10 ng/ml) in an average of 20.21 ± 1.27 min (n = 20). ETYA alone, or in the presence of indomethacin delayed the start of the contraction (Table 2). In contrast, Ro-3-1314 reduced the time of onset of contraction. Neither drug significantly altered the time to maximum contraction.

Maximum responses to histamine were always obtained when the bath concentration of the drug was 5.55 μg/ml base (5×10^{-5} M). ETYA (0.1 to 1.0 μg/ml) caused a 20% increase in the maximum response to histamine at all concentrations tested. Thus the effect was maximal at 0.1 μg/ml ETYA and unrelated to the degree of relaxation of basal tone. In the presence of indomethacin (5 μg/ml) ETYA (0.1 to

Table 1 Inhibition by ETYA of the antigen-induced contraction of actively sensitized guinea-pig trachea

ETYA concentration (ng/ml)	*% inhibition of the control response to antigen*		
	Concentration of antigen (ng/ml)		
	1.0	5.0	10.0
(a) No indomethacin			
0.1	100 ± 0.00*	5.36 ± 16.76	10.38 ± 9.07
1.0	88.64 ± 13.30	22.27 ± 62.70	55.69 ± 26.69
(b) With indomethacin†			
0.1	84.78 ± 16.68*	57.49 ± 16.17*	27.63 ± 11.76
1.0	100.00 ± 0.00*	86.61 ± 9.99*	73.67 ± 13.71

Figures are the average ± s.e. mean calculated according to formula given in Methods. n = 5–8 for each group.
† Both spirals were treated with indomethacin (5 μg/ml).
* Response significantly different from paired control ($P < 0.05$).

1.0 μg/ml) had no effect on the maximum response to histamine. Ro-3-1314 did not affect the maximum response to histamine alone, or in the presence of indomethacin.

Experiments with chopped lung fragments

It is well established that ETYA inhibits the antigen-induced release of SRS-A from actively sensitized chopped guinea-pig lung (Hitchcock, 1978; Piper, Tippins, Morris & Taylor, 1979; Watanabe-Sohno & Parker, 1980) and that indomethacin stimulates SRS-A release (Engineer, Niederhauser, Piper & Sirois, 1978; Hitchcock 1978; Watanabe-Sohno & Parker, 1980). Since Ro-3-1314 had similar effects to indomethacin on the antigen-induced contraction of guinea-pig trachea it was of interest to determine the effects of this fatty acid on SRS-A release from chopped guinea-pig lung. Ro-3-1314 (0.9 nM to 0.9 μM) stimulated SRS-A release by 17 to 40% in a concentration-dependent manner. This concentration range also inhibited the *de novo* synthesis of malondialdehyde-like material in antigen-treated chopped lung (Figure 3). Malondialdehyde is a metabolite of prostaglandin endoperoxide and its formation used as an indicator of cyclo-oxygenase activity (Flower *et al.*, 1973; Hamburg, Svenson & Samuelson, 1974). ETYA also inhibited malondialdehyde synthesis but the concentration range required was 0.1 to 10 μM. The EC_{50} values for malondialdehyde inhibition by Ro 3-1314 and ETYA were 32 nM and 0.7 μM respectively. However, the dose-response curves were not parallel indicating the possibility of more than one enzyme leading to the antigen-induced synthesis of malondialdehyde.

Prostaglandin release by guinea-pig trachea

Prostaglandins E and $F_{2\alpha}$ are continuously released into the bath fluid when the tracheal spirals are at resting tension (Hitchcock, 1980). Under these con-

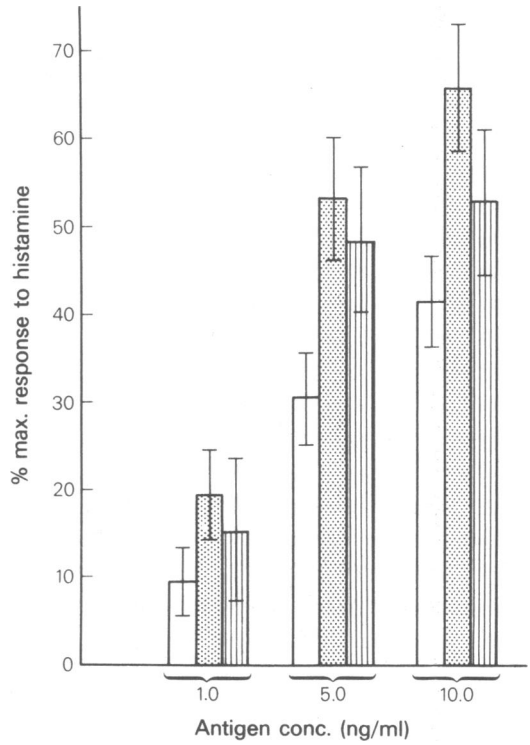

Figure 2 Comparison of the effects of Ro-3-1314 and indomethacin on the contraction of actively sensitized guinea-pig trachea to antigen. Open bars represent response of one half of the trachea in the absence of drug; stippled bars represent the response of the paired half in the presence of Ro-3-1314 (0.025 μg per ml bath). Striped bars represent the response of other tracheal spirals in the presence of indomethacin (5 μg per ml bath). Vertical lines show s.e. mean.

Table 2 Effect of ETYA and Ro-3-1314 on the time of onset and time to peak antigen-induced contraction of actively sensitized guinea-pig trachea

Drug treatment	Time to onset of contraction (min) Control	Time to onset of contraction (min) Drug-treated	Time to peak contraction (min) Control	Time to peak contraction (min) Drug-treated
ETYA (0.1 μg/ml)	3.75 ± 1.6	7.0 ± 0.5*	21.75 ± 4.5	23.75 ± 1.7
ETYA (1.0 μg/ml)	4.75 ± 1.5	9.0 ± 4.1	27.25 ± 2.4	26.0 ± 5.3
Indomethacin† plus ETYA (0.1 μg/ml)	4.67 ± 0.9	8.33 ± 2.3	19.67 ± 6.4	25.67 ± 4.8
Indomethacin† plus ETYA (1.0 μg/ml)	7.0 ± 0.7	10.33 ± 2.9	19.67 ± 6.4	25.67 ± 4.8
Ro-3-1314 (0.001 μg/ml)	2.33 ± 0.4	1.33 ± 0.4*	20.0 ± 1.9	20.0 ± 0.7
Ro-3-1314 (0.01 μg/ml)	3.67 ± 0.4	1.67 ± 0.4*	16.0 ± 4.4	14.67 ± 4.0
Ro-3-1314 (0.025 μg/ml)	3.50 ± 1.3	1.75 ± 0.5*	17.75 ± 1.6	18.0 ± 6.4

† Both spirals were treated with indomethacin (5 μg/ml)
* Significantly different from control ($P < 0.05$)
Figures are the average ± s.e. mean n = 5–8 for each group.

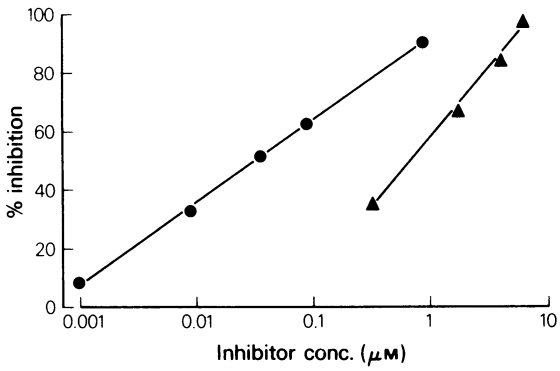

Figure 3 Inhibition of the immunological release of malondialdehyde from actively sensitized guinea-pig lung fragments by unsaturated fatty acids. Antigen concentration was 20 μg per ml. Weight of chopped lung fragments was 200 mg. (●) Ro-3-1314; (▲) ETYA. Each point represents mean results of five experiments. Control release in the absence of inhibitor was 1280 ± 160 fmol malondialdehyde equivalents released per 200 mg lung in 15 min. Data are corrected for spontaneous malondialdehyde release.

ditions the ratio of $PGE/PGF_{2\alpha}$ in the present series of experiments was 0.912 ± 0.106 (n = 10), which is very close to the previously published value of 1.03 (Hitchcock, 1980). ETYA (0.1 to 1 μg/ml) caused an incomplete inhibition of the resting efflux of prostaglandins E and $F_{2\alpha}$. The inhibition of prostaglandin E was greater than the inhibition of prostaglandin $F_{2\alpha}$ resulting in a decrease in the ratio of $PGE/PGF_{2\alpha}$. ETYA, 0.1 and 1 μg/ml inhibited prostaglandin E efflux by 62 and 79% respectively. Prostaglandin $F_{2\alpha}$ efflux was inhibited by 45% at both concentrations of ETYA. ETYA 0.1 and 1 μg/ml reduced the ratio of $PGE/PGF_{2\alpha}$ to 0.486 and 0.337 respectively. In contrast, Ro-3-1314 (0.001 to 0.025 μg/ml) caused a dose-related (40 to 70%) equal inhibition of the resting efflux of both prostaglandins with the result that the ratio of $PGE/PGF_{2\alpha}$ remained unchanged.

Discussion

The fatty acid 5,8,11,14-eicosatetraynoic acid (ETYA) is a competitive inhibitor of arachidonic acid metabolism and as such affects both cyclo-oxygenase and lipoxygenase. In this study we have demonstrated that ETYA inhibits the contractions of guinea-pig airway smooth muscle to threshold concentrations of antigen. Appropriate dose-response relationships between antigen, drug and degree of inhibition could only be obtained in the presence of indomethacin. The concentration of indomethacin used inhibited prostaglandin efflux and enhanced the size of the antigen-induced contraction (Hitchcock, 1980) possibly by redirection of the metabolism of arachidonic acid to favour bronchoconstrictor products of lipoxygenase. Under these conditions ETYA was probably acting on lipoxygenase, indicating that products of this enzyme contribute to the immunological response of guinea-pig airway smooth muscle. Conclusions with respect to the participation of constrictor products of lipoxygenase in antigen-induced contractions in the absence of cyclo-oxygenase inhibition requires the use of inhibitors which are specific for lipoxygenase. ETYA, even at the lowest concentration used caused some reduction in the resting efflux

of prostaglandins E and $F_{2\alpha}$ indicating partial inhibition of cyclo-oxygenase. This may have contributed to the inconsistent effects of ETYA on the response to higher concentrations of antigen.

The response of sensitized airway smooth muscle to antigen can be inhibited by low concentrations of FPL 55712 (Adams & Lichtenstein, 1979; Hitchcock, 1980). This information taken in conjunction with the data presented in this paper point to a role for SRS-A in antigen-induced contractions. Immunologically generated SRS-A from chopped guinea-pig lung has recently been identified as leukotriene D (Morris *et al.*, 1980; Watanable-Kohno & Parker, 1980). The response of guinea-pig airway smooth muscle to non-immunological contractile stimuli is unaffected by FPL 77512 (Adams & Lichtenstein 1979; Adcock & Garland, 1980; Hitchcock, 1980). Thus leukotriene D may not be produced under these conditions.

Products of lipoxygenase other than leukotrienes may contribute to the contraction of airway smooth muscle. Adcock & Garland (1980) demonstrated that two compounds which have mixed cyclo-oxygenase and lipoxygenase inhibitory activity reversed the indomethacin-induced increased reactivity to histamine. The concentrations of ETYA used in the experiments described in this paper did not have such an effect. Thus constrictor products of lipoxygenase other than leukotrienes may be less sensitive to inhibition of ETYA. Alternatively, the compounds used by Adcock & Garland (1980) may be non specific for arachidonate lipoxygenase and may act by inhibiting the production of constrictor products of other fatty acid lipoxygenases.

Data from this study and previous studies point to an emerging pattern of modulation of the immunological response of guinea-pig lung tissues by drugs that affect arachidonic acid metabolism. Drugs which inhibit cyclo-oxygenase stimulate the release of SRS-A (characterized by bioassay and likely to be predominantly leukotriene D) from chopped guinea-pig lung tissue (Engineer *et al.*, 1978; Hitchcock, 1978) and enhance the response of airway smooth muscle to antigen (Hitchcock, 1980). The effects of Ro-3-1314 are similar to those of drugs which selectively inhibit cyclo-oxygenase. The release of SRS-A from chopped guinea-pig lung can also be stimulated by fatty acid hydroperoxides (Adcock, Garland, Moncada & Salmon, 1978). Since these compounds are also products of the lipoxygenase pathway, they may also participate in the indomethacin-induced enhanced response of airway smooth muscle to antigen. Experiments with synthetic materials are required in order to assess the relative contribution of the different products of lipoxygenase to immunological and non-immunological bronchoconstriction.

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