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THE EFFECT OF INHIBITORS OF ARACHIDONIC ACID METABOLISM ON DRUG-INDUCED CONTRACTIONS IN ISOLATED TRACHEAL SMOOTH MUSCLE OF THE PIG

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1 The regulation of drug-induced tone in airways smooth muscle was examined in an isolated preparation of swine tracheal smooth muscle.

2 The trachea contracted (isometric) to histamine, 2-pyridylethylamine (2-PEA), acetylcholine and K^+ but no responses to histamine H_2-receptor agonists were observed.

3 Histamine-induced contractions (100 μM) were potentiated by 213.3% by indomethacin (1 μM) and by 126.9% by sodium salicylate (250 μM). These inhibitors had only slight or no effects on acetylcholine-induced tone. 2-PEA responses were also potentiated by indomethacin but there were no changes in the response to H_2-receptor agonists in the presence of indomethacin. The indomethacin-mediated potentiation of histamine was blocked by 5, 8, 11, 14-eicosatetraynoic acid (10 μM). FPL 55712 had no effect on these responses.

4 Mepacrine (100 μM) inhibited responses to histamine but not those to acetylcholine. No effect was observed with dexamethasone (up to 100 μM).

5 Prostaglandin E_2 caused relaxation but arachidonic acid did not.

6 The possibility that histamine H_1-agonist-induced contractions are regulated by contractile products of the arachidonic acid lipoxygenase pathway is discussed.

Introduction

There is good evidence that contractile agonists act not only directly on airways smooth muscle but that they also exert an indirect effect via arachidonic acid metabolism. Products of the cyclo-oxygenase pathway for arachidonic acid such as prostaglandin E_2 (PGE_2) relax isolated airways smooth muscle whereas other prostaglandins, thromboxane A_2 (TXA_2) and prostaglandin endoperoxides are usually contractile (Svensson, Strandberg, Tuvemo & Hamberg, 1977). Also the lipoxygenase products leukotriene C_4 and leukotriene D_4 (slow reacting substances) are potent contractors of airways smooth muscle (Dahlén, Hedqvist, Hammarström & Samuelsson, 1980; Hedqvist, Dahlén, Gustafsson, Hammarström & Samuelsson, 1980). Further, when guinea-pig isolated airways and lungs are perfused with arachidonic acid, tonal changes are induced, probably reflecting the elaboration of prostaglandins or other metabolites from this substrate (Lambley & Smith, 1975; Mitchell & Denborough, 1980).

Hitherto a number of physiological and pathophysiological stimuli have been shown to cause the release of some of the above products of arachidonic acid. Pertinent to the present study however, are observations that some contracting drugs liberate prostaglandins from guinea-pig (Orehek, Douglas & Bouhuys, 1975; Gryglewski, Dembinska-Kiec, Grodzinska & Panczenko, 1976) dog (Yamaguchi, Hitzig & Coburn, 1976; Anderson, Krzanowski, Polson & Szentivanyi, 1979a) and human (Steel, Platshon & Kaliner, 1979) airways. It has been argued that relaxant prostaglandins (PGE_2) are produced in response to muscle contraction because inhibition of prostaglandin synthesis with indomethacin and aspirin leads to larger contractions in response to drugs (Orehek *et al.*, 1975; Anderson, Krzanowski, Polson & Szentivanyi, 1979b; Krzanowski, Anderson, Polson & Szentivanyi, 1980). However, an alternative explanation might be that indomethacin diverts arachidonic acid metabolism through the lipoxygenase pathway. This action of indomethacin has been shown to occur with the immunological release of SRS-A from perfused guinea-pig lungs (Engineer, Niederhauser, Piper & Sirois, 1978). If the lipoxygenase products thus formed were contractile then this could partly explain why indomethacin can enhance contractile responses in tracheal tissues.

In a recent publication, Adcock & Garland (1980) showed that, in the guinea-pig trachea,

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indomethacin-induced potentiation of histamine contractions is blocked by drugs with anti-lipoxygenase and anti-cyclo-oxygenase activity. This observation suggests that lipoxygenase products may play a role in regulating histamine-induced tone in the airways. The data described in the present paper provide additional evidence on a role for these substances in drug-induced tone in swine airways *in vitro*. Some of the data in this paper have been presented as a communication to the British Pharmacological Society (Mitchell, 1981).

Methods

The experiments were carried out with tracheal tissue obtained from 6 month old, mixed breed swine (Landrace-Large White). Tissues were obtained from animals which had been freshly slaughtered (~15 min) at the Sheffield Corporation Abattoir. Only those tracheae without excessive or blood stained mucous were taken. The tracheae were placed in chilled (~4°C) Krebs solution in a thermos flask and then transported to the laboratory. The composition of the Krebs solution was the same as previously described (Mitchell & Denborough, 1979). In the potassium depolarizing solution the NaCl was replaced with 80 mM K_2SO_4.

Preparation of isolated tracheal tissue

Four rings, 5 mm wide, were cut from each trachea. The smooth muscle and mucosa were freed of surrounding tissue, then ligated with threads and dissected away from the cartilaginous ring. The smooth muscle could not easily be distinguished from the thick mucosal layer, therefore its presence was confirmed by standard histological techniques. Each preparation was suspended in a jacketed organ bath which was filled with Krebs solution, and maintained at 37°C and gassed with 95% O_2/5% CO_2 mixture. One of the threads was attached to a UF1 force transducer for recording isometric tension. The transducers were coupled to a Lectromed pen recorder. Initially a load of 2 g was placed on each tissue. After a 1 h equilibration period the tissues were stimulated several times with acetylcholine, then the tension was readjusted to 2 g.

Experimental procedures

Drug solutions were added to organ baths in small volumes (usually $< 100\ \mu l$) delivered by microsyringes. When a contraction was to be recorded a drug solution was left in contact with the tissue until tension was judged to have reached a plateau. The organ bath was then flushed twice with fresh Krebs solution. A period of at least 5 min was then allowed to elapse before the next dose was given. In most cases 4 organ baths were run simultaneously. In experiments where the effect of an inhibitor on drug-induced contractions was to be tested, 2 tracheal preparations served as controls and 2 received the test substance. Control tissues were dosed with vehicle (Krebs solution or ethanol) and were only used to test for any change in the sensitivity of the preparations to contractile drugs during the course of an incubation period. Correction was made in the responses of the test preparation where sensitivity changes occurred. Three of the inhibitors used (indomethacin, dexamethasone and ETYA) were dissolved in ethanol. In the majority of experiments (i.e. those with indomethacin) the final bath concentration of ethanol was 0.01% v/v. Changes in size of contractile responses before and after the inhibitor drug in test preparations are expressed as percentages. Statistical analysis was by the paired t test unless stated otherwise: $P < 0.05$ was regarded as significant. Values given are means ± s.e.mean.

Drugs

The following drugs were used: acetylcholine chloride (BDH), histamine acid phosphate (BDH), dimaprit dihydrochloride (Smith Kline & French), 2-pyridylethylamine hydrochloride (2-PEA) (Smith Kline & French), impromidine trihydrochloride (Smith Kline & French), mepyramine maleate (May & Baker), indomethacin (Sigma), sodium salicylate (BDH), mepacrine hydrochloride (Boots Company), dexamethasone (Sigma), 5, 8, 11, 14-eicosatetraynoic acid (ETYA) (Roche), prostaglandin E_2 (Upjohn Company), FPL 55712 sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate, (Fisons) and arachidonic acid (Sigma). A stock solution of the potassium salt of arachidonic acid was prepared by dissolving the acid in alcoholic KOH. This potassium arachidonate was stored under N_2 at −20°C and in the dark. Unless otherwise specified stock solutions were made up in either distilled water or Krebs solution. Concentrations refer to the free base or acid.

Results

Responses to potassium, acetylcholine and histamine receptor agonists

Following initial dosings with acetylcholine (see Methods) tracheal preparations relaxed back to a tension of less than 0.5 g and they did not exhibit

intrinsic tone, i.e. they could not be relaxed further by drugs.

Swine tracheae were contracted by K^+ (80 mM), acetylcholine (0.1–1000 μM) and histamine (10–1000 μM). Mean cumulative responses obtained in tissues from 3 pigs are shown in Figure 1. Histamine was less potent than acetylcholine. Maximum responses to histamine (1 mM) were about 60% of the maximum acetylcholine-induced contractions. The response to K^+ 80 mM was near maximal (~90%) for that agent. Histamine-induced contractions showed several characteristic features (Figure 2a). Firstly responses were usually slow to develop (~5 min for to reach plateau) and rhythmic oscillations in tone were always observed. These 'slow waves' varied in different preparations, but typically they were of about 3 per min in frequency and 0.5 g in amplitude. Secondly, after the first administration tissues became rapidly desensitized to histamine but not to acetylcholine or K^+. Because of this the mean maximum response to histamine 1 mM shown in Figure 2 is an underestimate. When histamine 1 mM was tested on tissues which had not previously been exposed to histamine, responses averaging 70–75% of the maximum to acetylcholine were obtained. Following washout of the histamine, tone returned to the baseline and the 'slow waves' stopped within a minute or so; however, subsequent acetylcholine or K^+ contractions were then potentiated by about 40%. This potentiation persisted for two or three acetylcholine or K^+ doses (over a 15–30 min period). Contractions, with the same characteristics as histamine, were obtained with 2-PEA, a selective H_1-receptor agonist (Durant, Ganellin & Parsons, 1975) (Figure 2b). Histamine-induced contractions were blocked by mepryamine 1 μM ($n=4$).

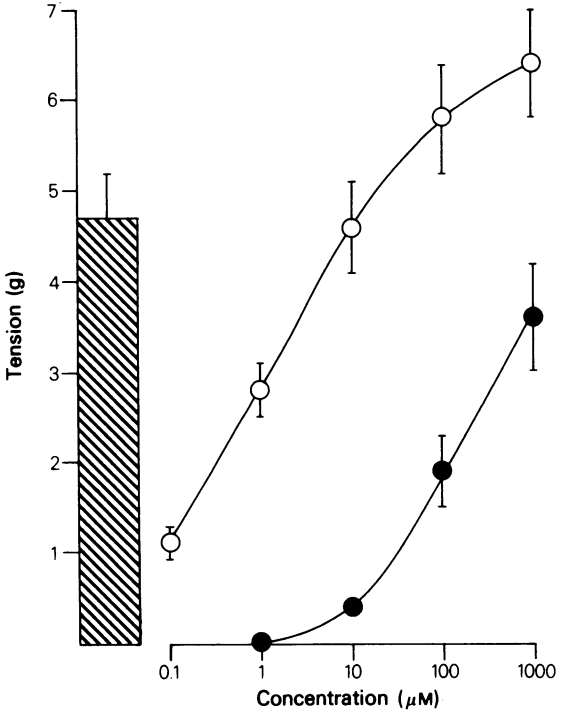

Figure 1 Contractile responses to K^+ 80 mM (hatched histogram), acetylcholine (○) and histamine (●) on swine isolated trachea. Each point is the mean from 8–12 preparations; vertical lines show s.e.mean and are only included when they exceed the dimensions of the symbols used.

In contrast to the above findings, histamine H_2-agonists were without effect on tracheal tone. Dimaprit (Parsons, Owen, Ganellin & Durant, 1977), in concentrations up to 1 mM failed to affect either the resting level of tone or tone induced with submaximal concentrations of acetylcholine or K^+ ($n=4$). Impromidine (Durant, Duncan, Ganellin, Parsons, Blakemore & Rasmussen, 1978) was likewise without effect on acetylcholine-induced tone ($n=4$) when used in concentrations up to 30 μM.

Effect of indomethacin and sodium salicylate

Preliminary experiments at the John Curtin School of Medical Research, Canberra, Australia (with the co-operation of Dr M.A. Denborough) revealed that histamine, but not acetylcholine or K^+-induced contractions in pig tracheae were markedly potentiated by exposure to indomethacin 10 μM. This observation has now been more fully investigated using indomethacin 1 μM and also sodium salicylate 250 μM.

The effect of indomethacin (1 h incubation) was tested on concentrations of acetylcholine (3 μM and 10 μM) giving about 50% and 70% of the maximum response. Because tissues became desensitized to histamine only a single concentration (100 μM), producing a 50% response to that agonist, was used. The results are shown in Table 1. Acetylcholine (3 μM) contractions were not significantly altered by indomethacin whereas at 10 μM a modest potentiation was observed. In contrast, histamine-induced responses were markedly potentiated. Similarly sodium salicylate had no effect on responses to acetylcholine 3 μM but enhanced those to histamine. Further, contractions to 2-PEA 1.6 mM were increased by 90.2 ± 9.0% ($n=4$, $P<0.01$) in the presence of indomethacin. This concentration of 2-PEA gave contractions of similar size to histamine 100 μM. Examples of experiments with indomethacin are shown in Figure 2a and b. Neither indomethacin nor sodium salicylate affected the generation of the 'slow waves' seen with histamine or 2-PEA. No responses

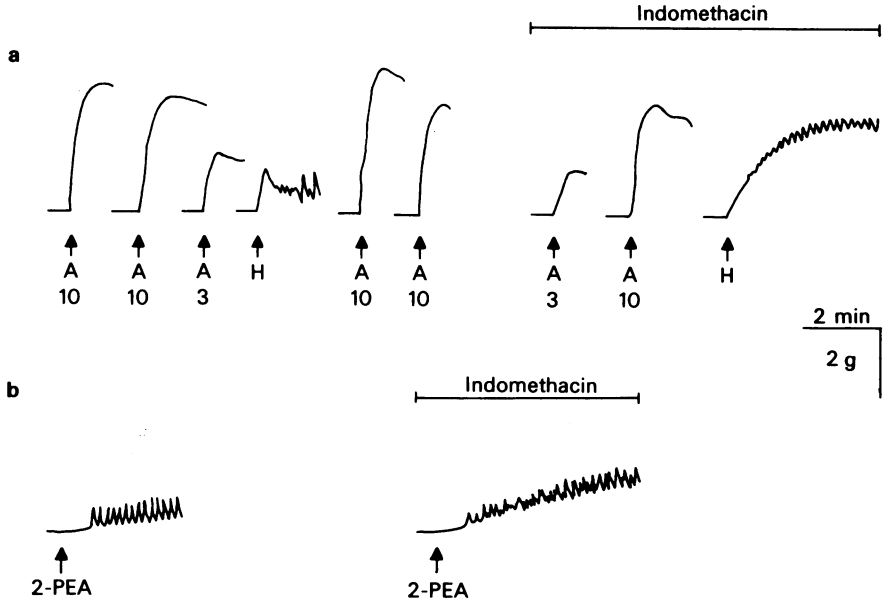

Figure 2 Tracing showing the effect of indomethacin on swine isolated tracheal preparations. (a) Control contractions were first elicited to acetylcholine (A) 10 and 3 μM and to histamine 100 μM (H). Acetylcholine responses show some potentiation after histamine. In the presence of indomethacin (1 μM) the histamine-induced contractions are markedly potentiated. (b) Shows the effect of indomethacin (1 μM) on contractions to 2-pyridylethylamine (2-PEA, 1.6 mM) in a different preparation.

were observed to dimaprit (100 μM) in the presence of indomethacin ($n = 3$).

Effect of prostaglandin E_2 and arachidonic acid

PGE_2 was a relaxant in the swine trachea. When given prior to submaximal concentrations of acetylcholine it inhibited subsequent contractions (Figure 3a). The threshold concentration was found to be in the range 1–3 μM PGE_2 ($n = 8$). Further, PGE_2 (0.1–3 μM) relaxed tissues already contracted by acetylcholine ($n = 4$). When administered during histamine-induced contractions, PGE_2 reduced tone and also eliminated the 'slow wave' activity ($n = 4$) (Figure 3b). Arachidonic acid (100 μM) on the other hand, did not affect acetylcholine ($n = 4$), or histamine-induced tone ($n = 8$) (Figure 3b).

Effect of mepacrine, dexamethasone and 5, 8, 11, 14-eicosatetraynoic acid (ETYA)

A 15 min incubation with mepacrine 100 μM almost abolished histamine-induced contractions but had no effect on acetylcholine-induced (3 μM) contractions (Table 1). In contrast no demonstrable effect was obtained with dexamethasone (1 h incubation) (Table 1).

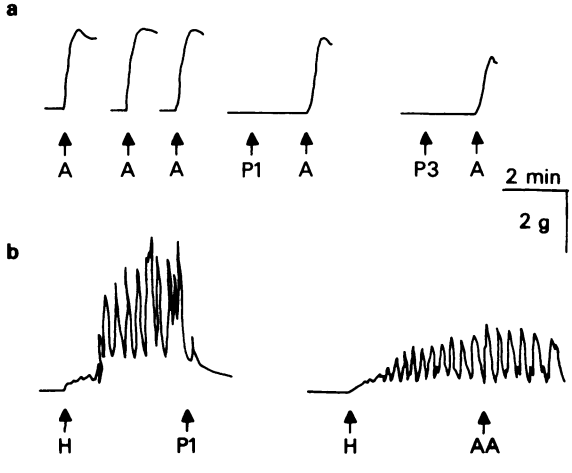

Figure 3 Traces showing the effect of prostaglandin E_2 (PGE_2) (a) and arachidonic acid (b) on swine isolated tracheal preparations. (a) Control contractions to acetylcholine 3 μM (A) which were then repeated after a 2 min incubation with PGE_2 1 μM (P1) and 3 μM (P3) added cumulatively. (b) Shows the effect of PGE_2 1 μM and arachidonic acid 100 μM (AA) on histamine-induced contractions (H, 100 μM) in a different preparation.

Table 1 Effect of inhibitors of arachidonic acid metabolism on drug-induced contractions in the swine isolated trachea

	Acetylcholine 3 μM	*Acetylcholine* 10 μM	*Histamine* 100 μM
Indomethacin 1 μM	+33.5 ± 22.8 (6)	+42.6 ± 14.9* (6)	+213.3 ± 50.1** (6)
Indomethacin 1 μM + ETYA 10 μM	+0.3 ± 11.7 (6)	NT	−31.5 ± 14.1^{+} (6)
Sodium salicylate 250 μM	+0.9 ± 1.3 (4)	NT	+126.9 ± 41.0* (6)
Mepacrine 100 μM	−5.5 ± 4.6 (4)	NT	−85.5 ± 9.7*** (4)
Dexamethasone 50 μM	−3.5 ± 13.5 (4)	NT	+37.0 ± 58.4 (4)
Dexamethasone 100 μM	+24.0 (2)	NT	+13.8 (2)

Values shown are the percentage changes (mean ± s.e.mean) in drug-induced contractions in the presence of an inhibitor. Number of experiments in parentheses.
*$P<0.05$; **$P<0.02$; ***$P<0.01$ compared to pre-inhibitor responses; $^{+}P<0.001$ compared to indomethacin alone (unpaired t test). NT, not tested.

ETYA (10 μM) was used in conjunction with indomethacin 1 μM to determine its effect on indomethacin-induced potentiation of histamine (Table 1). Histamine-induced contractions in the presence of ETYA and indomethacin were significantly less than those in the presence of indomethacin alone, whereas acetylcholine-induced contractions remained unchanged.

Responses in mucosa-free preparations

A series of experiments were carried out on tracheal preparations from which the mucosal layer had been stripped. The procedure was relatively simple since the mucosa is thick and strong and may be gently teased from the underlying smooth muscle.

Mucosa-free tissues responded in a fashion characteristic of the intact preparations. Thus (a) they contracted to acetylcholine and histamine, (b) the histamine responses were weak and displayed 'slow wave' activity and (c) histamine potentiated subsequent acetylcholine-induced contractions. Furthermore, indomethacin increased histamine-induced contractions. However, this potentiating effect was less consistent than that in the intact preparations. In 5 out of 7 experiments indomethacin enhanced histamine-induced contractions (mean 94.2 ± 28.4%; $P<0.05$; $n=5$). In the remaining 2 experiments indomethacin was without effect. No effect was observed on acetylcholine-induced contractions.

Discussion

Results from this study not only confirm previously reported observations that airways smooth muscle tone is modified by drugs with known effects on the metabolism of arachidonic acid, but further extend them by virtue of the observations that histamine-induced contractions are selectively sensitive to phospholipase and lipoxygenase inhibition.

There is a body of evidence that suggests that tonal changes in airways smooth muscle can be regulated by relaxant prostaglandins (Orehek *et al.*, 1975; Anderson *et al.*, 1979b; Krzanowski *et al.*, 1980). In the swine tracheal preparations, responses to histamine are potentiated by the cyclo-oxygenase inhibitors indomethacin and sodium salicylate. Little or no such potentiation was observed with acetylcholine or K^{+}-induced (unpublished) contractions. Indomethacin has a number of non-specific electrical effects which could affect the results (Yamaguchi *et al.*, 1976). However, the concentration used in the present study (1 μM) was less than the threshold level (~3 μM) producing electrical changes in canine tissue. Furthermore, the effect of indomethacin, and its relative specificity on histamine-induced contractions can not be attributed to possible tissue trauma during the slaughter procedure. Similar unpublished results to those reported here were obtained at the John Curtin School of Medical Research, Canberra, with tracheal tissue obtained during surgery from animals which were anaesthetized with barbiturate

and nitrous oxide. Present results then, with indomethacin and sodium salicylate in the swine trachea could be taken to provide further support for the hypothesis of prostaglandin-mediated regulation of drug-induced tone. That indomethacin could still exert an effect on mucosa-free preparations shows that the action of histamine and indomethacin is on the airway smooth muscle and not the mucosa.

However, it is difficult to reconcile the results obtained with the phospholipase A_2 inhibitor, mepacrine (Vargaftig & Hai, 1972; Blackwell, Flower, Nijkamp & Vane, 1978) with this hypothesis. Mepacrine almost abolished histamine (but not acetylcholine)-induced contractions in the trachea. If arachidonic acid is normally converted to PGE_2, then histamine-induced contractions would be enhanced by an antiphospholipase drug since this is the rate limiting stage in arachidonic acid metabolism. Dexamethasone, a drug that inhibits phospholipase A_2 by a protein synthesis-dependent mechanism in lungs (Flower & Blackwell, 1979) was without effect on the contractile responses. A similar lack of effect was reported by Gryglewski *et al.* (1976) who found that the release of prostaglandin and TXA_2-like substances was unaffected by hydrocortisone in the guinea-pig isolated lung strip and trachea stimulated with histamine. It might be that cellular synthetic activity is impaired or changed in isolated tissues used in these types of studies. If mepacrine acts selectively to inhibit the mobilization of arachidonic acid in the swine trachea then the results obtained here indicate that the histamine response (contraction) is normally regulated, in part, by the elaboration of contractile products of arachidonic acid.

Contractile products of the lipoxygenase pathway (leukotrienes/slow reacting substance of anaphylaxis (SRS-A)) are thought to be involved in the immunological (Adams & Lichtenstein, 1977; 1979; Mitchell & Denborough, 1979; Hitchcock & Kokolis, 1981) and complement-induced (Regal & Pickering, 1981) contractile responses in guinea-pig tracheae and lung strips. These slow reacting substances are constrictors of animal (Hedqvist *et al.*, 1980) and human (Dahlen *et al.*, 1980; Ghelani, Holroyde & Sheard, 1980) airways and lung strips. The results in the swine trachea however provide evidence that a lipoxygenase product(s) is involved in *drug-induced* contractions. The evidence is that (a) mepacrine inhibits contractions to histamine and (b) the mixed lipoxygenase/cyclo-oxygenase inhibitor ETYA (Downing, Ahern & Bachta, 1970; Hamberg, 1976) prevents all indomethacin-mediated potentiation of the histamine responses. The concentration of ETYA used in the present study is similar to that required to inhibit lipoxygenase/cyclo-oxygenase (Downing *et al.*, 1970), to inhibit SRS-A release from guinea-pig lungs (Morris, Piper, Taylor & Tippins, 1979) and to block arachidonic acid-induced contractions in the guinea-pig lung strip (Mitchell & Denborough, 1980). In the guinea-pig, Adcock & Garland (1980) have shown that indomethacin-mediated potentiation of histamine-induced tone in the isolated trachea is blocked by anti-lipoxygenase compounds. Furthermore, in guinea-pig lung strips, high concentrations of indomethacin (10 μM) only partially inhibit arachidonic acid-induced contractions whereas they are abolished by ETYA (Mitchell & Denborough 1980). These findings suggest that contractile lipoxygenase metabolites may be important in regulating tone in guinea-pig airways. However, although Hitchcock (1980) showed that the increased tracheal reactivity to histamine (observed in the presence of indomethacin) was not directly related to inhibition of $PGE/PGF_{2\alpha}$ release, neither was it prevented by ETYA (Hitchcock & Kokolis, 1981). Thus the role of lipoxygenase products in that species remains obscure. In the swine trachea the effect of indomethacin was not blocked by FPL 55712. Known bronchocontrictor products of the lipoxygenase pathway (leukotrienes) are blocked by FPL 55712 (Dahlén *et al.*, 1980). An exception here is leukotriene B_4 whose effects on the lung are FPL 55712-resistant. However, the effect of this metabolite is abolished by indomethacin (Sirois, Borgeat, Jeanson, Roy & Girard, 1980). Therefore the tracheal reactivity observed with indomethacin does not appear to be due to the above mentioned substances. A number of possibilities exist: (a) different metabolites are produced in response to different stimuli (antigen and histamine for example), (b) there are species specific metabolites, (c) active products are rapidly degraded and (d) the action of ETYA in blocking the effects of indomethacin is unrelated to inhibition of lipoxygenase.

Arachidonic acid itself had no effect on tone in the swine trachea. Weak and variable effects have been described, with arachidonic acid, on the guinea-pig trachea (Lambley & Smith, 1975; Mitchell & Denborough, 1980). In contrast, PGE_2 inhibited drug-induced tone and it abolished the rhythmic 'slow waves' seen with histamine. If PGE_2 were a principal metabolite produced in response to histamine then the wave activity seen with histamine would not be expected normally, whereas it would be expected in the presence of a cyclo-oxygenase inhibitor. In fact there was no apparent difference in the frequency or magnitude of the 'slow waves' with or without indomethacin, providing additional indirect evidence against a role for PGE_2 in this species.

The mechanisms described in this study appear to be relatively specific for agents which occupy histamine H_1-receptors (histamine and 2-PEA). No response-contraction or relaxation was noted to H_2-

agonists either alone or in the presence of indomethacin. Although indomethacin caused a modest enhancement of acetylcholine-induced contractions, none of the other inhibitors affected this agonist. Moreover, preliminary experiments indicated that K^+-induced contractions were not markedly affected by indomethacin (unpublished). This strongly suggests that the responses to the inhibitor drugs were not due to non-specific effects on cellular function (e.g. on Ca^{2+} metabolism) but to a specific action directly related to the mechanism of action of histamine.

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