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Pharmacological studies into cyclo-oxygenase, lipoxygenase and phospholipase in smooth muscle contraction in the isolated trachea

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1 Indomethacin (1 μM) enhanced histamine-induced contractions in pig and guinea-pig isolated tracheae. Mepacrine (30–50 μM) abolished this effect of indomethacin suggesting that contractile metabolites of arachidonate are involved in the response to indomethacin.

2 Mepacrine (100 μM) in the absence of indomethacin also markedly reduced histamine-induced contractions (81.2% inhibition) in the pig trachea, without affecting responses to acetylcholine. Histamine-induced responses in the guinea-pig trachea were similarly reduced, but with a higher concentration of mepacrine (300 μM).

3 BW755c (226 μM) enhanced histamine-induced contractions in some pig tracheal preparations and caused inhibition in others. These effects of BW755c were negatively correlated to the initial reactivity of the muscle to histamine such that weak contractions were potentiated and strong contractions were inhibited. A similar effect was seen with phenidone (100 μM).

4 The results with BW755c and phenidone suggest that muscle reactivity (to histamine) may be partly determined by the balance between the release of inhibitory and contractile arachidonate metabolites. Mepacrine exerts a different effect indicating that histamine-induced contractions are regulated by a mepacrine-sensitive process which appears to be separate from the metabolism of arachidonate.

Introduction

The translation of receptor activation to the response in a variety of cell types, including smooth muscle, can be modulated by the metabolism of fatty acids and probably phospholipids (reviewed by Michell & Kirk, 1981; Lapetina, 1982). For example in the case of airways smooth muscle, observations by Adcock & Garland (1980) and Mitchell (1982a, b) have shown that the cyclo-oxygenase inhibitor, indomethacin, enhances muscle contraction in response to histamine by some mechanism which appears to be dependent on the integrity of lipoxygenases since drugs which inhibit lipoxygenase block the hyperreactivity.

Arachidonate can be hydrolysed from phospholipids in lungs by phospholipase A_2 and this enzyme is inhibited by mepacrine (Vargaftig & Hai, 1972; Blackwell *et al.*, 1978; Franson & Weir, 1982). In the sensitized (IgE/IgG) guinea-pig, mepacrine inhibits anaphylactic bronchospasm unmasking the role of arachidonate metabolites in certain immunological responses (Andersson, 1982). In the pig isolated trachea it was shown that mepacrine inhibits contractions to histamine but not those to acetylcholine (Mitchell, 1982a) from which the involvement of arachidonate metabolites in drug-induced muscle contractions might be inferred. It has been noted, however, that anti-lipoxygenase and cyclo-oxygenase drugs do not inhibit histamine contractions but this was in the guinea-pig trachea (Adcock & Garland, 1980; Burka & Paterson, 1980; Hitchcock & Kokolis, 1981). If the inhibitory effect of mepacrine on drug-induced contractions in the pig trachea is due ultimately to the abolition of excitatory prostaglandins or hydroxy acids via inhibition of phospholipase A_2 then its effect should be mimicked by antagonists of cyclo-oxygenase and lipoxygenase.

The aims of the work presented in this paper were two fold. The first aim was to determine whether the augmentation of muscle contraction seen with indomethacin could be abolished by a phospholipase A_2 inhibitor (mepacrine). The second aim was to examine the physiological role of lipoxygenase/-cyclo-oxygenase processed metabolites, and phospholipase A_2 activity, in tracheal smooth muscle contraction, in the absence of indomethacin. This was

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partially achieved by comparing the effects of two lipoxygenase/cyclo-oxygenase inhibitors (BW755c and phenidone) and mepacrine on contractions elicited by histamine.

Methods

Experiments with the pig trachea

The methods used for recording isometric tension responses in pig isolated tracheae were the same as previously described (Mitchell, 1982a). Pig tracheae were obtained from local abattoirs; they were usually used fresh but in a few experiments they were first stored in Krebs solution at 4°C for 4–24 h. Tracheal strips were placed in organ baths, under a passive tension of 2 g. Experiments were begun once tissues were showing consistent responses to repeated challenges with a submaximal concentration of acetylcholine (>60 min). Because pig tracheae rapidly desensitize to the effects of sequential dosings of histamine the preparations used in the experiments shown in Figures 1, 2 and 3 were dosed only once with a concentration of histamine (100 μM) which produces about half the maximum response to histamine. However, the three-point concentration-response graphs shown in Figure 4 were obtained using a cumulative dosing schedule. Where the effects of mepacrine and BW755c on histamine-induced contractions were to be compared (Figure 2) responses to histamine were usually obtained in two pairs of tracheal preparations from a pig; one tissue from each pair was dosed with mepacrine or BW755c and the other served as a control. The second pair of tracheal preparations was likewise treated using the other inhibitor (mepacrine or BW755c) in the test preparation. Where appropriate, histamine-induced contractions were normalized by comparing them with a maximal contraction obtained to acetylcholine at the beginning of the experiment and in the absence of other drugs.

Experiments with the guinea-pig trachea

Guinea-pig isolated tracheae were obtained from animals of either sex weighing between 500–1000 g. Tracheal rings, opened along the cartilage, were suspended in organ baths containing Krebs solution of the same composition as that used in the pig experiment. The muscle was placed under 0.5 g passive tension. Desensitization to the effects of histamine did not occur in the guinea-pig trachea, therefore, each tissue could serve as its own control and the effect of indomethacin or mepacrine could be expressed as a percentage increase or decrease of the control contractions.

Drugs and statistics

The drugs used were histamine acid phosphate (BDH), acetylcholine chloride (BDH), indomethacin (Sigma, made up in ethanol), mepacrine hydrochloride (a gift from Dr V. R. Holland, The Boots Company), phenidone (1-phenyl-3-pyrazolidone, Sigma), BW755c (3-amino-1-[*m*-(trifluoromethyl) phenyl]-2-pyrazoline, a gift from J. J. Adcock, Wellcome Labs.) and arachidonic acid (Sigma). Arachidonic acid was made up in 0.02 M ammonia solution.

The results are expressed as means ± s.e.mean. Means were compared using Student's *t* test for unpaired data except where specified in the text. $P < 0.05$ was regarded as significant.

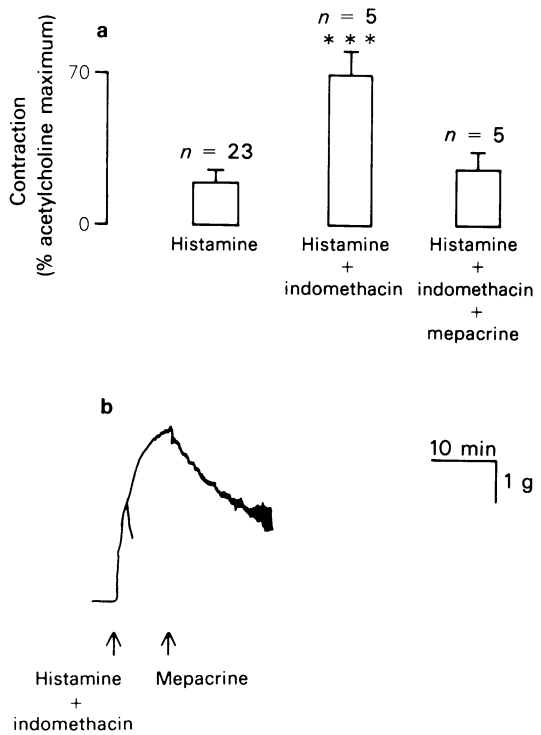

Figure 1 The effect of mepacrine on the indomethacin-induced hyper-reactivity to histamine in the pig isolated trachea. (a) The mean contractions to histamine (100 μM), histamine and indomethacin (1 μM for 1 h) and histamine, indomethacin and mepacrine (100 μM, for 15 min before histamine), ***$P < 0.01$, compared to histamine alone. (b) A histamine-induced contraction in the presence of indomethacin. Mepacrine (50 μM) was then added to the organ bath at the peak of the histamine response causing the muscle to relax.

Results

Effect of mepacrine on histamine-induced contractions augmented by indomethacin

In the pig trachea Histamine (100 μM) caused contractions in the tracheal preparations, which were slow in their development and rhythmic oscillations in tone were frequently observed (Figures 1 and 3). When compared to the maximum contraction obtained to acetylcholine the mean histamine-induced response was 18.8 ± 5.3% ($n = 23$). In contrast, histamine-induced contractions in the presence of indomethacin (1 μM for 1 h) were 69.2 ± 8.9% ($n = 5$, $P < 0.01$).

The augmentation of histamine-induced contractions by indomethacin was reduced or abolished by pre-incubation with mepacrine (50 μM for 15 min before histamine), so that the mean histamine-induced response was 25.0 ± 7.1% ($n = 5$) of the acetylcholine maximum (Figure 1). In addition to the inhibitory effect of a mepacrine pre-incubation, mepacrine also reduced the tone of tracheae previously contracted by histamine, in the presence of indomethacin ($n = 4$) (Figure 1). The onset of the relaxation occurred within 1 min of the mepacrine injection and was not due to muscle fatigue because histamine-induced muscle tone is normally well sustained. Arachidonate, in concentrations up to 2 mM, had no effect on tracheae in the absence or presence of indomethacin ($n = 4$) or on the mepacrine-relaxed tissues described above ($n = 3$).

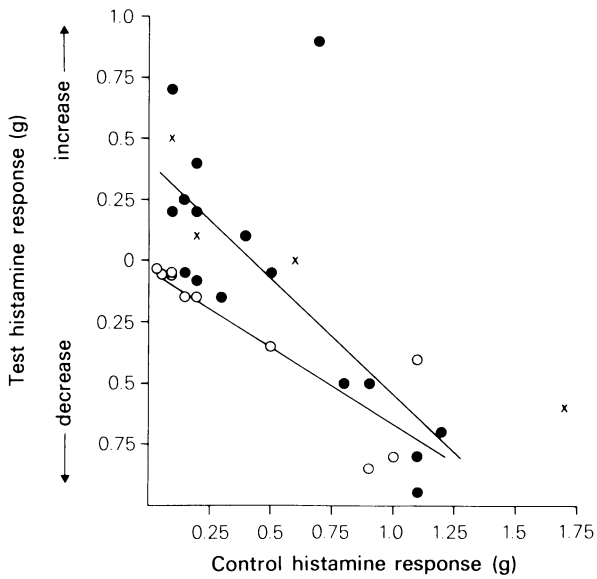

Figure 2 The effect of mepacrine, BW755c and phenidone on histamine-induced contractions in the pig isolated trachea. Each point compares the size of a contraction to histamine (100 μM) obtained in a control tracheal preparation (abscissa) and, on the ordinate, in a matched test preparation in the presence of either mepacrine 100 μM (○), BW755c 226 μM (●) or phenidone 100 μM (×). The response in the test preparation was calculated as the difference between the contraction in the test and control preparations. The correlation coefficient for BW755c was −0.73 and for mepacrine it was −0.88. The lines of best fit were calculated by the method of least squares.

In the guinea-pig trachea Two concentrations of histamine were used to give responses which were approximately 30% (10 μM) and 75% (30 μM) of the maximum histamine-induced contraction. Indomethacin (1 μM for 1 h) potentiated the responses to histamine 10 μM and 30 μM by 237 ± 59.5% ($n = 4$, $P < 0.05$, Student's paired t test) and 184.5 ± 64.1%, respectively. On the other hand, when tracheal preparations were pre-incubated in mepacrine (30 μM for 15 min before histamine) then indomethacin did not augment the responses. In the presence of indomethacin and mepacrine the contractions to histamine (10 μM and 30 μM) were only altered by + 14.9 ± 29.3% and −10.6 ± 11.8%, respectively ($n = 4$).

As with the pig trachea, mepacrine (30 μM) partially relaxed guinea-pig tracheal preparations previously contracted by histamine, in the presence of indomethacin ($n = 4$).

Effect of mepacrine on histamine-induced contractions without indomethacin

In the pig trachea In 10 pairs of tissues histamine-induced (100 μM) contractions were found to be substantially reduced by pre-incubation with mepacrine (100 μM for 15 min). From the results shown in Figure 2 it is seen that the degree of inhibition in a tracheal preparation (difference between the histamine-induced contraction, in grams, in the presence of mepacrine and that obtained in the appropriate control) was related to the size of the control contraction to histamine ($r = -0.88$, $P < 0.01$). As such the mepacrine-induced inhibition in a preparation (control) responding relatively weakly to histamine was less than that in a strongly responding preparation. However, on a percentage basis the inhibition was of a similar proportion (81.2 ± 6.9% inhibition, $n = 10$).

In one experiment (Figure 4) mepacrine (100 μM) was shown to inhibit histamine-induced contractions over a range of histamine concentrations (10–1000 μM). Moreover, when injected into an organ bath during a histamine-induced contraction, mepacrine (50–100 μM) caused relaxation ($n = 4$). The rhythmic oscillations in tone often seen in re-

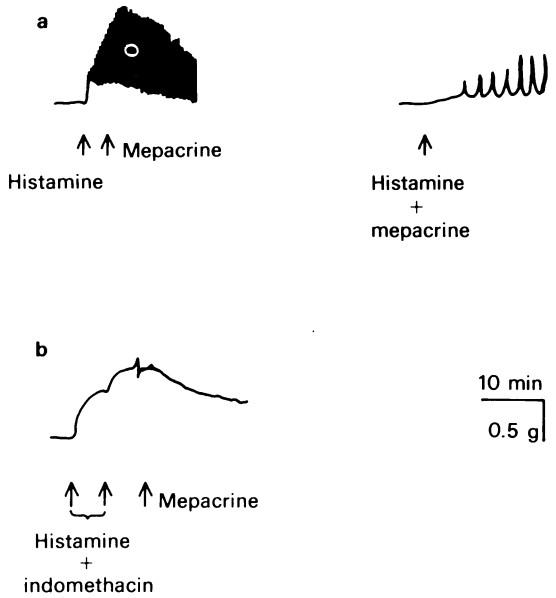

Figure 3 Examples of tracings showing the effect of mepacrine on histamine-induced contractions in the pig (a) and guinea-pig (b) isolated trachea. (a) In the first (left hand) example, mepacrine (50 μM) was injected into the organ bath containing the tissue at the peak of a contraction to histamine. The active tension was reduced and there was only a partial reduction in the size of the rhythmic tension oscillations. In the second example the tissue was pre-incubated in mepacrine (100 μM for 15 min). On challenge with histamine there was only a small increase in tone, with superimposed slow waves. The histamine concentration was 100 μM in both examples. (b) The guinea-pig preparation was pre-incubated in indomethacin (1 μM for 1 h) then dosed with two concentrations of histamine (10 then 30 μM). Mepacrine (30 μM) was then added, resulting in partial relaxation.

sponses to histamine, on the other hand, did not appear to be markedly affected by mepacrine (Figure 3). Mepacrine (100 μM) had no effect on contractions elicited by acetylcholine (3 μM, $n=3$) either in the presence or absence of indomethacin.

In the guinea-pig trachea The guinea-pig trachea was less sensitive to mepacrine than pig trachea. In these tissues 30 μM and 50 μM mepacrine had no effect on submaximal contractions to histamine (30 μM). However, at 100 μM mepacrine (for 15 min) there was a 13.1 ± 6.3% inhibition and 300 μM mepacrine nearly abolished the responses to histamine (Figure 5) (84.8 ± 2.4% inhibition, $n=4$, $P<0.001$, Student's paired t test). Mepacrine also reduced the intrinsic tone of the guinea-pig trachea and both of these effects of mepacrine were readily reversible after a 10 min washing period (Figure 5).

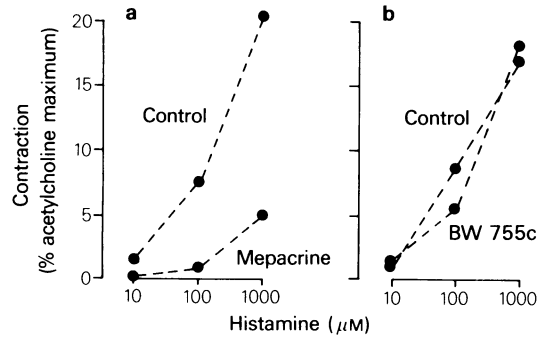

Figure 4 Concentration-effect curves for histamine in the pig isolated trachea. Each point is the average response obtained to histamine in a pair of tissues. Two tissues were used as controls and further pairs of tracheal preparations were incubated in either (a) mepacrine (100 μM for 15 min) or (b) BW755c (226 μM for 30 min).

Effect of BW755c and phenidone on histamine-induced contractions in the pig trachea

The effects of BW755c (226 μM for 30 min) and phenidone (100 μM for 30 min) on histamine-induced contractions (100 μM) are shown in Figure 2. The effect of BW755c was complex and depended on the reactivity of the muscle to histamine. In pairs of tracheal preparations responding relatively strongly to histamine (> 0.4 g), BW755c usually caused an inhibition of the histamine-induced contractions. On the other hand, where weaker control contractions were obtained then an increase in the histamine-induced contraction was generally seen ($r=-0.73$, $P<0.01$) in the presence of BW755c. On a percentage basis the potentiation by BW755c in the preparations responding weakly to histamine was large, as can be readily estimated from Figure 2. Furthermore, some preparations appeared to be unaffected by BW755c. The effect of BW755c in one such experiment is shown in Figure 4.

In order to ascertain whether higher concentrations of BW755c might have additional effects, 500 μM BW755c was administered to 4 preparations which were already contracted by histamine. In 3 cases there was no change in muscle tension and in 1 experiment there was a 30% relaxation.

Phenidone was tested in 4 pairs of tracheal preparations and there was a wide variation in its effect on histamine-induced contractions. There was however, an apparent relationship between the initial reactivity of the muscle, to histamine and the effect of phenidone (Figure 2).

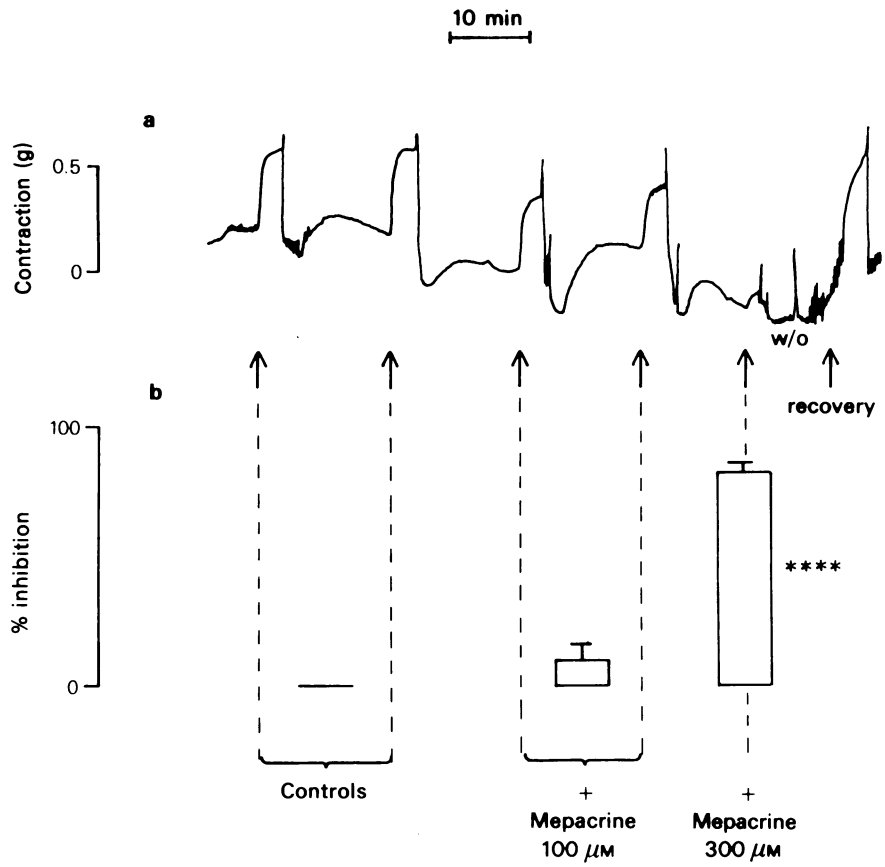

Figure 5 The effect of mepacrine on histamine-induced contractions in the guinea-pig isolated trachea. (a) Illustrates the effect of mepacrine (100 μM and 300 μM) on histamine-induced contractions (30 μM, injected at arrows) in one experiment. The mepacrine was washed from the organ bath at w/o to show recovery. (b) Columns show the mean results obtained in 4 experiments, $^{****}P < 0.001$ compared to controls (Student's paired t test).

Discussion

The experiments described here show, in tracheal preparations from two species, that mepacrine reduces or abolishes the action of indomethacin, which was to augment muscle contraction to histamine. It was shown previously that indomethacin has little or no effect on contractions to acetylcholine in the pig trachea (Mitchell, 1982a). Indomethacin has been found to enhance drug-induced contractions of airways smooth muscle by numerous authors and it has been proposed that this effect is due to the removal of the influence of relaxant prostaglandins (e.g. Orehek *et al.*, 1975) because prostaglandin E_2 has been demonstrated to be released from contracting airways. It is now apparent, however, that the enhancement by indomethacin may also be associated with activity of a lipoxygenase (Adcock & Garland, 1980; Mitchell, 1982a, b), which possibly catalyses the formation of excitatory metabolites of arachidonate. Arachidonic acid can be hydrolysed from phospholipids by a phospholipase A_2, an effect which is inhibited by mepacrine (Vargaftig & Hai, 1972; Blackwell *et al.*, 1978; Franson & Weir, 1982). Therefore, inhibition of phospholipid hydrolysis at this stage will effectively reduce or stop the production of prostaglandins, other cyclo-oxygenase products, hydroxy acids (produced via a lipoxygenase) and any other metabolite of arachidonate, except where a pre-existing store of free arachidonate may occur. In this study mepacrine at relatively low concentrations (Blackwell *et al.*, 1978) largely abolished the effect of indomethacin on contractions to histamine. This occurred whether tissues were pre-incubated in mepacrine or whether mepacrine was administered later on during a muscle contraction.

Submaximal responses to acetylcholine, either with or without indomethacin, were unaffected by mepacrine (present study; Mitchell, 1982a) suggesting that non-specific toxic effects of mepacrine are not involved in its effect on the histamine response. These observations further reinforce the suggestion that during the histamine-induced contraction (and in the presence of indomethacin) a lipoxygenase product(s) of arachidonate augments muscle contraction. That the relaxant action of mepacrine on pre-existing muscle tone is so rapid ($\sim$ 1 min) indicates a rapid disappearance of the excitatory product(s). In pig tissues this is supported by the observation that arachidonate fails to contract the trachea when in the presence of indomethacin.

The results discussed above indicate that arachidonate metabolism may be important in regulating muscle tension in the presence of indomethacin. The question arises, however, as to the physiological role of these processes when muscles are stimulated hormonally (e.g. histamine) in the absence of indomethacin. The approach taken in the present work to investigate this point was to attempt to inhibit the conversion of arachidonate to all putative regulators of muscle tension (cyclo-oxygenase and lipoxygenase products) using BW755c and phenidone (Blackwell & Flower, 1978; Higgs *et al.*, 1979). Indeed the interpretation of the biological responses obtained to BW755c and phenidone have been based on an assumption that these drugs, in the concentrations used, act on arachidonate metabolism. The concentration of BW755c used in the present study was about five times greater than that which blocks cyclo-oxygenase and lipoxygenase systems in platelet homogenates (Higgs *et al.*, 1979), but it is similar to the concentrations which block or inhibit the indomethacin-induced hyper-reactivity in strips of pig and guinea-pig airways (Adcock & Garland, 1980; Mitchell, 1982b). It has been previously found that mixed inhibitors of cyclo-oxygenase and lipoxygenase have only a little or no overall effect on histamine-induced contractions in the guinea-pig trachea (Adcock & Garland, 1980; Burka & Paterson, 1980; Hitchcock & Kokolis, 1981). The present data also indicate that neither BW755c nor phenidone have an effect on histamine-induced contractions in the pig trachea. This might be explained, however, by the observations that 'weak responders' tend to show potentiated contractions to histamine whereas 'strong responders' tend to show diminished contractions. In cases where there is a potentiated response to histamine in the presence of BW755c one could speculate that there might be a reduction in the release or effect of inhibitory arachidonate products. At the other extreme where a dimunition in reactivity was observed there may be a reduction in the release of an excitatory product of arachidonate metabolism. The results suggest, therefore, that physiological (histamine-induced) contractions in the pig isolated trachea may be influenced by the release and balance between excitatory and inhibitory arachidonate metabolites. Clearly however, direct biochemical measurements are required to examine these possibilities.

The effect of mepacrine on histamine-induced contractions was quite different from that of the mixed cyclo-oxygenase and lipoxygenase inhibitors, even though predictably they should have the same effect on muscle contraction in the context of the simple models discussed above. Mepacrine reduced all histamine-induced contractions (this study; Mitchell, 1982a) regardless of the initial reactivity of the muscle. Even contractions in the 'weak responders', which are markedly enhanced by BW755c, were inhibited by mepacrine. Therefore, mepacrine probably has an effect on muscle contraction which is independent of, or perhaps additional to, arachidonate metabolism. Alternatively arachidonate (or other products of phospholipid hydrolysis) may be metabolized to active substances via pathways other than those which are inhibited by BW755c and phenidone. Somewhat higher concentrations of mepacrine were required to inhibit histamine-induced contractions compared to the reversal of the indomethacin effect, particularly in the guinea-pig trachea, suggesting that some non-specific inhibitory action of mepacrine might contribute to its effect. As mentioned above, however, in the case of pig smooth muscle, comparable contractions to acetylcholine were not affected by the concentration of mepacrine which almost abolished the responses to histamine (this study; Mitchell, 1982a). Mepacrine does however, inhibit lung phospholipase A_2 in concentrations similar to those used to inhibit the histamine-induced contraction. Therefore, the present results indicate that muscle contraction to histamine may be regulated by a mepacrine-sensitive stage, possibly involving a phospholipase of the A_2 type. This step in muscle contraction appears to be distinct from that involving prostaglandin or hydroxy acid formation.

References

ADCOCK, J.J. & GARLAND, L.G. (1980). A possible role for lipoxygenase products as regulators of airway smooth muscle reactivity. *Br. J. Pharmac.*, **69**, 167–169.

ANDERSSON, P. (1982). Effects of inhibitors of anaphylactic mediators in two models of bronchial anaphylaxis in anaesthetised guinea-pigs. *Br. J. Pharmac.*, **77**, 301–307.

BLACKWELL, G.J. & FLOWER, R.J. (1978). 1-Phenyl-3-

pyrazolidone: an inhibitor of cyclo-oxygenase and lipoxygenase pathways in lung and platelets. *Prostaglandins*, **16**, 417–425.

BLACKWELL, G.J., FLOWER, R.J., NIJKAMP, F.P. & VANE, J.R. (1978). Phospholipase A_2 activity of guinea-pig isolated perfused lungs: stimulation and inhibition by anti-inflammatory steroids. *Br. J. Pharmac.*, **62**, 79–80.

BURKA, J.F. & PATERSON, N.A.M. (1980). Evidence for lipoxygenase pathway involvement in allergic tracheal contraction. *Prostaglandins*, **19**, 499–515.

DOWNING, D.T., AHERN, D.G. & BACHTA, M. (1970). Enzyme inhibition by acetylenic compounds. *Biochem. biophys. Res. Cummun.*, **40**, 218–223.

FRANSON, R.C. & WEIR, D.L. (1982). Isolation and characterisation of a membrane-associated Ca^{2+} -dependent phospholipase A_2 from rabbit lung. *Lung*, **160**, 275.

HIGGS, G.A., FLOWER, R.J. & VANE, J.R. (1979). A new approach to anti-inflammatory drugs. *Biochem. Pharmac.*, **28**, 1959–1961.

HITCHCOCK, M. & KOKOLIS, N.A. (1981). Arachidonic acid metabolism and modulation of *in vitro* anaphylaxis by 5, 8, 11, 14-eicosatetraynoic acid and 9a, 12-octadecadiynoic acid. *Br. J. Pharmac.*, **72**, 689–695.

LAPETINA, E.G. (1982). Regulation of arachidonic acid production: role of phospholipase C and A_2. *Trends Pharmac. Sci.*, **3**, 115–118.

MICHELL, R.H. & KIRK, C.J. (1981). Why is phosphatidylinositol degraded in response to stimulation of certain receptors? *Trends Pharmac. Sci.*, **2**, 86–89.

MITCHELL, H.W. (1982a) The effect of inhibitors of arachidonic acid metabolism on drug-induced contractions in isolated tracheal smooth muscle of the pig. *Br. J. Pharmac.*, **75**, 129–136.

MITCHELL, H.W. (1982b) Effect of mixed inhibitors of cyclo-oxygenase and lipoxygenase on the indomethacin-induced hyper-reactivity in the isolated trachea of the pig. *Br. J. Pharmac.*, **77**, 701–705.

OREHEK, J. DOUGLAS, J.S. & BOUHUYS, A. (1975). Contractile responses of the guinea-pig trachea in vitro: modification by prostaglandin synthesis-inhibiting drugs. *J. Pharmac. exp. Ther.*, **194**, 554–564.

VARGAFTIG, B.B. & HAI, N.D. (1972). Selective inhibition by mepacrine of the release of rabbit aorta contracting substance evoked by the administration of bradykinin. *J. Pharm. Pharmac.*, **24**, 159–161.

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