Phorbol myristate acetate causes in guinea-pig lung parenchymal strip a maintained spasm which is relatively resistant to isoprenaline

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The effect of phorbol myristate acetate (PMA) was compared with that of histamine on the guinea-pig lung parenchymal strip. PMA, 10^{-5} M, caused a slowly developing sustained contraction which had approximately the same magnitude as the maximal histamine contraction. Isoprenaline, at 10^{-5} M, caused 86% relaxation of the histamine contraction but only 22% relaxation of the PMA contraction. Forskolin, at 10^{-5} M had a similar action to isoprenaline on the effects of both spasmogens while aminophylline, 5×10^{-4} M, was considerably less effective. Sodium nitroprusside had little effect on the histamine contraction and actually increased the PMA spasm. It is suggested that protein kinase C may have a role in the tonic phase of the contraction of bronchiolar smooth muscle. These findings could have relevance for the delayed phase of asthma, which is known to be insensitive to β-agonists.

Polyphosphoinositide *Phorbol ester* *Smooth muscle* *Asthma* *Protein kinase*

1. INTRODUCTION

There is increasing evidence that in cells in which calcium is an intracellular messenger, an early event following receptor stimulation is breakdown of polyphosphoinositides with generation of diacylglyceryol, which activates protein kinase C, and inositol trisphosphate, which mobilizes calcium (reviews in [1,2]). Recent findings suggest that this process may be involved in signal transduction in smooth muscle. Inositol trisphosphate releases calcium from intracellular stores in skinned muscle fibres from porcine arteries [3] and turnover of polyphosphoinositides occurs during stimulus-activation coupling in guinea-pig ileal smooth muscle [4,5] and in canine tracheal muscle [6]. The involvement of the protein kinase C pathway is indicated by the fact that PMA has been found to cause tonic contraction in rat and rabbit vascular smooth muscle [7,8].

Since a sustained contraction of bronchiolar smooth muscle is implicated in the pathogenesis of asthma we thought it of interest to investigate the effect of PMA on the lung parenchymal strip and to compare its effects with that of histamine, which, released from mast cells, is considered by many to be an important mediator of bronchial constriction in asthma. In the guinea-pig, histamine is present within the granules of the mast cell in a concentration of approx. 0.2 M. When the mast cell releases its contents after an antigen/antibody reaction it will produce a very high local concentration of histamine, likely to be of the order of 10^{-5}–10^{-4} M – a concentration which could be expected to result in a maximal or near-maximal contraction of the bronchiolar smooth muscle in the vicinity. We set out to reproduce in vitro the effect of such histamine release, then to determine the concentration of PMA which produced an equivalent contraction and then to compare the characteristics of the two responses. We

Abbreviations: PMA, phorbol myristate acetate; DMSO, dimethyl sulphoxide

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also thought it of interest to examine the effect of the β-receptor agonist, isoprenaline, on both the histamine-mediated and the PMA-mediated response and to compare its effects with agents which acted at a post-receptor level to increase cyclic AMP levels (forskolin, aminophylline) or cyclic GMP levels (sodium nitroprusside). β-Adrenoceptor agonists and aminophylline are used in the treatment of asthma.

2. MATERIALS AND METHODS

Male Hartley guinea-pigs (350–400 g) were used. Parenchymal strips were cut from the distal edges of the lung lobes [9] and suspended under 0.5 g tension in either 5 ml or 50 ml baths in Krebs-Henseleit solution, maintained at 37°C and aerated with 95% O_2 and 5% CO_2. Contractions were measured isotonically using UFI transducers (Lab-Data Instruments) and Servo Scribe Pen recorders (Services UK).

Tissues were incubated in the organ baths for 1 h before use. Contractions to histamine were obtained using concentrations of 10^{-6} M followed by 10^{-5} M, leaving the drug in the bath until the maximum response occurred. PMA was added to the bath to give a final concentration of 10^{-5} M and was left in the bath for a minimum of 2 h to allow the contraction to reach maximum. The PMA was made up in DMSO and the final concentration of DMSO in the bath did not exceed 0.1%. At this concentration DMSO did not cause contraction or result in inhibition of either PMA or histamine responses. It is not possible to use an isometric recording technique with lung parenchymal strip preparations since the tissue is too delicate, and thus it is not possible to express a contraction in terms of g of tension. PMA-induced contractions were therefore expressed as % of the maximum histamine response.

The reagents used were histamine acid phosphate, isoprenaline sulphate, theophylline ethylene diamine (aminophylline) and sodium nitroprusside (all obtained from Sigma, England) and forskolin (obtained from Calbiochem, England).

The composition of the Krebs-Henseleit solution was as follows ($g \cdot l^{-1}$): NaCl, 6.9; KCl, 0.35; $MgSO_4 \cdot 7H_2O$, 0.29; KH_2PO_4, 0.16; $NaHCO_3$, 2.1; glucose, 2.0; $CaCl_2$, 0.55 (all chemicals being from BDH, England).

3. RESULTS

The mean normalized results of 20 experiments are given in fig.1. Since normalizing and averaging results does not give a clear picture of the original data, examples of the results of individual experiments are given in fig.2.

Addition of histamine to the lung parenchymal strip caused an immediate contraction, the maximum response occurring with 10^{-5} M. PMA caused a slowly-developing sustained contraction (fig.2) which was concentration-dependent over the range 3×10^{-8}–10^{-5} M. The concentration of 10^{-5} M was selected for the experiments reported here since it gave approximately the same magnitude of contraction as the maximum histamine response. In 20 strips (from 10 guinea-pigs) this contraction was 112% (SE 6.4%) of the maximum histamine response. It took between 2 and 3 h to reach maximum and washing the tissue

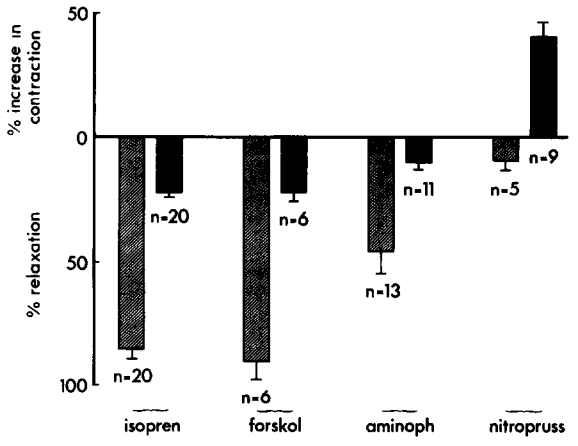

Fig.1. The change in the histamine-induced and the PMA-induced contraction of the guinea-pig parenchymal lung strip obtained with various relaxants. The change is expressed as % relaxation or % increase of the maximum contraction given by histamine, 10^{-5} M (hatched columns) or PMA, 10^{-5} M (doubly hatched columns). The 'relaxants' used were isoprenaline 10^{-6} M; forskolin, 10^{-6} M; aminophylline, 5×10^{-4} M; and sodium nitroprusside, 10^{-5} M. The PMA contraction was 112% (SE 6.4%) of the maximum histamine contraction (n = 20). The bars represent standard errors.

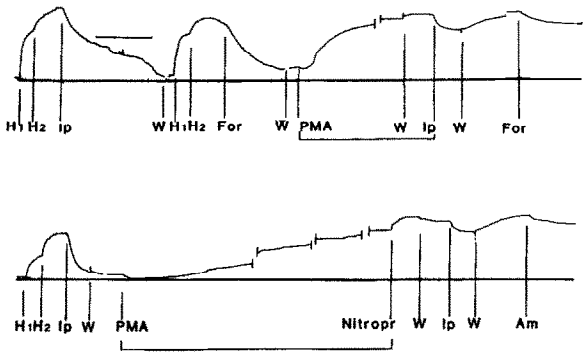

Fig.2. Examples of records obtained with guinea-pig lung parenchymal strips, showing the cumulative dose-response curve with histamine (H), the contractile response to phorbol myristate acetate 10^{-5} M (PMA), and the relaxant effects of various agents. (The relaxant effect of isoprenaline was included in all experiments, as a standard.) The bar underneath the record of the PMA response represents the length of time PMA was left in the bath before the first addition of a relaxant; this was 2 h 10 min in the upper record and 3 h 50 min in the lower. The bar above the contraction tracing in the upper record represents 30 min. Upper record: comparison of the effect of isoprenaline and forskolin on the H- and PMA-induced contractions. Lower record: comparison of the effect of isoprenaline on the H-induced contraction with the effect of isoprenaline, aminophylline and sodium nitroprusside on the PMA-induced contraction. H1, 10^{-6} M histamine; H2, 10^{-5} M histamine; Ip, 10^{-6} M isoprenaline; for, 10^{-6} M forskolin; Nitropr, 10^{-5} M sodium nitroprusside; Am, aminophylline.

did not decrease the contraction. In fact there was frequently an increase in tone when a strip was washed, and in the 10 strips in which the experiment was continued for an additional 40–60 min, the response gradually increased from 108% (SE 9.0%) of the histamine maximum to 134% (SE 11.3%) even though the strips were washed repeatedly.

Both the histamine-induced and the PMA-induced contraction required the presence of calcium in the surrounding fluid.

Since it was possible that histamine, released from mast cells by the PMA, could have been contributing to the PMA-induced muscle contraction, and since the effect of histamine, but not that of PMA, disappeared on washing, most parenchymal strips were washed with Krebs-Henseleit solution before the effect of the relaxants was tested on the PMA contraction.

Isoprenaline, 10^{-6} M, caused a rapid and virtually complete relaxation of the histamine-induced contraction (figs 1,2a and b) as did forskolin, 10^{-6} M (figs 1 and 2a). However, isoprenaline, 10^{-6} M, had relatively little effect on the PMA-induced contraction (figs 1,2a and b). Forskolin, 10^{-6} M, a concentration which had approximately the same effect as isoprenaline on the histamine contraction, also had relatively little effect on the PMA contraction (figs 1 and 2a). If forskolin, 10^{-6} M, was given before PMA, it markedly inhibited the subsequent response to PMA, 10^{-5} M, which then reached only 50% of the maximum histamine contraction even if given after an interval of 60 min, during which the tissue was repeatedly washed (not shown).

Aminophylline at 5×10^{-4} M was only partially effective in causing relaxation of the histamine-induced contraction and was virtually ineffective against the PMA-induced contraction, having no effect at all in 4 of 11 experiments (figs 1 and 2b). Sodium nitroprusside, 10^{-5} M, had very little effect on the histamine contraction but, unexpectedly, exacerbated the PMA-induced contraction in all 9 experiments in which it was tested (figs 1 and 2b): in one experiment by as much as 70%.

The difference between the effect on the histamine-induced contraction and that on the PMA-induced contraction was statistically significant at the level $p = 0.001$, on Student's t-test, for all 'relaxants' used.

4. DISCUSSION

It has recently been shown that when agonist action in vascular smooth muscle produces a sustained contraction, there is, concomitantly, a 2-stage change in calcium concentration, consisting of an initial large calcium transient followed by a smaller maintained calcium response [10]. Other results obtained [11] have given rise to the hypothesis that there are 2 calcium-dependent processes involved in vascular smooth muscle contraction: an initial phosphorylation of myosin, necessary for force development, followed by a second process which is necessary for force maintenance but which has a lower calcium requirement [11]. Rasmussen et al. [7] have sug-

gested that protein kinase C may be involved in this second process in vascular smooth muscle. Protein kinase C has been shown to phosphorylate both smooth muscle heavy meromyosin [12] and myosin light chain kinase [13] the latter action resulting in a reduced affinity for Ca^{2+}/calmodulin.

Here we set out to see whether a protein kinase C-activator such as PMA would produce a tonic spasm in the smooth muscle of the lung parenchymal strip, and if so, to compare the PMA response with the histamine response. We also wished to examine the effect of various agents known to relax smooth muscle, on both types of contraction.

The results showed that PMA, 10^{-5} M, caused a slowly-developing, sustained contraction of the parenchymal strip. The smooth muscle in the strip is considered to be predominantly that of the smaller bronchioles and there is evidence from work on vascular tissue [7,8] that PMA is able to cause a similar slow contraction by a direct effect on smooth muscle itself. However, the lung parenchymal strip is a complex tissue, containing many other types of cell, including mast cells, endothelial cells, leucocytes and platelets and it is known that PMA causes mediator release from many of these cells. Thus the contraction may have an indirect as well as a direct component. The contribution of these mediators, however, would seem to be of relatively minor importance since the contraction is maintained even after repeated, thorough washing of the tissue.

The β-adrenoceptor agonist, isoprenaline, 10^{-6} M, caused a marked relaxation of the histamine-induced contraction but had only a minor effect on the PMA-induced contraction. β-Adrenoceptor-mediated relaxation is generally considered to be due to activation of adenylate cyclase resulting in increased cyclic AMP (review [14]). There is evidence that the cyclic AMP-dependent kinase phosphorylates myosin light chain kinase apoenzyme and renders it less sensitive to activation by calcium/calmodulin [15], though other mechanisms for the relaxant effect of cyclic AMP on smooth muscle have been proposed [14].

We thought it possible that the decreased effect of isoprenaline on the PMA- as compared with the histamine-induced contraction was due to a PMA-induced phosphorylation of the β-receptor resulting in its desensitization [16,17]. We therefore expected that forskolin, a direct activator of adenylate cyclase, would be a more effective relaxant of the PMA-induced spasm than isoprenaline. Surprisingly, however, forskolin at a concentration of 10^{-6} M, which was approximately equipotent with isoprenaline in relaxing the histamine contraction, was also only minimally effective against the PMA spasm. The similarity in the actions of isoprenaline and forskolin on the effects of both spasmogens was rather striking and suggested that desensitization of the β-receptor might not be involved in the reduced effect of isoprenaline on PMA spasm.

Aminophylline was only moderately active in relaxing the histamine-induced contraction, and minimally active in relaxing the PMA-induced spasm, even when used in the high concentration of 5×10^{-4} M. Since this agent inhibits phosphodiesterase and results in an increase in cyclic AMP it was surprising that it was so much less effective than isoprenaline and forskolin.

Sodium nitroprusside stimulates guanylate cyclase and increases cyclic GMP, an action believed to be the basis of the well-known relaxant effect of sodium nitroprusside on vascular smooth muscle (reviews [18,19]). This agent, at a concentration of 10^{-5} M has also been reported to cause marked relaxation (77%) of bovine tracheal muscle pre-contracted with carbachol [20]. It was unexpected therefore, to find that sodium nitroprusside had only a minimal relaxant effect on the guinea-pig lung parenchymal strip pre-contracted with histamine and that it actually increased the PMA-induced spasm of this tissue. Protein kinase C thus appears to abrogate the relaxant action of smooth muscle guanylate cyclase, although it apparently enhances the activity of the guanylate cyclase from rat brain [21].

These results may have relevance for the pathogenesis of asthma, and for the action of drugs used to treat asthma. Constriction of the bronchioles is responsible for the difficulty in breathing in the first phase of asthma. This constriction is considered by most workers to be due largely to histamine and is rapidly reversed by β-adrenoceptor agonist drugs. A second, more severe increase in airways resistance, maximal 6–8 h after exposure to allergen, occurs frequently and is dif-

ficult to treat. The pathogenesis of this delayed phase, which is not adequately relieved by β-adrenoceptor agonists, is not understood. We suggest that it is worth considering whether the phenomenon described here (protein kinase C-mediated spasm, relatively insusceptible to β-agonists) might be implicated in the late phase of asthma, i.e. whether a tonic spasm produced by inappropriately stimulated protein kinase C might contribute to this phase. In this context, it is of interest that the late phase of asthma can be prevented by prior administration of glucocorticoids: agents that direct the synthesis of a mediator protein, lipocortin, which, though primarily a phospholipase A_2 inhibitor, also inhibits phospholipase C [22] and thus could inhibit the generation of diacylglycerol (the endogenous protein kinase C activator) and prevent the tonic spasm which follows activation of this enzyme.

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