Inhibition of histamine-stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle

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1 The effect on histamine-stimulated [³H]-inositol phosphate accumulation of a range of agents which increase the accumulation, or mimic the actions, of cyclic AMP has been investigated in bovine tracheal smooth muscle.

2 Salbutamol (1 μ M), forskolin (1 μ M) and vasoactive intestinal peptide (VIP, 1 μ M) inhibited the inositol phosphate response to 0.1 mM histamine and increased the accumulation of [³H]-cyclic AMP in [³H]-adenine-labelled slices of bovine tracheal smooth muscle. The effect on inositol phospholipid hydrolysis was mimicked by the membrane permeant analogues of cyclic AMP, dibutyl-cyclic AMP (1 mM) and 8-bromo-cyclic AMP (1 mM).

3 In contrast to salbutamol, which was equally effective at producing the two effects, forskolin produced large increases in [³H]-cyclic AMP accumulation ($EC_{50} = 1.2 \mu$ M) at much higher concentrations than those required for inhibition of histamine-stimulated [³H]-inositol phosphate accumulation ($EC_{50} = 0.09 \mu$ M). However, significant increases in [³H]-cyclic AMP accumulation, of similar magnitude to those obtained with salbutamol and VIP, were observed over the concentration range appropriate for inhibition of the inositol phosphate response to histamine.

4 In the presence of histamine (0.1 mM), isobutylmethylxanthine (IBMX, 1 mM) and rolipram (0.1 mM) both significantly ($P < 0.05$) elevated tissue [³H]-cyclic AMP levels. IBMX, rolipram and (to a lesser extent) SKF 94120 significantly ($P < 0.05$) reduced histamine-stimulated [³H]-inositol phosphate accumulation by 81%, 68% and 20%, respectively. M&B 22948 was without a significant effect on either [³H]-cyclic AMP or histamine-induced [³H]-inositol phosphate accumulation.

5 Both rolipram and forskolin reduced the increase in incorporation of [³H]-inositol into membrane phospholipids which followed stimulation with histamine. However, a significant inhibition of [³H]-inositol phosphate accumulation could be demonstrated under conditions in which there was no change in the level of [³H]-inositol incorporation.

Introduction

Histamine H_1 -receptor stimulation in tracheal smooth muscle produces a contractile response which appears to be initiated via the mobilization of calcium from intracellular stores (Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987) and maintained by the activation of protein kinase C (Park & Rasmussen, 1985; Dale & Obianime, 1985; Takuwa *et al.*, 1987). Studies in canine tracheal smooth muscle (Hashimoto *et al.*, 1985) and other tissues (Berridge, 1987) have shown that inositol-1,4,5-trisphosphate, which is produced following agonist-stimulated inositol phospholipid hydrolysis, can mobilise calcium ions from intracellular stores whilst 1,2-dia-

cylglycerol, another product of inositol phospholipid hydrolysis, has been shown to activate protein kinase C (Nishizuka, 1986; Berridge, 1987).

Histamine H_1 -receptor-activation in bovine tracheal smooth muscle stimulates the accumulation of [³H]-inositol phosphates following prelabelling of tracheal slices with [³H]-myo-inositol (Barnes *et al.*, 1986; Hall & Hill, 1988). β_2 -Adrenoceptor agonists are potent relaxants of airway smooth muscle (Barnes, 1986a) and we have shown previously that β_2 -adrenoceptor stimulation can markedly inhibit the hydrolysis of inositol phospholipids elicited by histamine in bovine tracheal smooth muscle (Hall & Hill, 1988). It is thought that the relaxant effects of β_2 -agonists on airway smooth muscle tone are

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related to their stimulating effects on adenylate cyclase activity leading to an increase in the intracellular levels of cyclic AMP (Rinard *et al.*, 1983; Barnes, 1986a; Fujiwara *et al.*, 1988) and subsequent activation of the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (Torphy *et al.*, 1982). The present study was therefore undertaken to determine whether the inhibitory effects of β -agonists on histamine-stimulated inositol phospholipid hydrolysis are mediated via the production and action of cyclic AMP. We now demonstrate that a number of agents, which either increase cyclic AMP accumulation or mimic the actions of cyclic AMP in bovine tracheal smooth muscle, also inhibit the histamine-induced accumulation of [3 H]-inositol phosphates in this tissue. A preliminary account of this work has been presented to the British Pharmacological Society (Hall *et al.*, 1988).

Methods

Accumulation of [3 H]-inositol phosphates

The accumulation of [3 H]-inositol phosphates in slices ($300 \times 300 \mu\text{m}$) of bovine tracheal smooth muscle was measured as described by Hall & Hill (1988). Briefly, washed slices were labelled in a minimal volume of Krebs-Henseleit buffer containing $30 \mu\text{Ci}$ [3 H]-myo-inositol (final concentration $0.4 \mu\text{M}$, total volume 8 ml) for 75 min at 37°C under an atmosphere of $95\% \text{ O}_2/5\% \text{ CO}_2$. The slices were then washed in Krebs-Henseleit buffer containing 5 mM lithium chloride and resuspended in 8 ml of medium. One hundred μl aliquots of the slice suspension were then transferred to flat-bottomed insert vials containing Krebs-Henseleit buffer and 5 mM LiCl (final volume $300 \mu\text{l}$). The vials were gassed with $95\% \text{ O}_2/5\% \text{ CO}_2$, capped and incubated for 20 min at 37°C in the presence or absence of phosphodiesterase inhibitors. Agonists or cyclic AMP analogues were finally added in $10 \mu\text{l}$ of medium and the incubation terminated after 45 min by the addition of $100 \mu\text{l}$ of ice-cold perchloric acid ($10\% \text{ w/v}$). Samples were neutralised with 0.75 ml KOH (0.15 M), centrifuged ($3000g$, 10 min , 4°C) and 0.75 ml aliquots of the supernatant were diluted to 3 ml with 50 mM Tris buffer ($\text{pH } 7.0$). Total [3 H]-inositol phosphates were finally separated from free [3 H]-myo-inositol by anion-exchange chromatography (Hill & Kendall, 1987).

For measurement of the incorporation of [3 H]-inositol into inositol-containing phospholipids, 1.2 ml chloroform/methanol/ 10 M HCl ($100:200:1 \text{ v/v/v}$) was added to each sample after removal of the supernatant as described above. The tubes were vortexed and left to stand for 30 min . Chloroform

(0.4 ml) and water (0.4 ml) were then added and the tubes centrifuged at *circa* $1500g$ for 5 min to separate the phases. A portion ($200 \mu\text{l}$) of the chloroform phase was then removed into a scintillation vial and dried overnight. Tritium was determined by liquid scintillation counting. In some experiments histamine (0.1 mM) was added during the last 45 min of the 75 min labelling period with [3 H]-inositol in order to stimulate incorporation. The slices were then washed with histamine-free Krebs medium and the experiment continued as described above for measurement of inositol phosphate accumulation.

Accumulation of [3 H]-cyclic AMP

Cyclic AMP production was measured essentially as described previously for brain slices (Donaldson *et al.*, 1988a). Washed slices ($300 \times 300 \mu\text{m}$) of bovine tracheal smooth muscle were incubated for 40 min at 37°C in 20 ml Krebs-Henseleit medium containing $0.08 \mu\text{M}$ ($40 \mu\text{Ci}$) [3 H]-adenine under an atmosphere of $95\% \text{ O}_2/5\% \text{ CO}_2$. The prelabelled slices were then washed with Krebs solution and resuspended in 8 ml of medium. Aliquots ($100 \mu\text{l}$) of the slice suspension were then added to insert vials containing Krebs medium and where appropriate phosphodiesterase inhibitors (final volume $300 \mu\text{l}$) and incubated for 20 min as described above. Agonists were finally added in $10 \mu\text{l}$ of medium and the incubations terminated after 10 min by the addition of $200 \mu\text{l}$ of 1 M HCl . Samples were left on ice for at least 5 min before being diluted with 0.75 ml distilled water. Slices were precipitated by centrifugation at $1500g$ for 10 min and 1 ml samples of the aqueous supernatant were taken for analysis of [3 H]-cyclic AMP by column chromatography as described by Donaldson *et al.* (1988b). An additional $100 \mu\text{l}$ sample of the aqueous supernatant was taken to determine the total radioactivity present in each sample. These 'totals' were used to correct for variations in the numbers of slices in each sample.

In studies of the time course of [3 H]-cyclic AMP accumulation, slices (3 ml of slice suspension) which had been prelabelled with [3 H]-adenine were transferred to a 20 ml glass vial containing 6 ml Krebs-Henseleit solution and where appropriate 1 mM 3-isobutyl-1-methylxanthine (IBMX). The slices and incubation medium were stirred magnetically at a rate sufficient to buoy up the slices and were gassed with $95\% \text{ O}_2/5\% \text{ CO}_2$ via a glass Pasteur pipette as described previously (Donaldson *et al.*, 1988b). Following agonist addition, $300 \mu\text{l}$ aliquots of the slice-containing incubation medium were taken at appropriate times and added to tubes containing $200 \mu\text{l}$ of ice-cold mHCl . Samples were then processed as described above.

Figure 1 The effect of salbutamol on the inositol phosphate response to 0.1 mM histamine in slices of bovine tracheal smooth muscle pre-labelled with [^3H]-myo-inositol. Histamine (H) was added at time zero and 1 μM salbutamol (Sal) (○) or 10 μl Krebs medium (●) was added 20 min later. The basal accumulation of [^3H]-inositol phosphates after 50 min in the absence of histamine is also shown (■). Each data point represents the mean of triplicate determinations and vertical lines indicate s.e.mean. If not shown the s.e.mean lies within the symbol. The experiment was repeated twice more with similar results.

Data analysis

Statistical analysis of the effect of various agents on inositol phospholipid hydrolysis was normally performed by both the Wilcoxon signed rank test and the paired t test. Dose-response curves were drawn by inspection and, where appropriate, EC_{50} values were compared by unpaired t test. All values in the text of the results section represent mean $\pm$ s.e.mean of n separate experiments.

Chemicals

Dowex 50 W, H^+ -form (200–400 mesh), Dowex-1 (X8, 100–200 mesh, chloride form) neutral alumina (type WN-3), imidazole, IBMX, salbutamol, $\text{N}^6,2'$ -O-dibutyryl cyclic AMP, 8-bromo-cyclic AMP and forskolin were obtained from Sigma. Histamine dihydrochloride was purchased from BDH and vasoactive intestinal peptide (VIP) from Peninsula Laboratories. 8- $[\text{^3H}]$ -adenine (specific activity 26 Ci mmol^{-1}) was obtained from Amersham and $[\text{^3H}]$ -myo-inositol ($16.5 \text{ Ci mmol}^{-1}$) and 8- $[\text{^14C}]$ -cyclic AMP (specific activity $42.4 \text{ mCi mmol}^{-1}$) from N.E.N. Gifts of rolipram (Schering AG), zaprinast (M&B 22948, May and Baker) and SKF 94120 ($\{5-(4\text{-acetamido-phenyl})\text{pyrazin-2-[1H]-one}\}$, Smith, Kline and French) are gratefully acknowledged.

Results

Effect of salbutamol on inositol phosphate and cyclic AMP accumulation

We have shown previously that addition of salbutamol markedly reduces the inositol phosphate response to histamine, when the two agonists are added simultaneously (Hall & Hill, 1988). In the present study, addition of 1 μM salbutamol 20 min after 0.1 mM histamine produced a significant and rapid reduction in the accumulation of [^3H]-inositol phosphates elicited by histamine alone (Figure 1). This effect of salbutamol in bovine tracheal smooth muscle slices was accompanied by a significant ($P < 0.05$) 3.2 $\pm$ 0.3 fold ($n = 3$) stimulation of the accumulation of [^3H]-cyclic AMP (Figure 2) which reached a steady level within 5 min and was maintained for at least a further 40 min (Figure 3). Histamine (0.1 mM) produced only a small effect (1.52 ± 0.11 fold, basal = 1.0, $n = 19$) on cyclic AMP levels in this tissue. There was no significant difference between the EC_{50} values for salbutamol-stimulated cyclic AMP accumulation when measured in the presence ($\text{EC}_{50} = 3.5 \pm 1.3 \times 10^{-7} \text{ M}$, $n = 3$) or absence ($\text{EC}_{50} = 2.0 \pm 0.6 \times 10^{-7} \text{ M}$, $n = 3$) of 0.1 mM histamine. These values were in excellent agreement with the EC_{50} value ($2.9 \times 10^{-7} \text{ M}$) obtained previously for salbutamol from inhibition of histamine-stimulated [^3H]-inositol phosphate accumulation (Hall & Hill, 1988). [^3H]-cyclic AMP levels rose rapidly following stimulation of β_2 -adrenoceptors with salbutamol (1 μM) in the presence or absence of the phosphodiesterase inhibitor IBMX (1 mM, Figure 3). The large effect of IBMX on the size of the cyclic AMP response to salbutamol suggests that phosphodiesterase enzymes are very active in this tissue. The half-times ($t_{1/2}$) for the rise in cyclic AMP levels elicited by salbutamol (1 μM) were similar in the presence (0.72 ± 0.11 min) and absence (0.63 ± 0.09 min, $n = 3$) of IBMX.

Effect of other cyclic AMP stimulants and analogues.

The rapid nature of the stimulation of cyclic AMP accumulation produced by salbutamol and the similarity in the dose-response characteristics for the effect of this β -agonist on cyclic AMP and inositol phosphate accumulation prompted us to examine the effect of other agents, known to stimulate the formation or mimic the actions of cyclic AMP in this tissue, on histamine-stimulated [^3H]-inositol phosphate accumulation. Vasoactive intestinal polypeptide (1 μM , VIP), forskolin (1 μM) and two analogues of cyclic AMP (8-bromo-cyclic AMP and dibutyryl-cyclic AMP, both at 1 mM) all significantly attenuated the inositol phosphate response to 0.1 mM

Figure 2 The effect of increasing concentrations of salbutamol on the accumulation of [^3H]-cyclic AMP in [^3H]-adenine-labelled slices of bovine tracheal smooth muscle. The basal accumulation of [^3H]-cyclic AMP in the absence of salbutamol is given by the column marked C. Values represent the mean of triplicate determinations and vertical lines show s.e.mean. Similar data were obtained in two other experiments.

histamine (Figure 4 and Table 1). Parallel studies in [^3H]-adenine-labelled slices confirmed that both $1\text{ }\mu\text{M}$ VIP and $1\text{ }\mu\text{M}$ forskolin elevated tissue levels of [^3H]-cyclic AMP in the presence or absence of 0.1 mM histamine (Figure 5). Studies of the time course of the cyclic AMP response to $1\text{ }\mu\text{M}$ forskolin in the presence of 0.1 mM histamine in two experiments showed that it was essentially similar to that found for salbutamol (Figure 3). A maximum

Figure 3 Time course of the accumulation of [^3H]-cyclic AMP in bovine tracheal smooth muscle after the addition of $1\text{ }\mu\text{M}$ salbutamol in the presence (○) or absence (●) of 1 mM isobutyl methylxanthine (IBMX). Where appropriate (○) slices were incubated for 20 min in the presence of IBMX before agonist administration. Data were obtained in a single experiment. Similar results were obtained in two other experiments.

Figure 4 Inhibition of histamine-induced [^3H]-inositol phosphate accumulation by agents which raise, or mimic the actions of, cyclic AMP. Histograms show the basal (C) and histamine-stimulated (H, 0.1 mM) levels of [^3H]-inositol phosphates. (a) Shows the inhibition of the response to 0.1 mM histamine produced by $1\text{ }\mu\text{M}$ salbutamol (Sal), 1 mM dibutyryl-cyclic AMP (dbcAMP) and 1 mM 8-bromo-cyclic AMP (8Br cAMP). (b) Shows the inositol phosphate responses obtained with $1\text{ }\mu\text{M}$ forskolin (For) and $1\text{ }\mu\text{M}$ vasoactive intestinal peptide (VIP) in the presence of 0.1 mM histamine. Values represent mean ($\pm$ s.e. mean, shown by bars) of triplicate determinations. *Values significantly different from the response to histamine alone ($P < 0.05$, ANOVA). Similar data were obtained for each agent in 3–14 other experiments (see Table 1).

response was obtained within 6 min and cyclic AMP levels remained elevated for 45 min. However, it was notable that, although $1\text{ }\mu\text{M}$ forskolin produced an inhibition of histamine-stimulated [^3H]-inositol

Table 1 Inhibition of histamine-induced [^3H]-inositol phosphate accumulation in bovine tracheal smooth muscle

Agent	% inhibition	(n)
Salbutamol ($1\text{ }\mu\text{M}$)	66 ± 3	(15)
Dibutyryl-cyclic AMP (1 mM)	33 ± 7	(6)
8-Bromo-cyclic AMP (1 mM)	49 ± 6	(6)
Forskolin ($1\text{ }\mu\text{M}$)	48 ± 6	(8)
VIP ($1\text{ }\mu\text{M}$)	60 ± 6	(4)

Values represent mean $\pm$ s.e.mean. The number of separate experiments with each agent is given in parentheses. VIP = vasoactive intestinal peptide.

Figure 5 Effect of salbutamol (Sal, $1 \mu\text{M}$), Vasoactive intestinal peptide (VIP, $1 \mu\text{M}$) and forskolin (For, $1 \mu\text{M}$) on the accumulation of [^3H]-cyclic AMP in the presence (hatched columns) or absence (open columns) of 0.1 mM histamine. The basal accumulations of [^3H]-cyclic AMP in the presence (hatched column) or absence (open column) of 0.1 mM histamine is shown by the columns marked C. Values represent the means of triplicate determinations. Vertical lines indicate s.e. mean. Similar results were obtained in two further experiments.

Figure 6 Concentration-response curve for the inhibition of histamine-stimulated [^3H]-inositol phosphate accumulation by the diterpine forskolin. The basal and control histamine (0.1 mM) responses are shown by columns C and H, respectively. Where appropriate forskolin and histamine (0.1 mM) were added simultaneously. Data represent the mean of triplicate determinations in a single experiment and vertical lines indicate s.e. mean. Each experiment was repeated twice more with similar results.

Figure 7 Effect of forskolin (●) on the accumulation of [^3H]-cyclic AMP in [^3H]-adenine-labelled slices of bovine tracheal smooth muscle. All incubations contained 0.1 mM histamine. Data represent the mean of triplicate determinations in a single experiment. Vertical lines show s.e. mean. If not shown the s.e. mean lies within the symbol. The basal accumulation of [^3H]-cyclic AMP in this experiment was $433 \pm 50 \text{ d.p.m.}$ and the levels obtained with 1 , 10 and 100 nM forskolin (in the presence of 0.1 mM histamine) were 752 ± 46 , 1047 ± 117 and $2130 \pm 154 \text{ d.p.m.}$, respectively. Similar data were obtained in two further experiments. The dashed line shows the calculated mass-action curve (0 to 100% maximal response, right ordinate scale) based on the mean EC_{50} ($0.09 \pm 0.01 \mu\text{M}$) obtained for forskolin-mediated inhibition of histamine-stimulated [^3H]-inositol phosphate accumulation

phosphate accumulation of similar magnitude to that obtained with $1 \mu\text{M}$ salbutamol (Table 1), there was a large difference in the relative sizes of the cyclic AMP responses obtained with these agents in the presence of histamine (Figure 5).

Analysis of the concentration-response characteristics of the inhibitory effect of forskolin on histamine-stimulated [^3H]-inositol phosphate accumulation indicated that the EC_{50} for forskolin was $0.09 \pm 0.01 \mu\text{M}$ ($n = 3$, Figure 6). This value was an order of magnitude lower than the EC_{50} value ($1.2 \pm 0.3 \mu\text{M}$, $n = 3$) obtained for forskolin-mediated stimulation of [^3H]-cyclic AMP accumulation in the presence of histamine (0.1 mM) (Figure 7). It should be noted, however, that although there was a very large maximal stimulation of cyclic AMP accumulation produced by forskolin in the presence of histamine (Figure 7), significant accumulations of [^3H]-cyclic AMP over basal levels (see legend to Figure 7) were still achieved over the forskolin concentration range appropriate for inhibition of inositol phospholipid hydrolysis (1 , 10 and 100 nM , respectively).

Effect of phosphodiesterase inhibitors

An alternative way of elevating tissue cyclic AMP levels in bovine tracheal smooth muscle is to inhibit the breakdown of this cyclic nucleotide with phosphodiesterase (PDE) inhibitors. In this study we have investigated the effect on cyclic AMP and inositol phosphate accumulation of the isozyme-selective PDE inhibitors rolipram (an inhibitor of the cyclic AMP-specific PDE IV, Reeves *et al.*, 1987), SKF 94120 (an inhibitor of the other cyclic AMP selective isozyme PDE III, Reeves *et al.*, 1987) and M & B 22948 (which inhibits calcium-calmodulin stimulated PDE I activity and the cyclic GMP selective isoform PDE II, Frossard *et al.*, 1981; Weishaar *et al.*, 1986), and the non-selective PDE inhibitor IBMX. The concentrations chosen were those that would produce the largest effect on the target PDE-isoform without influencing the activity of the other PDE isoenzymes (Stanley *et al.*, 1989). In the absence of 0.1 mM histamine, only IBMX produced a significant elevation of [3 H]-cyclic AMP over basal levels (Table 2), while in the presence of histamine both IBMX and rolipram significantly increased cyclic AMP levels over control and histamine-stimulated basal levels (Table 2). IBMX, rolipram and to a lesser extent SKF 94120 significantly reduced the accumulation of [3 H]-inositol phosphates elicited by histamine in bovine tracheal slices (Figure 8). The mean percentage inhibitions obtained with these PDE inhibitors were for IBMX (1 mM) $81 \pm 6\%$ ($P < 0.001$, paired *t* and Wilcoxon signed rank tests, 8 experiments); rolipram (0.1 mM) $68 \pm 9\%$ ($P < 0.001$, 7 experiments) and SKF 94120 (0.1 mM) $20 \pm 5\%$ ($P < 0.05$, 4 experiments). M & B 22948

Table 2 The effect of phosphodiesterase (PDE) inhibitors on the accumulation of [3 H]-cyclic AMP in [3 H]-adenine-labelled slices of bovine tracheal smooth muscle

Agent	[3 H]-cyclic AMP Basal	accumulation + Histamine
Control	1.0	1.5 ± 0.2
IBMX (1 mM)	$4.0 \pm 0.6^*$	$8.6 \pm 1.2^*$
Rolipram (0.1 mM)	1.6 ± 0.1	$3.1 \pm 0.5^*$
SKF 94120 (0.1 mM)	1.4 ± 0.1	1.9 ± 0.2
M & B 22948 (0.1 mM)	1.0 ± 0.2	1.8 ± 0.3

Values represent mean $\pm$ approximate s.e.mean of the accumulation of [3 H]-cyclic AMP, expressed relative to the basal accumulation (=1), in four identical experiments in which individual measurements were made in quadruplicate.

* $P < 0.05$ with respect to the basal accumulation (2-way analysis of variance with Newman Keuls *post hoc* test). IBMX = isobutyl methylxanthine

Figure 8 Effect of isoenzyme-selective inhibitors of phosphodiesterase (PDE) on histamine-stimulated [3 H]-inositol phosphate accumulation in bovine tracheal smooth muscle. Columns show the basal (C) and histamine-stimulated (H, 0.1 mM) levels, together with the effects of 1 mM isobutyl methylxanthine (IBMX), 0.1 mM rolipram (Rol), M & B 22948 (M & B) and SKF 94120 (SKF) measured in the presence of 0.1 mM histamine. PDE inhibitors were added 20 min before the administration of histamine. Data represent the means of triplicate determinations in a single experiment and vertical lines show s.e.mean. Similar results were obtained with each PDE inhibitor in 3–7 other experiments. * $P < 0.05$, ** $P < 0.01$, with respect to the response to 0.1 mM histamine alone (ANOVAR)

(0.1 mM) was without significant effect on the histamine-induced inositol phosphate response ($n = 4$).

Effects on the incorporation of [3 H]-inositol

A striking characteristic of the effect of agonists on inositol phospholipid hydrolysis in bovine tracheal smooth muscle is that the increase in accumulation of [3 H]-inositol phosphates is accompanied by a stimulation of the incorporation of [3 H]-inositol into membrane inositol phospholipids (Figure 9; Chilvers *et al.*, 1988; Hall & Hill, 1988). We have shown previously that although under normal conditions the stimulation of [3 H]-inositol incorporation by histamine can be inhibited by salbutamol, a marked inhibition of [3 H]-inositol phosphate accumulation by salbutamol can still be demonstrated under conditions in which there is no change in the level of [3 H]-inositol incorporation (i.e. when histamine is used to stimulate maximally incorporation during the prelabelling period, Hall & Hill, 1988). In the present study, under normal conditions, both rolipram (0.1 mM) and forskolin (Figure 9) produced a significant inhibition of the histamine-stimulated [3 H]-inositol incorporation. The mean EC_{50} value obtained for forskolin for this effect over three experiments ($0.06 \pm 0.05 \mu\text{M}$) was similar to that

Figure 9 The effect of forskolin on the histamine-stimulated incorporation of [³H]-inositol into inositol-containing phospholipids in slices of bovine tracheal smooth muscle. Columns show the incorporation under basal (C) conditions and following stimulation with 0.1 mM histamine in the absence (H) or presence of 0.1 mM rolipram (H + Rol). The concentration-response curve for forskolin (●) was obtained in the presence of histamine (0.1 mM). Values represent the means of triplicate determinations in a single experiment and vertical lines show s.e.mean. Similar data were obtained in two other experiments.

obtained when [³H]-inositol phosphate accumulation was measured (Figure 6). However, the incorporation of [³H]-inositol obtained in the presence of 0.1 mM histamine in three experiments ($172 \pm 15\%$ of basal) was not significantly altered by 0.1 mM SKF 94120 ($167 \pm 30\%$ of basal).

When histamine (0.1 mM) was present during the final 45 min of the prelabelling period with [³H]-inositol, there was no further stimulation by histamine of [³H]-inositol incorporation during the experimental period (Figure 10). Under these conditions, the initial lag period normally observed in the time course for histamine-stimulated [³H]-inositol phosphate (Figure 1) was abolished (Figure 10). Furthermore, as observed previously with β -agonists, a significant attenuation of the histamine-stimulated [³H]-inositol phosphate response by rolipram could be demonstrated under conditions in which rolipram did not interfere with the incorporation of [³H]-inositol into membrane phospholipids (Figure 10).

Discussion

The present study confirms our previous observation (Hall & Hill, 1988), that β_2 -agonist stimulation can markedly attenuate the accumulation of [³H]-inositol phosphates elicited by histamine in slices of

bovine tracheal smooth muscle, and indicates that salbutamol can produce a rapid effect even when added 20 min after the addition of histamine. The time course of this effect on inositol phosphate accumulation is similar to the rapid relaxation produced by β -agonists in bovine and canine tracheal smooth muscle strips which have been previously contracted by smooth muscle spasmogens (Fujiwara *et al.*, 1988; Takuwa *et al.* 1988). This raises the possibility that inhibition of histamine-stimulated inositol phospholipid hydrolysis by β_2 -agonists may contribute to the relaxation of pre-contracted (with histamine) airway smooth muscle by preventing the formation of either the calcium-mobilising inositol-1,4,5-trisphosphate or 1,2-diacylglycerol, which appears to be responsible for the maintenance of the contractile state via the activation of protein kinase C (Park & Rasmussen, 1985; Dale & Obianime, 1985; Takuwa *et al.*, 1987).

The effect of salbutamol on inositol phospholipid hydrolysis was paralleled by an increase in the accumulation of [³H]-cyclic AMP in [³H]-adenine-labelled slices of bovine tracheal smooth muscle. The rapid nature of the cyclic AMP response to salbutamol ($t_{1/2} = 0.5$ min), and the close similarity in the EC_{50} values for the two separate second messenger responses, suggested that the inhibition of histamine-stimulated inositol phosphate accumulation produced by salbutamol might be secondary to the change in the intracellular levels of the cyclic nucleotide. This hypothesis was supported by the fact that both the diterpine forskolin (a direct activator of adenylate cyclase, Seamon *et al.*, 1981) and VIP (which raises cyclic AMP levels in airway smooth muscle via an interaction with specific VIP receptors; Barnes, 1986b) increased [³H]-cyclic AMP levels and inhibited histamine-stimulated [³H]-inositol phosphate accumulation in this tissue. In addition two membrane permeant analogues of cyclic AMP, dibutyryl-cyclic AMP and 8-bromo-cyclic AMP, were also able to inhibit significantly the histamine-stimulated inositol phosphate response.

A notable feature of the data obtained with forskolin was that the cyclic AMP response obtained in the presence of 0.1 mM histamine was substantially greater than that obtained with the diterpine alone. No similar effect was seen with either salbutamol or VIP suggesting that forskolin, as in other tissues (Daly *et al.*, 1982; Barovsky *et al.*, 1984), raises intracellular cyclic AMP levels in airway smooth muscle by two mechanisms: (1) by a direct activation of adenylate cyclase and (2) by a potentiation of receptor-stimulated cyclic AMP accumulation. However, the large difference in maximal cyclic AMP responses observed in the presence of histamine between forskolin and other agonists which elevate cyclic AMP levels in tracheal slices, under conditions in which their relative effects on the

Figure 10 Effect of rolipram (0.1 mM) on the accumulation of $[^3\text{H}]\text{-inositol phosphates}$ obtained in the presence of 0.1 mM histamine under conditions in which the incorporation of $[^3\text{H}]\text{-inositol}$ is not affected. (a) Histamine-stimulated accumulation $[^3\text{H}]\text{-inositol phosphates}$ in the presence (○) or absence (●) of 0.1 mM rolipram. (b) Incorporation of $[^3\text{H}]\text{-inositol}$ into inositol phospholipids under basal conditions (45 min incubation, C) and following 45 min stimulation with 0.1 mM histamine in the absence (H) and presence (H + Rol) of 0.1 mM rolipram. Values represent mean of triplicate determinations in a single experiment and vertical lines show s.e.mean. If not shown s.e.mean lies within the symbol. Similar data were obtained in two further experiments.

histamine-induced inositol phosphate response are similar, does require explanation if cyclic AMP is proposed to mediate this latter response.

The simplest explanation is that the high levels of cyclic AMP achieved in the presence of a combination of histamine and forskolin (at concentrations of the diterpine above $1\ \mu\text{M}$) are supramaximal, and that the maximal inhibitory effect on inositol phospholipid hydrolysis is achieved at much lower cyclic AMP levels. If this is the case then one would predict that the concentration-response curve, obtained from measurement of cyclic AMP accumulation, for forskolin should lie to the right of the curve obtained for inositol phosphate accumulation. Consistent with this hypothesis, the EC_{50} values obtained for forskolin, but not salbutamol, on cyclic AMP accumulation (in the presence of 0.1 mM histamine) were one order of magnitude higher than the corresponding values obtained from measurements of inositol phosphate accumulation (Figure 7). Furthermore, the size of the increases in cyclic AMP obtained with forskolin (in the presence of histamine), over the concentration range appropriate for effects on inositol phosphate accumulation, were similar to those obtained with both VIP and salbutamol under similar conditions.

Cyclic AMP levels can also be raised in airway smooth muscle by inhibiting the PDE enzymes which break down cyclic AMP. The large extent to which the PDE enzymes are active in this tissue is illustrated by the data in Figure 3 for the time course of the effect of salbutamol on cyclic AMP levels in the presence and absence of IBMX. A new steady level of cyclic AMP is obtained with salbutamol in the presence of IBMX suggesting that, as for rolipram in guinea-pig brain slices (Donaldson *et al.*, 1988a), the inhibition of PDE can be eventually reversed by the higher intracellular levels of cyclic AMP produced in the presence of IBMX.

A number of different PDE isozymes have been described in various tissues which have different substrate specificities and sensitivities to various PDE inhibitors (Davis, 1984; Weishaar *et al.*, 1986; Reeves *et al.*, 1987; Silver *et al.*, 1988). Three PDE activities have been identified in broken cell preparations of tracheal smooth muscle (Torphy *et al.*, 1987; Silver *et al.*, 1988): (1) a calcium-calmodulin stimulated activity (PDE I), which hydrolyses both cyclic AMP and cyclic GMP with similar potencies, and is inhibitable by M & B 22948 (Weishaar *et al.*, 1986; Torphy *et al.*, 1987; Silver *et al.*, 1988); (2) a cyclic AMP-specific activity (PDE III) which is sensitive to

SKF 94120 (Gristwood *et al.*, 1986; Reeves *et al.*, 1987) and (3) a rolipram-sensitive activity, specific for cyclic AMP, with a higher Michaelis constant (K_m) for the substrate cyclic nucleotide (Torphy *et al.*, 1987; Reeves *et al.*, 1987; Silver *et al.*, 1988). Studies with partially purified isozymes suggest that the calcium-sensitive PDE I accounts for all of the cyclic GMP and 85% of the cyclic AMP metabolising activity in homogenates of tracheal smooth muscle (Torphy *et al.*, 1987). These studies also indicate that PDE III and PDE IV account for the remaining 5% and 10% respectively of the cyclic AMP metabolising activity in this tissue.

Studies with isozyme selective inhibitors in the present study, however, indicate that only rolipram and IBMX significantly raise [3 H]-cyclic AMP levels in intact tracheal slices incubated in the presence of 0.1 mM histamine. This is consistent with previous results obtained in brain slices (Stanley *et al.*, 1989; Challis & Nicholson, 1988) and suggests that PDE IV, although a minor component of many cells, may still have a major role in metabolising cyclic AMP in intact cells. A lack of effect of the PDE I inhibitor M & B 22948 on cyclic AMP accumulation has also been observed in canine tracheal smooth muscle, where the isoprenaline-induced smooth muscle relaxation is similarly insensitive to M & B 22948 (Torphy *et al.*, 1987). Thus, although PDE I accounts for 85% of cyclic AMP hydrolysis in the broken cell preparation of tracheal smooth muscle, it does not have a major role in intact cells. This suggests that the different PDE isozymes may be distributed within different cellular compartments in tracheal smooth muscle. The difference in the extent of the increases in cyclic AMP accumulation obtained with rolipram and IBMX (which inhibits all PDE isozymes) are difficult to explain given the lack of effect of SKF 94120 and M & B 22948, but they may be related to differences in tissue penetration or due to other properties of IBMX such as adenosine antagonism (Green & Stanberry, 1976) or antagonism of the inhibitory regulatory protein (G_i) of adenylate cyclase (Parsons *et al.*, 1988).

It is clear, however, that those PDE inhibitors which produce significant increases in cyclic AMP accumulation, i.e., rolipram and IBMX but not M & B 22948, produce a marked inhibition of histamine-stimulated [3 H]-cyclic AMP accumulation. The similar extent of the effects of rolipram and IBMX on inositol phospholipid hydrolysis, under conditions in which the size of the cyclic AMP responses differs, is also consistent with the hypothesis, put forward to explain the forskolin data above, that the effects of cyclic AMP on [3 H]-inositol phosphate accumulation are saturable.

It was notable that a small but significant inhibition of histamine-stimulated inositol phosphate

accumulation was observed with SKF 94120 (Figure 8), which had no significant effect on [3 H]-cyclic AMP accumulation (Table 2). Small or negligible increases in cyclic AMP accumulation have been obtained with SKF 94120 in other tissues (Gristwood & Owen, 1986), under conditions where it is still possible to demonstrate activation of cyclic AMP-dependent protein kinase A (Murray *et al.*, 1987) and a pharmacological response (i.e. inotropic effect; Gristwood *et al.*, 1986) mediated by intracellular cyclic AMP. Thus, it remains a possibility that the effects of SKF 94120 on inositol phosphate accumulation are mediated via a small rise in cyclic AMP in a minor compartment of tracheal smooth muscle cells. Interestingly, a small effect of SKF 94120 on cyclic AMP accumulation and a potentiation of the isoprenaline-induced relaxation has been observed in canine tracheal smooth muscle strips (Torphy *et al.*, 1987).

A relation between intracellular cyclic AMP and inhibition of agonist-induced inositol phospholipid breakdown has been observed in a number of other tissues, including platelets (Takai *et al.*, 1982; Watson *et al.*, 1984), neutrophils (Della Bianca *et al.*, 1986), gastric mucosal cells (Puurunen *et al.*, 1987) and slices of rat kidney (Neylon & Summers, 1988). Preliminary studies by other groups in canine tracheal smooth muscle have also confirmed our observations that forskolin can inhibit histamine-induced inositol phospholipid hydrolysis (Madison & Brown, 1988; Murray *et al.*, 1988). The site of action of the cyclic AMP is at present unknown but phospholipase C (which hydrolyses phosphatidylinositol-4,5-bisphosphate, PIP_2) and the inositol phospholipid kinases (which convert phosphatidylinositol to phosphatidylinositol-4-phosphate and then to PIP_2) are the most likely sites of action.

In tracheal smooth muscle, histamine stimulates the incorporation of [3 H]-inositol into inositol phospholipids in addition to activating phospholipase C. This effect can be reduced by salbutamol (Hall & Hill, 1988), forskolin or rolipram, which suggests that the inositol phospholipid kinases may be the primary target for cyclic AMP. However, it seems very likely that the stimulation by histamine of [3 H]-inositol incorporation in airway smooth muscle is due to changes in the specific radioactivity of the inositol phospholipids that follow histamine-stimulated inositol phospholipid breakdown, as a consequence of the low turnover of inositol phospholipids under basal conditions. This means that the cyclic AMP elevating drugs have an effect on [3 H]-inositol incorporation which is secondary to their effect on inositol phospholipid breakdown. This suggestion is supported by the fact that inclusion of histamine in the medium during the preincubation period with [3 H]-inositol still revealed changes in

[³H]-inositol phosphate accumulation, under conditions in which the incorporation of [³H]-inositol into the phospholipid layer did not change (see Hall & Hill, 1988 and Results above). The exact site of action of cyclic AMP on the inositol phospholipid cycle in tracheal smooth muscle therefore remains to be established.

In summary, the present results show that a variety of agents which can raise (via PDE inhibition, receptor activation or direct activation of

adenylate cyclase), or mimic the action of, intracellular cyclic AMP can inhibit histamine-stimulated inositol phospholipid hydrolysis. These findings may be relevant to the mechanisms by which raised intracellular cyclic AMP levels lead to relaxation of airway smooth muscle which has been pre-contracted with histamine.

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References

- BARNES, P.J. (1986a) Bronchodilator mechanisms. In *Asthma: Clinical Pharmacology and Therapeutic Progress*, ed. Kay, A.B. pp. 146–160. Oxford: Blackwell.
- BARNES, P.J. (1986b) Neuropeptides in the airways: Functional significance. In *Asthma: Clinical Pharmacology and Therapeutic Progress*, ed. Kay, A.B. pp. 58–72. Oxford: Blackwell.
- BARNES, P.J., CUSS, F.M. & GRANDORY, B.M. (1986). Spasmogens and phosphatidylinositol breakdown in bovine trachealis smooth muscle. *Br. J. Pharmacol.*, **87**, 65P.
- BAROVSKY, K., PEDONE, C. & BROOKER, G. (1984). Distinct mechanisms of forskolin-stimulated cyclic AMP accumulation and forskolin-potentiated hormone responses in C6-2B cells. *Molec. Pharmacol.*, **25**, 256–260.
- BERRIDGE, M.J. (1987). Inositol trisphosphate and diacylglycerol: Two interacting second messengers. *Ann. Rev. Biochem.*, **56**, 159–93.
- CHALLIS, R.A.J. & NICHOLSON, C.D. (1988). Effects of selective phosphodiesterase inhibitors on cyclic AMP hydrolysis in rat cerebral cortical slices. *Br. J. Pharmacol.*, **95**, 553P.
- CHILVERS, E.R., BARNES, P.J. & NAHORSKI, S.R. (1988). Muscarinic receptor stimulated turnover of polyphosphoinositides and inositol polyphosphates in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **95**, 778P.
- DALE, M.M. & OBIANIME, W. (1985). Phorbol myristate acetate causes in guinea-pig lung parenchymal strip a maintained spasm which is relatively resistant to isoprenaline. *FEBS Lett.*, **190**, 6–10.
- DALY, J.W., PADGETT, W. & SEAMON, K.B. (1982). Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpine forskolin: Augmentation of receptor-mediated responses. *J. Neurochem.*, **38**, 532–544.
- DAVIS, C.W. (1984). Assessment of selective inhibition of rat cerebral cortical calcium-independent and calcium-dependent phosphodiesterases in crude extracts using deoxycyclic AMP and potassium ions. *Biochim. Biophys. Acta*, **797**, 354–362.
- DELLA BIANCA, V., DE TONGNI, P., GRZESKOWIAK, M., VINCENTINI, L.M. & DI VIRGILIO, F. (1986). Cyclic AMP inhibition of phosphoinositide turnover in human neutrophils. *Biochim. Biophys. Acta*, **886**, 441–447.
- DONALDSON, J., BROWN, A.M. & HILL, S.J. (1988a). Influence of rolipram on the cyclic 3',5'-adenosine monophosphate response to histamine and adenosine in slices of guinea-pig cerebral cortex. *Biochem. Pharmacol.*, **37**, 715–723.
- DONALDSON, J., HILL, S.J. & BROWN, A.M. (1988b). Kinetic studies on the mechanism by which histamine H₁-receptors potentiate cyclic AMP accumulation in guinea-pig cerebral cortical slices. *Molec. Pharmacol.*, **33**, 626–633.
- FROSSARD, N., LANDRY, Y., PAULI, G. & RUCKSTUHL, M. (1981). Effects of cyclic AMP- and cyclic GMP-phosphodiesterase inhibitors on immunological release of histamine and on lung contraction. *Br. J. Pharmacol.*, **73**, 933–938.
- FUJIWARA, T., SUMIMOTO, K., ITOH, T., SUZUKI, H. & KURIYAMA, H. (1988). Relaxing actions of procaterol, a β_2 -adrenoceptor stimulant, on smooth muscle cells of the dog trachea. *Br. J. Pharmacol.*, **93**, 199–209.
- GREEN, R.D. & STANBERRY, L.R. (1976). Elevation of cyclic AMP in C-1300 murine neuroblastoma by adenosine and related compounds and the antagonism of this response by methylxanthines. *Biochem. Pharmacol.*, **26**, 37–44.
- GRISTWOOD, R.W., EDEN, R.J., OWEN, D.A.A. & TAYLOR, E.M. (1986). Pharmacological studies with SKF 94120, a novel positive inotropic agent with vasodilator activity. *J. Pharm. Pharmacol.*, **38**, 452–459.
- GRISTWOOD, R.W. & OWEN, D.A.A. (1986) Effects of rolipram on guinea-pig ventricles *in vitro*: Evidence of an unexpected synergism with SK & F 94120. *Br. J. Pharmacol.*, **87**, 91P.
- HALL, I.P. & HILL, S.J. (1988). β_2 -adrenoceptor stimulation inhibits histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **95**, 1204–1212.
- HALL, I.P., DONALDSON, J. & HILL, S.J. (1988). Cyclic AMP-mediated inhibition of histamine-induced inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **95**, 748P.
- HASHIMOTO, T., HIRATA, M. & ITO, Y. (1985). A role for inositol 1,4,5-trisphosphate in the initiation of agonist-induced contractions of dog tracheal smooth muscle. *Br. J. Pharmacol.*, **86**, 191–201.
- HILL, S.J. & KENDALL, D.A. (1987). Studies on the adenosine-receptor mediating the augmentation of histamine-induced inositol phospholipid hydrolysis in guinea-pig cerebral cortex. *Br. J. Pharmacol.*, **91**, 661–669.
- KOTLIKOFF, M.I., MURRAY, R.K. & REYNOLDS, E.E. (1987). Histamine-induced calcium release and phorbol

- antagonism in cultured airway smooth muscle cells. *Am. J. Physiol.*, **253**, C561–C566.
- MADISON, J.M. & BROWN, J.K. (1988). Differential effects of forskolin on agonist-induced phosphoinositide hydrolysis in canine tracheal smooth muscle. *Am. Rev. Resp. Dis.*, **137**, A308.
- MURRAY, K.J., ENGLAND, P.J. & REEVES, M.L. (1987). Positive inotropic actions of SKF 94120 occur via a cyclic AMP-dependent mechanism: activation of cAMP-protein kinase. *Br. J. Pharmacol.*, **92**, 755P.
- MURRAY, R.K., FLUHARTY, S.J. & KOTLIKOFF, M.L. (1988). Phorbol ester blocks histamine-induced inositol triphosphate production in cultured airway smooth muscle. *Am. Rev. Resp. Dis.*, **137**, A309.
- NEYLON, C.M. & SUMMERS, R.J. (1988). Inhibition by cAMP of the phosphoinositide response to α_1 -adrenoceptor stimulation in rat kidney. *Eur. J. Pharmacol.*, **148**, 441–444.
- NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science*, **233**, 305–312.
- PARK, S. & RASMUSSEN, H. (1985). Activation of tracheal smooth muscle contraction: synergism between Ca^{2+} and activators of protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 8835–8839.
- PARSONS, W.J., RAMKUMAR, V. & STILES, G.L. (1988). Isobutylmethylxanthine stimulates adenylate cyclase by blocking the inhibitory regulatory protein, G_i . *Molec. Pharmacol.*, **34**, 37–41.
- PUURUNEN, J., LOHSE, M.J. & SCHWABE, U. (1987). Interactions between intracellular cyclic AMP and agonist-induced inositol phospholipid breakdown in isolated gastric mucosal cells of the rat. *Naunyn-Schmiedeberg Arch. Pharmacol.*, **336**, 471–477.
- REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 537–543.
- RINARD, G.A., JENSEN, A. & PUCKETT, A.M. (1983). Hydrocortisone and isoproterenol effects on tracheal cAMP and relaxation. *J. Appl. Physiol.*, **55**, 1609–1613.
- SEAMON, K.B., PADGETT, W. & DALY, J.W. (1981). Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3363–3367.
- SILVER, P.J., HAMEL, L.T., PERRONE, M.H., BENTLEY, R.G., BUSHOVER, C.R. & EVANS, D.B. (1988). Differential pharmacologic sensitivity of cyclic nucleotide phosphodiesterase isozymes isolated from cardiac muscle, arterial and airway smooth muscle. *Eur. J. Pharmacol.*, **150**, 85–94.
- STANLEY, C., BROWN, A.M. & HILL, S.J. (1989). The effect of isozyme-selective inhibitors of phosphodiesterase on histamine-stimulated cyclic AMP accumulation in guinea-pig hippocampus. *J. Neurochem.*, **52**, 671–676.
- TAKAI, Y., KAIBUCHI, K., SANO, K. & NISHIZUKA, Y. (1982). Counteraction of calcium-activated phospholipid-dependent protein kinase activation by adenosine-3',5'-monophosphate and guanine-3',5'-monophosphate in platelets. *J. Biochem.*, **91**, 403–416.
- TAKUWA, Y., TAKUWA, N. & RASMUSSEN, H. (1987). Measurement of cytoplasmic free Ca^{2+} concentration in bovine tracheal smooth muscle using aequorin. *Am. J. Physiol.*, **253**, C817–C827.
- TAKUWA, Y., TAKUWA, N. & RASMUSSEN, H. (1988). The effects of isoproterenol on intracellular calcium concentration. *J. Biol. Chem.*, **263**, 762–768.
- TORPHY, T.J., FREESE, W.B., RINARD, G.A., BRUNTON, L.L. & MAYER, S.E. (1982). Cyclic nucleotide-dependent protein kinases in airway smooth muscle. *J. Biol. Chem.*, **257**, 11609–11616.
- TORPHY, T.J., BURMAN, M., HUANG, L.B.F., HOROHONICH, S. & CIESLINSKI, L.B. (1987). Regulation of cAMP content and cAMP-dependent protein kinase activity in airway smooth muscle. *Prog. Clin. Biol. Res.*, **245**, 263–275.
- WATSON, S.P., MCCONNELL, R.T. & LAPETINA, E.G. (1984). The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.*, **259**, 13199–13203.
- WEISHAAR, R.E., BURROWS, S.D., KOBYLARZ, D.C., QUADE, M.M. & EVANS, D.B. (1986). Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets: isolation, characterisation and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. *Biochem. Pharmacol.*, **35**, 787–800.

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