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MODULATION OF CARBACHOL-INDUCED INOSITOL PHOSPHATE FORMATION IN BOVINE TRACHEAL SMOOTH MUSCLE BY CYCLIC AMP PHOSPHODIESTERASE INHIBITORS

IAN P. HALL, JILL DONALDSON and STEPHEN J. HILL*
Department of Physiology and Pharmacology, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

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Abstract—An investigation was made of a range of agents capable of elevating tissue cyclic AMP levels, or acting as a stable analogue of cyclic AMP, upon carbachol induced inositol phosphate responses in bovine tracheal smooth muscle slices. Whereas the β_2 adrenoceptor agonist salbutamol (1 μM) and the membrane permeable analogue of cyclic AMP, 8-bromo-cyclic AMP (1 mM) were without effect upon total [^{3}H]inositol phosphate formation induced by carbachol, 3-iso-butyl-1-methylaxanthine (IBMX) (EC_{50} 140 μM), the high K_m, cyclic AMP selective phosphodiesterase inhibitor rolipram (EC_{50} 41 μM) and theophylline (EC_{50} 76 μM) all inhibited the inositol phosphate response to low (1 μM) concentrations of carbachol. IBMX (IC_{50} 13 μM), rolipram (IC_{50} 4.6 μM) and theophylline (IC_{50} 180 μM) all relaxed bovine tracheal muscle strips precontracted with methacholine (1 μM). The adenylate cyclase activator forskolin (1 μM), produced a much smaller (10% inhibition) effect upon inositol phosphate formation induced by carbachol. Carbachol (1 μM–1 mM) did not inhibit forskolin induced [^{3}H]cyclic AMP formation. An inhibitor of the cyclic GMP preferring phosphodiesterase isozyme, M&B 22948 (1–100 μM), was without effect upon either carbachol induced inositol phosphate formation or trachealis tone. It is concluded that IBMX, rolipram and theophylline inhibit carbachol stimulated inositol phosphate formation, possibly through a cyclic AMP independent mechanism.

The contraction of airway smooth muscle in response to the spasmogens histamine and carbachol is thought to involve the hydrolysis of phosphatidylinositol 4,5 bisphosphate to produce the intracellular second messengers inositol 1,4,5 trisphosphate (Ins 1,4,5 P_3), which can mobilize calcium from internal stores, and diacylglycerol, which can activate protein kinase C [1–4]. Mobilization of intracellular calcium is thought to be responsible for the initiation of the contractile response, while protein kinase C activation has been implicated in the maintained tonic contractile response of airway smooth muscle [5–7]. We have previously shown that a variety of smooth muscle relaxant agents capable of elevating tissue cyclic AMP levels through differing mechanisms are able to inhibit the histamine-induced inositol phosphate response in bovine tracheal smooth muscle [8, 9]. A striking feature of this response was the inability of noradrenaline to inhibit carbachol-induced inositol phosphate formation [9]. In this report we have further studied the inositol phosphate response to carbachol in bovine tracheal smooth muscle and report here on the effects of a range of isozyme selective inhibitors of phosphodiesterase and other agents capable of elevating intracellular cyclic AMP levels upon the inositol phosphate response to varying concentrations of carbachol. A preliminary account of this work has been presented to the British Pharmacological Society [10].

MATERIALS AND METHODS

Tissue preparation. Trachea were obtained from freshly slaughtered young bullocks and tracheal smooth muscle dissected free from mucosa and surrounding connective tissue and then chopped (300 × 300 μm slices) using a McIlwain tissue chopper.

Accumulation of total [^{3}H]*inositol phosphates.* This was measured in slices of bovine tracheal smooth muscle as reported previously [8]. Washed slices were incubated for 30 min in 25 mL of Krebs–Henseleit buffer at 37° under an atmosphere of 95% O_2/5% CO_2, and then labelled in a minimal volume of Krebs–Henseleit buffer containing 30 μCi [^{3}H]myo-inositol (final concentration 0.4 μM, total volume 8 mL) for a further 75 min under the same conditions. The slices were then washed in Krebs–Henseleit buffer containing 5 mM lithium chloride and resuspended in 8 mL of medium. Aliquots (100 μM) of the slice suspension were then transferred to flat-bottomed insert vials containing Krebs–Henseleit buffer and 5 mM LiCl (final volume 300 μL). The vials were gassed with 95% O_2/5% CO_2, capped and incubated for 20 min at 37° in the presence or absence of phosphodiesterase inhibitors. Agonists were finally added in 10 μL of medium and the incubation terminated after 45 min by the addition of 100 μL of ice-cold perchloric acid (10% w/v). Samples were neutralized with 0.75 mL KOH (0.15 M), centrifuged (3000 *g*, 10 min, 4°) and 0.75-mL aliquots of the supernatant were diluted to 3 mL with 50 mM Tris buffer (pH 7.0). Total [^{3}H]inositol phosphates were finally separated from free [^{3}H]myo-inositol by anion-exchange chromatography [11].

In relevant experiments designed to examine the

* To whom correspondence should be addressed.

effects of varying calcium concentrations upon inhibitions of carbachol induced inositol phosphate responses, the protocol outlined above was followed until the prelabelling period was finished. Then slices were washed in LiCl (5 mM) Krebs–Henseleit containing: (i) 2.5 mM calcium, (ii) without added calcium chloride; or (iii) without added calcium chloride and with 100 μM EGTA added to give a range of extracellular calcium concentrations. Experiments were then continued as above using the relevant media throughout.

In some experiments, carbachol (1 μM) was included during the last 45 min of the prelabelling period in order to maximally stimulate incorporation of [^{3}H]myo-inositol into tissue slices [12]. The slices were then washed thoroughly in Krebs–Henseleit containing 5 mM LiCl and the experiments continued as outlined above.

Incorporation of [^{3}H]*inositol into membrane phospholipids.* In order to measure incorporation of [^{3}H]inositol into inositol-containing phospholipids, 1.2 mL chloroform/methanol/10 M HCl (100:200:1 v/v/v) were 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 *ca.* 1500 *g* for 5 min to separate the phases. A volume of 200 μL of the chloroform phase was then removed into a scintillation vial and dried overnight. Tritium was determined by liquid scintillation counting.

Accumulation of [^{3}H]*cyclic AMP.* Formation of [^{3}H]cyclic AMP by slices of bovine tracheal smooth muscle was measured as previously described [9]. Essentially, washed slices prepared as described above were incubated for 40 min at 37° in 20 mL of Krebs–Henseleit medium containing 0.08 μM (40 μCi) [^{3}H]adenine under an atmosphere of 95% O_2/5% CO_2. These slices were then washed with Krebs–Henseleit, resuspended in 8 mL of medium, and 100 μL aliquots of slice suspension were added to flat bottomed insert vials containing Krebs–Henseleit medium. Agonists were then added in 10 μL of medium and the incubations terminated after 10 min by the addition of 200 μL of 1 M HCl. After leaving the samples on ice for 5 min, 0.75 mL of distilled water was added to dilute the samples, and slices precipitated by centrifugation at 1500 *g* for 10 min. Aliquots (1 mL) of the supernatant were then analysed for [^{3}H]cyclic AMP by column chromatography as described previously [13]. Additional 100 μL samples were taken from the supernatant and used to determine the total radioactivity present in each sample; these "totals" were then used to correct for variations in the amount of slices present in each sample.

Relaxant responses to phosphodiesterase inhibitors. These were examined using strips of bovine tracheal smooth muscle (1 × 0.3 cm) denuded of epithelium and mucosa suspended in an organ bath containing Krebs–Henseleit and continuously gassed with O_2/CO_2 (95%/5%). Isometric recordings of tension were made using standard techniques. After an initial period of 60 min equilibrium under 2 g of tension, tissue strips were contracted with 1 μM methacholine and then cumulative relaxation responses to phosphodiesterase inhibitors constructed.

Chemicals. Dowex 50W, H^+-form (200–400 mesh), Dowex-1 (X8, 100–200 mesh, chloride form), neutral alumina (type WN-3), carbachol, methacholine, imidazole, IBMX, theophylline, salbutamol, 8-bromocyclic AMP, and forskolin were obtained from the Sigma Chemical Co. (Poole, U.K.). [^{3}H]Myoinositol (16.5 μCi/mmol) and 8-[^{14}C]cyclic AMP (sp. act. 42.4 μCi/mmol) was purchased from New England Nuclear (Stevenage, U.K.), and 8-[^{3}H]adenine (sp. act. 26 Ci/mmol) was obtained from Amersham (Bucks, U.K.). M&B 22948 and rolipram were gifts from May and Baker (Dagenham, U.K.) and Schering AG (Berlin, F.R.G.), respectively. Stock solutions (30 mM) of M&B 22948 and rolipram were prepared in ethanol, and appropriate serial dilutions prepared freshly on each day of usage. The corresponding vehicles had no effect alone at the relevant concentrations.

Data analysis. The effect of the various agents on inositol phosphate formation was performed using paired *t*-tests, and in addition with the Wilcoxon signed rank test where applicable. Concentration–response curves were either drawn by inspection or fitted to a logistic equation using the non-linear program ALLFIT [8]. The equation fitted was:

$$\frac{\%\ \text{Inhibition}}{(\text{contraction or inositol phosphates})} = \frac{100 \times X^n}{EC_{50} + X^n}$$

where X is the concentration of PDE inhibitor, n is the Hill coefficient and EC_{50} is the concentration producing 50% inhibition of methacholine-induced smooth muscle contraction or 50% inhibition of carbachol-induced inositol phospholipid hydrolysis. In the case of theophylline-induced inhibition of inositol phospholipid hydrolysis, where the maximal response did not reach 100%, the data were fitted to the following expression:

$$\%\ \text{Inhibition} = \frac{M \times X}{EC_{50} + X}$$

where M is the maximal percentage inhibition and the other parameters are as defined above.

All values given in the text or figure legends represent mean ± SE of N separate experiments.

RESULTS

Effect of salbutamol and IBMX on carbachol induced [^{3}H]*inositol phosphate formation*

Carbachol produced concentration-related [^{3}H]inositol phosphate formation in slices of bovine tracheal smooth muscle as has been previously described [8, 14], with an EC_{50} of 5.9 ± 2.3 μM (N = 5). We have previously described how the [^{3}H]inositol phosphate response to histamine in bovine tracheal smooth muscle can be inhibited by agents capable of elevating cyclic AMP, but that noradrenaline was unable to alter the [^{3}H]inositol phosphate response to carbachol. The effect of the β_2-adrenoreceptor agonist salbutamol (1 μM) upon the inositol phosphate response to varying concentrations of carbachol is shown in Fig. 1. Salbutamol was without significant effect upon the accumulation of [^{3}H]inositol phosphates induced by a range of

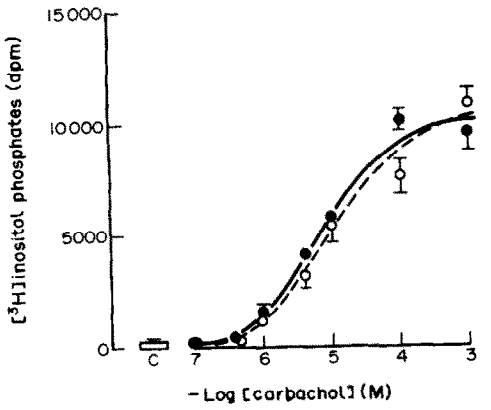

Fig. 1. Effect of salbutamol on the [^{3}H]inositol phosphate response to carbachol. Data were obtained in the presence (○) or absence (●) of salbutamol (1 μM). The basal accumulation of total [^{3}H]inositol phosphates in each case is given by the histogram marked C. Values represent means ± SE of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

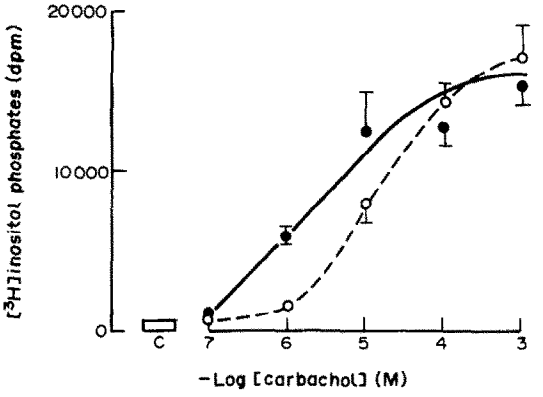

Fig. 2. Effect of IBMX on the [^{3}H]inositol phosphate response to carbachol. Data were obtained in the presence (○) or absence (●) of IBMX (1 mM). The basal accumulation of total [^{3}H]inositol phosphates in each case is given by the histogram marked C. Values represent means ± SE of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

Table 1. Effect of agents raising tissue cyclic AMP levels or mimicking the action of cyclic AMP upon agonist induced [^{3}H]inositol phosphate formation in bovine tracheal smooth muscle

	Agonist		
	Carbachol		
Agent	1 μM	100 μM	Histamine (100 μM)*
Salbutamol (1 μM)	13 ± 7 (6)	5 ± 6 (6)	66 ± 3† (15)
Forskolin (1 μM)	10 ± 2† (6)	ND	48 ± 6† (8)
8br-cAMP (1 mM)	9 ± 6 (6)	ND	49 ± 6† (6)
IBMX (1 mM)	88 ± 2† (12)	0 ± 5 (6)	81 ± 6† (8)
Rolipram (0.1 mM)	50 ± 6† (9)	ND	68 ± 9† (4)
Theophylline (1 mM)	41 ± 3† (6)	ND	65 ± 5† (3)

* Data from Hall *et al.* [9].
† $P < 0.05$.
ND, not determined.
Number of experiments given in parentheses. Values represent % inhibition.

concentrations of carbachol (Fig. 1, Table 1). In contrast, the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM) was able to modulate the [^{3}H]inositol phosphate response to low concentrations (<10 μM) carbachol (Fig. 2). IBMX had no significant effect upon the accumulation of [^{3}H]inositol phosphates induced by high (>10 μM) concentrations of carbachol (Fig. 2, Table 1). In these studies, 1 and 100 μM carbachol produced 17 ± 2.3-fold and 59 ± 9.1-fold increases in total [^{3}H]inositol phosphate formation in slices of bovine tracheal smooth muscle in the absence of other drugs (N = 10).

Effects of rolipram, theophylline and M&B 22948 on carbachol induced [^{3}H]inositol phosphate formation

In order to further examine this effect of IBMX, and the possible mechanisms underlying it, we looked at the effect of theophylline (a non-selective phosphodiesterase inhibitor), the high K_m cAMP-selective type IV phosphodiesterase isozyme inhibitor rolipram [15] and the cyclic GMP-selective phosphodiesterase inhibitor M&B 22948 [15] upon the [^{3}H]inositol response to 1 μM CCh (Fig. 3, Table 2). It can be seen that both rolipram and IBMX produce concentration-related inhibition of the [^{3}H]inositol phosphate response induced by 1 μM CCh and that this effect is mimicked, although to a lesser extent, by theophylline. In contrast, M&B 22948 (1 μM–1 mM) was without significant effect upon the inositol phosphate response to 1 μM carbachol in six experiments.

Effects of phosphodiesterase inhibitors on bovine tracheal muscle tone

The effect of these agents upon the tone of bovine

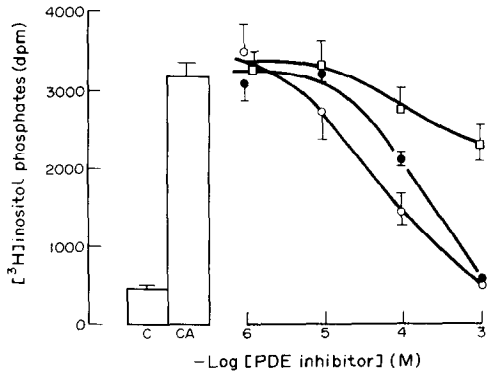

Fig. 3. Inhibition by phosphodiesterase inhibitors of the accumulation of [^{3}H]inositol phosphates elicited by 1 μM carbachol in bovine tracheal smooth muscle. [^{3}H]Inositol phosphate accumulation was measured in the presence of carbachol (1 μM) and (●) IBMX, (○) rolipram or (□) theophylline. The basal and control carbachol responses are shown by the two histograms marked C and CA, respectively. Values represent means ± SE of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

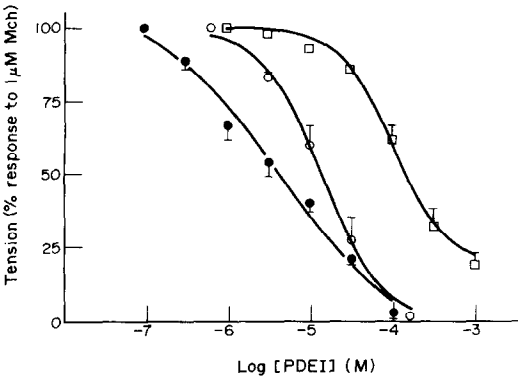

Fig. 4. The effect of theophylline (□), rolipram (●), and IBMX (○) on tension in strips of bovine tracheal smooth muscle precontracted with 1 μM methacholine. EC_{50} values are given in Table 2. Data represent mean ± SE of 4–6 experiments.

tracheal smooth muscle strips precontracted with 1 μM methacholine is shown in Fig. 4. Rolipram, IBMX and theophylline all produced concentration related relaxation of tissue strips (Table 2). In contrast, M&B 22948 (1 μM–1 mM) was without relaxant effect.

Effect of IBMX on incorporation of [^{3}H]*myoinositol into membrane phospholipids*

It has been shown previously that carbachol increases incorporation of [^{3}H]myoinositol into membrane phospholipids in bovine tracheal smooth muscle, and that this effect appears to be prevented by prelabelling slices in the presence of low concentrations of carbachol [8, 12]. In order to exclude the possibility that the inhibitory effect of IBMX was related to an inhibition of this agonist-stimulated labelling effect, we looked at the ability of IBMX to inhibit the carbachol (1 μM)-induced inositol phosphate response under conditions where no change in the degree of tissue incorporation of [^{3}H]inositol occurs (see Materials and Methods). Under conditions where no significant ($P > 0.5$, $N = 3$) agonist induced increase in membrane phospholipid labelling is apparent, there still existed a profound inhibitory effect of IBMX upon the carbachol response ($P < 0.005$, $N = 3$) suggesting that the effect of IBMX upon carbachol induced inositol phosphate formation is not being exerted at the level of incorporation of [^{3}H]myoinositol into the tissue slices (Fig. 5).

Effect of forskolin and 8-bromocyclic AMP on carbachol induced [^{3}H]*inositol phosphate formation*

The most likely candidate for intracellular messenger for mediating this inhibitory effect of IBMX, theophylline and rolipram would seem, at first sight, to be cyclic AMP. The lack of effect of salbutamol could in theory be explained by invoking muscarinic inhibition of adenylate cyclase [16, 17]. In order to examine this possibility further, we studied the effects on carbachol (1 μM) induced inositol phosphate formation of forskolin, which activates adenylate cyclase directly, and the stable membrane

Table 2. Comparison of effects of phosphodiesterase inhibitors on carbachol induced [^{3}H]inositol phosphate formation and tension in bovine tracheal smooth muscle

	Tension		[^{3}H]IP formation	
Agent	EC_{50} (μM)	n	EC_{50} (μM)	n
Theophylline	180 ± 30	1.0 ± 0.1 (4)	100, 51*	(2)
IBMX	13 ± 2	1.3 ± 0.1 (4)	140 ± 20	1.2 ± 0.3 (3)
Rolipram	4.6 ± 0.7	0.73 ± 0.02 (4)	41 ± 10	1.0 ± 0.1 (3)
M&B 22948	NE	(4)	NE	(3)

* Maximum inhibitions in these two experiments were 36 and 37%, respectively (determined as described in Materials and Methods). In a third experiment no inhibition of inositol phospholipid hydrolysis was detected at concentrations below 1 mM. At 1 mM 41% inhibition was observed.

NE, no effect at concentrations up to 100 μM.

Number of experiments given in parentheses.

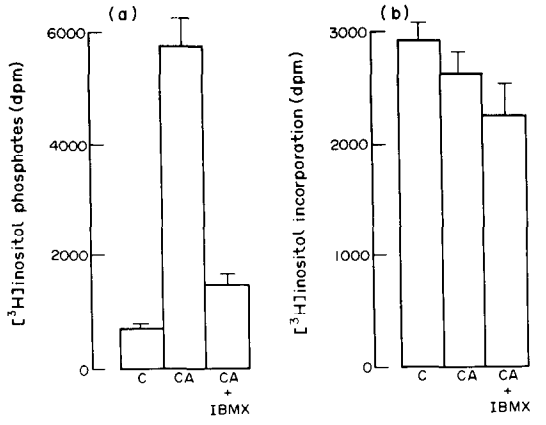

Fig. 5. A comparison of the effect of carbachol and IBMX on [^{3}H]myo-inositol incorporation and [^{3}H]inositol phosphate accumulation in bovine tracheal smooth muscle. Values represent mean ± SE of six replicate determinations in a single experiment. Measurement of (a) [^{3}H]inositol phosphate accumulation and (b) [^{3}H]inositol incorporation into membrane phospholipids was made under basal (C) conditions and in the presence of 1 μM carbachol (CA). Similar data were obtained in two further experiments.

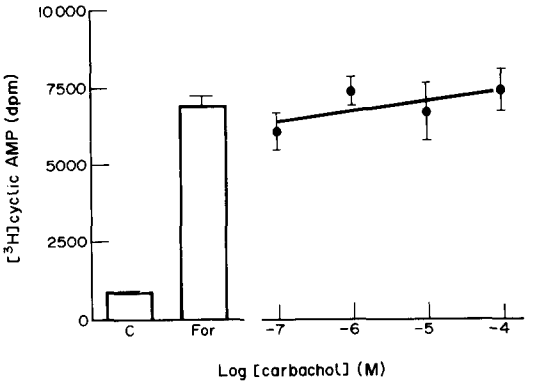

Fig. 6. Lack of effect of carbachol (0.1–100 μM) on forskolin induced [^{3}H]cyclic AMP formation. The bar marked C represents the basal (unstimulated) accumulation of [^{3}H]cyclic AMP, and the bar marked For the response to 1 μM forskolin. The lack of effect of varying concentrations of carbachol upon the response to forskolin is shown in the concentration–response curve (filled circles). Each data point represents the mean (± SE) of triplicate determinations. The experiment was repeated twice with similar results.

permeable analogue of cyclic AMP, 8-bromo-cyclic AMP. Concentrations of these agents were chosen which we have previously shown to be capable of inhibiting the histamine induced inositol phosphate response [9], and, in the case of forskolin, to produce marked increases in tissue cyclic AMP levels [9]. Forskolin produced a small (10 ± 2%, $P < 0.05$, $N = 6$) inhibition of the [^{3}H]inositol phosphate response to 1 μM carbachol, whereas 8-bromo-cyclic AMP was without significant effect upon the carbachol response (9 ± 6% inhibition, $P > 0.05$, $N = 6$). Table 1 shows a comparison of the effects of the agents studied upon histamine and carbachol induced inositol phosphate responses. From these results, it seems probable that the effect of IBMX, theophylline and rolipram are being mediated through some other mechanism than elevation of whole tissue cyclic AMP content. Further evidence in support of this hypothesis is provided by the inability of carbachol to significantly inhibit forskolin induced [^{3}H]cyclic AMP formation (Fig. 6) ($P > 0.05$ at all concentrations of carbachol, $N = 3$) in bovine tracheal smooth muscle slices.

Effect of altering extracellular calcium on IBMX induced inhibition of inositol phosphate response to carbachol

Another potential mechanism through which IBMX could be altering carbachol induced inositol phosphate formation is through alteration in intracellular calcium levels, either directly by altering the release of intracellular calcium, or by preventing entry of calcium from outside the cell. The muscarinic inositol phosphate response has previously been reported to be sensitive to changes in extracellular calcium [18]. Hence we examined the ability of IBMX to inhibit the inositol phosphate response to carbachol under a range of conditions designed to vary extracellular calcium concentrations. As previously reported [18], when extracellular calcium levels are reduced by the use of calcium free medium (to the approximate range 1 to 10 μM), reduction of the size of the inositol phosphate response to carbachol is seen. Further reduction of the inositol phosphate response is seen when EGTA (100 μM), which chelates extracellular calcium, is added to the media. However, significant accumulation of inositol phosphates was seen in response to carbachol under all conditions examined, and, in addition, the ability of IBMX to significantly inhibit the response to carbachol was maintained (Fig. 7), ($P < 0.05$ for IBMX induced inhibition under all conditions, $N = 3$).

DISCUSSION

In the experiments described here we have used total [^{3}H]inositol phosphate formation as an index of agonist induced activation of phosphoinositidase C in order to examine the modulation of the inositol phosphate response to carbachol in bovine trachealis smooth muscle by a range of different agents. The results presented demonstrate that the inositol phosphate response to 1 μM carbachol in bovine tracheal smooth muscle is sensitive to inhibition by non-selective phosphodiesterase inhibition (with IBMX and theophylline) and by rolipram, an inhibitor of the high K_m cyclic AMP selective (type IV) phosphodiesterase isozyme present in this tissue [15, 19, 20]. These agents are all capable of relaxing bovine airway smooth muscle and demonstrate the same relative potencies in their relaxant effects as for their effects upon carbachol induced inositol phosphate formation. In contrast M&B 22948, an inhibitor of the cyclic GMP preferring phosphodiesterase isozyme [19], was without effect upon either carbachol induced inositol phosphate formation or airway smooth muscle tone. It is noteworthy that the EC_{50}

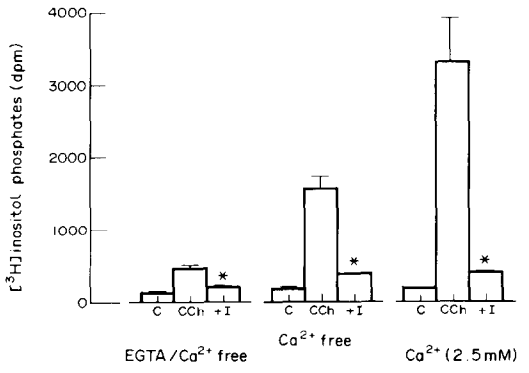

Fig. 7. The inhibition of carbachol induced [^{3}H]inositol phosphate formation by 1 mM IBMX under different extracellular calcium concentrations. In each histogram, the bar marked C represents the basal (unstimulated) accumulation of total [^{3}H]inositol phosphates, and the bar marked CCh the response to 1 μM carbachol. The response obtained with 1 μM carbachol in the presence of IBMX is shown in the bar marked +I. Measurements were made in: (a) 2.5 mM Ca^{2+}; (b) nominally calcium-free Krebs medium and (c) calcium-free Krebs + 100 μM EGTA. Data are from a single experiment, which was repeated twice with similar results. *Significant reduction ($P < 0.05$) of carbachol response by IBMX.

values for inhibition of carbachol induced inositol phosphate formation by rolipram and IBMX were approximately 10-fold greater than for the relaxant effects of these agents.

The mechanisms whereby phosphodiesterase inhibitors relax airway smooth muscle remain unclear. At least part of their mode of action appears to be related to their ability to elevate tissue cyclic AMP content. Elevation of tissue cyclic AMP content can lead to tissue relaxation through a range of different mechanisms including phosphorylation of myosin light chain kinase [21], membrane hyperpolarization [22], activation of Ca^{2+} gated K^+ channels [23], sequestration of intracellular calcium [24], and, when histamine is used as the contractile agonist, inhibition of inositol phospholipid hydrolysis [9].

Inhibition of carbachol induced inositol phosphate formation occurred at concentrations of phosphodiesterase inhibitors causing submaximal tissue relaxation (cf. Figs 3 and 4), suggesting that the effects observed are likely to be of physiological relevance. The other point of note is that the inositol phosphate response to high (>10 μM) concentrations of carbachol was not modulated by any of the agents studied. Concentrations of carbachol and methacholine above 100 μM are supramaximal for tissue contraction in bovine tracheal smooth muscle (unpublished data). The mechanism underlying this differential sensitivity of the muscarinic response to inhibition at different concentrations of carbachol remains to be determined. However, it is interesting to note that the relaxant effects of a range of smooth muscle relaxants become less marked as the concentration of muscarinic agonists used to induce contraction of airway smooth muscle preparations is increased [25].

We have previously demonstrated that the inositol phosphate response to histamine in bovine tracheal smooth muscle is inhibited by elevation of tissue cyclic AMP content [9]. This would hence appear to be the most likely mechanism to underlie the effects of IBMX, rolipram and theophylline on carbachol induced inositol phosphate formation. Further support for this hypothesis comes from the observation that rolipram and IBMX are able to elevate tissue cyclic AMP content in bovine tracheal smooth muscle [9]. However, the lack of effect of a range of other relaxant agents including salbutamol and forskolin, which also elevate tissue cyclic AMP content [9], and a membrane permeable stable analogue of cyclic AMP makes this explanation unlikely. Similar differential inhibitory effects of forskolin, β_2 agonists and other stable analogue of cyclic AMP have also been reported in canine tracheal smooth muscle [26].

The involvement of cyclic AMP in this response to IBMX could still be postulated if muscarinic receptor stimulation in bovine airway smooth muscle is capable of inhibiting cyclic AMP accumulation, thus reducing the effectiveness of agents which work through stimulation of adenylate cyclase. However, we were unable to demonstrate inhibition of cyclic AMP accumulation by carbachol when forskolin was used to stimulate adenylate cyclase. These findings are in agreement with other work in canine tracheal smooth muscle [16, 25]. In addition, the lack of effect of the stable analogues of cyclic AMP upon the carbachol (cf. histamine) response would also require explanation.

We have examined two further possible modes of action of IBMX in this study. It is well recognized that agonists increase the incorporation of [^{3}H]inositol into tissue slices in airway smooth muscle. This is probably due to the low basal rate of turnover of the membrane inositol phospholipid pool under unstimulated conditions [8, 12]. If IBMX were to inhibit this agonist induced increase in [^{3}H]inositol incorporation, this would have the effect of reducing the subsequent accumulation of inositol phosphates seen after stimulation with carbachol. This potential explanation for the mode of action of IBMX is unlikely, however, as the inhibitory effect of IBMX was maintained under conditions where increased tissue labelling did not occur.

The dependence of the magnitude of the inositol phosphate response to carbachol upon extracellular calcium [18] provides another potential site for the action of IBMX. If IBMX were to prevent calcium entry into the cell this might be expected to have a similar effect to lowering extracellular calcium, i.e. reducing the size of the inositol phosphate response. However, under conditions where extracellular calcium is reduced to very low levels (i.e. in the presence of EGTA) significant inhibition of the inositol phosphate response to carbachol is still observed. The mechanism whereby these phosphodiesterase inhibitors produce their inhibitory effect upon the inositol phosphate response to low concentrations of carbachol thus remains undefined, but may be due to a direct effect on phosphoinositidase C.

The observation that elevation of tissue cyclic AMP content is able to switch off the generation of

inositol phosphates induced by histamine, suggests that this mechanism may in part be responsible for the relaxant effects of a range of bronchodilators which elevate cyclic AMP [9]. The relaxant response of agents elevating tissue cyclic AMP content when contraction of airway smooth muscle is induced by muscarinic stimulation is probably due to a combination of all the mechanisms listed above. We have described here another route through which IBMX, rolipram and theophylline can potentially mediate tissue relaxation where tone is maintained by muscarinic receptor stimulation. It is also tempting to speculate that the relative insensitivity of airway smooth muscle contractile responses induced by muscarinic agonists to reversal with β_2 agonists [25] is due to the lack of effect of salbutamol and related compounds upon muscarinic stimulated inositol phosphate formation.

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