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Modulation of fluoroaluminate-induced inositol phosphate formation by increases in tissue cyclic AMP content in bovine tracheal smooth muscle

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1 The effect of fluoroaluminate complexes ($AlCl_3$ plus NaF) upon smooth muscle tone, [^{3}H]-inositol phosphate accumulation and [^{3}H]-cyclic AMP accumulation has been investigated in slices of bovine tracheal smooth muscle.

2 Fluoroaluminate (10 μM $AlCl_3$ + various concentrations of NaF) elicited concentration-dependent contractions of bovine tracheal smooth muscle strips at concentrations of NaF in the range 1–10 mM. The resultant contractile response was reversed by isoprenaline (50 nM) and was preserved in calcium-free medium.

3 Fluoroaluminate stimulated [^{3}H]-inositol phosphate formation at concentrations of NaF over 1 mM. The response to 20 mM NaF + 10 μM $AlCl_3$ was 164 ± 29% of the response to 1 mM histamine. Fluoroaluminate also increased the incorporation of [^{3}H]-*myo*-inositol into membrane phospholipids.

4 Fluoroaluminate produced a small rise in [^{3}H]-cyclic AMP levels (2.1 fold increase over basal with 20 mM NaF). The response to forskolin (1 μM, 8.6 fold over basal) was reduced by fluoroaluminate in a concentration-dependent manner, but still remained significantly ($P < 0.05$) elevated over the response to fluoroaluminate alone.

5 The [^{3}H]-inositol phosphate response to fluoroaluminate was inhibited by salbutamol (maximum inhibition 60%, IC_{50} = 0.08 μM), forskolin (1 μM, 46% inhibition) and isobutylmethylxanthine (1 mM, 73% inhibition).

6 These data suggest that inhibition of agonist-induced inositol phospholipid turnover by cyclic AMP in this tissue can occur at the post-receptor level.

Introduction

Histamine H_1-receptor stimulation in bovine tracheal smooth muscle is associated with inositol phospholipid hydrolysis and a contractile response (Barnes, 1986; Hall & Hill, 1988; Hall *et al.*, 1989). The second messenger products of inositol phospholipid hydrolysis, inositol 1,4,5-trisphosphate and diacylglycerol, have both been implicated as mediators of the contractile response to histamine in this tissue. Inositol 1,4,5-trisphosphate is thought to be involved in the initiation of the contractile response by mobilising calcium from intracellular stores (Hashimoto *et al.*, 1985; Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987; Berridge, 1987), whilst diacylglycerol has been proposed to have a role in the maintenance of the tonic contractile phase by activating protein kinase C (Park & Rasmussen, 1985; Takuwa *et al.*, 1986).

We have previously demonstrated that the inositol phosphate response induced by histamine in bovine tracheal smooth muscle can be inhibited by a range of smooth muscle relaxants which are capable of elevating tissue adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels through a variety of different mechanisms, including β_2-adrenoceptor stimulation, direct stimulation of adenylate cyclase and phosphodiesterase inhibition (Hall & Hill, 1988; Hall *et al.*, 1989). One possible explanation of this effect is that cyclic AMP is phosphorylating some component(s) of the inositol phospholipid receptor-effector system, via the activation of protein kinase A, leading to inhibition of receptor-mediated inositol phospholipid hydrolysis. This could occur either at the H_1-receptor itself, or at the post receptor (G protein/phosphoinositidase C) level.

There is increasing evidence that a guanosine 5'-triphosphate (GTP)-binding protein (termed G_p) is responsible for transducing information between calcium mobilising receptors, such as the H_1-receptor, and phosphoinositidase C (Cockcroft & Gomperts, 1985; Berridge 1987; Cockcroft & Stutchfield, 1988; Claro *et al.*, 1989). In intact tissues and membrane preparations from a number of tissues, phosphoinositidase C activity can be stimulated by the fluoroaluminate complex AlF_4^- formed when Al^{3+} and F^- are present in solution together (Sternweis & Gilman, 1982). AlF_4^- is believed to stimulate phosphoinositidase C activity by activating G_p directly via the GTP binding site (Blackmore *et al.*, 1985; Harden, 1989). In addition, fluoroaluminates have been shown to induce both contraction and inositol phosphate formation in uterine smooth muscle (Marc *et al.*, 1988).

In this study we have utilized the ability of fluoroaluminates to stimulate phosphoinositidase C activity via activation of G_p, in order to examine the potential of the inositol phospholipid system in airway smooth msucle for modulation by raised intracellular cyclic AMP levels at the post-receptor level. A preliminary account of this work has been presented to the British Pharmacological Society (Hall & Hill, 1989a).

Methods

Contractile responses

The contractile response of strips of bovine tracheal smooth muscle (1 cm by 0.3 cm), denuded of epithelium and mucosa, was measured by isometric recording in a 20 ml organ bath by use of Grass FT03 isometric transducers. The tissue was allowed to equilibrate under 1 g of tension in Krebs-Henseleit buffer, continuously gassed with 95% O_2/5% CO_2 for 1 h, before the addition of 10 μM $AlCl_3$ and various concentrations of NaF. In some experiments, the medium was changed to a nominally calcium-free Krebs-Henseleit buffer, and contractile responses monitored again after a 30 min equilibration period.

Formation of [^{3}H]-inositol phosphates

Accumulation of total [^{3}H]-inositol phosphates in response to stimulation with histamine was measured in slices of bovine tracheal smooth muscle as describe previously (Hall & Hill, 1988). In brief, the trachealis muscle was dissected free from

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surrounding tissue in trachea obtained from freshly slaughtered bullocks, and smooth muscle slices (300 μm) prepared with a McIlwain tissue chopper. Washed slices were incubated for 30 min in 25 ml of Krebs-Henseleit buffer at 37°C under an atomosphere 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. One hundred μl aliquots of the slice suspension were 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°C in the presence or absence of phosphodiesterase inhibitors. Agonists or cyclic AMP analogues 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 neutralised with 0.75 ml KOH (0.15 M), centrifuged (3000 g, 10 min, 4°C) and 0.75 ml aliquots of the supernatant 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 (Hill & Kendall, 1987).

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) 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* 1500 g for 5 min to separate the phases. Two hundred 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 (Hall *et al.*, 1989). Essentially, washed slices prepared as described above were incubated for 40 min at 37°C 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 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 the samples had been left 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. One ml aliquots of the supernatant were analysed for [^{3}H]-cyclic AMP by column chromatography, as described previously (Donaldson *et al.*, 1988). Recovery of [^{3}H]-cyclic AMP from the columns was corrected for by spiking the samples with [^{14}C]-cyclic AMP (Donaldson *et al.*, 1988). 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 tissue present in each sample.

Data analysis

The effect of the various agents on inositol phosphate formation was performed by paired t tests, and in addition with the Wilcoxon signed rank test where applicable. Concentration-response curves were drawn by inspection. All values given in the text or figure legends represent mean ± s.e.mean of n separate experiments.

Chemicals

Dowex 50W, H^+-form (200–400 mesh), Dowex-1 (X8, 100–200 mesh, chloride form), neutral alumina (type WN-3), imidazole, isobutylmethylxanthine (IBMX), salbutamol, forskolin, $AlCl_3$ and NaF were obtained from Sigma. Histamine dihydrochloride was purchased from BDH. [^{3}H]-*myo*-inositol (16.5 Ci mmol^{-1}) and 8-[^{14}C]-cyclic AMP (specific activity 42.4 mCi mmol^{-1}) were purchased from N.E.N., and 8-[^{3}H]-adenine (specific activity 26 Ci mmol^{-1}) was obtained from Amersham.

Results

Effect of fluoroaluminate on tracheal smooth muscle strips

A combination of $AlCl_3$ (10 μM) and NaF elicited a marked contraction of bovine tracheal smooth muscle strips at concentrations of NaF above 1 mM NaF (Figure 1). The maximum contractile response seen (10 mM NaF) accounted for 59 ± 7% of the response to a maximal dose of carbachol (10 μM; $n = 4$). The contractile response to fluoroaluminate was reversed by isoprenaline (50 nM) (Figure 1a and c). Switching to a nominally calcium-free buffer before the addition of fluoroaluminate had little effect upon the ensuing contractile response in the majority of experiments (Figure 1d), although in one experiment some increase in the contractile response was noted (Figure 1b). The contractile response to fluoroaluminate was usually delayed by a period of 2–4 min after the addition of fluoroaluminate in calcium-containing medium (but see Figure 1c), and this delay was increased in the absence of added calcium (Figure 1). A similar delay has been noted in the contractile response of uterine smooth muscle (Marc *et al.*, 1988).

Effect of fluoroaluminate on [^{3}H]-inositol phosphate formation

The effect of varying the concentration of the fluoroaluminate ion upon total [^{3}H]-inositol phosphate formation in slices of bovine tracheal smooth muscle is shown in Figure 2. It can be seen that at concentrations of fluoroaluminate ≥ 5 mM there is marked stimulation of [^{3}H]-inositol phosphate formation,

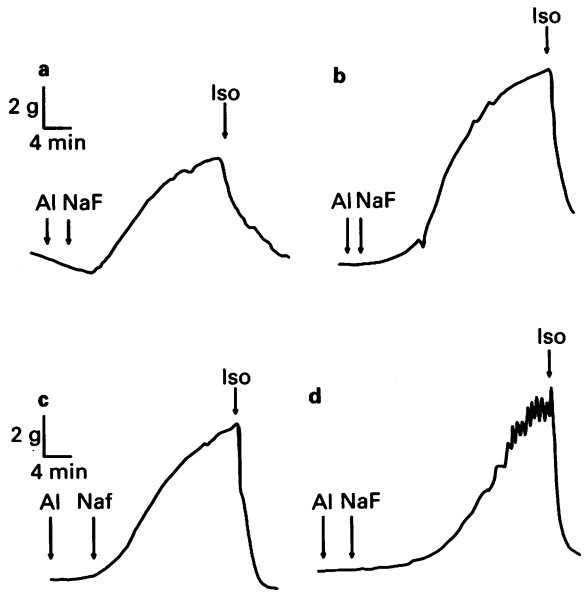

Figure 1 The effect of 4 mM NaF, in the presence of 10 μM $AlCl_3$ (Al), on the smooth muscle tone of bovine tracheal smooth muscle strips, and the inhibition of this response by 50 nM isoprenaline (Iso). Traces show the response to NaF obtained in calcium-containing (a and c) or calcium-free (b and d) Krebs-Henseleit medium. Traces (a and b) or (c and d) were obtained on the same tracheal smooth muscle strip.

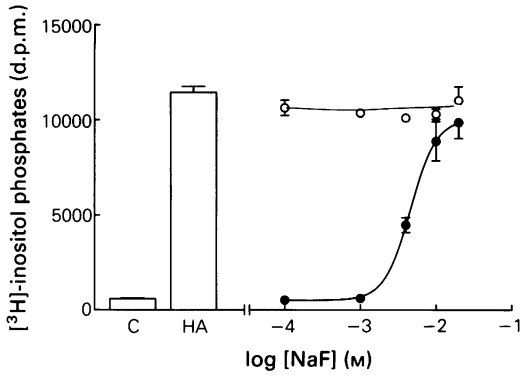

Figure 2 The effect of increasing concentrations of NaF in the presence of 10 μM $AlCl_3$ (●) on the accumulation of total [^{3}H]-inositol phosphates in slices of bovine tracheal smooth muscle. The control (unstimulated) response is shown by the column marked C, and the response to 1 mM histamine by the column marked HA. (○) Simultaneous addition of NaF, 10 μM $AlCl_3$ and 1 mM histamine. Each data point represents the mean of triplicate determinations and vertical lines show s.e.mean. Where no error bars are shown they are within the size of the symbol. Data are from a single experiment repeated twice more with similar results.

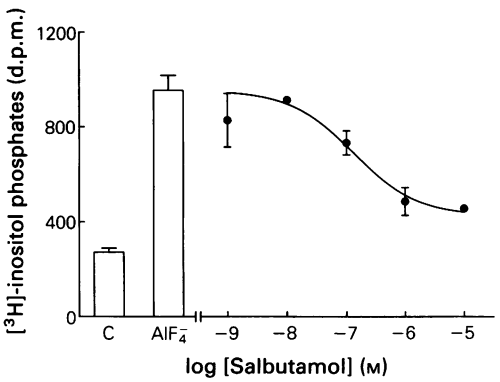

Figure 3 Inhibition of the inositol phosphate response to 20 mM NaF and 10 μM $AlCl_3$ by salbutamol. The basal (unstimulated response) is shown by the column marked C, and the response to fluoraluminate by the column marked AlF_4^-. The effect of adding various concentrations of salbutamol with fluoroaluminate is shown in the graph on the right of the figure. Each data point shows the mean of triplicate determinations in a representative experiment and vertical lines show s.e.mean. The experiment was repeated twice with similar results.

compatible with fluoroaluminate stimulating hydrolysis of phosphatidylinositol 4,5-bisphosphate via its action on the G protein coupled to phosphoinositidase C. The relative sizes of the responses obtained with 1 mM histamine and a standard dose of fluoroaluminate (10 μM $AlCl_3$ + 20 mM NaF) varied quite widely between preparations. The relative size of the fluoroaluminate response (10 μM $AlCl_3$ + 20 mM NaF) ranged from 60 to 345% of the response to 1 mM histamine with a mean value of 164 ± 29% (n = 11). Figure 2 also indicates that the inositol phosphate responses to histamine and fluoroaluminate ions were not additive. As has been demonstrated for other agonists in this tissue (Hall & Hill, 1988; 1989b; Chilvers *et al.*, 1989), 20 mM fluoroaluminate also induced an increase in the incorporation of [^{3}H]-*myo*-inositol into tissue slices to 171 ± 32% (n = 4) compared with control (unstimulated, = 100%) levels.

Modulation of inositol phosphate response to fluoroaluminate by cyclic AMP

To investigate the ability of changes in tissue cyclic AMP content to modulate the [^{3}H]-inositol phosphate response to fluoroaluminate, we examined the effect of varying the concentration of the β_2-adrenoceptor agonist salbutamol upon the [^{3}H]-inositol phosphate response to 20 mM fluoroaluminate. A typical example of this series of experiments is shown in Figure 3, demonstrating that concentration-dependent inhibition of the response is seen with salbutamol. The calculated IC_{50} value for salbutamol was 0.08 ± 0.02 μM (n = 3), which is in excellent agreement with the previously obtained IC_{50} for salbutamol-induced inhibition of the inositol phosphate response to histamine (0.03 μM) in this tissue (Hall & Hill, 1988). The mean inhibition seen with 1 μM salbutamol was 60 ± 6% (n = 8, $P < 0.05$), again agreeing well with the inhibitory effect of 1 μM salbutamol on histamine-stimulated inositol phosphate formation.

It is notable that the absolute magnitude (in terms of d.p.m.) of the inositol phosphate response to fluoroaluminate and histamine varied extensively between experiments (cf Figures 2 and 3 which show respectively the largest and smallest fluoroaluminate responses observed in this study). The reason for this is unclear, but it probably reflects inter-animal differences in both the size of the response and the extent to which [^{3}H]-inositol is taken up by different bovine tracheal slice preparations. However, within individual experiments reproducibility was good (Figures 2 and 3) and the extent of the inhibition produced by cyclic AMP-elevating agents (in percentage terms) was consistent between experiments (Table 1).

The effect of a range of agents capable of elevating tissue cyclic AMP levels by (a) phosphodiesterase inhibition (IBMX, a non-selective phosphodiesterase inhibitor, Reeves *et al.*, 1987), (b) direct stimulation of adenylate cyclase (forskolin) or (c) receptor mediated stimulation of adenylate cyclase (salbutamol) is shown in Table 1, together with results previously obtained for the inhibitory effect of these agents on histamine-stimulated inositol phosphate formation (Hall *et al.*, 1989). These results show that the inositol phosphate response to fluoroaluminate is subject to modulation in a similar manner to the histamine-induced inositol phosphate response.

Effect of fluoroaluminate on G_s and G_i

In addition to stimulating the G protein linked to phosphoinositidase C, fluoroaluminate might be expected to stimulate other G proteins present in the tissue including G_s and G_i linked to adenylate cyclase. This might in turn be expected to modulate the effect of agents working through adenylate cyclase on the inositol phosphate response to fluoroaluminate. In order to examine this possibility the effect of various concentrations of fluoroaluminate on [^{3}H]-cyclic AMP formation was quantified. Alone, fluoroaluminate produced a small, but significant increase in tissue cyclic AMP levels at the highest concentration examined (20 mM, 2.1 ± 0.4 fold stimulation over basal, n = 8, $P < 0.05$) (Figure 4). Forskolin (1 μM) produced a marked stimulation (8.6 ± 1.7 fold, n = 7) of [^{3}H]-

Table 1 Inhibition of fluoroaluminate- and histamine-stimulated [^{3}H]-inositol phosphate formation in bovine tracheal smooth muscle

	% inhibition of response to:	
Agent	*AlF_4^- 20 mM*	*Histamine 0.1 mM†*
Salbutamol (1 μM)	60 ± 6* (8)	66 ± 3* (15)
IBMX (1 mM)	73 ± 2* (6)	81 ± 6* (8)
Forskolin (1 μM)	46 ± 5* (6)	48 ± 6* (8)

Data shown are means ± s.e.mean; n = number in parentheses.
* $P < 0.05$ (paired t and Wilcoxon signed rank tests). IBMX = 3-isobutyl-1-methylxanthine. AlF_4^- 20 mM represents a mixture of 10 μM $AlCl_3$ and 20 mM NaF.
† Data from Hall *et al.* (1989).

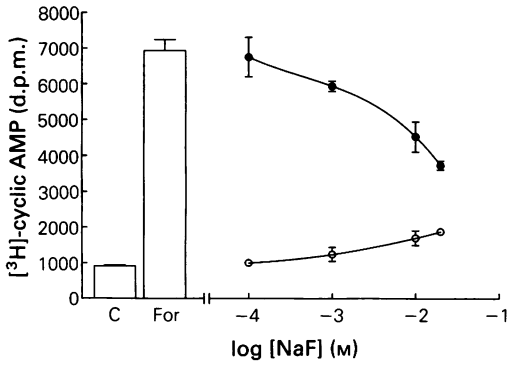

Figure 4 The effect of varying concentrations of NaF in the presence of 10 μM $AlCl_3$ on the accumulation of [^{3}H]-cyclic AMP in slices of bovine tracheal smooth muscle in the presence and absence of 1 μM forskolin. The basal (unstimulated) response is shown by the column marked C, and the response to forskolin alone by the column marked For. The responses to various concentrations of NaF in the presence of 10 μM $AlCl_3$ in the absence (○) and presence (●) of forskolin are shown. Each data point represents the mean of triplicate determinations in a representative experiment repeated at least twice with similar results. Vertical lines represent s.e.mean and where no error bars are shown they are within the size of the symbol.

cyclic AMP formation as previously demonstrated (Hall *et al.*, 1989). When 20 mM fluoroaluminate and 1 μM forskolin were added together, the net effect of fluoroaluminate seen was a significant ($P < 0.05$) inhibition of the [^{3}H]-cyclic AMP response to forskolin, the fold stimulation being reduced to 3.7 ± 0.8 ($n = 7$) (see Figure 4). However, the net effect of forskolin and fluoroaluminate was still a significant ($P < 0.05$) accumulation of cyclic AMP over basal levels. At lower concentrations of fluoroaluminate, a smaller stimulation of [^{3}H]-cyclic AMP accumulation was observed in the absence of forskolin, and the inhibitory effect on the response to 1 μM forskolin was less marked (Figure 4).

Discussion

We have previously shown that elevation of tissue cyclic AMP levels through a variety of different mechanisms, with a range of agents capable of relaxing airway smooth muscle, can inhibit the inositol phosphate responses to histamine and 5-hydroxytryptamine in bovine tracheal smooth muscle (Hall *et al.*, 1989; Hall & Hill, 1989b). The studies described here provide further insight into the mechanisms underlying the modulatory effect of elevation of tissue cyclic AMP on inositol phosphate responses.

Fluoroaluminate (AlF_4^-) is thought to induce inositol phosphate responses in smooth muscle and other tissues by a receptor-independent mechanism involving stimulation of G_p, the G protein which acts as a link in the signal transduction pathway between agonist receptors and phosphoinositidase C (Sternweis *et al.*, 1982; Cockcroft & Gomperts, 1985; Blackmore *et al.*, 1985; Gilman, 1987; Cockcroft & Stutchfield, 1988). This is probably a result of the fluoroaluminate complex substituting for GTP and activating the G protein, causing dissociation of α from $\beta\gamma$ subunits in a manner similar to the proposed mechanism of action of the stable analogue of GTP, GTPγS (Harden, 1989). Fluoride is able to activate membrane phosphoinositidase activity at similar concentrations (>1 mM) to those activating G proteins (Cockcroft & Stutchfield, 1988), and has been shown to induce inositol phosphate formation in uterine smooth muscle (Marc *et al.*, 1988). As shown above, at the concentrations at which it is effective in stimulating phosphoinositidase C activity in other tissues, fluoroaluminate causes bovine tracheal smooth muscle contraction and stimulates [^{3}H]-inositol phosphate formation, implicating the involvement of a G protein in this response, as in many other tissues which have been studied (Harden, 1989). These results therefore provide support for the involvement of the phosphoinositide system in pharmaco-mechanical coupling in this tissue. It was notable, particularly in calcium-free conditions, that a delay of 2–4 min was seen before the contractile response commenced: the explanation for this remains at present uncertain. A similar observation has been made in uterine smooth muscle (Marc *et al.*, 1988).

A relationship between intracellular cyclic AMP and inhibition of agonist-induced inositol phosphate formation has been observed in a number of tissues including bovine and canine tracheal smooth muscle (Hall & Hill, 1988; Madison & Brown, 1988; Hall *et al.*, 1989), neutrophils (Della Bianca *et al.*, 1986), platelets (Takai *et al.*, 1982; Watson *et al.*, 1984), gastric mucosal cells (Puurunen *et al.*, 1987) and rat kidney (Neylon & Summers, 1988), but little is known of the mechanism underlying the interaction between these two intracellular second messenger pathways. The present study suggests that, in bovine tracheal smooth muscle, cyclic AMP exerts a modulatory effect on inositol phosphate responses at the post-receptor level. Thus, the inositol phosphate response, induced by direct stimulation by fluoroaluminate of the G protein linked to phosphoinositidase C, is inhibited by agents which elevate cyclic AMP levels in this tissue. The most likely target for cyclic AMP would therefore seem to be either the G protein itself or phosphoinositidase C.

In addition to stimulating the G protein coupled to phosphoinositidase C, fluoroaluminate would be expected to stimulate other G proteins present in the tissue, including G_s and/or G_i linked to adenylate cyclase. When added on its own in bovine tracheal smooth muscle, fluoroaluminate produced a small stimulation of [^{3}H]-cyclic AMP formation at the higher concentration used. However, when added with the adenylate cyclase activator forskolin, the net effect of fluoroaluminate was an inhibition of the cyclic AMP response to forskolin at concentrations of fluoroaluminate above 10 mM, suggesting that fluoroaluminate inhibits adenylate cyclase activity when this is stimulated with other agents in intact tissues, as has been previously demonstrated (Marc *et al.*, 1988). This effect is presumably due to stimulation of G_i by fluoroaluminate. The combination of forskolin and fluoroaluminate, however, still produced a significant stimulation of cyclic AMP accumulation over basal levels (i.e. in the presence of fluoroaluminate alone) of a magnitude that we have previously shown to be associated with inhibition of histamine-stimulated inositol phosphate (Hall *et al.*, 1989). Hence the net effect of adding forskolin with fluoroaluminate on [^{3}H]-inositol phosphate formation would be expected to be the cyclic AMP-mediated inhibition that was observed.

The increase in cyclic AMP accumulation observed in the presence of fluoroaluminate alone (Figure 4) is within the range that one would expect to observe some inhibition of inositol phospholipid hydrolysis (Hall *et al.*, 1989). This suggests that the fluoroaluminate inositol phosphate response is blunted to some extent by the effect of this ion on the cyclic AMP messenger system. No marked inhibition of the inositol phosphate response to histamine was obvious when increasing concentrations of fluoroaluminate were added simultaneously (Figure 1). However, the possible presence of an inhibitory cyclic AMP-dependent component in the combined fluoroaluminate/histamine response makes it difficult to draw any conclusions regarding the additivity of the two responses and *vice versa*. The fact that further decrements in fluoroaluminate-induced inositol phosphate responses can be achieved by salbutamol, IBMX and forskolin does, however, indicate that the raised cyclic AMP levels produced by fluoroaluminate alone are not sufficient to produce a maximal inhibition of the inositol phosphate response.

In summary, this study suggests that a G protein is likely to be involved in the signal transduction pathway thought to be responsible for pharmaco-mechanical coupling in bovine airway smooth muscle, and that stimulation of the G protein

with fluoroaluminate produces an inositol phosphate response in this tissue, which is subject to regulation by changes in tissue cyclic AMP content. The target of action of cyclic AMP in this response is likely to be either phosphoinositidase C or the G protein itself. These observations lend support to the proposal that part of the relaxant properties of agents, which elevate tissue cyclic AMP levels against histamine- or 5-hydroxytryptamine-contracted airway smooth muscle, is due to the inhibitory effect of raised cyclic AMP levels on inositol phosphate production at the post-receptor level.

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