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Modulation of spasmogen-stimulated Ins(1,4,5)P_3 generation and functional responses by selective inhibitors of types 3 and 4 phosphodiesterase in airways smooth muscle

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1 The effects of isoenzyme-selective inhibitors of phosphodiesterases PDE3 and PDE4 on cyclic AMP concentration, two indices of phosphoinositide hydrolysis, and contractile responses to spasmogens have been investigated in bovine tracheal smooth muscle (BTSM).

2 Neither the PDE3-selective inhibitor ORG 9935, nor the PDE4-selective inhibitor rolipram increased cyclic AMP levels in BTSM. However, rolipram addition in the presence of PDE3 inhibition (ORG 9935; 1 μM) concentration-dependently ($-\log EC_{50}$ (M), 6.55 ± 0.15; $n=3$) increased cyclic AMP levels to about 70% of the maximal response to the β-adrenoceptor agonist isoprenaline.

3 Rolipram *per se* inhibited histamine-stimulated [^{3}H]-inositol (poly)phosphate ([^{3}H]-InsP_X) accumulation by >80% ($-\log EC_{50}$ (M), 6.92 ± 0.11; $n=3$). Although ORG 9935 (1 μM) had little effect on histamine-stimulated [^{3}H]-InsP_X accumulation alone it greatly facilitated the inhibitory action of rolipram ($-\log EC_{50}$ (M), 8.82 ± 0.39; $n=3$). The effects of PDE3 and/or PDE4 inhibition on [^{3}H]-InsP_X accumulation stimulated by muscarinic acetylcholine (mACh) receptor activation were less marked. However, combined PDE3/4 inhibition significantly decreased this response at a submaximal concentration of mACh receptor agonist (carbachol; 1 μM).

4 The greater-than-additive effect of combined PDE3/4 inhibition was also observed at the level of contractile responses to histamine and carbachol. In experiments designed to investigate the effects of PDE3 and/or 4 inhibitors on the carbachol-mediated phasic contraction, additions of rolipram (10 μM) or ORG 9935 (1 μM) were without effect, whereas added together the inhibitors caused a significant ($P<0.01$) 40% reduction in the peak phasic contractile response.

5 The effect on contraction correlated with a substantial inhibitory effect of PDE3/4 inhibition on the initial increase in inositol 1,4,5-trisphosphate (InsP_3) accumulation stimulated by spasmogen. Thus, in the presence of ORG 9935 (1 μM) rolipram concentration-dependently inhibited carbachol-stimulated InsP_3 accumulation by $\geq$50% ($-\log EC_{50}$ (M), 6.77 ± 0.21; $n=4$).

6 Carbachol (100 μM) addition caused a rapid decrease (by 67% at 10 s) in BTSM cyclic AMP level in the presence of PDE3/4 inhibition. However, omission of Ca^{2+} from the incubation medium prevented the carbachol-evoked decrease in cyclic AMP and this coincided with a greater inhibition ($\geq$80%) of the carbachol-stimulated InsP_3 response.

7 These data indicate that combined PDE3 and PDE4 inhibition has greater-than-additive effects on second messenger and functional responses to spasmogens in BTSM. Furthermore, the ability of PDE3/4 inhibition significantly to attenuate mACh receptor-mediated contractile responses, may be, at least in part, attributed to an effect exerted at the level of InsP_3 generation.

Keywords: Phosphodiesterases; rolipram; ORG 9935; cyclic AMP; Ins(1,4,5)P_3; phosphoinositide hydrolysis; phasic contraction; smooth muscle relaxation; bovine tracheal smooth muscle

Introduction

The generation of the second messengers inositol 1,4,5-trisphosphate (InsP_3) and *sn*-1,2-diacylglycerol (DAG) plays a central role in the initiation and maintenance phases of airways smooth muscle contraction in response to stimulation of cell-surface G protein-coupled receptors (Takuwa *et al*., 1986; Miller-Hance *et al*., 1988; Roffel *et al*., 1990; Chilvers *et al*., 1991; Schramm & Grunstein, 1992). For example, activation of receptors for acetylcholine (M_3-mACh receptors) and histamine (H_1-receptors) results in a G protein-dependent activation of phosphoinositide-specific phospholipase C (PLC) to generate InsP_3 and DAG, which mobilize sarcoplasmic reticular Ca^{2+}-stores (Hashimoto *et al*., 1985; Chilvers *et al*., 1990; Hoiting *et al*., 1996), and activate protein kinase C (Langlands & Diamond, 1992; Yang & Black, 1995), respectively.

Contraction of airways smooth muscle can be prevented (anti-spasmogenic effect) or reversed (spasmolytic effect) by agents which increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels within the tissue (Scheid *et al*., 1979; Giembycz & Raeburn, 1991), such as β-adrenoceptor agonists (Torphy, 1994; Kotlikoff & Kamm, 1996) and cyclic nucleotide phosphodiesterase (PDE) inhibitors (Nicholson *et al*., 1991; Beavo, 1995). A number of mechanisms have been proposed to account for the relaxant effect of cyclic AMP (see Giembycz & Raeburn, 1991; Kotlikoff & Kamm, 1996), although which of these is physiologically most significant remains unresolved (Rasmussen *et al*., 1990; Torphy, 1994). One potentially important mechanism involves the ability of cyclic AMP-elevating agents to inhibit receptor-mediated phosphoinositide turnover (Hall *et al*., 1989a; Madison & Brown, 1988). This

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action seems to be spasmogen-dependent as, like the modulation of contractile responses (Torphy *et al*., 1985), mACh receptor-stimulated phosphoinositide responses are relatively resistant to cyclic AMP-elevation compared to those elicited by other spasmogens (Hall *et al*., 1989a; Madison & Brown, 1988; Offer *et al*., 1991).

Biochemical studies have demonstrated the presence of multiple PDE isoenzymes in airways smooth muscle, and isoenzyme-selective inhibitors have been used to define which PDEs may play a physiologically important role, in different species including man, in regulating tissue cyclic AMP concentration (Torphy & Cieslinski, 1990; Giembycz & Barnes, 1991; Shahid *et al*., 1991; de Boer *et al*., 1992; Rabe *et al*., 1993; Torphy *et al*., 1993). In the present study we have investigated the effect of inhibition of the low K_m, cyclic AMP-specific (PDE4) isoenzyme, and its enhancement by concurrent inhibition of the guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-inhibited (PDE3) isoenzyme, on tissue cyclic AMP levels, spasmogen-stimulated phosphoinositide hydrolysis and contractile responses in bovine tracheal smooth muscle (BTSM). In particular, we have concentrated upon the ability of combined PDE3/4 inhibition to affect Ins(1,4,5)P_3 generation and tissue responses to mACh receptor activation.

Methods

Incubation methods

Bovine trachealis muscle was obtained and prepared as described previously (Chilvers *et al*., 1989a). Tissue was maintained at 4°C in a modified Krebs-Henseleit buffer (KHB; composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 1.3, K_2HPO_4 1.2, $MgSO_4$ 1.2, HEPES 5, glucose 10; equilibrated with O_2/CO_2 (19:1 v/v) and adjusted to pH 7.4) during transportation to the laboratory and dissection to remove the epithelium and connective tissue. For contractile studies, BTSM was cut into strips (approx. $2 \times 2 \times 10$ mm) and mounted for tension recording as described previously (Ellis *et al*., 1995). For second messenger experiments, the bovine tracheal smooth muscle (BTSM) was cross-chopped (300×300 μm) with a McIlwain tissue-chopper, and slices incubated with multiple changes of KHB (100 ml) at 37°C for 45 – 60 min. Where indicated slices were labelled with *myo*-[^{3}H]-inositol (1 μCi ml^{-1}) for 24 h as described previously (Chilvers *et al*., 1994).

Contractile studies

BTSM strips were mounted in 30 ml organ baths containing KHB equilibrated with O_2/CO_2 at 37°C under a resting tension of 0.5 g. After a 60 min stabilization period, during which time the tissue was washed with multiple KHB changes and tension adjusted back to 0.5 g, anti-spasmogenic experiments were performed as described previously (Ellis *et al*., 1995) to investigate the ability of pretreatment (for 30 min) with PDE3 and/or 4 inhibitors to affect the contraction elicited by either carbachol (0.001 – 3 μM) or histamine (1 – 300 μM).

To study phasic contraction studies were performed as above, except that single concentrations of spasmogen were studied, and 10 min before spasmogen addition BTSM strips were exposed to Ca^{2+}-free KHB containing 2 mM EGTA (Tagliente *et al*., 1992). Under these conditions a rapid phasic contraction to spasmogen was observed. Within 30 s the tension declined to resting levels and the tissue was extensively washed with normal KHB and allowed to re-stabilize for 30 min before further manipulation.

Spasmogen-stimulated total [^{3}H]-inositol phosphate accumulation

After the 24 h labelling period BTSM slices were washed back into KHB and 75 μl packed BTSM slices transferred to 400 μl KHB containing 1 μCi ml^{-1} *myo*-[^{3}H]-inositol and 5 mM LiCl (final concentrations). Drug additions were made as indicated in the Results section and incubations terminated by addition of 500 μl ice-cold 10% perchloric acid and immediate transfer of samples to an icebath. After 30 min samples were centrifuged ($4000 \times g$, 15 min, 4°C) and the supernatant neutralized with 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (Chilvers *et al*., 1994). The total [^{3}H]-inositol phosphate ([^{3}H]-InsP_X) fraction was recovered by Dowex (Cl^- form) ion exchange chromatography (Challiss *et al*., 1992).

Spasmogen-stimulated changes in Ins(1,4,5)P_3 and effects of PDE inhibition on cyclic AMP levels

The effects of isoenzyme-selective PDE inhibitors or vehicle on cyclic AMP and Ins(1,4,5)P_3 mass levels in BTSM slices were assessed in the absence or presence of a subsequent spasmogen challenge. Incubations were terminated as described above for [^{3}H]-InsP_X recovery. Cyclic AMP levels were quantified in neutral tissue extracts by the method of Brown *et al*. (1971). Ins(1,4,5)P_3 levels were quantified by the method of Challiss *et al*. (1988).

Materials

Myo-[^{3}H]-inositol (12-20 Ci mmol^{-1}), [^{3}H]-Ins(1,4,5)P_3 (17 – 20 Ci mmol^{-1}) and cyclic AMP (50 Ci mmol^{-1}) were purchases or gifts from NEN DuPont (U.K.) Ltd. (Stevenage Herts). Histamine, carbachol, isoprenaline, milrinone and Dowex 1-X8 (100 – 200 mesh; Cl^- form) were from Sigma Chemical Co. Ltd. (Poole, Dorset). Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone and cilostamide were from Tocris Cookson Ltd. (Langford, Bristol). The following were obtained as gifts: ORG 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien - 2-yl -5-methyl -3(2H) - pyridazinone), (Organon Scientific Development Group, Newhouse, Scotland); rolipram (Schering Health Care Ltd., Burgess Hill, West Sussex); denbufylline and siguazodan (SK&F 94836), (SmithKline Beecham Pharmaceuticals, Welwyn, Herts).

Data analysis

All data are presented as means ± s.e.mean for the indicated number of separate experiments, performed in triplicate unless otherwise stated. Concentration-effect curves were analysed by use of a commercially available program (InPlot, GraphPad Software, San Diego, CA, U.S.A.) and used to generate EC_{50}/IC_{50} values. Statistical comparisons between individual group means were by two-tailed, unpaired Student's *t* test unless otherwise stated.

Results

Effects of isoenzyme-selective PDE inhibitors on cyclic AMP accumulation

A number of PDE3- and PDE4-selective PDE inhibitors were assessed for their ability to increase basal cyclic AMP accumulation in BTSM slices. Neither PDE3-selective (siguazodan, cilostamide, ORG 9935), nor PDE4-selective (Ro 20-

1724, denbufylline, rolipram) inhibitors used at isoenzyme-specific concentrations (Torphy & Cieslinski, 1990; Shahid *et al*., 1991; Qian *et al*., 1993; Beavo, 1995) produced a significant elevation in cyclic AMP concentration during a 30 min exposure time (data not shown). However, in the presence of PDE3 inhibition a PDE4 inhibitor could significantly and concentration-dependently increase cyclic AMP accumulation. For example, in the presence of 1 μM ORG 9935, rolipram caused a concentration-dependent ($-\log EC_{50}$ (M), 6.55 ± 0.15; $n=3$) increase in cyclic AMP accumulation which amounted to a 6 fold-over-basal increase at 100 μM rolipram and which was about 70% of the response to a maximally-effective concentration of isoprenaline (Figure 1).

Comparison of the effects of rolipram on spasmogen-stimulated phosphoinositide turnover in the absence and presence of PDE3 inhibition

Previous studies have demonstrated that rolipram inhibits both inositol phospholipid hydrolysis (Hall *et al*., 1989b) and contractile responses (Shahid *et al*., 1991) to histamine in BTSM. The inhibitory effect of rolipram on histamine-stimulated [^{3}H]-$InsP_X$ accumulation in BTSM slices is shown in Figure 2. Rolipram caused a concentration-dependent ($-\log EC_{50}$ (M), 6.92 ± 0.11; $n=3$) inhibition which amounted to $>80\%$ at a maximally-effective concentration (Figure 2). The PDE3-selective inhibitor ORG 9935 (1 μM) caused only a small ($\leqslant 10\%$) inhibition of histamine-stimulated [^{3}H]-$InsP_X$ accumulation *per se*, but dramatically leftward-shifted the concentration-effect curve for the inhibitory effect of rolipram ($-\log EC_{50}$ (M), 8.82 ± 0.39; $n=3$) (Figure 2).

The effects of rolipram, in the absence and presence of ORG 9935, on carbachol-stimulated [^{3}H]-$InsP_X$ accumulation were much less pronounced (Figure 3), consistent with previous findings on differential sensitivities of muscarinic- and histamine-receptor-mediated phosphoinositide responses to cyclic AMP-elevating agents (Hall & Hill, 1988; Madison & Brown, 1988; Offer *et al*., 1991). Thus a small rightward-shift in the concentration-effect curve for carbachol-stimulated [^{3}H]-$InsP_X$ accumulation was observed, but only at submaximal

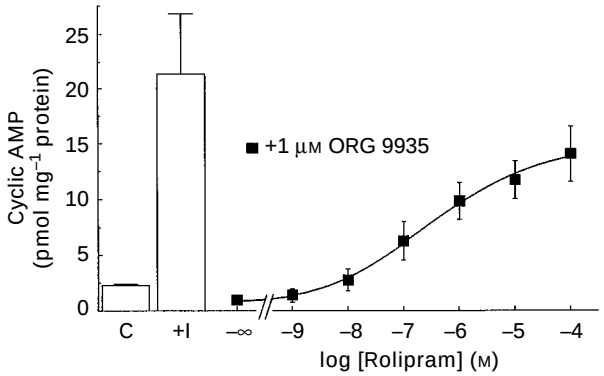

Figure 1 Effect of PDE3/4 inhibition on cyclic AMP accumulation. BTSM slices were prepared as described in the Methods section. Slices were incubated in the absence or presence of ORG 9935 (1 μM) and the indicated concentration of rolipram for 30 min. For comparison, the effect of a maximally-effective concentration of isoprenaline (+I; 10 μM for 10 min) was also assessed. Incubations were terminated and cyclic AMP concentration determined as described in the Methods section. Neither ORG 9935 (1 μM), nor rolipram (100 μM) alone significantly affected basal cyclic AMP levels (C). Data are shown as means with vertical lines indicating s.e.mean for at least 4 separate experiments performed in duplicate or triplicate.

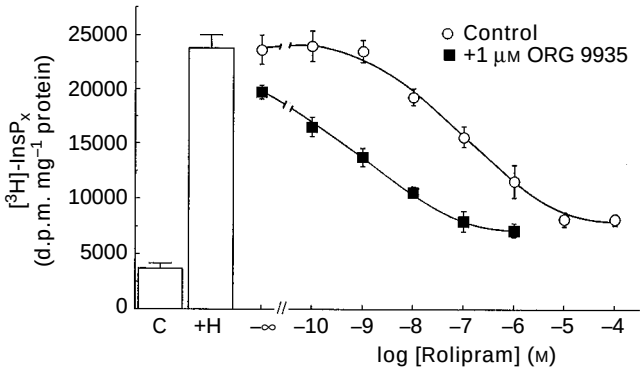

Figure 2 Effect of PDE3/4 inhibition on histamine-stimulated [^{3}H]-$InsP_X$ accumulation. BTSM slices were prepared and radiolabelled with [^{3}H]-inositol as described in the Methods section. Slices were incubated in KHB (containing 5 mM LiCl) in the absence (control) or presence of ORG 9935 (1 μM) and the indicated concentrations of rolipram for 30 min before addition of histamine (+H; 100 μM) for a further 30 min. Incubations were terminated and [^{3}H]-$InsP_X$ accumulation determined as described in the Methods section. Data are shown as means with vertical lines indicating s.e.mean for 3 separate experiments performed in triplicate.

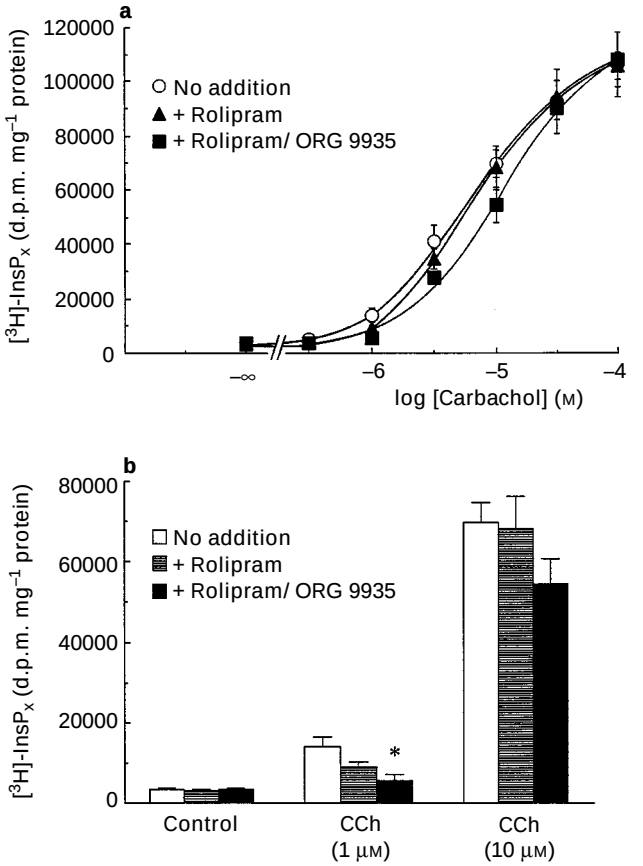

Figure 3 Effect of PDE3/4 inhibition on carbachol-stimulated [^{3}H]-$InsP_X$ accumulation. BTSM slices were prepared and radiolabelled with [^{3}H]-inositol as described in the Methods section. Slices were incubated in KHB (containing 5 mM LiCl) in the absence or presence of ORG 9935 (1 μM) and rolipram (100 μM) for 30 min before addition of the indicated concentrations of carbachol for a further 30 min. (a) The effects of PDE3 and/or PDE4 inhibition on the concentration-response curves for carbachol. (b) The data for 0, 1 and 10 μM carbachol (CCh). Incubations were terminated and [^{3}H]-$InsP_X$ accumulation determined as described in the Methods section. Data are shown as means with vertical lines indicating s.e.mean for 3 separate experiments performed in triplicate. A statistically significant effect of rolipram/ORG 9935 on the [^{3}H]-$InsP_X$ accumulation stimulated by carbachol is indicated as $^*P<0.05$.

concentrations of the spasmogen (Figure 3a). At 1 μM carbachol, rolipram (100 μM), and rolipram (100 μM) + ORG 9935 (1 μM), caused 44 and 82% decreases in the [^{3}H]-InsP_X response, respectively, although only the latter effect attained statistical significance (Figure 3b). Despite the modest size of the inhibitory effect of rolipram in the presence of the PDE3 inhibitor, the effect appeared to be concentration-dependent and evident at very low concentrations of the PDE4-selective inhibitor (1–10 nM; Figure 4).

Effect of rolipram on spasmogen-stimulated contraction in the absence and presence of PDE3 inhibition under normal and Ca^{2+}-free conditions

Concentration-effect curves to carbachol and histamine were constructed by incremental addition of either spasmogen to BTSM strips. In general, full spasmogen-stimulated concentration-effect curves were constructed before exposure to PDE inhibitor(s) and then the incremental protocol repeated in the presence of ORG 9935 and/or rolipram, or vehicle (to act as a time-matched control). Figure 5a shows data for the effects of PDE3/4 inhibition at single concentrations of carbachol (1 μM) or histamine (30 μM). ORG 9935 (1 μM) had no effect on either carbachol- or histamine-stimulated contraction. Rolipram was without effect on the carbachol-mediated contractile response, but had a significant inhibitory effect on the response to histamine. However, the presence of both PDE inhibitors caused essentially 100% inhibition (N.B. resting tension 0.5 g) of the histamine-evoked contraction, and a significant 44% inhibition of that evoked by carbachol (Figure 5a).

In the absence of extracellular Ca^{2+} (Ca^{2+}_e) both carbachol (1 μM) and histamine (30 μM) caused transient, phasic contractions of BTSM strips. Pretreatment with rolipram (100 μM), or ORG 9935 (1 μM) had no significant effect on the subsequent phasic contraction elicited by either spasmogen (Figure 5b). However, co-addition of rolipram + ORG 9935 significantly attenuated the contractions elicited by carbachol and histamine by 40% and >90%, respectively (Figure 5b). As the phasic contraction of airways smooth muscle is considered to involve the generation of Ins(1,4,5)P_3 and mobilization of intracellular Ca^{2+}-stores (Hashimoto *et al*., 1985; Miller-Hance *et al*., 1988; Chilvers *et al*,. 1989b), we investigated whether the attenuation of this response by combined PDE inhibition might involve an action at the level of Ins(1,4,5)P_3 mass generation.

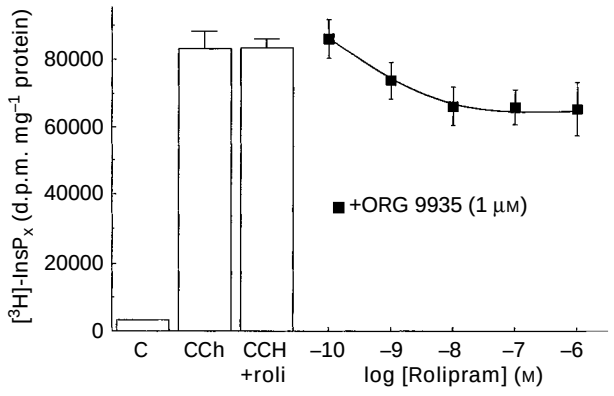

Figure 4 Concentration-dependence of the effect of PDE3/4 inhibition on carbachol-stimulated [^{3}H]-InsP_X accumulation. BTSM slices were prepared and radiolabelled with [^{3}H]-inositol as described in the Methods section. Slices were incubated in KHB (containing 5 mM LiCl) and 100 μM rolipram (CCh + roli), or ORG 9935 (1 μM) and the indicated concentrations of rolipram for 30 min before addition of carbachol (10 μM) for a further 30 min. The effects of vehicle (C) or carbachol-only (CCh) additions are also shown. Incubations were terminated and [^{3}H]-InsP_X accumulation determined as described in the Methods section. Data are shown as means with vertical lines indicating s.e.mean for 3 separate experiments performed in triplicate.

Inhibitory effects of combined PDE3/PDE4 inhibition on spasmogen-stimulated Ins(1,4,5)P_3 mass generation

As previously shown (Chilvers *et al*., 1989b; 1991), carbachol (100 μM) caused a rapid, transient increase in Ins(1,4,5)P_3 mass which was maximal within 5 s and had returned to basal levels by 60 s (Figure 6). In contrast to the extremely transient nature of the second messenger response, it is known that total [^{3}H]-InsP_X accumulation continues at an apparently constant rate for >30 min (Chilvers *et al*., 1991). Pretreatment with rolipram (100 μM), or ORG 9935 (1 μM) had no significant effect on the basal Ins(1,4,5)P_3 level or the subsequent Ins(1,4,5)P_3 response to carbachol (at 5 s: +CCh, 22.1 ± 1.2;

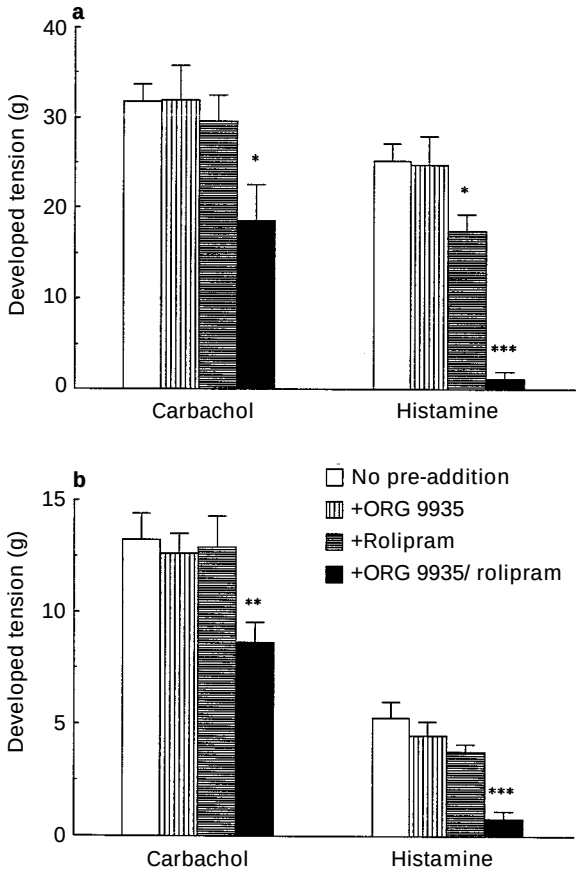

Figure 5 Effects of PDE3/4 inhibition on contractile responses to carbachol and histamine. Contractile responses of BTSM strips were monitored as described in the Methods section. In (a), strips were incubated with ORG 9935 (1 μM), rolipram (10 μM) or a combination of both agents (ORG 9935/rolipram) for 30 min before construction of concentration-effect curves for carbachol or histamine. Only data for the effects seen at 1 μM carbachol and 30 μM histamine are shown. In (b), strips also were incubated with ORG 9935 (1 μM), rolipram (10 μM) or a combination of both agents (ORG 9935/rolipram) for 30 min. However, for the final 10 min the medium was changed to a Ca^{2+}-free KHB containing 2 mM EGTA. Carbachol (1 μM) or histamine (30 μM) was added and the initial peak of the phasic contractions recorded. Data are shown as means ± s.e.mean for at least 3 separate BTSM preparations performed in triplicate. Statistically significant differences from the respective control spasmogen-stimulated contractile response are indicated as $*P<0.05$; $**P<0.01$; $***P<0.001$.

+ORG 9935/CCh, 22.4±1.7; +rolipram/CCh, 20.2±1.6 pmol mg^{-1} protein; $n=4$). However, co-addition of rolipram+ORG 9935 significantly attenuated the Ins(1,4,5)P_3 response (Figure 6), decreasing the initial response to carbachol by at least 50%.

The effect of PDE3/PDE4 inhibition was evident at all concentrations of carbachol examined (Figure 7a), and in the presence of ORG 9935 (1 μM), rolipram caused a concentration-dependent inhibition of carbachol-stimulated Ins(1,4,5)P_3 response ($-\log EC_{50}$ (M), 6.77±0.21; $n=4$: Figure 7b).

Effects of $[Ca^{2+}]_e$ and PDE3/4 inhibition on Ins(1,4,5)P_3 and cyclic AMP responses to carbachol

Comparison of the inhibitory effects of ORG 9935 and rolipram on carbachol-stimulated Ins(1,4,5)P_3 generation over an initial 30 s exposure period was also made in the presence and absence of $Ca^{2+}{}_e$ (Table 1). In nominally Ca^{2+}-free

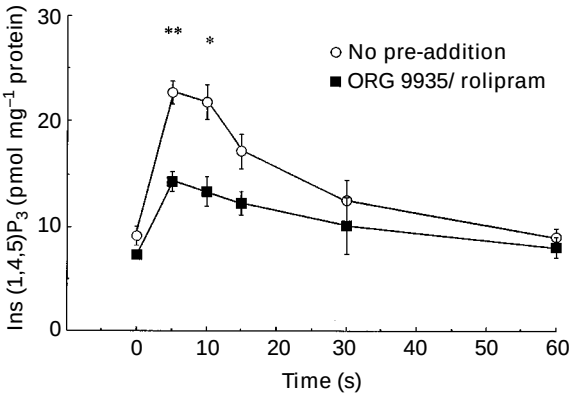

Figure 6 Effect of PDE3/4 inhibition on the time-course of carbachol-stimulated Ins(1,4,5)P_3 mass accumulation. BTSM slices were prepared as described in the Methods section. Slices were incubated in the absence or presence of rolipram (100 μM)+ORG 9935 (1 μM) for 30 min, followed by challenge with carbachol (100 μM) for the times indicated. Incubations were terminated and Ins(1,4,5)P_3 concentration determined as described in the Methods section. Data are shown as means with vertical lines indicating s.e.mean for 5 separate experiments performed in triplicate. Statistically significant differences for ±PDE3/4 inhibition at a given time-point are indicated as $*P<0.05$; $**P<0.01$.

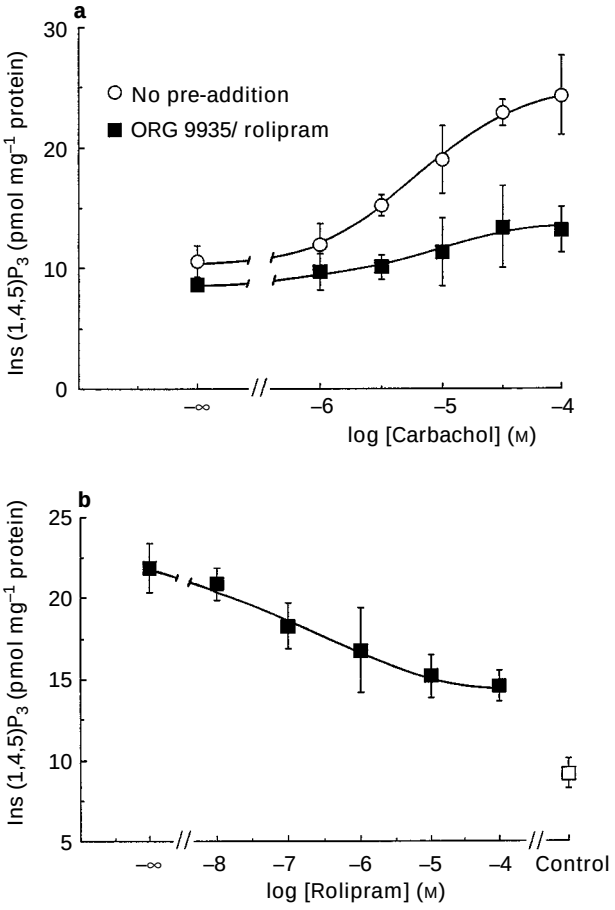

Figure 7 Characterization of the effects of PDE3/4 inhibition on carbachol-stimulated Ins(1,4,5)P_3 mass accumulation. BTSM slices were prepared as described in the Methods section. In (a), slices were incubated in the absence or presence of rolipram (100 μM)+ORG 9935 (1 μM) for 30 min, followed by challenge with the indicated concentrations of carbachol for 10 s. In (b), slices were incubated with ORG 9935 (1 μM) and the indicated concentration of rolipram for 30 min before addition of carbachol (100 μM) for 10 s. The basal level of Ins(1,4,5)P_3 is also shown (Control). Incubations were terminated and Ins(1,4,5)P_3 concentration determined as described in the Methods section. Data are shown as means with vertical lines indicating s.e.mean for 3 (a) or 4 (b) separate experiments performed in triplicate.

Table 1 The effects of PDE3/4 inhibition and extracellular Ca^{2+} concentration on the levels of Ins(1,4,5)P_3 and cyclic AMP immediately after addition of carbachol to bovine tracheal smooth muscle slices

	$[Ca^{2+}]_e$=1.3 mM		*Nominally Ca^{2+}-free*	
	No pre-addition	*+Rolipram/ORG 9935*	*No pre-addition*	*+Rolipram/ORG 9935*
Ins (1,4,5)P_3 (pmol mg^{-1} protein)				
Control	9.2±0.9	7.4±0.4	5.9±1.2	6.6±0.8
+CCh 5 s	22.6±1.1	14.3±0.9**	18.3±2.1	8.8±1.6*
+CCh 10 s	21.7±1.6	13.4±1.4**	16.9±1.9	8.4±2.2*
+CCh 30 s	9.1±0.8	8.1±1.0	N/D	N/D
Cyclic AMP (pmol mg^{-1} protein)				
Control	3.4±0.2	12.0±0.8	4.2±0.5	18.8±1.4
+CCh 5 s	2.8±0.4	9.4±1.6	3.6±0.4	16.2±2.0
+CCh 10 s	3.0±0.3	5.8±0.7††	4.0±0.4	17.9±2.0
+CCH 30 s	3.8±0.5	10.6±1.0	3.9±0.4	18.4±2.4

Bovine tracheal smooth muscle slices were incubated in the absence or presence of rolipram (100 μM)+ORG 9935 (1 μM) for 30 min before addition of carbachol (CCh; 100 μM) or vehicle for the times indicated. Neutralized tissue extracts were analysed for Ins (1,4,5)P_3 and cyclic AMP content as described in the Methods section. Data are shown as means±s.e.mean for 4 ($+Ca^{2+}$) or 3 ($-Ca^{2+}$) separate experiments performed in triplicate. Statistical analysis was performed by use of Student's t test (unpaired observations). Statistically significant differences with respect to the effects of rolipram+ORG 9935 on carbachol-stimulated increases in Ins (1,4,5)P_3 are shown as $*P<0.05$; $**P<0.01$; and for the effect of carbachol addition on cyclic AMP levels as $††P<0.01$.

medium basal and carbachol-stimulated Ins(1,4,5)P_3 levels were slightly lower in BTSM slices, although a rapid and robust 2–3 fold increase in second messenger concentration was observed under both conditions. The presence of PDE3/4 inhibitors exerted a more profound effect on carbachol-stimulated Ins(1,4,5)P_3 generation in the absence (82% inhibition) compared to the presence (49% inhibition) of $Ca^{2+}{}_e$ (Table 1). In addition to monitoring changes in Ins(1,4,5)P_3, cyclic AMP levels were also assessed. The presence of ORG 9935 and rolipram appeared to cause a greater increase in cyclic AMP levels in the absence, compared to the presence, of $Ca^{2+}{}_e$. Furthermore, whilst under normal conditions ($[Ca^{2+}]_e = 1.3$ mM) carbachol addition caused an immediate and marked (67% at 10 s) decrease in BTSM cyclic AMP levels in the presence of PDE3/4 inhibition, when Ca^{2+} was removed from the medium carbachol addition failed to decrease cyclic AMP concentration (Table 1). Thus, it is possible that the effectiveness of PDE3/4 inhibition to attenuate carbachol-stimulated Ins(1,4,5)P_3 accumulation may depend on whether carbachol addition *per se* can exert an action (dependent upon $[Ca^{2+}]_e$) to decrease BTSM cyclic AMP concentration.

Discussion

Previous studies by a number of groups have catalogued the expression of multiple isoenzymes of cyclic nucleotide PDE in airways smooth muscle from various species, including dog (Torphy & Cieslinski, 1990), cow (Giembycz & Barnes, 1991; Shahid *et al*., 1991) and man (de Boer *et al*., 1992; Rabe *et al*., 1993; Torphy *et al*., 1993). In addition, functional consequences of isoenzyme-selective PDE inhibition on inherent and spasmogen-stimulated tone have been investigated in airways smooth muscle preparations. Although the relative importance of PDE3 and PDE4 in regulating cyclic AMP levels varies between species (Tomkinson *et al*., 1993), it is clear from studies in bovine and human airways that combined inhibition of PDE3/4 is more effective in relaxing inherent (Rabe *et al*., 1993) or spasmogen-induced tone (Shahid *et al*., 1991; de Boer *et al*., 1992; Torphy *et al*., 1993) than inhibition of either isoenzyme alone. Similar synergistic PDE3/4 interactions have also been observed in vascular smooth muscle (Eckly & Lugnier, 1994; Pan *et al*., 1994).

In the present study we have used isoenzyme-selective inhibitors to investigate the effects of types 3 and 4 PDE inhibition on cyclic AMP levels and phosphoinositide responses, in conjunction with functional assessment in BTSM. In agreement with previous functional studies, and the limited information available regarding second messenger regulation by PDE inhibitors (Hall *et al*., 1989a,b; Offer *et al*., 1991), we have shown that combined PDE3 and PDE4 inhibition has greater-than-additive effects upon all responses measured. Thus, whilst either PDE3 or PDE4 inhibition alone did not significantly effect cyclic AMP levels in BTSM, combined PDE3/4 inhibition resulted in a marked increase in this second messenger. Similarly, although the PDE4-selective inhibitor rolipram at high concentrations caused a similar maximal inhibition of histamine-stimulated [^{3}H]-InsP_X accumulation to that elicited by combined PDE3/4 inhibition, the IC_{50} for the action of this agent was 70–80 fold lower in the presence, compared to the absence, of the PDE-selective inhibitor ORG 9935. The effects of PDE inhibition were less dramatic with respect to the carbachol-stimulated [^{3}H]-InsP_X response. However, a significant inhibitory effect was observed in the presence of both PDE3- and 4-selective inhibitors, which was not seen in the presence of either agent alone.

Despite the modest effects of PDE inhibition (or any cyclic AMP-elevating manipulation) on mACh receptor-mediated responses in BTSM, we have demonstrated for the first time that the carbachol-stimulated Ins(1,4,5)P_3 mass response can be significantly affected by combined PDE3/4 inhibition, whilst selective inhibition of either PDE3 or PDE4 was without effect. The initial spasmogen-evoked increase in Ins(1,4,5)P_3 was attenuated by 50% in the absence of maximally-effective concentrations of rolipram and ORG 9935, and this correlated with a similar marked effect of combined PDE3/4 inhibition on carbachol-stimulated phasic contraction in BTSM strips. Furthermore, if the conditions under which the functional effects were examined were more closely adhered to in the second messenger studies (i.e. in the absence of $Ca^{2+}{}_e$) a greater (>80%) inhibitory effect on spasmogen-stimulated Ins(1,4,5)P_3 accumulation could be observed. Thus, in contrast to previous (and present) observations made by measuring [^{3}H]-InsP_X accumulation (assessed over a 30 min period in the presence of Li^+), where this index of phosphoinositide turnover was relatively unaffected by PDE inhibition, the initial generation of the pathway second messenger was much more dramatically affected by combined PDE3/4 inhibition.

Why should these two indices of phosphoinositide turnover provide such contrasting conclusions regarding the susceptibility of the spasmogen-stimulated response to PDE inhibition? It has been proposed that the inhibition of histamine-stimulated [^{3}H]-InsP_X accumulation by different agents, including β-adrenoceptor agonists, PDE inhibitors, K^+-channel openers and voltage-gated Ca^{2+}-channel antagonists, occurs via a common mechanism (Challiss *et al*., 1992; 1993). The discrepant effectiveness of such agents on histamine- and carbachol-stimulated [^{3}H]-InsP_X accumulation is at least in part explained by the fact that the sustained phosphoinositide hydrolysis caused by mACh receptors is much less sensitive to changes in $Ca^{2+}{}_e$ (Chilvers *et al*., 1994). Furthermore, it should be noted that time-course studies have shown that inhibition of [^{3}H]-InsP_X accumulation only becomes evident 2–10 min after histamine challenge, even if BTSM is preincubated (for ⩽30 min) with agents which elevate cyclic AMP levels or exert other inhibitory effects (Hall & Hill, 1988; Challiss *et al*., 1992). This suggests that whilst agents which limit Ca^{2+}-entry can inhibit sustained agonist-stimulated [^{3}H]-InsP_X accumulation, the relevance of this to the modulation of functional smooth muscle responses remains unclear. We have shown previously that whilst agonist-stimulated [^{3}H]-InsP_X accumulation (in the presence of Li^+) and inositol phospholipid hydrolysis are sustained for long periods (⩾30 min), the agonist-stimulated increase in Ins(1,4,5)P_3 occurs only transiently and returns to basal or sub-basal levels within 30–60 s of agonist addition (Chilvers *et al*., 1989b; 1991). Thus although the modest inhibition of carbachol-stimulated [^{3}H]-InsP_X accumulation by ORG 9935 and rolipram may be brought about through a direct or indirect inhibitory effect on Ca^{2+}-entry during the sustained phase of phosphoinositide hydrolysis, such a mechanism cannot explain the more dramatic effect on Ins(1,4,5)P_3 generation.

At present it is unclear how PDE3/4 inhibition results in an attenuation of Ins(1,4,5)P_3 generation stimulated by mACh receptor activation. An inhibitory effect of cyclic AMP-elevating agents on muscarinic receptor-stimulated Ca^{2+}-mobilization has been demonstrated in bovine and canine airways smooth muscle (Takuwa *et al*., 1988; Ozaki *et al*., 1990; Hoiting *et al*., 1996). However, this effect has been considered to occur independently of changes in muscarinic receptor-stimulated Ins(1,4,5)P_3 generation, as this process has been assumed, until now, to be insensitive to modulation by

cyclic AMP. The present data strongly suggests that modulation of Ins(1,4,5)P_3 concentration may also contribute to the attenuation of spasmogen-stimulated Ca^{2+}-mobilization. It is interesting to note that changes in both Ins(1,4,5)P_3 and cyclic AMP levels occur when BTSM is challenged with carbachol in the presence of ORG 9935 and rolipram. Thus, whilst PDE3/4 inhibition attenuates carbachol-stimulated Ins(1,4,5)P_3 generation, mACh receptor activation also appears simultaneously to affect tissue cyclic AMP levels, causing a rapid and transient decrease. It is possible that the reduction in cyclic AMP concentration results from activation of M_2-mACh receptor leading to inhibition of adenylyl cyclase activity (Challiss *et al*., 1993). However, the inhibitory effect of carbachol appears to require the presence of $Ca^{2+}{}_e$ as no significant decrease in cyclic AMP levels was observed when BTSM was exposed to carbachol in nominally Ca^{2+}-free medium. Furthermore, the lack of a suppressant effect of carbachol on cyclic AMP levels coincides with a greater inhibitory effect of PDE3/4 inhibition on spasmogen-stimulated Ins(1,4,5)P_3 generation. One possible explanation for these observations is that increases in cyclic AMP caused by selective inhibition of PDE3/4 can profoundly inhibit carbachol-stimulated Ins(1,4,5)P_3, but this effect is fully observed only under Ca^{2+}-free conditions. Thus, in the presence of $Ca^{2+}{}_e$ mACh receptor activation causes a decrease in cyclic AMP levels and this reduces the extent of the inhibitory effect on agonist-stimulated Ins(1,4,5)P_3 generation. The Ca^{2+}-dependent modulation of cyclic AMP by mACh receptors could result from effects on cyclic AMP synthesis by adenylyl cyclase or degradation, for example by Ca^{2+}-stimulated PDE (Nicholson *et al*., 1991).

In conclusion, the present study has demonstrated that the pathway by which muscarinic receptor activation can generate Ins(1,4,5)P_3, leading to Ca^{2+}-mobilization and airways smooth muscle contraction, can be inhibited by combined isoenzyme-selective inhibition of PDE3 and 4 activities, but not separate inhibition of either activity alone. Thus, in contrast to a recent study in mesangial cells where PDE3 and 4 activities were shown to modulate distinct signalling pathways through compartmentation of PDE activities to cellular cyclic AMP microenvironments (Chini *et al*., 1997), inhibition of PDE3 and 4 activities can modulate a common cyclic AMP pool in airways smooth muscle. Although the precise locus at which cyclic AMP modulates the initial rate of phosphoinositide hydrolysis has not been defined, the present data suggest that selective PDE inhibition can modify the contractile response in airways smooth muscle via effects on Ins(1,4,5)P_3 generation.

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References

BEAVO, J.A. (1995). Cyclic nucleotide phosphodiesterases: Functional implications of multiple isoforms. *Physiol. Rev.,* **75,** 725–748.

BROWN, B.L., ALBANO, J.D.M., ELKINS, R.P., SGHERZI, A.M. & TAMPION, W. (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3′,5′-cyclic monophosphate. *Biochem. J.,* **121,** 561–562.

CHALLISS, R.A.J., ADAMS, D., MISTRY, R. & BOYLE, J.P. (1993). Second messenger and ionic modulation of agonist-stimulated phosphoinositide turnover in airway smooth muscle. *Biochem. Soc. Trans.,* **21,** 1138–1145.

CHALLISS, R.A.J., BATTY, I.H. & NAHORSKI, S.R. (1988). Mass measurements of inositol 1,4,5-trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem. Biophys. Res. Commun.,* **157,** 684–691.

CHALLISS, R.A.J., PATEL, N. & ARCH, J.R.S. (1992). Comparative effects of BRL 38227, nitrendipine and isoprenaline on carbachol- and histamine-stimulated phosphoinositide metabolism in airway smooth muscle. *Br. J. Pharmacol.,* **105,** 997–1003.

CHILVERS, E.R., BARNES, P.J. & NAHORSKI, S.R. (1989a). Characterization of agonist-stimulated incorporation of *myo*-[^{3}H]-inositol into inositol phospholipids and [^{3}H]-inositol phosphate formation in tracheal smooth muscle. *Biochem. J.,* **262,** 739–746.

CHILVERS, E.R., BATTY, I.H., CHALLISS, R.A.J., BARNES, P.J. & NAHORSKI, S.R. (1991). Determination of mass changes in phosphatidylinositol 4,5-bisphosphate and evidence for agonist-stimulated metabolism of inositol 1,4,5-trisphosphate in airway smooth muscle. *Biochem. J.,* **275,** 373–379.

CHILVERS, E.R., CHALLISS, R.A.J., BARNES, P.J. & NAHORSKI, S.R. (1989b). Mass changes in inositol 1,4,5-trisphosphate in trachealis muscle following agonist stimulation. *Eur. J. Pharmacol.,* **164,** 587–590.

CHILVERS, E.R., CHALLISS, R.A.J., WILLCOCKS, A.L., POTTER, B.V.L., BARNES, P.J. & NAHORSKI, S.R. (1990). Characterization of stereospecific binding sites for inositol 1,4,5-trisphosphate in airway smooth muscle. *Br. J. Pharmacol.,* **99,** 297–302.

CHILVERS, E.R., LYNCH, B.J., OFFER, G.J. & CHALLISS, R.A.J. (1994). Effects of membrane depolarization and changes in intra- and extracellular calcium concentration on phosphoinositide hydrolysis in bovine tracheal smooth muscle. *Biochem. Pharmacol.,* **47,** 2171–2179.

CHINI, C.C.S., GRANDE, J.P., CHINI, E.N. & DOUSA, T.P. (1997). Compartmentalization of cyclic AMP signalling in mesangial cells by phosphodiesterase isozymes PDE3 and PDE4. *J. Biol. Chem.,* **272,** 9854–9859.

DE BOER, J., PHILPOTT, A.J., VAN AMSTERDAM, R.G.M., SHAHID, M., ZAAGSMA, J. & NICHOLSON, C.D. (1992). Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: biochemical and pharmacological analysis using selective inhibitors. *Br. J. Pharmacol.,* **106,** 1028–1034.

ECKLY, A.E. & LUGNIER, C. (1994). Role of phosphodiesterase III and IV in the modulation of vascular cyclic AMP content by the NO/cyclic GMP pathway. *Br. J. Pharmacol.,* **113,** 445–450.

ELLIS, K.E., MISTRY, R., BOYLE, J.P. & CHALLISS, R.A.J. (1995). Correlation of cyclic AMP accumulation and relaxant actions of salmeterol and salbutamol in bovine tracheal smooth muscle. *Br. J. Pharmacol.,* **116,** 2510–2516.

GIEMBYCZ, M.A. & BARNES, P.J. (1991). Selective inhibition of a high affinity type IV cyclic AMP phosphodiesterase in bovine trachealis by AH-21-132. *Biochem. Pharmacol.,* **42,** 663–677.

GIEMBYCZ, M.A. & RAEBURN, D. (1991). Putative substrates for cyclic nucleotide-dependent protein kinases and the control of airway smooth muscle tone. *J. Auton. Pharmacol.,* **11,** 365–398.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1989a). Inhibition of histamine-stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle. *Br. J. Pharmacol.,* **97,** 603–613.

HALL, I.P., DONALDSON, J. & HILL, S.J. (1989b). Modulation of carbachol-induced inositol phosphate formation in bovine tracheal smooth muscle by cyclic AMP phosphodiesterase inhibitors. *Biochem. Pharmacol.,* **39,** 1357–1363.

HALL, I.P. & HILL, S.J. (1988). β-Adrenoceptor stimulation inhibits histamine-stimulated inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br. J. Pharmacol.,* **95,** 1204–1212.

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,** 191–199.

HOITING, B.H., MEURS, H., SCHUILING, M., KUIPERS, R., ELZINGA, C.R.S. & ZAAGSMA, J. (1996). Modulation of agonist-induced phosphoinositide metabolism, Ca^{2+}-signalling and contraction of airway smooth muscle by cyclic AMP-dependent mechanisms. *Br. J. Pharmacol.,* **117,** 419–426.

KOTLIKOFF, M.I. & KAMM, K.E. (1996). Molecular mechanisms of β-adrenergic relaxation of airway smooth muscle. *Annu. Rev. Physiol.,* **58,** 115–141.

LANGLANDS, J.M. & DIAMOND, J. (1992). Translocation of protein kinase C in bovine tracheal smooth muscle strips: the effect of methacholine and isoprenaline. *Eur. J. Pharmacol.,* **227,** 131–138.

MADISON, J.M. & BROWN, J.K. (1988). Differential inhibitory effects of forskolin, isoproterenol, and dibutyrykl cyclic adenosine monophosphate on phosphoinositide hydrolysis in canine tracheal smooth muscle. *J. Clin. Invest.,* **82,** 1462–1465.

MILLER-HANCE, W.C., MILLER, J.R., WELLS, J.N., STULL, J.T. & KAMM, K.E. (1988). Biochemical events associated with activation of smooth muscle contraction. *J. Biol. Chem.,* **263,** 13979–13982.

NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.,* **12,** 19–27.

OFFER, G.J., CHILVERS, E.R. & NAHORSKI, S.R. (1991). β-Adrenoceptor-induced inhibition of muscarinic receptor-stimulated phosphoinositide metabolism is agonist specific in bovine tracheal smooth muscle. *Eur. J. Pharmacol.,* **207,** 243–248.

OZAKI, H., KWON, S.C., TAJIMI, M. & KARAKI, H. (1990). Changes in cytosolic Ca^{2+} and contraction induced by various stimulants and relaxants in canine tracheal smooth muscle. *Pflügers Arch.,* **416,** 351–359.

PAN, X., ARAUZ, E., KRZANOWSKI, J.J., FITZPATRICK, D.F. & POLSON, J.B. (1994). Synergistic interactions between selective pharmacological inhibitors of phosphodiesterase isozyme families PDE III and PDE IV to attenuate proliferation of rat vascular smooth muscle cells. *Biochem. Pharmacol.,* **48,** 827–835.

QIAN, Y., NALINE, E., KARLSSON, J.-A., RAEBURN, D. & ADVENIER, C. (1993). Effects of rolipram and siguazodan on the human isolated bronchus and their interaction with isoprenaline and sodium nitroprusside. *Br. J. Pharmacol.,* **109,** 774–778.

RABE, K.F., TENOR, H., DENT, G., SCHUDT, C., LIEBIG, S. & MAGNUSSEN, H. (1993). Phosphodiesterase isozymes modulating inherent tone in human airways: identification and characterization. *Am. J. Physiol.,* **264,** L458–L464.

RASMUSSEN, H., KELLEY, G. & DOUGLAS, J.S. (1990). Interactions between Ca^{2+} and cyclic AMP messenger systems in regulation of airway smooth muscle contraction. *Am. J. Physiol.,* **258,** L279–L288.

ROFFEL, A.F., MEURS, H., ELZINGA, C.R.S. & ZAAGSMA, J. (1990). Characterization of the muscarinic receptor subtype involved in phosphoinositide metabolism in bovine tracheal smooth muscle. *Br. J. Pharmacol.,* **99,** 293–296.

SCHEID, C.R., HONEYMAN, T.W. & FAY, F.S. (1979). Mechanisms of β-adrenergic relaxation of smooth muscle. *Nature,* **277,** 32–36.

SCHRAMM, C.M. & GRUNSTEIN, M.M. (1992). Assessment of signal transduction mechanisms regulating airway smooth muscle contractility. *Am. J. Physiol.,* **262,** L119–L139.

SHAHID, M., VAN AMSTERDAM, R.G.M., DE BOER, J., TEN BERGE, R.E., NICHOLSON, C.D. & ZAAGSMA, J. (1991). The present of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br. J. Pharmacol.,* **104,** 471–477.

TAGLIENTE, T.M., DALTON, B.A. & BEN-HARARI, R.R. (1992). Phasic responses to carbachol in isolated guinea-pig trachea are augmented by cooling and inhibited by nifedipine. *J. Pharmacol. Exp. Ther.,* **261,** 755–763.

TAKUWA, Y., TAKUWA, N. & RASMUSSEN, H. (1986). Carbachol induces a rapid and sustained hydrolysis of polyphosphoinositide in bovine tracheal smooth muscle: Measurements of the mass of polyphosphoinositides, 1,2-diacylglycerol, and phosphatidic acid. *J. Biol. Chem.,* **261,** 14670–14675.

TAKUWA, Y., TAKUWA, N. & RASMUSSEN, H. (1988). The effects of isoproterenol on intracellular calcium concentration. *J. Biol. Chem.,* **263,** 762–768.

TOMKINSON, A., KARLSSON, J.-A. & RAEBURN, D. (1993). Comparison of the effects of selective inhibitors of phosphodiesterase types III and IV in airways smooth muscle with differing β-adrenoceptor subtypes. *Br. J. Pharmacol.,* **108,** 57–61.

TORPHY, T.J. (1994). β-adrenoceptors, cyclic AMP and airway smooth muscle relaxation: Challenges to the dogma. *Trends Pharmacol. Sci.,* **15,** 370–373.

TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol. Pharmacol.,* **37,** 206–214.

TORPHY, T.J., UNDEM, B.J., CIESLINSKI, L.B., LUTTMANN, M.A., REEVES, M.L. & HAY, D.W.P. (1993). Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. *J. Pharmacol. Exp. Ther.,* **265,** 1213–1223.

TORPHY, T.J., ZHENG, C., PETERSON, S.M., FISCUS, R.R., RINARD, G.A. & MAYER, S.E. (1985). Inhibitory effect of methacholine on drug-induced relaxation, cyclic AMP accumulation, and cyclic AMP-dependent protein kinase activation in canine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.,* **233,** 409–417.

YANG, K.X.F. & BLACK, J.L. (1995). The involvement of protein kinase C in the contraction of human airway smooth muscle. *Eur. J. Pharmacol.,* **275,** 283–289.

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