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Modulation of agonist-induced phosphoinositide metabolism, Ca^{2+} signalling and contraction of airway smooth muscle by cyclic AMP-dependent mechanisms

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1 The effects of increased cellular cyclic AMP levels induced by isoprenaline, forskolin and 8-bromo-adenosine 3':5'-cyclic monophosphate (8-Br-cyclic AMP) on phosphoinositide metabolism and changes in intracellular Ca^{2+} elicited by methacholine and histamine were examined in bovine isolated tracheal smooth muscle (BTSM) cells.

2 Isoprenaline (pD_2 ($-\log_{10} EC_{50}$) $= 6.32 \pm 0.24$) and forskolin ($pD_2 = 5.6 \pm 0.05$) enhanced cyclic AMP levels in a concentration-dependent fashion in these cells, while methacholine ($pD_2 = 5.64 \pm 0.12$) and histamine ($pD_2 = 4.90 \pm 0.04$) caused a concentration-related increase in [^{3}H]-inositol phosphates (IP) accumulation in the presence of 10 mM LiCl.

3 Preincubation of the cells (5 min, 37°C) with isoprenaline (1 μM), forskolin (10 μM) and 8-Br-cyclic AMP (1 mM) did not affect the IP accumulation induced by methacholine, but significantly reduced the maximal IP production by histamine (1 mM). However, the effect of isoprenaline was small (15.0 ± 0.6% inhibition) and insignificant at histamine concentrations between 0.1 and 100 μM.

4 Both methacholine and histamine induced a fast (max. in 0.5-2 s) and transient increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a sustained phase lasting several minutes. EGTA (5 mM) attenuated the sustained phase, indicating that this phase depends on extracellular Ca^{2+}.

5 Preincubation of the cells (5 min, 37°C) with isoprenaline (1 μM), forskolin (10 μM) and 8-Br-cyclic AMP (1 μM) significantly attenuated both the Ca^{2+}-transient and the sustained phase generated at equipotent IP producing concentrations of 1 μM methacholine and 100 μM histamine (approx. 40% of maximal methacholine-induced IP response), but did not affect changes in $[Ca^{2+}]_i$ induced by 100 μM methacholine (95.2 ± 3.5% of maximal methacholine-induced IP response).

6 Significant correlations were found between the isoprenaline-induced inhibition of BTSM contraction and inhibition of Ca^{2+} mobilization or influx induced by methacholine and histamine, that were similar for each contractile agonist.

7 These data indicate that (a) cyclic AMP-dependent inhibition of Ca^{2+} mobilization in BTSM cells is not primarily caused by attenuation of IP production, suggesting that cyclic AMP induced protein kinase A (PKA) activation is effective at a different level in the $[Ca^{2+}]_i$ homeostasis, (b) that attenuation of intracellular Ca^{2+} concentration plays a major role in β-adrenoceptor-mediated relaxation of methacholine- and histamine-induced airway smooth muscle contraction, and (c) that the relative resistance of the muscarinic agonist-induced contraction to β-adrenoceptor agonists, especially at (supra) maximal contractile concentrations is largely determined by its higher potency in inducing intracellular Ca^{2+} changes.

Keywords: Phosphoinositide metabolism; intracellular calcium; contraction; cyclic AMP; methacholine; histamine; isoprenaline; forskolin; 8-Br-cyclic AMP; airway smooth muscle cells

Introduction

Both in animal (Baron *et al.*, 1984; Meurs *et al.*, 1988; Van Amsterdam *et al.*, 1989; Roffel *et al.*, 1990) and in human (Meurs *et al.*, 1989; Van Amsterdam *et al.*, 1990) airway smooth muscle it has been well established that phosphoinositide (PI) metabolism is involved in the contraction induced by muscarinic agonists and histamine. Muscarinic M_3 or histamine H_1 receptor stimulation leads to activation of phosphoinositide-specific phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 triggers the release of Ca^{2+} from intracellular stores, producing a transient rise in $[Ca^{2+}]_i$ which initiates contraction via activation of a Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK) and subsequent phosphorylation of myosin (Hashimoto *et al.*, 1985; Murray & Kotlikoff, 1991; De Lanerolle *et al.*, 1991). Receptor-mediated Ca^{2+} influx causes a sustained rise in $[Ca^{2+}]_i$, which is considered to be involved in the tonic component of contraction (Murray & Kotlikoff, 1991).

Conversely, elevation of intracellular cyclic AMP concentration and subsequent activation of PKA is involved in airway smooth muscle relaxation (Zhou *et al.*, 1992), which may involve phosphorylation of a number of effector proteins that cause either reduction of $[Ca^{2+}]_i$ and/or reduction of MLCK sensitivity to Ca^{2+}-calmodulin (De Lanerolle *et al.*, 1991; Abdel-Latif, 1991).

Both in canine (Madison & Brown, 1988) and bovine (Hall & Hill, 1988; Hall *et al.*, 1989; Offer *et al.*, 1991) tracheal smooth muscle slices, it has recently been demonstrated that β-adrenoceptor agonists and other cyclic AMP elevating agents such as forskolin and dibutyryl cyclic AMP cause a marked attenuation of histamine-induced IP formation. By contrast, no such effects were observed when IP formation was induced

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by full muscarinic agonists such as methacholine and carbachol, even at low agonist concentrations (Hall & Hill, 1988; Madison & Brown, 1988; Hall *et al.*, 1989; Offer *et al.*, 1991). Since airway smooth muscle contractions to these muscarinic agonists are relatively resistant to relaxation by β-adrenoceptor agonists compared with contractions induced by histamine (Russell, 1984; Jenne *et al.*, 1987; Van Amsterdam *et al.*, 1989), it may be suggested that this resistance is related to the lack of effect of β-agonists on muscarinic agonist-induced IP formation.

However, the contribution of inhibition of PI metabolism to a reduced intracellular Ca^{2+} mobilization and subsequent relaxation by cyclic AMP-elevating agents is at present unclear. In the present study, we addressed this question by investigating the effects of isoprenaline, forskolin and 8-Br-cyclic AMP on IP production and intracellular Ca^{2+} mobilization induced by methacholine and histamine in isolated BTSM cells. In addition, we investigated the functional relevance of the isoprenaline-induced biochemical effects for the β-agonist-induced relaxation of methacholine- and histamine-induced contraction of isolated BTSM strips.

Methods

Isolation of tracheal smooth muscle cells

Fresh bovine tracheae were obtained from the slaughter house and were transported to the laboratory within 30 min at room temperature in Krebs-Henseleit (KH) buffer of the following composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25.0, glucose 5.5, pregassed with 95% O_2 and 5% CO_2; pH 7.4. The tracheal smooth muscle was dissected carefully and smooth muscle strips were prepared free of mucosa and connective tissue in KH buffer at room temperature. The following procedure was performed under sterile conditions. The strips were chopped with a McIlwain tissue chopper, twice at a setting of 500 μm, followed by three times at a setting of 100 μm. The tissue particles were washed four times (600 *g*, 5 min) in sterile Krebs-Ringer-Henseleit (KRH) buffer containing (mM); NaCl 125.0, KCl 6.0, $MgCl_2$ 2.5, $CaCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.0, HEPES 25.0, pH 7.4, supplemented with 2.0 mM ($\pm$)-dithiothreitol (DTT) and were then resuspended in a mixture of collagenase (62 u ml^{-1}), papain (30 u ml^{-1}) and trypsin-inhibitor (1 mg ml^{-1}) in KRH buffer. This suspension was incubated at 37°C in an incubator shaker (Innova 4000) at 55 r.p.m. After 20 min, the cells were gently dispersed with a wide-bored pipette and the suspension was incubated for another 10 min at 37°C at 77 r.p.m. Subsequently, the suspension was filtered over a 50 μm gauze and the cells were collected by centrifugation (1000 *g*, 10 min). The isolated BTSM cells were washed three times in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 7 mM $NaHCO_3$ and 10 mM HEPES, pH 7.4 at 37°C and cultured overnight (18–20 h) in a suspension of $0.5-1.5 \times 10^6$ cells ml^{-1} at 37°C. The cell viability was >90% as assessed by trypan blue exclusion.

Mechanical responses

Trachealis muscle was dissected carefully and smooth muscle strips (12 × 3 mm) were prepared free of mucosa and serosal connective tissue. These strips were mounted for isotonic recording in KH-buffer solution (gassed with 95% O_2/5% CO_2, 37°C, pH 7.4) with a preload of 500 mg. After a 60 min equilibration period the strips were precontracted twice with methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 μM, respectively) with 60 min washing periods in between. Maximal relaxation was established with isoprenaline (0.1 μM), immediately followed by a 30 min washing period. To construct dose-relaxation curves the strips were precontracted with different concentrations of methacholine (1 and 100 μM) or histamine (100 μM), building up the smooth muscle tone in four to eight concentration steps. When the muscle tone was established, isoprenaline was added cumulatively. When the maximal response to isoprenaline had been obtained, the preparations were washed twice and maximal relaxation was re-established with 10 and 100 μM isoprenaline.

Determination of cyclic AMP

Cells were washed two times (1000 *g*, 5 min) and resuspended in DMEM without FCS at a density of $5-8 \times 10^6$ cells ml^{-1}. Cell samples (50 μl) were incubated with various concentrations of isoprenaline or forskolin in DMEM (10 μl) for 5 min at 37°C. The reaction was stopped by addition of 60 μl 3% perchloric acid (PCA). The samples were neutralized with 120 μl of a 50% saturated $KHCO_3$ solution. The neutralized samples were centrifuged (2000 *g*, 15 min) and cyclic AMP was determined in the supernatant by a competitive protein-binding assay (Brown *et al.*, 1971)

[^{3}H]-inositol phosphates determination

Freshly isolated cells ($15-20 \times 10^6$ in 10 ml) were loaded with [^{3}H]-inositol (100 μCi) to steady state in inositol-free DMEM containing 10% FCS for 20 h at 37°C.

After the labelling period, cells were washed (1000 *g*, 5 min) in DMEM and resuspended in the same buffer containing 10 mM LiCl at a density 8×10^6 cells ml^{-1}. Aliquots of 50 μl cell suspension were preincubated for 10 min at 37°C, followed by the addition of 50 μl of various concentrations of methacholine or histamine in DMEM as indicated. Where used, isoprenaline, forskolin and 8-Br-cyclic AMP were added 5 min before the contractile agonists. Reactions were terminated at 25 min after methacholine or histamine addition by adding 750 μl ice-cold chloroform/methanol/HCl (20:40:1, v/v/v) and the samples were stored at −20°C. Prior to the analysis, 300 μl water was added to the samples. After vigorous vortex mixing and centrifugation (2000 *g*, 5 min), 800 μl of the upper phase, containing the [^{3}H]-IP, was removed and applied to a column, containing 500 μl Dowex AG 1X8 anion exchange resin in the formate form. Columns were washed with 10 ml of water to remove [^{3}H]-inositol, followed by a wash with 10 ml 5 mM disodium tetraborate/30 mM sodium formate to remove [^{3}H]-glycerophosphoinositol. Total ^{3}H-labelled IP was eluted with 10 ml 1.2 M ammonium formate/0.2 M formic acid. One ml of this eluate was added to 4 ml of scintillation cocktail (Plasmasol, Packard, Groningen, The Netherlands) and counted for radioactivity.

Intracellular Ca^{2+} measurements

Cells were washed three times (1000 *g*, 5 min) with KRH, pH 7.4, supplemented with 2.5 mg ml^{-1} bovine serum albumin (KRH/BSA). The cells ($1-2 \times 10^6$ ml^{-1}) were resuspended in KRH/BSA and were incubated with the fluorescent dye, Fura-2/AM (3 μM) for 30 min at 37°C. After being loaded, the cells were washed three times in KRH/BSA and resuspended to a density of 3×10^5 cells ml^{-1}. The cells were kept at room temperature and used within 2–4 h, during which they remained viable and responsive. The Fura-2 fluorescence of the cells (excitation wavelengths: 340 and 380 nm; emission wavelength: 510 nm) was measured at 37°C with a Perkin Elmer Spectrometer (LS-50B). Each cuvette contained 2 ml of magnetically stirred cell suspension and contractile agonists were added in a volume of 20 μl. Where used, cyclic AMP-elevating agents were added 5 min prior to the contractile agonists. The $[Ca^{2+}]_i$ was calculated every 0.2 s according to Grynkiewicz *et al.* (1985). At the end of the experiment the maximal fluorescence ratio (R_{max}) was determined after adding 0.01% of Triton X-100 as a permeabilizing agent. The minimal fluorescence ratio (R_{min}) was determined by addition of 5 mM EGTA to the permeabilized cells.

Data analysis

The transient rise of $[Ca^{2+}]_i$ was expressed both as the maximal increase of $[Ca^{2+}]_i$ above basal and as the area under the $[Ca^{2+}]_i$-time curve above basal during 1 min after addition of agonist ($AUC_{0-60\,s}$), calculated by trapezoid integration over 0.2 s periods. The plateau level of $[Ca^{2+}]_i$ was expressed as the value obtained 2 min after contractile agonist additon.

All data are presented as means ± s.e. mean from n separate experiments. The statistical significance of differences between data was assessed by Student's t test for paired or unpaired observations as appropriate. The level of significance was set at $P=0.05$.

Materials

Fura 2/AM, papain and 8-Br-cyclic AMP were obtained from Boehringer Mannheim (Germany). Collagenase (type 1A), methacholine chloride, histamine dihydrochloride, (−)-isoprenaline and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.) and foetal calf serum from GIBCO BRL Life Technologies (Paisley, U.K.). [^{3}H]-myoinositol (specific radioactivity, 45–60 Ci mmol^{-1}) and [^{3}H]-cyclic AMP (specific radioactivity, 50–60 Ci mmol^{-1}) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of analytical grade.

Results

Isoprenaline- and forskolin-induced cyclic AMP production in BTSM cells

Incubation of the BTSM cells with isoprenaline (10 nM–100 μM) and forskolin (10 nM–100 μM) resulted in a dose-dependent production of cyclic AMP (Table 1). In order to study the effect of these agents on methacholine- and histamine-induced IP and Ca^{2+} responses, submaximal cyclic AMP-generating concentrations of isoprenaline (1 μM) and forskolin (10 μM) were used. The cyclic AMP responses at these concentrations were 5.3 ± 1.8 and 24.7 ± 7.0 pmol cyclic AMP per 10^6 cells above basal (5.7 ± 0.5 pmol per 10^6 cells), respectively.

Effect of isoprenaline, forskolin and 8-Br cyclic AMP on the methacholine- and histamine-induced IP accumulation in BTSM cells

Table 2 shows the maximal responses and pD_2 values of methacholine (100 nM–1 mM)- and histamine (100 nM–1 mM)-induced IP accumulation in BTSM cells. As previously found in BTSM slices (Van Amsterdam *et al.*, 1989), methacholine showed a higher efficacy accumulating IP than histamine, with the maximal response of histamine being 48.3 ± 5.3% of the maximal response to methacholine.

Table 2 Maximal effect (E_{max}) and potency (pD_2) of methacholine- and histamine-induced total inositol phosphates accumulation in bovine isolated tracheal smooth muscle cells

Stimulus	E_{max} (△ d.p.s. per 10^6 cells)	pD_2 (−log M)
Methacholine	4719 ± 403	5.64 ± 0.12
Histamine	1961 ± 421	4.90 ± 0.04

Results are means ± s.e.mean of 4–5 experiments. E_{max} is expressed as d.p.s. per 10^6 cells above basal (2391 ± 486 d.p.s. per 10^6 cells) and represents the response to the maximal effective concentration of both agonists (1 mM).

Table 1 Maximal effect (E_{max}) and potency (pD_2) of isoprenaline- and forskolin-induced cyclic AMP production in bovine isolated tracheal smooth muscle cells

Stimulus	E_{max} (△ pmol per 10^6 cells)	pD_2 (−log M)
Isoprenaline	8.3 ± 0.5	6.32 ± 0.24
Forskolin	27.6 ± 8.7	5.63 ± 0.05

Results are means ± s.e.mean of 5 experiments. E_{max} is expressed as pmol cyclic AMP per 10^6 cells above basal cyclic AMP level (5.7 ± 0.5 pmol per 10^6 cells) and represents the response to the maximal effective concentration of both agonists (100 μM).

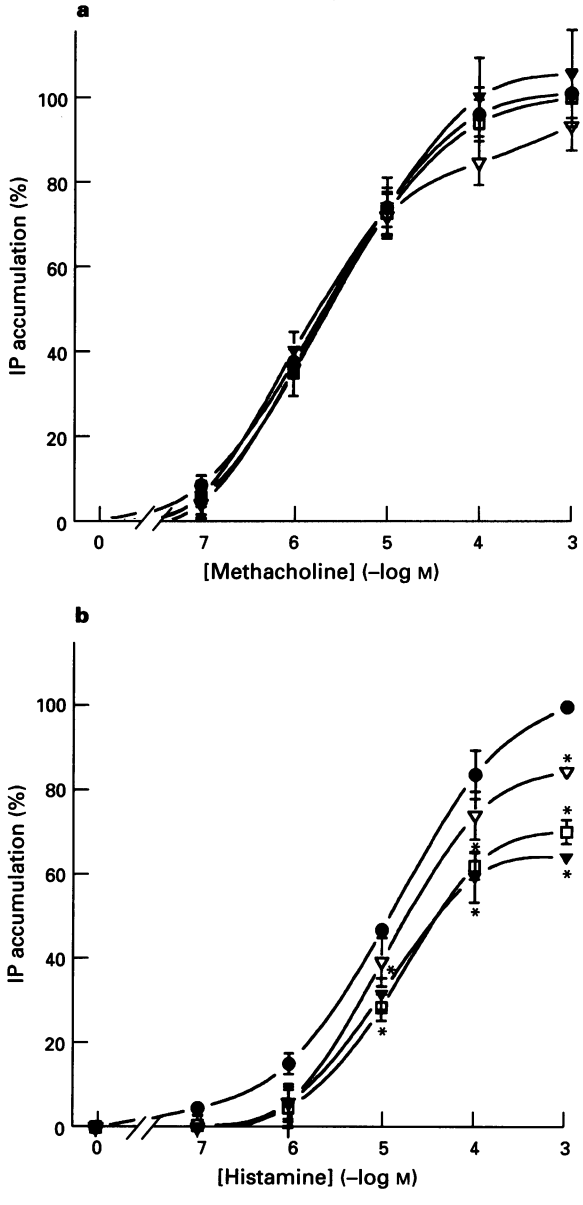

Figure 1 The effects of isoprenaline, forskolin and 8-Br-cyclic AMP on methacholine- (a) and histamine- (b) induced IP accumulation in bovine isolated tracheal smooth muscle cells. Cells were preincubated for 5 min in the absence (●) or presence of 1 μM isoprenaline (▽), 10 μM forskolin (▼) or 1 mM 8-Br-cyclic AMP (□) followed by the addition of various concentrations of methacholine or histamine as indicated; 100% IP production in the presence of methacholine and histamine was 4719 ± 403 and 1961 ± 421 d.p.s. per 10^6 cells above basal level (2391 ± 486 d.p.s. per 10^6 cells), respectively. *$P<0.05$ compared with controls. Means ± s.e. mean of 4–5 experiments.

Preincubation of the cells with isoprenaline (1 μM), forskolin (10 μM) and 8-Br-cyclic AMP (1 mM) did not significantly affect methacholine-induced IP accumulation at any agonist concentration used (Figure 1a), neither was there an effect on basal IP accumulation in the absence of contractile agonist. By contrast, the maximal IP response to histamine was significantly reduced by these agents to 85.0±0.6% ($n=4$; $P<0.05$), 64.4±1.3% ($n=4$; $P<0.05$) and 70.5±2.9% ($n=4$; $P<0.05$), respectively (Figure 1b), without a significant effect on the pD_2-value for histamine. However, in contrast to for-

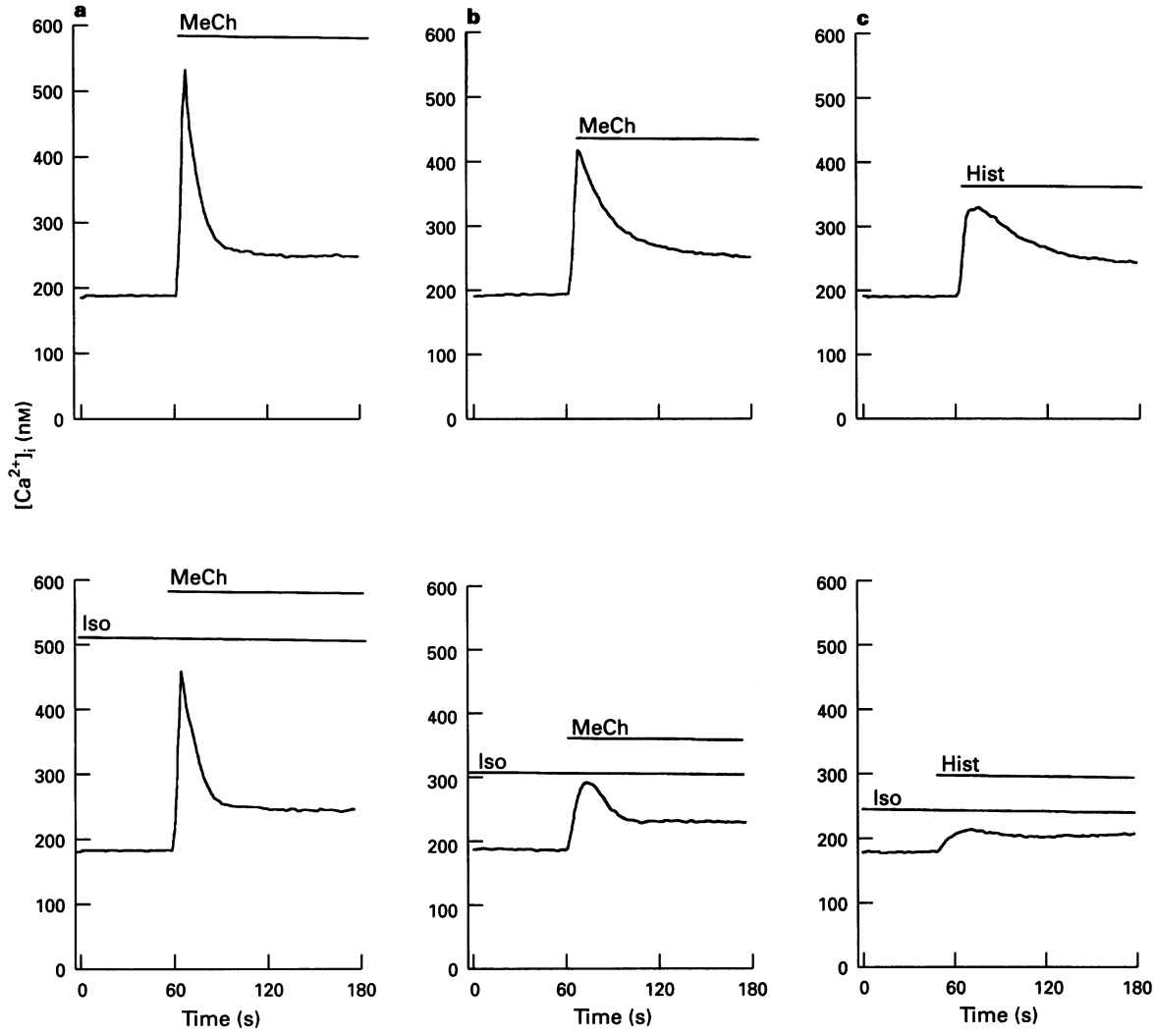

Figure 2 The effects of 1 μM isoprenaline (Iso) on 100 μM (a) and 1 μM (b) methacholine (MeCh)- and 100 μM histamine (Hist)- (c) induced changes in $[Ca^{2+}]_i$ in bovine isolated tracheal smooth muscle cells. The presence of the agonists is indicated by the horizontal bars.

Table 3 Transient and sustained rise in $[Ca^{2+}]_i$ in bovine isolated tracheal smooth muscle cells induced by methacholine and histamine

	Transient rise in $[Ca^{2+}]_i$		*Sustained rise in $[Ca^{2+}]_i$*
Stimulus	*Peak rise* (△ nM)	*AUC_{0-60s}* (nM s)	(△ nM)
Methacholine (100 μM)	417±55	38938±2356	89±10
Methacholine (1 μM)	185±24**	28052±3209*	80±9
Histamine (100 μM)	128±20***/#	21873±4146**/#	62±9*/#

Results are means±s.e.mean of 9–10 experiments. Peak rise and sustained rise in $[Ca^{2+}]_i$ are expressed as nM above basal $[Ca^{2+}]_i$ (188±22 nM). AUC_{0-60s}: area under the curve above prestimulatory level of the Ca^{2+} transient between 0 and 60 s after agonist addition. Statistical analysis: *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to 100 μM methacholine; # not significant compared to 1 μM methacholine.

skolin and 8-Br-cyclic AMP, the isoprenaline-induced inhibition of IP accumulation was statistically significant only at the highest concentration of histamine (1 mM).

Effect of isoprenaline, forskolin and 8-Br-cyclic AMP on the methacholine- and histamine-induced changes in $[Ca^{2+}]_i$ in BTSM cells

Figure 2 (upper panels) shows representative Ca^{2+} responses of BTSM cells to methacholine (1 μM and 100 μM) and histamine (100 μM). Exposure of the cells to both agonists induced a rapid and transient rise in $[Ca^{2+}]_i$, reaching a peak after 0.5–2 s. For both agonists, the transient was followed by a sustained rise in $[Ca^{2+}]_i$ which was reached at approximately 60 s after agonist delivery and which was maintained for at least 15 min (not shown).

The Ca^{2+} transient (peak rise in $[Ca^{2+}]_i$ and $AUC_{0-60\,s}$) as well as the sustained rise in $[Ca^{2+}]_i$ for 100 μM histamine were significantly lower than those for 100 μM methacholine (Table 3), as may be expected from the observed difference in IP accumulation induced by these agonists (43.1±7.1% and 95.2±3.5% of methacholine maximum, respectively, $P<0.01$). For 1 μM methacholine, which was equipotent with 100 μM histamine with respect to IP accumulation (37.0±2.9% of methacholine maximum), the values of peak-rise in $[Ca^{2+}]_i$, $AUC_{0-60\,s}$ and sustained rise in $[Ca^{2+}]_i$ tended to be slightly higher than those of 100 μM histamine (Table 3).

Addition of 5 mM EGTA to the extracellular medium abolished the sustained phase of the Ca^{2+} responses to both methacholine and histamine (Figure 3), indicating that the latter phase depends on the influx of extracellular Ca^{2+}.

Incubation of the BTSM cells with isoprenaline (1 μM) caused a marked attenuation of both the Ca^{2+}-transient and the sustained rise in $[Ca^{2+}]_i$ induced by 1 μM methacholine and 100 μM histamine (Figures 2b and c, lower panels). This effect was virtually absent for the responses induced by 100 μM methacholine (Figure 2a, lower panel). Figure 4 shows that the Ca^{2+}-transient (Figure 4a) and the sustained rise in $[Ca^{2+}]_i$ (Figure 4b) induced by 100 μM histamine as well as 1 μM methacholine were significantly inhibited by isoprenaline (1 μM), forskolin (10 μM) and 8-Br-cyclic AMP (1 mM) to similar extents, whereas the responses induced by 100 μM methacholine were not significantly affected by these cyclic AMP-elevating agents; neither was there an effect of these agents on the basal $[Ca^{2+}]_i$ [188±22 nM versus 185±20, 180±25 and 180±32 nM in the presence of isoprenaline (1 μM), forskolin (10 μM) and 8-Br-cyclic AMP (1 mM), respectively].

Relationship between isoprenaline-induced inhibition of contraction and intracellular Ca^{2+} changes induced by methacholine and histamine

As is shown in Figure 5a, the potency (pD_2) and the maximal effect (E_{max}) of isoprenaline-induced relaxation of BTSM strips

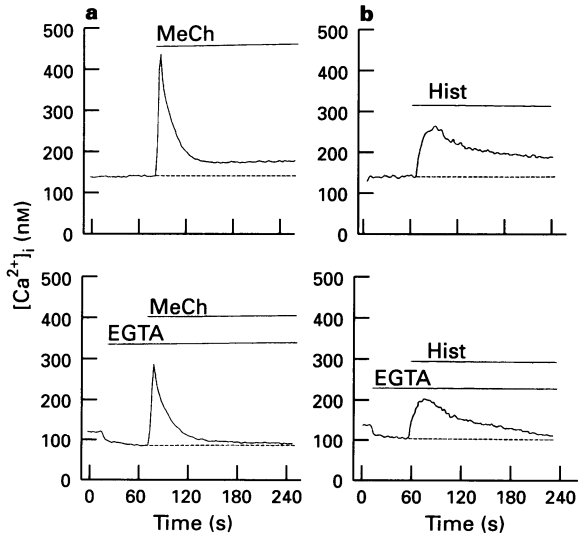

Figure 3 The effects of 5 mM EGTA on 1 μM methacholine (MeCh)- (a) and 100 μM histamine (Hist)- (b) induced changes in $[Ca^{2+}]_i$ in bovine isolated tracheal smooth muscle cells. The presence of EGTA and agonists is indicated by the horizontal bars.

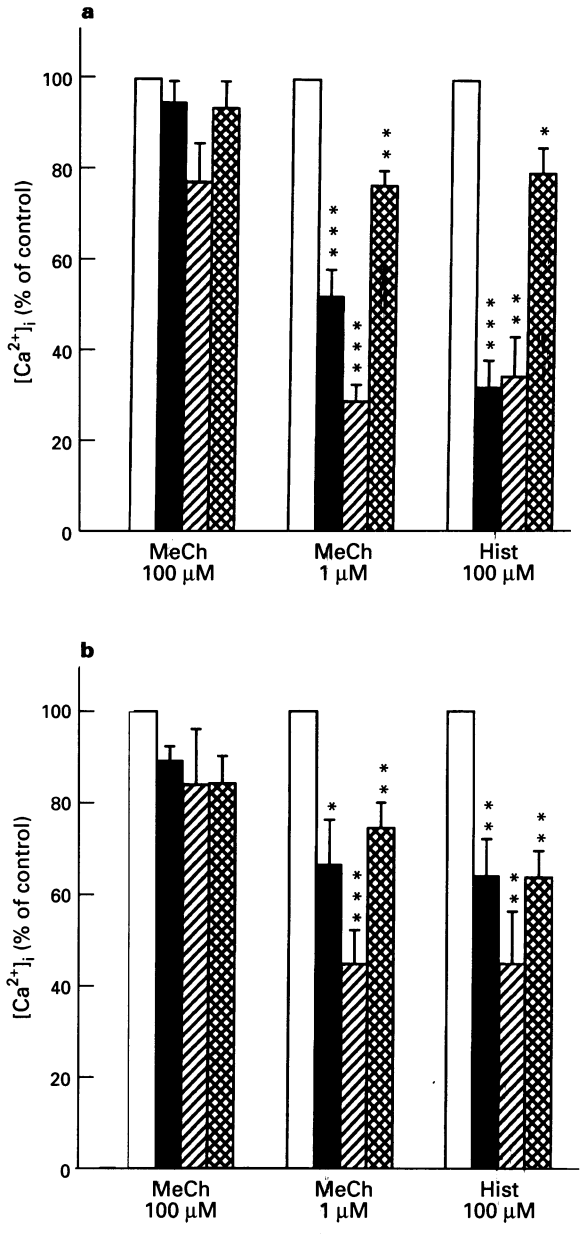

Figure 4 The effects of isoprenaline, forskolin and 8-Br-cyclic AMP on methacholine- and histamine-induced transient rise (a) and plateau level (b) of $[Ca^{2+}]_i$ in bovine isolated tracheal smooth muscle cells. Cells were preincubated for 5 min in the absence (open columns) or presence of 1 μM isoprenaline (solid columns), 10 μM forskolin (hatched columns) or 1 mM 8-Br-cyclic AMP (cross-hatched columns) followed by the addition of 100 μM or 1 μM methacholine (MeCh) or 100 μM histamine (Hist). The Ca^{2+}-transients were measured as an area under the curve above prestimulatory level between 0 and 60 s after addition of the contractile agonist. Plateau levels of intracellular calcium were measured 2 min after the addition of this agonist. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with controls. Means ±s.e. mean of 5 experiments.

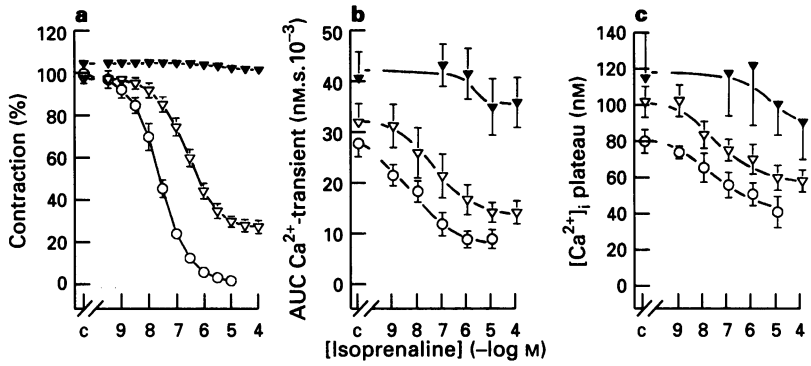

Figure 5 The dose-dependent effect of isoprenaline on 100 μM (▼) and 1 μM (▽) methacholine- and 100 μM histamine (○)-induced contraction (a), and transient rise (b) and plateau level (c) of $[Ca^{2+}]_i$ in bovine isolated tracheal smooth muscle cells. Contraction is expressed as percentage of the response to 0.1 mM methacholine in each experiment. The Ca^{2+}-transients were measured as an area under the curve above prestimulatory level between 0 and 60 s after addition of the contractile agonist. Plateau levels of intracellular calcium were measured 2 min after the addition of this agonist. Means ± s.e. mean of 4–5 experiments.

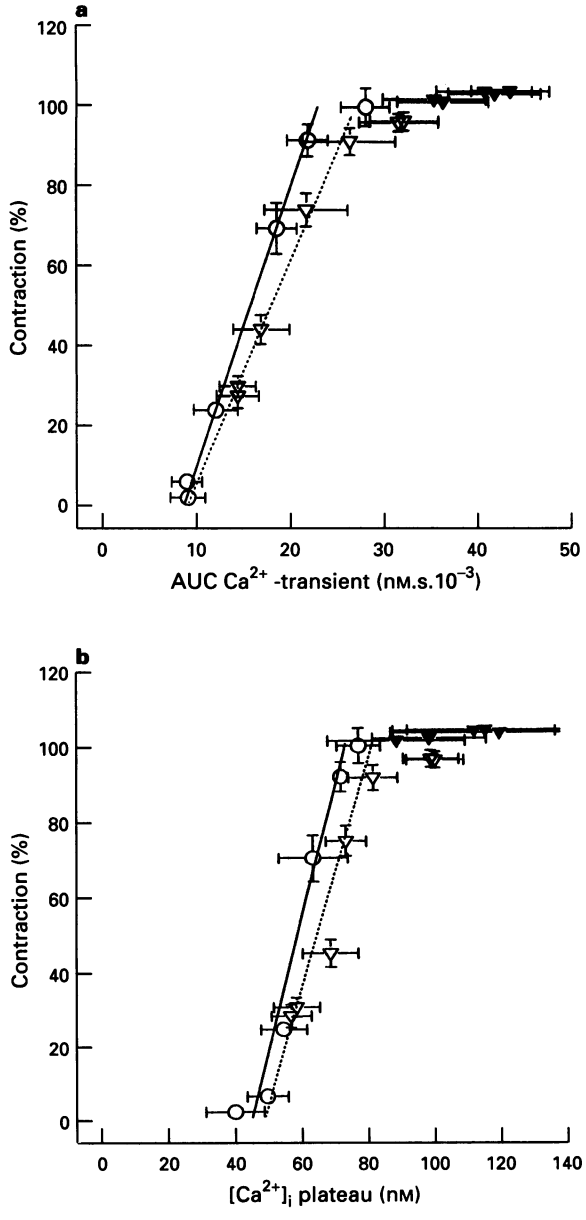

Figure 6 Relationship between isoprenaline-induced inhibition of contraction and inhibition of transient rise (a) or plateau level (b) of $[Ca^{2+}]_i$ in the presence of 100 μM (▼) and 1 μM (▽) methacholine and 100 μM (○) histamine. Increasing concentrations of isoprenaline were used as indicated in Figures 6b and 6c.

depended largely on the concentration and nature of the contractile agonist used. At comparable maximal contraction levels both the pD_2 values (8.1 ± 0.4 and 6.9 ± 0.2; $P<0.05$) and E_{max} values (99.9 ± 0.5% and 80.1 ± 3.2%; $P<0.05$) of isoprenaline were significantly different for 100 μM histamine and 1 μM methacholine, respectively (Figure 5a). At the supramaximal contractile concentration of 100 μM methacholine, only a very small relaxation was observed, with a pD_2 value of 5.4 ± 0.1 and an E_{max} of 7.6 ± 1.1% (Figure 5a). Figures 5b and 5c show the inhibition of 1 and 100 μM methacholine- and 100 μM histamine-induced Ca^{2+}-transient and plateau levels. As with the relaxation, the rank order of efficacy for isoprenaline in inhibiting the rise of contractile agonist-induced intracellular Ca^{2+} mobilization and influx was 100 μM histamine > 1 μM methacholine > 100 μM methacholine.

Figure 6 shows that significant linear correlations were found between the isoprenaline-induced inhibition of tracheal smooth muscle contraction and the isoprenaline-induced inhibition of the Ca^{2+}-transient as well as the plateau level induced by histamine ($r=0.999$, $P<0.001$ and $r=0.968$, $P<0.05$, respectively) and methacholine ($r=0.994$, $P<0.01$ and $r=0.972$, $P<0.05$, respectively). For both contractile agonists similar relationships were observed (Figure 6). It is also shown that inhibition of the Ca^{2+}-transient down to approximately 25,000 nM s and the Ca^{2+}-plateau down to approximately 30 nM does not result in relaxation, indicating the intracellular Ca^{2+} reserve for maximal contraction.

Discussion

In isolated BTSM cells that had been cultured overnight, we confirmed previous observations on bovine (Hall & Hill, 1988; Offer *et al.*, 1991) and canine (Madison & Brown, 1988) tracheal smooth muscle slices that β-adrenoceptor agonists such as isoprenaline and other agents which either raise intracellular cyclic AMP (forskolin) or act as a cyclic AMP analogue (8-Br-cyclic AMP) inhibit histamine-induced IP accumulation, whereas the IP response to full muscarinic agonists such as methacholine or carbachol is unaffected by these agents even at low concentrations of these agonists.

The cause of the differential susceptibility of agonist-stimulated PI metabolism to cyclic AMP elevating agents is at present unclear. The differential effects on histamine- and muscarinic agonist-induced PI metabolism cannot be explained by M_2-muscarinic receptor-mediated inhibition of adenylyl cyclase that has been observed in bovine (Meurs *et al.*, 1993) and canine (Jones *et al.*, 1987) tracheal smooth muscle cells, since methacholine-induced PI metabolism was also shown to be resistant to cyclic AMP elevations induced by the membrane permeant analogues of cyclic AMP, dibutyryl-cyclic

AMP and 8-Br-cyclic AMP (Madison & Brown, 1988; Hall *et al.*, 1989; this study). Interestingly, in BTSM slices it was recently shown that combined inhibition of type III and type IV phosphodiesterase can inhibit methacholine-induced IP_3 and total IP responses; however, the involvement of cyclic AMP in this process is as yet uncertain (Challiss *et al.*, 1995).

In BTSM tissue slices, a resistance to β-adrenoceptor inhibition was also found for the partial IP-producing muscarinic agonist, oxotremorine; however, the IP response to pilocarpine, another partial muscarinic agonist was inhibited (Offer *et al.*, 1991).

With the assumption that all muscarinic agonists act through the same (M_3-)receptor subtype, these results are difficult to explain. Based on the current receptor theory, one would predict a shift to the right of the full agonist concentration-response curves and a suppression of the maximal effects of partial agonists. One possible mechanism for the differential inhibition of PI metabolism by cyclic AMP could be the involvement of different G-proteins and/or PLC isoenzymes in the responses of histamine and the different muscarinic agonists, with only the histamine- and pilocarpine-stimulated G-protein(s) and/or PLC(s) being sensitive to PKA (Offer *et al.*, 1991).

As compared to the previous observations in bovine and canine tracheal smooth muscle slices, the inhibitory effect of isoprenaline (1 μM) on the histamine-induced IP accumulation was small and only significant at a histamine concentration of 1 mM ($15 \pm 0.6\%$, $P<0.05$). The cause of this discrepancy between isolated cells and tissue is at present unclear, but appears not to be related to a reduced isoprenaline-induced cyclic AMP production in the cells, since both the pD_2 (6.32 ± 0.24) and the E_{max} (2.21 ± 0.24 fold stimulation above basal cyclic AMP concentration) values were quite comparable to those found for bovine (our data, not shown) and canine (Zhou *et al.*, 1992) tracheal smooth muscle slices. In addition, we found that the pD_2 values and the relative E_{max} values of methacholine- and histamine-induced IP accumulation were similar to previous observations in BTSM slices (Van Amsterdam *et al.*, 1989), indicating that these cells are very useful for investigation of (cross-talk between) signal transduction mechanisms of histamine H_1 receptors, muscarinic receptors and β-adrenoceptors. This is in contrast to immobilized cell cultures, which selectively lose the M_3 muscarinic receptor function over one or more passages (Yang *et al.*, 1991; Daykin *et al.*, 1993).

In contrast to the effects of the different cyclic AMP stimulants on PI metabolism, we found a marked inhibitory effect on both methacholine- and histamine-induced Ca^{2+} mobilization. Methacholine and histamine induced a rapid transient increase in $[Ca^{2+}]_i$ in the BTSM cells, which was followed by a steady-state $[Ca^{2+}]_i$ that was substantially higher than the resting level. Both the transient phase (due to release from intracellular stores, as indicated by its resistance to 5 mM EGTA) and the sustained phase (due to influx of extracellular Ca^{2+}, as indicated by its attenuation by 5 mM EGTA) of methacholine- and histamine-induced Ca^{2+}-mobilization were inhibited by cyclic AMP-elevating agents. As observed with airway smooth muscle contraction in this and in previous (Russell, 1984; Jenne *et al.*, 1987; Van Amsterdam *et al.*, 1989) studies, the inhibitory effects were strongly dependent on the concentration and nature of the contractile agonist used, with the higher concentration of methacholine (100 μM) being resistant to cyclic AMP-mediated inhibition as compared to the same concentration of histamine (100 μM) and the lower concentration of methacholine (1 μM) used. Accordingly, we found a striking and similar correlation between the isoprenaline-induced inhibition of BTSM contraction and inhibition of Ca^{2+} mobilization or influx induced by both contractile agonists, indicating that attenuation of $[Ca^{2+}]_i$ plays a major role in β-adrenoceptor-mediated relaxation of methacholine- and histamine-induced airway smooth muscle contraction. The similarity of this relationship for methacholine- and histamine-induced contractions indicates that this mechanism occurs irrespective of the agonist and that the relative resistance of the muscarinic agonist-induced contraction to β-adrenoceptor agonists, especially at (supra)maximal contractile concentrations, is largely determined by its efficacy in inducing PI metabolism and subsequent changes in $[Ca^{2+}]_i$. As previously also shown for PI metabolism (Meurs *et al.*, 1988), we found evidence for a large reserve of Ca^{2+} mobilization and influx for methacholine-induced contraction at the supramaximal concentration of 100 μM, which is not inhibited sufficiently by the β-agonist to cause substantial relaxation.

Inhibition of muscarinic agonist-induced Ca^{2+} mobilization by isoprenaline and/or forskolin has been observed previously by Takuwa *et al.* (1988) and Taylor *et al.* (1989) in BTSM strips and cultured BTSM cells, respectively, and by Ozaki *et al.* (1990) in canine isolated tracheal smooth muscle strips. As also reported by Ozaki *et al.* (1990), we found that isoprenaline (1 μM) and forskolin (10 μM) did not significantly change the resting $[Ca^{2+}]_i$. This is in contrast to earlier reports of Takuwa *et al.* (1988) and Felbel *et al.* (1988) in bovine trachea, who found, in the absence of muscarinic agonist, a paradoxical increase in $[Ca^{2+}]_i$ induced by isoprenaline and/or forskolin, without stimulating contraction. The cause of the discrepancy is at present unclear, but could be related to preferential measurement of high $[Ca^{2+}]_i$ areas in the peripheral cytoplasm induced by β-agonists under the conditions used, thus leading to an overestimation of the average $[Ca^{2+}]_i$ (Ozaki *et al.*, 1990; Kajita & Yamaguchi, 1993; Yamaguchi *et al.*, 1995).

Since the β-adrenoceptor-mediated inhibition of 1 μM methacholine- and 100 μM histamine-induced Ca^{2+} responses as well as the forskolin- and 8-Br-cyclic AMP-induced inhibition of the 1 μM methacholine-induced Ca^{2+} responses were not associated with significant inhibitory effects on PI metabolism, it can be concluded that cyclic AMP-activated PKA is mainly effective at a different level of the Ca^{2+} homeostasis in BTSM. There are a number of possible mechanisms for PKA-induced attenuation of Ca^{2+} mobilization. First, PKA may lower $[Ca^{2+}]_i$ by stimulating Ca^{2+} extrusion from the cells via activation of the plasmalemmal Ca^{2+}-ATPase pump (Suematsu *et al* 1984; Velema *et al.*, 1983) or by sequestration of Ca^{2+} into the intracellular Ca^{2+} store (Twort *et al.*, 1989). In rat brain it was recently shown that PKA may phosphorylate and activate IP_3 3-kinase (Sim *et al.*, 1990), which may thus attenuate the accumulation of IP_3 (but not of total IP) and subsequently decrease the release of Ca^{2+}. However, the latter possibility seems not to be involved in BTSM, since it was recently shown that isoprenaline did not inhibit the histamine-induced IP_3 transient in BTSM slices (Challiss & Boyle, 1994). Another potential target for PKA regulation of agonist-induced Ca^{2+} mobilization is the IP_3-receptor. Thus, in rat brain it has been shown that PKA phosphorylates the IP_3-receptor on the intracellular stores, which did not markedly affect the IP_3 binding characteristics, but which caused a marked inhibition of the IP_3-mediated Ca^{2+} release from these stores (Volpe & Alderson-Lang, 1990). Very interestingly, it was recently shown that cyclic AMP elevating agents may cause inhibition of IP_3 binding to rabbit tracheal smooth muscle membranes (Grunstein *et al.*, 1994).

In conclusion, the results of the present study indicate that the cyclic AMP-dependent inhibition of Ca^{2+} mobilization and subsequent relaxation of BTSM are not primarily caused by attenuation of IP production, indicating that other mechanisms are involved in the differential sensitivity of muscarinic agonist and histamine-induced contractions to β-adrenoceptor mediated relaxation. These mechanisms may be related to the different potencies of both agonists in inducing PI metabolism and subsequent changes in $[Ca^{2+}]_i$.

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