The effects of elevated cyclic AMP levels on histamine-H₁-receptor-stimulated inositol phospholipid hydrolysis and calcium mobilization in the smooth-muscle cell line DDT₁MF-2

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The effects of raising cyclic AMP levels, by forskolin stimulation, β -adrenoceptor activation or cyclic AMP phosphodiesterase inhibition, on inositol phospholipid hydrolysis and increases in intracellular free $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) elicited by a range of agonists have been investigated in the hamster vas deferens smooth-muscle cell line DDT₁MF-2. Isoprenaline ($\log [EC_{50} (M)] = -7.7 \pm 0.2$), forskolin and the type IV cyclic AMP phosphodiesterase inhibitor rolipram elicited significant increases in the accumulation of cyclic $[^3H]AMP$. Pretreatment with forskolin (10 μM) attenuated histamine (100 μM)- and *N*⁶-cyclopentyladenosine (CPA; 300 nM)-induced release of intracellular Ca^{2+} , observed when cells are stimulated in Ca^{2+} -free buffer containing 0.1 mM EGTA. Forskolin had no effect on ATP (100 μM)- or bradykinin (1 μM)-stimulated release of intracellular Ca^{2+} . Histamine-induced intracellular Ca^{2+} release was also inhibited by pretreatment with rolipram (100 μM) or the membrane-permeant

cyclic AMP analogue (*S*_p)-adenosine 3',5'-monophosphothioate (100 μM). Isoprenaline (1 μM) pretreatment (in the presence of 10 μM rolipram, a concentration which on its own did not decrease the histamine response) attenuated histamine-induced intracellular Ca^{2+} release. Forskolin inhibited histamine (100 μM)- and CPA (100 nM) stimulated accumulation of $[^3H]$ -inositol phosphates, but was without effect on ATP or bradykinin responses. Addition of forskolin (in the presence of 100 μM rolipram) after the cells had been stimulated with histamine (in experiments initiated in Ca^{2+} -free buffer) inhibited the rise in $[Ca^{2+}]_i$ observed when extracellular Ca^{2+} (2 mM) was re-applied (owing to receptor-mediated Ca^{2+} influx). Finally, the refilling of intracellular Ca^{2+} stores (after receptor-mediated Ca^{2+} influx is blocked by mepyramine) can be demonstrated in the presence of raised cyclic AMP levels.

INTRODUCTION

In mammalian cells, numerous membrane-bound receptors are coupled to phospholipase C, via a regulatory G-protein, which upon activation hydrolyses the plasma-membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdInsP₂), producing the two second messengers inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol [1–3]. In smooth muscle, InsP₃ triggers the release of Ca^{2+} from intracellular stores, producing a rise in intracellular free $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) which activates Ca^{2+} -calmodulin-dependent myosin light-chain kinases [4,5]. The subsequent phosphorylation of the myosin light chain activates an actin-myosin ATPase and results in the initial phase of muscle contraction. The maintenance of muscle contraction (during prolonged agonist activation) is thought to involve the phosphorylation of a number of proteins (late phase) by protein kinase C, which is activated by diacylglycerol [6].

In contrast, elevating the intracellular cyclic AMP concentration ($[cAMP]_i$), via receptors coupled positively to adenylate cyclase (for example, histamine H₂- or β -adrenoceptors) activates cyclic AMP-dependent protein kinase A (PKA), producing smooth-muscle relaxation [5,7]. There are a variety of possible mechanisms by which PKA is able to inhibit smooth-muscle contraction (for reviews see [4,5,7]). These include: (1) the phosphorylation of myosin light-chain kinase, which decreases its affinity for the Ca^{2+} -calmodulin complex, resulting in a decrease in myosin light-chain phosphorylation [8]; (2) inhibition of late-phase protein phosphorylation, which may involve the

inhibition of several kinases and/or an activation of protein phosphatase activity [6]; (3) inhibition of receptor-mediated PtdInsP₂ hydrolysis and InsP₃ production by phosphorylating the receptor, G-protein or phospholipase C, which has been reported in a variety of tissue and cell types, including platelets [9], NG108-15 cells [10] and bovine tracheal [11–14] and iris sphincter smooth muscle [7,15]; and (4) phosphorylation of the InsP₃ receptor, preventing the InsP₃-mediated release of intracellular Ca^{2+} , as shown in rat brain [16] and human platelets [17]. Several effects of cyclic AMP on cellular Ca^{2+} metabolism have also been reported, including the inhibition of Ca^{2+} influx [18], the stimulation of Ca^{2+} efflux [19] and increases in Ca^{2+} uptake into intracellular storage sites [20]. Interestingly, in bovine tracheal smooth muscle it has been found that, in the absence of muscarinic agonists, isoprenaline stimulated increases in $[Ca^{2+}]_i$ (due to Ca^{2+} influx) without stimulating contraction [21,22].

We have previously measured $[Ca^{2+}]_i$ changes in response to histamine H₁- and adenosine A₁-receptor stimulation in monolayers of the hamster vas-deferens-derived smooth-muscle cell line DDT₁MF-2 [23–25]. Activation of these receptors stimulates the release of Ca^{2+} from intracellular stores (InsP₃-induced) and Ca^{2+} entry (influx) across the plasma membrane, which requires the continued presence of agonist on the receptor (receptor-mediated Ca^{2+} influx). Furthermore, the refilling of the intracellular Ca^{2+} store can occur independently of histamine H₁-receptor stimulation, i.e. mepyramine-insensitive, and occurs without an observable rise in cytosolic Ca^{2+} .

Previous studies have reported the effect of elevating $[cAMP]_i$

Abbreviations used: $[Ca^{2+}]_i$, intracellular free $[Ca^{2+}]$; CPA, *N*⁶-cyclopentyladenosine; $[cAMP]_i$, intracellular cAMP concn.; PKA, cyclic AMP-dependent protein kinase A.

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on inositol phosphate formation and Ca^{2+} mobilization in DDT₁MF-2 cells. Schachter et al. [26] reported that forskolin attenuates noradrenaline-induced Ca^{2+} responses without affecting noradrenaline-stimulated InsP_3 production. Recently, Gerwins and Fredholm [27] showed that forskolin pretreatment inhibited ATP-induced InsP_3 formation. In this paper we have investigated further the intracellular cross-talk between agents that elevate $[\text{cAMP}]_i$ and agonists coupled to phospholipase C and mobilization of intracellular Ca^{2+} in the smooth-muscle cell line DDT₁MF-2.

MATERIALS AND METHODS

Materials

The hamster vas deferens smooth-muscle cell line (DDT₁MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). Fura-2/AM, ionomycin and 1,9-dideoxyforskolin were from Calbiochem. Histamine, mepyramine, bradykinin, ATP, *N*⁶-cyclopentyladenosine (CPA), isoprenaline and forskolin were obtained from Sigma Chemical Co. (U.K.). The kind gifts of rolipram (Schering A.G., Berlin, Germany) and the cyclic AMP analogue (S_p)-cAMPS (S_p -adenosine 3',5'-cyclic monophosphothioate) from Research Biochemicals Inc. (Natick, U.S.A.) are acknowledged. *myo*-[2-³H]inositol (529 GBq/mmol) and [2,8-³H]adenine (1.1 Tbq/mmol) were obtained from New England Nuclear (Stevenage, Herts, U.K.), and cyclic [8-¹⁴C]AMP (11.43 Gbq/mmol) from Amersham International (Aylesbury, Bucks., U.K.). Dulbecco's modified Eagle's medium and foetal-calf serum were from Northumbria Biologicals (U.K.). All other chemicals were of analytical grade.

Cell culture

DDT₁MF-2 cells were cultured at 37 °C in a humidified air/CO₂ (9:1) atmosphere in 75 cm² flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal-calf serum. Cells were passaged twice a week (1/5 split ratio) by vigorous shaking of the flask and placed into 75 cm² flasks and fed with fresh growth medium every 48 h. Cells for [³H]inositol phosphate and cyclic [³H]AMP accumulation were finally grown in 24-well cluster dishes, and cells for $[\text{Ca}^{2+}]_i$ determinations were grown on 24 mm × 10 mm glass coverslips in 90 mm Petri dishes. All experiments were performed on confluent monolayers (passages 4–17, numbers assigned after receiving the cell line).

Accumulation of cyclic [³H]AMP

This was measured by loading confluent cell monolayers with [³H]adenine (37 kBq/well) for 2 h at 37 °C in 1 ml of Hanks/Hepes (20 mM) buffer (pH 7.4)/well. Prelabelled cell monolayers were then washed twice with 1 ml of Hanks/Hepes (20 mM) buffer (pH 7.4)/well, and where appropriate incubated with the cyclic AMP phosphodiesterase inhibitor rolipram (100 μM) for 15 min at 37 °C. Agonists were then added in 10 μl portions and the incubation continued for 10 min (unless otherwise stated). Incubations were terminated by addition of 50 μl of conc. HCl. Cyclic [³H]AMP was then isolated by sequential Dowex-alumina chromatography as previously described [28]. Before elution, samples were spiked with cyclic [¹⁴C]AMP to allow for percentage recovery correction. Eluates were collected

and the levels of cyclic [³H]- and [¹⁴C]-AMP determined by liquid-scintillation counting.

Inositol phospholipid hydrolysis

At confluence, monolayer cultures were loaded with *myo*-[³H]-inositol (37 kBq/well) for 24 h in 24-well cluster dishes in inositol-free Dulbecco's modified Eagle's medium containing 2 mM glutamine and 10% foetal-calf serum. Prelabelled cells were then washed with 2 × 1 ml of Hanks/Hepes (20 mM) buffer (pH 7.4)/well and incubated at 37 °C for 30 min in the presence of 20 mM LiCl, and where appropriate rolipram (100 μM). Isoprenaline (1 μM) and forskolin (10 μM) were added 15 min before agonist stimulation (histamine, bradykinin and ATP 10 min; CPA 45 min). Incubations were terminated by aspiration of the incubation medium and addition of 1 ml of cold (−20 °C) methanol/0.12 M HCl (1:1, v/v). Cells were left a minimum of 2 h at −20 °C before isolation of total [³H]inositol phosphates in the supernatant of the disrupted cell monolayers by anion-exchange chromatography [29]. [³H]inositol phosphate levels were determined by liquid-scintillation counting.

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured by loading confluent cell monolayers with the Ca^{2+} -sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm Petri dishes with 1 ml of physiological buffer (145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM MgSO₄, 10 mM-Hepes, 2 mM CaCl₂, pH 7.45) containing 10% foetal-calf serum and 3 μM fura-2/AM and incubated for 30 min at 37 °C. After this 'loading' period, the fura-2-containing buffer was replaced with fresh buffer, which was free of fura-2 and foetal-calf serum but contained 0.1% BSA, and left at 37 °C for a further 15 min. Loaded coverslips were then mounted in a specifically designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 ml of physiological buffer (drugs were added to the cuvettes in 100 μl portions) and fluorescence measurements were made at 37 °C with a Perkin-Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths, and the time taken to switch between 340 and 380 nm was 0.8 s. $[\text{Ca}^{2+}]_i$ was calculated every 1.6 s from the ratio (*R*) of 340 nm/380 nm fluorescence values by using the equation of Grynkiewicz et al. [30]:

$$[\text{Ca}^{2+}]_i = \frac{(R - R_{\min.})}{(R_{\max.} - R)} \times (S_{380, \min.}/S_{380, \max.}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37 °C) and $S_{380, \min.}/S_{380, \max.}$ is the ratio (β value) of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[\text{Ca}^{2+}]_i$. The maximum and minimum *R* values ($R_{\max.}$ and $R_{\min.}$) were determined on separate coverslips under saturating $[\text{Ca}^{2+}]_i$ (achieved by increasing the extracellular $[\text{Ca}^{2+}]$ to 20 mM, followed by 10 μM ionomycin, pH 7.45) and Ca^{2+} -free (achieved by using 8.3 mM EGTA, immediately followed by 25 μl of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 μM ionomycin) conditions respectively. Corrections for auto-fluorescence were made by measuring the fluorescence produced by coverslips that had not been loaded with fura-2. Where Ca^{2+} -free conditions were required, experiments were performed in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA.

Data analysis

Agonist-concentration-response curves were fitted to a logistic equation by using the non-linear regression program GraphPAD (ISI). Rises in $[Ca^{2+}]_i$ were evaluated by importing the fluorescence data into the spreadsheet AsEasyAs (TRIUS Inc). Basal $[Ca^{2+}]_i$ levels were determined by calculating the mean of the ten data points (measured every 1.9 s) before drug addition, whereas the maximum Ca^{2+} signal was deemed to be the largest Ca^{2+} response obtained immediately after drug addition. Owing to the fluctuations in basal $[Ca^{2+}]_i$, the mean of the ten data points measured immediately after drug addition were also calculated to determine whether the measured response was significantly different from basal levels (Mann-Whitney U test). Data are shown as means $\pm$ S.E.M. Statistical analysis was performed by Student's unpaired *t* test; $P < 0.05$ was considered as statistically significant. *n* in the text refers to the number of separate experiments.

RESULTS

β -Adrenoceptor- and forskolin-stimulated cyclic $[^3H]$ AMP accumulation in DDT₁MF-2 cells

β -Adrenoceptor activation or forskolin stimulation in the smooth-muscle cell line DDT₁MF-2 resulted in a dose-dependent increase in cyclic $[^3H]$ AMP accumulation (Figures 1a and 1c). Stimulation for 10 min with isoprenaline $\{1 \mu M$; $\log[EC_{50} (M)] = -7.7 \pm 0.2$; $n = 4\}$ and forskolin $\{10 \mu M$; $\log[EC_{50} (M)] = -6.2 \pm 0.1$; $n = 6\}$ elicited significant increases

in the accumulation of cyclic $[^3H]$ AMP over basal levels of 2.7 ± 0.3 -fold ($n = 4$; $P < 0.001$) and 4.0 ± 0.3 -fold ($n = 6$; $P < 0.001$) respectively. Preincubation with the type IV cyclic AMP-specific phosphodiesterase inhibitor rolipram $[31]$ ($100 \mu M$; 15 min) significantly increased the basal level of cyclic $[^3H]$ AMP (4.1 ± 0.5 -fold increase in basal cyclic $[^3H]$ AMP levels; $n = 15$; $P < 0.001$; Figure 1b). In the absence of rolipram, isoprenaline ($1 \mu M$) and forskolin ($10 \mu M$) produced rapid accumulations in cyclic $[^3H]$ AMP during the first 10 min of stimulation, which began to reach a plateau between 20 and 40 min (see Figures 1b and 1d). In the presence of rolipram, however, both isoprenaline and forskolin stimulation resulted in a significantly larger ($P < 0.05$) accumulation of cyclic $[^3H]$ AMP at all time points up to 40 min (see Figures 1b and 1d).

Effects of cyclic AMP on agonist-stimulated increases in $[Ca^{2+}]_i$

Our previous studies have shown that in DDT₁MF-2 cells histamine H_1 - and adenosine A_1 -receptor stimulation results in an increase in $[Ca^{2+}]_i$ that comprises two distinct components: (1) release of Ca^{2+} from intracellular stores, which is mediated by the second messenger $InsP_3$ $[32]$; and (2) influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane $[23-25]$.

Figures 2(a) and 3(a) show typical responses to histamine ($100 \mu M$) and CPA ($300 nM$) respectively. These profiles were obtained by initially stimulating the cells in nominally Ca^{2+} -free buffer containing $0.1 mM$ EGTA, after which extracellular Ca^{2+} ($2 mM$) was re-applied. The rise in $[Ca^{2+}]_i$ in the absence of exogenous Ca^{2+} is due to the release of Ca^{2+} from intracellular

Figure 1 Isoprenaline- and forskolin-stimulated cyclic $[^3H]$ AMP accumulation in DDT₁MF-2 cells

Concentration-response curves for isoprenaline (a) and forskolin (c) in the absence of rolipram (10 min stimulation), and time-course profiles for $1 \mu M$ isoprenaline (b)- and $10 \mu M$ forskolin (d)-stimulated cyclic $[^3H]$ AMP accumulation in the absence (●) or presence (○) of rolipram. The basal accumulation of cyclic $[^3H]$ AMP in the absence or presence of rolipram are shown by the histograms marked C (10 min for the concentration-response curves and 1 min for the time-course profiles) or C + ROL (16 min) respectively. Values represent means $\pm$ S.E.M. of quadruplicate determinations in a single experiment. Similar data were obtained in at least three other experiments.

Figure 2 Effect of forskolin on histamine-induced increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

(a) Cells were stimulated with histamine in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA, after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 10 μ M forskolin. The control experiment (a) contained 0.1% ethanol to eliminate forskolin vehicle effects. Histamine (HA; 100 μ M) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in 25 other experiments.

Figure 3 Effect of forskolin on CPA-induced increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

(a) Cells were stimulated with CPA in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA, after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 10 μ M forskolin. The control experiment (a) contained 0.1% ethanol to eliminate forskolin vehicle effects. CPA (300 nM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in 15 other experiments.

stores, whereas the increase in $[Ca^{2+}]_i$ obtained when extracellular Ca^{2+} is re-applied is attributed to Ca^{2+} influx. Figures 2(b) and 3(b) show the effect of forskolin pretreatment on histamine- and CPA-mediated increases in $[Ca^{2+}]_i$. Forskolin pretreatment (10 μ M; 15 min) significantly attenuated the release of intracellular Ca^{2+} mediated by histamine and CPA by $75 \pm 3\%$ ($n = 33$; $P < 0.001$) and $84 \pm 6\%$ ($n = 16$; $P < 0.001$) respectively. In addition to attenuating the release of intracellular Ca^{2+} , forskolin pretreatment inhibited histamine- and CPA-induced Ca^{2+} influx (observed when exogenous Ca^{2+} was re-applied) by $59 \pm 7\%$ ($n = 26$) and $60 \pm 10\%$ ($n = 16$) respectively (Figure 2a compared with Figure 2b, and Figure 3a compared with Figure 3b). The data in Table 1 summarize the effect of forskolin pretreatment on histamine- and CPA-mediated intracellular Ca^{2+} release and Ca^{2+} influx. Furthermore, pretreatment with rolipram (100 μ M; 45 min) also inhibited histamine-induced intracellular Ca^{2+} release by $50 \pm 9\%$ ($n = 12$; see Table 2).

Figure 4(a) shows ATP (100 μ M)-stimulated increases in $[Ca^{2+}]_i$ in DDT₁MF-2 cells. The profile is similar to those obtained with histamine and CPA, i.e. ATP stimulates the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+} . However, forskolin pretreatment did not attenuate ATP-stimulated intracellular Ca^{2+} release or Ca^{2+} influx (compare Figure 4a with Figure 4b). The maximum peak Ca^{2+} responses to ATP (100 μ M) obtained in Ca^{2+} -free buffer and upon re-addition of extracellular Ca^{2+} (2 mM) in forskolin-treated cells were $96 \pm 6\%$ ($n = 12$) and $99 \pm 9\%$ ($n = 12$) respectively of those obtained in control experiments. In control experiments ATP (100 μ M)-stimulated intracellular Ca^{2+} release increased $[Ca^{2+}]_i$ from 110 ± 10 nM to 551 ± 24 nM ($n = 12$), whereas in forskolin-treated cells ATP increased $[Ca^{2+}]_i$ from 108 ± 11 nM to 580 ± 32 nM ($n = 12$). ATP (100 μ M)-mediated Ca^{2+} influx in control experiments increased $[Ca^{2+}]_i$ from 98 ± 7 nM to 535 ± 20 nM ($n = 12$), com-

pared with 105 ± 7 nM to 650 ± 30 nM ($n = 12$) in forskolin-treated cells. Similarly, forskolin pretreatment had no effect on bradykinin (1 μ M)-induced release of intracellular Ca^{2+} ($96 \pm 9\%$ of the maximum Ca^{2+} response measured in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA; $[Ca^{2+}]_i$ increased from 105 ± 4 nM to 268 ± 27 nM in control cells and from 113 ± 5 nM to 327 ± 9 nM in forskolin-treated cells; $n = 15$).

Pretreatment with isoprenaline (1 μ M; 15 min) had no significant effect on histamine (100 μ M)-induced release of intracellular Ca^{2+} ($87 \pm 8\%$ of the maximum Ca^{2+} response measured in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA; $n = 8$; see Table 2). However, if the cells were preincubated for 15 min with 1 μ M isoprenaline in the presence of 10 μ M rolipram, the histamine-stimulated release of intracellular Ca^{2+} was inhibited by $57 \pm 10\%$ ($n = 7$; compare Figure 5a with Figure 5b; see Table 2). Figure 5(c) shows that a control response was elicited by 100 μ M histamine when cells were preincubated for 15 min with 10 μ M rolipram alone ($125 \pm 25\%$ of the maximum response; $n = 7$; see Table 2). In the presence of 10 μ M rolipram, isoprenaline-stimulated cyclic [³H]AMP accumulation was increased to 9.5 ± 0.7 -fold over basal ($n = 2$), compared with 2.7 ± 0.3 -fold over basal in the absence of 10 μ M rolipram. Rolipram (10 μ M) alone caused a 2.0 ± 0.3 -fold (over basal; $n = 2$) increase in cyclic [³H]AMP accumulation. It seems likely that the inclusion of 10 μ M rolipram has raised the isoprenaline-induced stimulation of cyclic AMP to a level sufficient to attenuate histamine-induced release of intracellular Ca^{2+} .

As shown in Figure 2(b), histamine H_1 -receptor-mediated Ca^{2+} influx was also sensitive to cyclic AMP elevation. A similar result was obtained if forskolin (10 μ M; 10 min incubation and in the presence of 100 μ M rolipram) was added after the cells had been stimulated with histamine, in the experiments initiated in nominally Ca^{2+} -free buffer. With this protocol, the rise in $[Ca^{2+}]_i$ observed when extracellular Ca^{2+} was re-applied was $39 \pm 8\%$ ($n = 16$; $P < 0.001$) of that obtained in the control experiments

Table 1 Effect of forskolin pretreatment on histamine H_1 - and adenosine A_1 -receptor-stimulated increases in $[Ca^{2+}]_i$

Cells were preincubated with forskolin (10 μ M) for 15 min before stimulation with 100 μ M histamine or 300 nM CPA. The control Ca^{2+} responses (release and influx) were obtained by stimulating cells (with histamine or CPA) which had not been preincubated with forskolin. Experiments were initiated in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA (to measure intracellular Ca^{2+} release), after which exogenous Ca^{2+} (2 mM) was re-applied (to measure Ca^{2+} influx). The percentage inhibition of histamine H_1 - and adenosine A_1 -receptor-mediated intracellular Ca^{2+} release and Ca^{2+} influx by forskolin (see the Results section) represent the mean of the percentage inhibition from individual experiments (after subtracting basal $[Ca^{2+}]_i$ from the stimulated Ca^{2+} response). Values represent means $\pm$ S.E.M. of (*n*) experiments. Statistical analysis was performed (Student's unpaired *t* test) comparing the stimulated Ca^{2+} responses (after subtracting basal $[Ca^{2+}]_i$) obtained in control and forskolin-treated cells (for both histamine and CPA): * statistically significant (*P* < 0.05).

	$[Ca^{2+}]_i$ (nM)					
	Histamine			CPA		
	Basal	Stimulated	(<i>n</i>)	Basal	Stimulated	(<i>n</i>)
Ca^{2+} release						
Control	101 $\pm$ 8	318 $\pm$ 14	33	110 $\pm$ 12	265 $\pm$ 28	16
10 μ M forskolin	90 $\pm$ 9	157 $\pm$ 6*	33	94 $\pm$ 17	119 $\pm$ 13*	16
Ca^{2+} influx						
Control	86 $\pm$ 6	249 $\pm$ 23	26	101 $\pm$ 12	360 $\pm$ 62	16
10 μ M forskolin	98 $\pm$ 9	150 $\pm$ 10*	26	95 $\pm$ 9	173 $\pm$ 19*	16

Table 2 Effect of 1,9-dideoxyforskolin, rolipram, (S_p)-cAMPS and isoprenaline on histamine H_1 -receptor-mediated release of intracellular Ca^{2+}

Cells were preincubated for 15 min (experiments using 10 μ M 1,9-dideoxyforskolin, 1 μ M isoprenaline, 10 μ M rolipram, or 1 μ M isoprenaline in the presence of 10 μ M rolipram) or 45 min [experiments using 100 μ M rolipram or 100 μ M of the protein kinase A activator (S_p -cAMP)] before stimulation with 100 μ M histamine. The control Ca^{2+} responses (intracellular Ca^{2+} release) were obtained by stimulating cells which had not been preincubated with the appropriate drug(s). Experiments were performed in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA (to measure intracellular Ca^{2+} release). Values represent means $\pm$ S.E.M. of (*n*) experiments. Statistical analysis was performed (Student's unpaired *t* test) comparing the stimulated Ca^{2+} responses (after subtracting basal $[Ca^{2+}]_i$) obtained in control and treated cells: * statistically significant (*P* < 0.05).

	$[Ca^{2+}]_i$ (nM)		
	Basal	Stimulated	(<i>n</i>)
Control	108 $\pm$ 7	310 $\pm$ 23	12
1,9-Dideoxyforskolin	113 $\pm$ 6	295 $\pm$ 14	12
Control	120 $\pm$ 12	324 $\pm$ 28	12
Rolipram (100 μ M)	103 $\pm$ 8	213 $\pm$ 25*	12
Control	87 $\pm$ 8	274 $\pm$ 10	4
(S_p)-cAMPS (100 μ M)	93 $\pm$ 10	185 $\pm$ 26*	4
Control	98 $\pm$ 7	387 $\pm$ 21	8
Isoprenaline (1 μ M)	112 $\pm$ 7	332 $\pm$ 18	8
Control	116 $\pm$ 12	295 $\pm$ 24	7
10 μ M rolipram	115 $\pm$ 11	358 $\pm$ 64	7
10 μ M rolipram + 1 μ M isoprenaline	102 $\pm$ 7	179 $\pm$ 17*	7

(compare Figure 6a with Figure 6b). Ca^{2+} influx increased $[Ca^{2+}]_i$ from 110 $\pm$ 15 nM to 225 $\pm$ 12 nM (*n* = 16) in control cells, whereas in forskolin-treated cells Ca^{2+} influx increased $[Ca^{2+}]_i$ from 108 $\pm$ 16 nM to 143 $\pm$ 9 nM (*n* = 16).

It should be noted that pretreatment with the 1,9-dideoxy derivative of forskolin, which is unable to activate adenylate cyclase, did not inhibit histamine-stimulated increases in $[Ca^{2+}]_i$ (96 $\pm$ 6% of the maximum Ca^{2+} response measured in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA: *n* = 12; see Table 2). Finally, the effect of a membrane-permeant analogue of cyclic

Figure 4 Effect of forskolin on ATP-induced increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

(a) Cells were stimulated with ATP in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA, after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 10 μ M forskolin. The control experiment (a) contained 0.1% ethanol to eliminate forskolin vehicle effects. ATP (100 μ M) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in 15 other experiments.

AMP on histamine-stimulated increases in $[Ca^{2+}]_i$ was examined. The cyclic AMP analogue (R_p)-cAMPS is a competitive inhibitor of PKA, whereas its diastereoisomer (S_p)-cAMPS activates the kinase [33]. Pretreatment with (S_p)-cAMPS (100 μ M, 45 min) inhibited histamine-stimulated release of intracellular Ca^{2+} (51 $\pm$ 14% of the maximum histamine response; *n* = 4; *P* < 0.02; see Table 2).

Effects of cyclic AMP on the refilling of intracellular Ca^{2+} stores

To evaluate the effect of raising $[cAMP]_i$ on intracellular Ca^{2+} -store refilling, experiments were performed using ATP (whose

Figure 5 Effect of isoprenaline on HA-induced increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

Stimulation with histamine in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA in the absence (a) or presence of 1 μ M isoprenaline and 10 μ M rolipram (b) or 10 μ M rolipram alone (c). Cells were incubated with isoprenaline or rolipram for 15 min before stimulation with histamine. Histamine (HA; 100 μ M) was added where indicated. Similar results were obtained in six other experiments.

response is insensitive to forskolin) as a second Ca^{2+} -mobilizing agent. Figure 7(a) shows the effect of adding ATP (100 μ M) after the cells had been stimulated with 100 μ M histamine in nominally Ca^{2+} -free buffer. The ATP response was abolished under these

Figure 6 Effect of forskolin on histamine-induced $[Ca^{2+}]_i$ influx

(a) Cells were stimulated with histamine in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA, after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but 10 μ M forskolin (in the presence of 100 μ M rolipram) was added 10 min before re-addition of exogenous Ca^{2+} . Histamine (HA; 100 μ M), forskolin (FK; 10 μ M) or $CaCl_2$ (2 mM) was added where indicated. Similar results were obtained in 15 other experiments.

conditions, suggesting that exposure to histamine was sufficient to deplete the intracellular Ca^{2+} store. It should be noted in Figure 7(a) that 10 μ M mepyramine was added after the histamine response to act as an appropriate control for Figure 7(b) and to eliminate the possibility that the loss of an ATP response was due to heterologous desensitization of ATP-mediated intracellular Ca^{2+} release caused by prolonged H_1 -receptor activation. In addition, the glass coverslip was transferred to a fresh cuvette that contained Ca^{2+} -free (plus 0.1 mM EGTA) buffer before stimulation with ATP. In Figure 7(b) 10 μ M mepyramine was added after the histamine response (to block H_1 -receptor-activated Ca^{2+} influx) and followed 10 min later by a 5 min exposure to 2 mM Ca^{2+} . As previously reported [24] in these cells, mepyramine inhibited the rise in $[Ca^{2+}]_i$ (due to Ca^{2+} influx) normally observed when Ca^{2+} is re-applied after cells have been stimulated with histamine in nominally Ca^{2+} -free buffer (see Figure 2a). If the cells were then exposed to ATP (100 μ M) in the absence of extracellular Ca^{2+} , there was a rapid rise in $[Ca^{2+}]_i$ which is attributed to release of Ca^{2+} from intracellular stores. Thus, as previously reported in DDT₁MF-2 cells, the refilling of the internal Ca^{2+} store can occur in the presence of the H_1 -antagonist mepyramine via a pathway that does not involve $[Ca^{2+}]_i$ rising measurably above the basal level of 100 nM [24]. Finally, to determine whether refilling of intracellular Ca^{2+} stores is affected by the presence of raised intracellular cyclic AMP, forskolin was added at the same time as mepyramine, i.e. 10 min before addition of extracellular Ca^{2+} . Figure 7(c) shows the effect of elevating $[cAMP]_i$ by using 10 μ M forskolin (in the presence of 10 μ M mepyramine) for 10 min before the re-addition of extracellular Ca^{2+} (2 mM). When the cells are subsequently stimulated with ATP in the absence of extracellular Ca^{2+} , the response is similar to that obtained in Figure 7(b). These data suggest that in DDT₁MF-2 cells refilling of the intracellular Ca^{2+} stores can occur in the presence of elevated $[cAMP]_i$ levels.

Figure 7 Effect of forskolin on the refilling of Ca^{2+} stores

Except where indicated, experiments (a)–(c) were performed in nominally Ca^{2+} -free buffer containing 0.1 mM EGTA. The histamine and ATP responses were obtained in Ca^{2+} -free medium (0.1 mM EGTA). (a) Stimulation with 100 μ M histamine appears to deplete the intracellular Ca^{2+} store, since subsequent addition of ATP failed to elicit a response. Mepyramine (10 μ M) was added after the histamine response to eliminate the possibility that the absence of an ATP response is due to heterologous desensitization of ATP-mediated intracellular Ca^{2+} release. In addition, the glass coverslip was transferred to a fresh cuvette that contained nominally Ca^{2+} -free buffer before stimulation with ATP. (b) Addition of 10 μ M mepyramine after the histamine response (to block H_1 -receptor-activated Ca^{2+} influx) and followed 10 min later by a 5 min exposure to 2 mM Ca^{2+} resulted in a maximum ATP response. The 5 min exposure to extracellular Ca^{2+} was sufficient to refill the internal Ca^{2+} store. (c) The same protocol as in (b) but 10 μ M forskolin was added at the same time as mepyramine, i.e. 10 min before the re-addition of extracellular Ca^{2+} . The subsequent addition of ATP again produces a maximum Ca^{2+} response. The internal Ca^{2+} stores have refilled in the presence of elevated [cAMP]. The label R in profiles (a)–(c) is where the cuvette medium (2 mM Ca^{2+}) was replaced with nominally Ca^{2+} -free buffer, before stimulation with ATP. Histamine (HA; 100 μ M), ATP (100 μ M), $CaCl_2$, mepyramine (Mep; 10 μ M) or forskolin (FK; 10 μ M) was added where indicated. Similar results were obtained in five other experiments.

Effects of cyclic AMP on agonist-mediated $[^3H]$ inositol phosphate accumulation

We performed experiments to determine whether the cyclic AMP-mediated attenuation of histamine H_1 - and adenosine A_1 -receptor-induced rises in $[Ca^{2+}]_i$ are due to the inhibition of inositol phospholipid hydrolysis. Preincubation with isoprenaline (1 μ M, 15 min) or forskolin (10 μ M, 15 min) significantly inhibited the accumulation of $[^3H]$ inositol phosphates elicited by 100 μ M histamine by $46 \pm 7\%$ ($n = 4$) and $38 \pm 6\%$ ($n = 4$)

Figure 8 Effect of isoprenaline, forskolin and rolipram on histamine-induced $[^3H]$ inositol phosphate accumulation in DDT, MF-2 cells

Basal accumulation of $[^3H]$ inositol phosphates (C) was unaffected by pretreatment with rolipram (R; 100 μ M; 30 min), isoprenaline (ISO; 1 μ M; 15 min) or forskolin (FK; 10 μ M; 15 min). The control 100 μ M histamine response (10 min stimulation) is shown in the column marked HA. Responses to 100 μ M histamine after cells had been preincubated with 1 μ M isoprenaline (HA + ISO; 15 min), 10 μ M forskolin (HA + FK; 15 min) or 100 μ M rolipram (HA + R; 30 min) are shown. Data represent means $\pm$ S.E.M. of quadruplicate or triplicate (rolipram data) determinations in a single experiment. Similar results were obtained in three other experiments.

Figure 9 Effect of forskolin on histamine-, ATP-, CPA- and bradykinin-induced $[^3H]$ inositol phosphate accumulation in DDT, MF-2 cells

Control (C) and responses to histamine (HA; 100 μ M), ATP (100 μ M), CPA (100 nM) and bradykinin (BK; 100 nM) measured in the absence ($\square$) or presence (hatched) of 10 μ M forskolin. Data represent means $\pm$ S.E.M. of quadruplicate determinations in a single experiment. Similar results were obtained in four other experiments.

respectively (Figure 8). Furthermore, rolipram pretreatment (100 μ M, 30 min) alone significantly inhibited the histamine-induced hydrolysis of inositol phospholipids by $80 \pm 3\%$ ($n = 4$).

The effect of forskolin pretreatment on the accumulation of $[^3H]$ inositol phosphates elicited by a range of different agonists is shown in Figure 9. The response to CPA in forskolin-treated cells is $43 \pm 9\%$ ($n = 5$) of that obtained with untreated cells. In contrast, the maximum responses elicited by ATP ($90 \pm 7\%$; $n = 5$) and bradykinin ($137 \pm 37\%$; $n = 5$) are unaffected by pretreatment with forskolin.

DISCUSSION

The present study set out to investigate the effects of elevating $[cAMP]_i$ on histamine-induced inositol phospholipid hydrolysis and increases in $[Ca^{2+}]_i$ in the hamster vas deferens smooth-muscle cell line DDT₁MF-2. The data presented show that histamine H_1 - and adenosine A_1 -receptor-mediated increases in $[Ca^{2+}]_i$ and $[^3H]$ inositol phosphate accumulation are attenuated by agents that increase $[cAMP]_i$. In contrast, elevating $[cAMP]_i$ had no effect on the Ca^{2+} response or the accumulation of $[^3H]$ inositol phosphates elicited by bradykinin or ATP.

The inhibition of agonist-induced inositol phospholipid hydrolysis by β -adrenoceptor activation, cyclic AMP phosphodiesterase inhibition or direct stimulation of adenylate cyclase by forskolin in other cell types is well documented. For example, cyclic AMP inhibits inositol phospholipid hydrolysis in bovine tracheal [11–14] and iris sphincter [7,15] smooth muscle, platelets [9], neutrophils [34], glomerulosa cells [35] and NG108-15 cells [10]. The inhibitory effect of cyclic AMP could be due to PKA-mediated phosphorylation of the agonist receptor, G-protein or phospholipase c. In several studies, AlF_4^- or guanosine 5'-[γ -imido]triphosphate (activators of GTP-binding proteins)-stimulated $PtdInsP_2$ hydrolysis has been inhibited by isoprenaline or forskolin, indicating that PKA is exerting its effect at the post-receptor level [13,15]. Alternatively, elevations of cyclic AMP may inhibit agonist-induced inositol phospholipid hydrolysis by decreasing agonist receptor number, as has been reported in rat glomerulosa cells [35]. However, the precise site(s) of PKA action in inhibiting agonist-induced inositol phosphate accumulation in DDT₁MF-2 cells remain to be determined.

In addition to inhibiting the accumulation of $[^3H]$ inositol phosphates, elicited by histamine H_1 - and adenosine A_1 -receptor stimulation, elevated cyclic AMP levels decreased the Ca^{2+} responses observed when these two receptors were stimulated. In DDT₁MF-2 cells histamine H_1 - or adenosine A_1 -receptor activation causes the release of Ca^{2+} from intracellular stores ($InsP_3$ -induced) and Ca^{2+} entry (influx) across the plasma membrane, which requires the continued presence of agonist on the receptor, i.e. receptor-mediated Ca^{2+} influx [24,25]. The data presented in this study clearly show that raising cyclic AMP levels with forskolin attenuated histamine H_1 - and adenosine A_1 -receptor-induced rises in $[Ca^{2+}]_i$ obtained when cells were stimulated in the absence of extracellular Ca^{2+} . Under these conditions the increase in $[Ca^{2+}]_i$ is attributed to the release of intracellular Ca^{2+} , which is mediated by the second messenger $InsP_3$ [32]. Hence the decrease in the release of intracellular Ca^{2+} elicited by histamine or CPA may result from the decreased production of $InsP_3$. This seems likely, in view of the forskolin-induced inhibition of histamine- and CPA-stimulated $[^3H]$ inositol phosphate accumulation. These data are similar to those results obtained with the neuronal cell line NG108-15, where forskolin decreased bradykinin-induced $InsP_3$ formation and Ca^{2+} mobilization [10]. In contrast, data obtained with the neuroblastoma cell line NCB-20 showed that cyclic AMP elevation (by using forskolin) attenuated the duration of bradykinin-induced rises in $[Ca^{2+}]_i$ without affecting the initial peak Ca^{2+} response [36].

We also examined the effect of elevating cyclic AMP levels on agonist-induced Ca^{2+} influx. Histamine H_1 - and adenosine A_1 -receptor-mediated Ca^{2+} influx were attenuated if cells were pretreated with forskolin before addition of the Ca^{2+} -mobilizing agonist (see Figures 2 and 3). Similar results were obtained if $[cAMP]_i$ levels were raised after intracellular Ca^{2+} stores had been released by histamine (see Figure 6). In contrast, ATP-induced Ca^{2+} influx was unaffected by forskolin (see Figure 4). The mechanism(s) by which agonists activate receptor-mediated

Ca^{2+} influx have not been elucidated. However, several postulated mechanisms have been proposed. These include the following: (1) activation of a putative receptor-mediated Ca^{2+} channel by an unknown messenger that is generated as the intracellular Ca^{2+} stores are depleted [37]; (2) the receptor may be directly linked to a Ca^{2+} channel, an example of which is the ATP-activated Ca^{2+} channel found in rabbit arterial smooth muscle [38]; (3) a second-messenger-operated Ca^{2+} channel which may be activated by increases in $[Ca^{2+}]_i$ or by the putative second messenger $Ins(1,3,4,5)P_4$, as recently reported in bovine aorta endothelial cells [39]; or (4) a G-protein-coupled Ca^{2+} channel. However, the mechanism(s) involved in agonist-induced Ca^{2+} influx in DDT₁MF-2 cells and how cyclic AMP-dependent phosphorylation attenuates the Ca^{2+} -influx response remain to be established.

We have previously reported that in DDT₁MF-2 cells histamine H_1 -receptor activation also stimulates Ca^{2+} influx into the intracellular Ca^{2+} store via a mechanism which appears independent of H_1 -receptor occupancy [24]. The data presented in Figure 7 clearly showed that in the presence of raised $[cAMP]_i$, and under conditions where receptor-activated Ca^{2+} influx was blocked, refilling of the intracellular Ca^{2+} store still occurred. In these experiments we employed ATP as the second Ca^{2+} -mobilizing agent, since forskolin had no effect on ATP-stimulated release of intracellular Ca^{2+} .

There have been several reports in the literature detailing the effects of cyclic AMP on cellular Ca^{2+} signalling. Suppattapone et al. [16] reported that cyclic AMP-dependent phosphorylation of the rat brain $InsP_3$ receptor decreases its ability to release intracellular Ca^{2+} . PKA activation may also block Ca^{2+} -induced Ca^{2+} release [40]. Finally, McAtee and Dawson [41] proposed that PKA-mediated phosphorylation may inhibit Ca^{2+} -induced phospholipase C activation. This would decrease inositol phospholipid hydrolysis and attenuate the duration of the Ca^{2+} signal without affecting the initial release, as was reported for bradykinin-induced Ca^{2+} responses in NCB-20 cells [36]. Our previous studies [32] have shown that in the absence of added extracellular Ca^{2+} the accumulation of $[^3H]$ inositol phosphates elicited by histamine is $25 \pm 7\%$ of that obtained in the presence of 2.4 mM Ca^{2+} . This raises the possibility that Ca^{2+} -dependent activation of phospholipase C may contribute to the increase in $[Ca^{2+}]_i$ observed in DDT₁MF-2 cells when exogenous Ca^{2+} is re-applied. The inhibitory effects of elevated cyclic AMP levels on agonist-induced Ca^{2+} influx in DDT₁MF-2 cells may be a consequence of a decrease in Ca^{2+} -induced phospholipase C activation.

The non-selective β -adrenoreceptor agonist isoprenaline and the type IV cyclic AMP phosphodiesterase inhibitor rolipram also stimulated the accumulation of cyclic $[^3H]AMP$ in DDT₁MF-2 cells. Isoprenaline pretreatment alone attenuated histamine-induced $[^3H]$ inositol phosphate accumulation, but was without effect on histamine-stimulated release of intracellular Ca^{2+} . However, β -adrenoceptor-mediated inhibition of histamine-induced intracellular Ca^{2+} release was observed when cells were preincubated with isoprenaline in the presence of 10 μM rolipram (see Figure 6). This particular concentration of rolipram did not inhibit histamine-stimulated release of intracellular Ca^{2+} on its own. The inability of isoprenaline pretreatment alone to inhibit histamine-induced Ca^{2+} responses reflects the lower accumulations of cyclic $[^3H]AMP$ elicited by isoprenaline (2.7-fold over basal) when compared with those obtained with forskolin (4.0-fold over basal). However, in the presence of rolipram, β -adrenoceptor-stimulated cyclic AMP levels are enhanced sufficiently to inhibit the Ca^{2+} response. In addition the membrane-permeant analogue of cyclic AMP,

(S_p)-cAMPS, was also able to inhibit significantly histamine-stimulated intracellular Ca^{2+} release. These data support the contention that cyclic AMP is the mediator involved in forskolin-induced inhibition of agonist-induced inositol phosphate accumulation and intracellular Ca^{2+} responses.

A notable feature of the data presented in this study is the differing effects which elevating cytosolic cyclic AMP has on agonist-induced inositol phospholipid hydrolysis and intracellular Ca^{2+} responses. The reasons for the selective inhibition of histamine H_1 - and adenosine A_1 -receptor-mediated responses, as opposed to the bradykinin- or ATP-induced responses, which are unaffected, are unclear. One possibility is that the proteins involved in the signal-transduction pathways associated with bradykinin or ATP-receptor activation are not suitable substrates for PKA phosphorylation. A similar discriminative effect of cyclic AMP on histamine H_1 -receptor-mediated, but not muscarinic receptor-mediated, inositol phospholipid hydrolysis has been reported in airway smooth muscle [11].

In summary, the present study has shown that elevating $[cAMP]_i$ levels has varying effects on inositol phospholipid hydrolysis and increases in $[Ca^{2+}]_i$ elicited by a range of agonists in DDT₁MF-2 cells. The accumulation of $[^3H]$ inositol phosphates and Ca^{2+} responses (intracellular Ca^{2+} release and Ca^{2+} influx) produced by histamine H_1 - and adenosine A_1 -receptor stimulation are attenuated by increases in $[cAMP]_i$, whereas ATP- or bradykinin-induced $[^3H]$ inositol phosphate accumulation and intracellular Ca^{2+} responses are unaffected.

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