Protein kinase C-dependent cyclic AMP formation in airway smooth muscle: the role of type II adenylate cyclase and the blockade of extracellular-signal-regulated kinase-2 (ERK-2) activation

Nigel J. PYNE, Noreen MOUGHAL, Patricia A. STEVENS, Deborah TOLAN and Susan PYNE

Department of Physiology and Pharmacology, University of Strathclyde, 204 George Street, Glasgow G1 1XW, Scotland, U.K.

Bradykinin activates adenylate cyclase via a pathway that involves the 'up-stream' regulation of phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine and activation of protein kinase C (PKC) in airway smooth muscle [Stevens, Pyne, Grady and Pyne (1994) *Biochem. J.* **297**, 233–239]. Coincident signal ($G_s\alpha$ and PKC) amplification of the cyclic AMP response can be completely attenuated either by diverting PLD-derived phosphatidate or by inhibiting PKC. In this regard, the coincident signal detector type II adenylate cyclase is expressed as a 110/112 kDa polypeptide in these cells. PKC α is not involved in the activation of adenylate cyclase, since a B_2 -receptor antagonist (NPC567, 10 μ M) blocked its bradykinin-stimulated translocation to the membrane and was without effect against both bradykinin-stimulated PLD activity and cyclic AMP formation. Cyclic AMP formation can also be activated by platelet-derived growth factor (PDGF), via a PKC-dependent pathway, although

the magnitude of the response is less than that elicited by bradykinin. Nevertheless, these results indicate that multiple receptor types employ PKC to initiate cyclic AMP signals. PDGF (10 ng/ml) elicited the marked sustained activation of extracellular-signal-regulated kinase-2 (ERK-2), whereas bradykinin (1 μ M) provoked only modest transient activation of ERK-2. Deoxyadenosine (0.1 mM), a P-site inhibitor of adenylate cyclase, blocked bradykinin-stimulated cyclic AMP formation and converted the activation of ERK-2 into a sustained response. Thus the PKC-stimulated cyclic AMP response can limit the activation of ERK-2 in response to bradykinin. These studies indicate that the integration of distinct signal pathways by adenylate cyclase can determine the kinetics of ERK activation, an enzyme that appears to be important for mitogenic progression.

INTRODUCTION

The molecular cloning of adenylate cyclase has allowed identification of six isoforms (types I–VI) in mammalian cells [1]. Of these, only the type I, II and III isoforms appear to become activated upon phorbol ester stimulation of human embryonic kidney cells [2]. The type I and III adenylate cyclase isoforms are also activated by Ca^{2+} /calmodulin, whereas the type II isoform is insensitive. The coincident activation of both PKC and Ca^{2+} release may be the physiologically important route of type I and III isoform regulation. For this reason, it is possible that these isoforms specifically integrate PKC signals that are down-stream from PLC-catalysed hydrolysis of PtdIns(4,5) P_2 . In contrast, the airway smooth muscle adenylate cyclase integrates PKC signals that are down-stream of the bradykinin-stimulated PLD-catalysed hydrolysis of PtdCho [3]. The airway smooth-muscle adenylate cyclase can also detect and integrate coincident signals, i.e. $G_s\alpha$ and PKC [3].

Tyrosyl phosphorylation of proteins is an important early signalling event in the initiation of mitogenesis. In this regard, *Raf-1* and ERK-2 appear to play a pivotal role [4]. Furthermore, cyclic AMP-dependent protein kinase has been demonstrated to block activation of *Raf-1* by p21^{ras}, and this may underlie the cyclic AMP-dependent inhibition of mitogenesis in some cell types [5]. The relationship between the PKC-activated adenylate

cyclase, cyclic AMP and early signalling via ERK is unknown, but may have important consequences for determining the mitogenicity of an agonist.

In this paper, we show that: (i) coincident signal ($G_s\alpha$ and PKC) amplification of cyclic AMP can be completely attenuated by either diverting PLD-derived phosphatidate or inhibiting PKC, (ii) cross-regulation is unidirectional; (iii) the coincident signal detector type II adenylate cyclase is expressed in these cells; and (iv) ERK-2 activation is conditionally inhibited by the PKC-sensitive cyclic AMP pool. These studies are discussed in relation to the regulation of mitogenesis of airway smooth muscle.

MATERIALS AND METHODS

Materials

All biochemicals were from Boehringer Mannheim (Germany) and general chemicals were from Sigma Chemical Co. (U.K.). Cyclic [3H]AMP, [3H]palmitate and cyclic [3H]AMP-binding assay kit were from Amersham International (U.K.). All cell-culture media were from Gibco (U.K.) and ICN Flow (U.K.). Collagenase, elastase and soya-bean trypsin inhibitor were from Sigma (U.K.). Male Dunkin–Hartley guinea pigs (200–400 g) were used for isolation of tracheal smooth muscle. Anti-PKC α antibodies were purchased from Gibco (U.K.). Antibody to type

Abbreviations used: ERK-2, extracellular-signal-regulated kinase-2; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; NPC567, D-Arg-[Hyp³, D-Phe⁷]bradykinin; DME, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; DHS, donor horse serum; HRP, horseradish peroxidase.

II adenylylase cyclase (APC2-1077) was kindly given by Professor A. Gilman and Dr. W.-J. Tang (University of Texas, Dallas, TX, U.S.A.). Anti-ERK-2 antibody was from Affiniti (U.K.). ECL kits were from Amersham (U.K.). Ro318220 was kindly given by Dr. G. Lawton (Roche Products, U.K.).

Cell culture

The primary cultures of guinea-pig airway smooth-muscle cells were prepared as described previously [6]. The cells were maintained in DME containing 10% (v/v) FCS and 10% (v/v) DHS and were passaged twice, by using trypsin, before experimentation. Cells were grown to confluence on 24- or 6-well plates and routinely used 14–21 days after the initial preparation. Their identity was confirmed to be smooth muscle by the presence of α -actin, shown by using a smooth-muscle-specific mouse anti- α -actin monoclonal antibody [6].

Cyclic AMP assay

Cells were placed in DME containing 1% (v/v) FCS and 1% (v/v) DHS 18 h before use. Alternatively, cells were preincubated for 18 h with cholera toxin (0.5 ng/ml) in DME containing 1% FCS and 1% DHS as required. The cells were washed twice in gassed (O_2/CO_2 , 19:1) Krebs–Henseleit buffer, pH 7.4, and preincubated in Krebs–Henseleit buffer with 1% (w/v) BSA for 30 min at 37 °C in air/ CO_2 (19:1). The cells were then challenged with final concentrations of either bradykinin (10 pM–10 μ M) or PDGF (10–100 ng/ml) for 10 min at 37 °C. In some cases, the cells were pre-treated with either butan-1-ol (0.3%, v/v) for 10 min, or with Ro318220 (1 μ M, final concn.) or deoxyadenosine (0.1 mM) for 15 min before agonist stimulation. Incubations were terminated by precipitation of the cells in 80% (v/v) ethanol. Samples were harvested and centrifuged at 14000 g, and cyclic AMP was extracted into ethanol, which was then freeze-dried. Samples were taken for cyclic AMP determination by using the cyclic [3H]AMP binding assay with bovine heart cyclic AMP-binding protein that had been purified by us as described by Rubin et al. [7].

PKC detection

For PKC studies, Krebs–Henseleit medium, pH 7.4, containing 1% (w/v) BSA was removed from the cells after bradykinin (10 nM) stimulation and replaced with buffer A containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.4, 2 mM benzamide and 0.1 mM phenylmethanesulphonyl fluoride. In some cases, cells were pretreated with NPC567 (10 μ M) before bradykinin stimulation. Cells were scraped into 1 ml of buffer A and broken by passage through a syringe needle (2–3 strokes). Membranes were prepared by centrifugation at 4 °C in a Microfuge at 14000 g for 10 min. Protein was determined by using the Bio-Rad reagent. After equalizing protein concentrations, samples were subjected to SDS/PAGE [10% (w/v) acrylamide]. This was followed by immunoblotting with a specific polyclonal antibody raised to a unique non-conserved peptide region of PKC α . After transfer, the nitrocellulose sheets were blocked in 5% (w/v) gelatin and 10 mM phosphate-buffered saline, pH 7.4 (PBS), at 37 °C for 2 h. After washing with distilled water, the sheets were incubated with anti-PKC α antibody in PBS and 0.05% (v/v) Nonidet P40 for 12 h at 30 °C. Routinely a 1:5000 (v/v) dilution of antibody was used. After this incubation, the sheets were washed in PBS containing 0.05% (v/v) Nonidet P40 and then incubated with a horseradish-peroxidase-linked (HRP) anti-rabbit antibody [1:2000 (v/v) dilution] for 2 h at room temperature and then washed as above.

The ECL technique was used on the sheets in order to locate the immunoreactive bands. Quantification was performed by using densitometry to measure the absorbance of immunoreactive peptides.

Detection of adenylylase cyclase II

Immunoblotting of membranes with anti-(adenylylase cyclase II) antibodies was initiated by treating 20 μ l membrane samples (20–40 μ g) with 5 μ l of 1 mM dithiothreitol and 10% (w/v) SDS and heating at 80 °C for 5 min. After cooling, the sample was combined with 5 μ l of *N*-ethylmaleimide (300 mM) and incubated for 10 min at room temperature. Samples were then subjected to electrophoresis on SDS/8% (w/v)-polyacrylamide gels. The procedure for immunoblotting was identical with the PKC studies, except that 1% (v/v) Tween-20 was used instead of Nonidet P40. Anti-(adenylylase cyclase II) and HRP-anti-rabbit antibodies were used at 1:2000 (v/v) and 1:1000 (v/v) dilutions respectively.

PLD, Ins(1,4,5) P_3 mass and phosphatidate assays

Confluent airway smooth-muscle cells were preincubated with [3H]palmitate (2 μ Ci/ml) in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. This procedure has previously been shown to result in the preferential incorporation of radioactivity into PtdCho, which contained 82% of the total radioactivity associated with major phospholipids, in comparison with only 6% associated with phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine combined [6].

PLD activity was determined by using a transphosphatidyl-ation assay as described by us previously [6]. [3H]Phosphatidylbutanol, which is a direct indicator of PLD activity, routinely migrated with $R_f = 0.35$ and was quantified by excising the appropriate area from each lane and counting the associated radioactivity. [3H]Phosphatidate was determined in parallel, being identified by its co-migration with an authentic standard. The Ins(1,4,5) P_3 mass assay in unlabelled cells was performed as described in [8].

Detection of ERK-2

Cells were placed in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. After stimulation with either PDGF (10 ng/ml) or bradykinin (1 μ M), the medium was removed and the cells were washed in ice-cold 50 mM Hepes, pH 7.4, containing 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA and 2 mM Na_3VO_4 . Boiling sample buffer [0.125 M Tris/HCl, pH 6.7, 0.5 mM $Na_4P_2O_7$, 1.25 mM EDTA, 2.5% (v/v) glycerol, 0.5% (w/v) SDS, 25 mM dithiothreitol] was added and the cell lysates were harvested. Samples were boiled for 5 min and then subjected to SDS/PAGE. Immunoblotting, blocking of nitrocellulose and washing were performed as described for PKC detection. Nitrocellulose sheets were probed with anti-ERK-2 antibody [1:5000 (v/v) dilution] as described above, and ERK-2 reactivity was detected with a reporter HRP-linked anti-mouse antibody [1:2000 (v/v) dilution] and the ECL detection kit.

RESULTS

Coincident signal integration by adenylylase cyclase

We have previously demonstrated that the activation of adenylylase cyclase by bradykinin can be attenuated by the prior treatment of cells with 0.3% (v/v) butan-1-ol, implicating a role for PLD [3]. Butan-1-ol (0.3%, v/v) alone appeared to elicit cyclic AMP formation (approx. 2-fold increase in cyclic AMP; Figure 1) in

Figure 1 Effect of butan-1-ol on bradykinin-stimulated intracellular cyclic AMP formation in cholera-toxin-treated airway smooth-muscle cells

Effect of butan-1-ol (0.3%, v/v) on the bradykinin concentration–response curve for cyclic AMP accumulation in cholera-toxin-treated cells. Results, expressed as percentage of the maximal response in the absence of butan-1-ol, are means $\pm$ S.D. for experiments performed in triplicate on four separate cell preparations: $\square$, control; $\bullet$, butan-1-ol-pre-treated cells. The basal cyclic AMP level was 1.52 ± 0.31 pmol/ 0.25×10^6 in untreated cells and 3.82 ± 0.166 pmol/ 0.25×10^6 in cholera-toxin-treated cells (0.5 ng/ml, 18 h).

Table 1 Effect of PDGF and bradykinin on cyclic AMP accumulation

Unstimulated and cholera-toxin-treated cells were challenged with either PDGF (10 ng/ml) or bradykinin (1 μ M) for 10 min at 37 $^{\circ}$ C. Data shown are means $\pm$ S.D. for assays performed in triplicate on three separate cell preparations: * $P < 0.05$ compared with corresponding controls (Student t test).

	Cyclic AMP (pmol/ 0.25×10^6 cells)
Untreated cells	
Control	1.12 ± 0.05
PDGF	$3.26 \pm 0.2^*$
Bradykinin	$20 \pm 7^*$
Cholera-toxin-treated cells	
Control	8.82 ± 0.6
PDGF	$62.2 \pm 3.4^*$
Bradykinin	$157 \pm 6^*$

cells where G_s had been activated by cholera toxin [3]. Nevertheless, as in untreated cells, butan-1-ol was still capable of ameliorating the concentration-dependent bradykinin-stimulated cyclic AMP formation (Figure 1).

PDGF (10–100 ng/ml) elicited a cyclic AMP response which was appreciably smaller in magnitude compared with bradykinin stimulation in both non-treated and cholera-toxin-treated cells (Figure 2, Table 1). However, as with bradykinin, considerable amplification was achieved by coincident activation of G_s (Table 1). Pre-treatment of the cells with Ro318220 (1 μ M) completely abolished both the bradykinin- and PDGF-stimulated cyclic AMP responses in cholera-toxin-treated cells (Figure 2).

Bradykinin also elicits inhibition of cyclic AMP phosphodiesterase activity via a PKC-independent mechanism [3]. Thus, the complete inhibitory action of Ro318220 on bradykinin-stimulated cyclic AMP formation suggests that the role of

Figure 2 Effect of Ro318220 on bradykinin- and PDGF-stimulated intracellular cyclic AMP levels in cholera-toxin-treated airway smooth-muscle cells

Effect of Ro318220 (1 μ M) on the bradykinin (BK; 0.1 μ M)- and PDGF (100 ng/ml)-stimulated cyclic AMP accumulation in cholera-toxin-treated cells. The results are expressed as fold stimulation above basal cyclic AMP levels in cholera-toxin-treated cells and are means $\pm$ S.D. for experiments performed in triplicate assays on three separate cell preparations.

phosphodiesterase inhibition may be to augment increases in intracellular cyclic AMP that are already brought about by stimulation of adenylate cyclase activity.

Effect of butan-1-ol on phosphatidate levels

Bradykinin-stimulated [3 H]phosphatidate formation in [3 H]palmitate-labelled cells arises predominantly from PLC- and PLD-catalysed hydrolysis of PtdCho [8]. Butan-1-ol blocked bradykinin-stimulated [3 H]phosphatidate accumulation (57% inhibition at 0.1 μ M bradykinin; no butan-1-ol, control, 1763 $\pm$ 14 d.p.m.; plus bradykinin, 3859 $\pm$ 762 d.p.m.; plus butan-1-ol, control, 1459 $\pm$ 58 d.p.m.; plus bradykinin, 2369 $\pm$ 89 d.p.m.; $t = 10$ min, means $\pm$ S.D. for $n = 3$ separate experiments). Since 0.3% butan-1-ol is a maximal concentration for diverting the PLD-derived phosphatidate into phosphatidylbutanol [6], the remaining [3 H]phosphatidate is probably derived from PLC-catalysed hydrolysis of PtdCho, via the concerted action of diacylglycerol kinase [9]. Butan-1-ol (0.3%) also had no effect on bradykinin-stimulated Ins(1,4,5) P_3 formation (results not shown).

Effect of the activation of G_s on bradykinin-stimulated PLD activity and Ins(1,4,5) P_3 formation

Cholera-toxin pre-treatment elicited the sustained accumulation of cyclic AMP (Table 1, and [3]), but was without effect on maximal PMA-, bradykinin- and PDGF-stimulated PLD activity [control: 613 $\pm$ 7 d.p.m.; bradykinin (0.1 μ M), 1543 $\pm$ 278 d.p.m.; PDGF (50 ng/ml), 1083 $\pm$ 60 d.p.m.; PMA (0.1 μ M), 3063 $\pm$ 280 d.p.m.; cholera-toxin-treated: control, 643 $\pm$ 22 d.p.m.; bradykinin (0.1 μ M), 1887 $\pm$ 174 d.p.m.; PDGF (50 ng/ml), 1042 $\pm$ 127 d.p.m.; PMA (0.1 μ M), 3102 $\pm$ 262 d.p.m.; $t = 10$ min, means $\pm$ S.D. for $n = 3$ separate cell preparations] and had no

Figure 3 Effect of cholera toxin on bradykinin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ formation

Changes in $\text{Ins}(1,4,5)\text{P}_3$ mass in response to bradykinin at 10 s in control ($\square$) and cholera-toxin-treated cells (0.5 ng/ml; $\bullet$). Results, expressed as pmol/ 0.25×10^6 cells, are means $\pm$ S.D. for experiments performed in triplicate on three separate cell preparations. Basal $\text{Ins}(1,4,5)\text{P}_3$ levels were 2.31 ± 0.68 pmol/ 0.25×10^6 control cells and 1.43 ± 0.62 pmol/ 0.25×10^6 cholera-toxin-treated cells.

Figure 4 Concentration-dependent stimulation of PLD activity by PDGF

The concentration-dependent effect of PDGF on $[^3\text{H}]$ phosphatidylbutanol accumulation (10 min) in cultured guinea-pig airway smooth-muscle cells. Results are means $\pm$ S.D. ($n = 3$) from a representative experiment and are expressed as d.p.m./ 0.25×10^6 cells.

effect on bradykinin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ formation (Figure 3). Therefore, the cross-talk regulation appears selective for the modulation of adenylate cyclase and is not bi-directional.

PDGF and PLD activity

PDGF elicited a concentration-dependent stimulation of PLD activity (Figure 4), the maximal response of which was less than that elicited by bradykinin. This is similar to the relative effects of these agonists on cyclic AMP mass [3,6].

Figure 5 Effect of bradykinin and the B_2 -receptor antagonist on $\text{PKC}\alpha$ translocation

(a) The time course of bradykinin (10 nM)-stimulated $\text{PKC}\alpha$ translocation and (b) the effect of pre-treatment with a B_2 -receptor antagonist (NPC567; $10 \mu\text{M}$) on bradykinin-stimulated translocation. Time points are: (1) zero time; (2) 1 min; (3) 2 min; (4) 3 min; (5) 5 min; (6) 15 min. This is a representative result of an experiment performed on four separate cell preparations. Positions of molecular-mass markers (kDa) are shown, and the position of $\text{PKC}\alpha$ is denoted with an arrow.

Figure 6 Quantification of $\text{PKC}\alpha$ translocation in response to bradykinin and B_2 -receptor antagonist

Absorbances, measured as fold increase above basal, of $\text{PKC}\alpha$ detected in response to bradykinin (10 nM) in the absence ($\square$) and presence ($\bullet$) of NPC567 ($10 \mu\text{M}$) were determined by densitometry. Results are expressed as means $\pm$ S.D. for an experiment performed on three different cell preparations.

Effect of bradykinin and B_2 -receptor antagonists on $\text{PKC}\alpha$ translocation

Immunoblotting with a PKC -isoform-specific antibody revealed the presence of $\text{PKC}\alpha$ in both particulate (Figure 5a) and cytosolic fractions (results not shown). $\text{PKC}\alpha$ migrated on SDS/PAGE at 71 kDa. Bradykinin (10 nM) induced the rapid translocation of $\text{PKC}\alpha$ to the membrane (Figures 5a and 6). We have previously demonstrated that the B_2 -receptor antagonist NPC567 ($10 \mu\text{M}$) completely blocked the bradykinin-stimulated

Figure 7 Immunodetection of type II adenylate cyclase

Immunoblotting of cell membranes with anti-(adenylate cyclase II) antibodies. Lane 1, airway smooth-muscle membranes, 10 µg; lane 2, 20 µg; lane 3, Sf9-cell membranes containing adenylate cyclase II, 2 µg; lane 4, 4 µg; lane 5, 10 µg; lane 6, 20 µg; lane 7, 40 µg. This is a representative result of an experiment performed on five separate airway smooth-muscle cell preparations. Positions of molecular-mass markers (kDa) are shown, and the position of type II adenylate cyclase is denoted with an arrow.

Ins(1,4,5) P_3 response, yet was ineffective against bradykinin-stimulated PLD activity and cyclic AMP accumulation [3,10]. NPC567 (10 µM) was also effective at blocking the bradykinin-stimulated translocation of PKC α (Figures 5b and 6), and this is consistent with PKC α acting as a target for lipid activators derived from bradykinin-stimulated PtdIns(4,5) P_2 hydrolysis. These studies suggest that the translocated pool of PKC α may not be involved in the positive modulation of adenylate cyclase activity.

Immunodetection of type II adenylate cyclase

Immunoblotting of airway smooth-muscle membranes with anti-(type II adenylate cyclase) antibody (APC2-1077) revealed the presence of a major band of 110 kDa polypeptide (with a minor polypeptide at 112 kDa; Figure 7). These immunoreactive polypeptides migrated slightly faster than a similar type II adenylate cyclase doublet that was expressed in insect Sf9-cell membranes (pcDNA-1 vector containing cDNA for type II adenylate cyclase transfected in Sf9 cells [11]). Recombinant type II adenylate cyclase does migrate slightly slower than native enzyme. The protein doublet has been previously interpreted as representing different extents of glycosylation of the native protein [11]. Immunoblotting of total cell lysates with the antibody for type II adenylate cyclase, without prior treatment with *N*-ethylmaleimide, also enabled detection of the 110/112 kDa polypeptides. These polypeptides were detected as a broad immunoreactive band, which is typical electrophoretic behaviour of glycosylated proteins (results not shown). These studies indicate that the type II adenylate cyclase is expressed in airway smooth muscle and supports the proposal that it may provide a target for PKC-dependent regulation.

ERK-2 activation

ERK-2 was detected as a 42 kDa protein on immunoblots of electrophoresed cell lysates (Figure 8). PDGF (10 ng/ml) elicited a sustained shift in the electrophoretic mobility of ERK-2, due to its complete activation (Figure 8a). In contrast, bradykinin (1 µM) provoked only a very modest mobility shift, due to the partial activation of the available pool of ERK-2 (Figure 8b), leading to poor resolution of both activated and non-activated ERK-2. In addition, activation of ERK-2 by bradykinin was transient (Figure 8b). It has previously been demonstrated that

Figure 8 Immunodetection of ERK-2 in response to PDGF and bradykinin

(a) Time course of PDGF (10 ng/ml)-stimulated ERK-2 activation; (b) the time course of bradykinin (1 µM)-stimulated ERK-2 activation. Time points are: (1) zero time; (2) 2 min; (3) 5 min; (4) 7.5 min; (5) 10 min; (6) 15 min. This is a representative result of an experiment performed on five separate cell preparations. The arrow indicates the position of ERK-2.

Figure 9 Immunodetection of ERK-2 in response to bradykinin and deoxyadenosine

Time course of bradykinin (1 µM)-stimulated ERK-2 activation in cells pre-treated with deoxyadenosine (0.1 mM). Time points are: (1) zero time; (2) 2 min; (3) 5 min; (4) 10 min; (5) 15 min. This is a representative result of an experiment performed on five separate cell preparations. The arrow indicates the position of ERK-2.

phosphorylation of ERK-2 results in its retarded mobility on SDS/PAGE [5].

Deoxyadenosine (0.1 mM), a P-site-specific inhibitor of adenylate cyclase, completely blocked the bradykinin-stimulated cyclic AMP response in cells where G_s was coincidentally activated with cholera toxin. Inhibition is complete despite the magnitude of the cyclic AMP response, which is substantial [deoxyadenosine, 1.5 ± 0.1 pmol/ 0.25×10^6 cells; bradykinin (1 µM), 148 ± 20 pmol/ 0.25×10^6 cells; bradykinin plus deoxyadenosine, 2 ± 0.5 pmol/ 0.25×10^6 cells; $n = 3$]. The inhibition of bradykinin-stimulated adenylate cyclase activity and cyclic AMP formation in intact cells was associated with the complete and sustained activation of ERK-2 (Figure 9).

DISCUSSION

PKC and signal integration by adenylate cyclase

PKC activates the type I, II and III adenylate cyclase isoforms [2]. Of these isoforms, only the type II adenylate cyclase has been demonstrated to be phosphorylated by PKC in intact cells, and this elicits activation of basal activity [12]. The type II isoform can also detect and integrate the coincident signals, $G_s\alpha$ and PKC, leading to synergistic cyclic AMP formation by virtue of a

G_{α} -dependent amplification of PKC-activated adenylate cyclase activity [13]. This is identical with the effect of PKC-stimulating agents in airway smooth-muscle cells that contain cholera-toxin-activated G_{α} . Moreover, the coincident signal detector type II adenylate cyclase is expressed in these cells. In contrast, the type I isoform is activated by PKC, but cannot integrate the coincident G_{α} signal, i.e. cholera toxin will not amplify the response [2]. The type III isoform appears to be predominantly expressed in olfactory neuroepithelial cells [1,14]. Nevertheless, PKC activation of the type III isoform does not prevent G_{α} from inhibiting it [15]. In contrast, airway smooth-muscle adenylate cyclase is no longer inhibited by G_{α} when it is activated by PKC [16] and behaves similarly to the type II isoform in this respect [14]. We have not been able to functionally detect either the type I or III isoforms in airway smooth muscle (results not shown).

We consider that adenylate cyclase is directly activated by PKC in airway smooth muscle. This is substantiated by the following: (i) both PMA and bradykinin elicit the activation of basal adenylate cyclase [3] and (ii) PKC-stimulated cyclic AMP formation also occurs in cells where G_{α} has not been activated [3].

Butan-1-ol was shown to divert PLD-derived phosphatidate into phosphatidylbutan-1-ol, and completely ablated the PKC-stimulated cyclic AMP response in cells where G_{α} has not been activated [3]. The amplification of the cyclic AMP response by G_{α} and PKC remains totally sensitive to butan-1-ol and is also completely blocked by a PKC inhibitor. This excludes a possible additional role for G-protein $\beta\gamma$ -subunit integration, which is conditional on the activation of G_{α} and could have contributed to the cyclic AMP response in cholera-toxin-treated cells [17]. Cyclic AMP formation can also be activated by PDGF, via a PKC-dependent pathway, although the magnitude of the response is decreased compared with that by bradykinin. Nevertheless, these results indicate that multiple receptor types employ a PKC pathway to initiate cyclic AMP signals.

The role of phosphatidate

A proportion of the phosphatidate that is formed in response to bradykinin is derived from PtdCho-specific PLC activity and diacylglycerol kinase and is therefore insensitive to blockade by butan-1-ol [9]. This is substantiated by the observation that PKC inhibitors can completely block bradykinin-stimulated PLD activity, yet have no effect against phosphatidate that is formed in butan-1-ol treated cells (results not shown). Butan-1-ol can completely block bradykinin-stimulated cyclic AMP formation, suggesting that the PLC-derived phosphatidate cannot induce the cyclic AMP response. From our previous study [3], we suggested that PLD-derived phosphatidate may facilitate cross-regulation with adenylate cyclase. From the present study it is apparent that cross-regulation may be highly dependent on the correct species of phosphatidate and its specificity for different PKC isoforms.

Adenylate cyclase and ERK-2

PDGF elicited complete activation of the available pool of ERK-2. In contrast, bradykinin provoked only a modest transient activation. In this regard, both intrinsic tyrosine kinase and G-protein-linked receptors can stimulate p21^{ras} and *Raf-1* [18] and also provoke integration of PKC-dependent regulation at the level of *Raf-1* [19,20]. It is possible that the PKC-sensitive cyclic AMP pool may limit the extent of ERK-2 activation in response to bradykinin and may be insufficient to block the PDGF-stimulated ERK-2 pathway. This is substantiated by our observation that inhibition of bradykinin-stimulated adenylate cyclase activity converts the transient activation of ERK-2 into a sustained response. In this regard, cyclic AMP has been shown to catalyse phosphorylation of *Raf-1* [5,21].

In summary, we have provided evidence that adenylate cyclase detects and integrates PKC signals and that this can limit the activation of ERK-2. It remains to be determined if the type II adenylate cyclase can function to regulate the subsequent entry of the cells into the cell cycle.

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REFERENCES

- 1 Iyengar, R. (1993) *FASEB J.* **7**, 768–775
- 2 Jacobowitz, O., Chen, J., Premont, R. T. and Iyengar, R. (1993) *J. Biol. Chem.* **268**, 3829–3832
- 3 Stevens, P., Pyne, S., Grady, M. and Pyne, N. J. (1994) *Biochem. J.* **297**, 233–239
- 4 Boulton, T. G. and Cobb, M. H. (1991) *Cell. Regul.* **2**, 357
- 5 Cook, S. J. and McCormick, F. (1993) *Science* **262**, 1069–1072
- 6 Pyne, S. and Pyne, N. J. (1993) *Biochem. Pharmacol.* **45**, 593–603
- 7 Rubin, C. S., Erlichman, J. and Rosen, O. M. (1974) *Methods Enzymol.* **38**, 308–315
- 8 Palmer, S. and Wakelam, M. J. O. (1990) in *Methods in Inositol Research* (Irvine, R. F., ed.), pp. 147–153, Raven Press, New York
- 9 Pyne, S. and Pyne, N. J. (1994) *Cell. Signalling* **6**, 269–277
- 10 Pyne, S. and Pyne, N. J. (1993) *Br. J. Pharmacol.* **110**, 477–481
- 11 Gao, B. and Gilman, A. G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10178–10182
- 12 Jacobowitz, O. and Iyengar, R. (1994) *Proc. Natl. Acad. Sci. U.S.A.*, in the press
- 13 Lustig, K. D., Conklin, B. R., Herzmark, P., Taussig, R. and Bourne, H. R. (1993) *J. Biol. Chem.* **268**, 13900–13905
- 14 Xia, Z. G., Choi, E. J., Fan, W., Blazynski, C. and Storm, D. R. (1993) *J. Neurochem.* **60**, 305–308
- 15 Chen, J. and Iyengar, R. (1993) *J. Biol. Chem.* **268**, 12253–12256
- 16 Grady, M., Stevens, P., Pyne, S. and Pyne, N. J. (1993) *Biochim. Biophys. Acta* **1176**, 313–320
- 17 Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R. and Bourne, H. R. (1992) *Nature (London)* **356**, 159–161
- 18 Howe, L. R. and Marshall, C. J. (1993) *J. Biol. Chem.* **268**, 20717–20720
- 19 Kolch, W., Heiddeckar, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993) *Nature (London)* **364**, 249–252
- 20 Sozeri, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, G. E. and Stabel, S. (1992) *Oncogene* **7**, 3345–3356
- 21 Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. and Sturgill, T. W. (1993) *Science* **262**, 1065–1069