Adenylate cyclase, cyclic AMP and extracellular-signal-regulated kinase-2 in airway smooth muscle: modulation by protein kinase C and growth serum

Noreen MOUGHAL, Patricia A. STEVENS, Dixon KONG, Susan PYNE and Nigel J. PYNE*

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

Bradykinin and phorbol 12-myristate 13-acetate stimulate adenylate cyclase activity in serum-depleted cultured airway smooth muscle via a protein kinase C (PKC)-dependent pathway. The probable target is the type II adenylate cyclase, which can integrate coincident signals from both PKC and G_s . Therefore, activation of G_s (by cholera-toxin pre-treatment) amplified the bradykinin-stimulated cyclic AMP signal and concurrently attenuated the partial activation of extracellular-signal-regulated kinase-2 (ERK-2) by bradykinin. We have previously demonstrated that, in order to induce full activation of ERK-2 with bradykinin, it is necessary to obliterate PKC-stimulated cyclic AMP formation. We concluded that the cyclic AMP signal limits the magnitude of ERK-2 activation [Pyne, Moughal, Stevens, Tolan and Pyne (1994) *Biochem. J.* **304**, 611–616]. The present study indicates that the bradykinin-stimulated ERK-2 pathway is entirely cyclic AMP-sensitive, and suggests that coincident signal detection by adenylate cyclase may be an important

physiological route for the modulation of early mitogenic signalling. Furthermore, the direct inhibition of adenylate cyclase activity enables bradykinin to induce DNA synthesis, indicating that the PKC-dependent activation of adenylate cyclase limits entry of cells into the cell cycle. These studies suggest that the mitogenicity of an agonist may be governed, in part, by its ability to stimulate an inhibitory cyclic AMP signal pathway in the cell. The activation of adenylate cyclase by PKC appears to be downstream of phospholipase D. However, in cells that were maintained in growth serum (i.e. were not growth-arrested), bradykinin was unable to elicit a PKC-stimulated cyclic AMP response. The lesion in the signal-response coupling was not at the level of either the receptor or phospholipase D, which remain functionally operative and suggests modification occurs at either PKC or adenylate cyclase itself. These studies are discussed with respect to the cell signal regulation of mitogenesis in airway smooth muscle.

INTRODUCTION

We have previously demonstrated that bradykinin, phorbol 12-myristate 13-acetate (PMA) and, to a substantially lesser extent, platelet-derived growth factor (PDGF), stimulate cyclic AMP formation in serum-depleted cultured airway smooth-muscle cells via a protein kinase C (PKC)-dependent pathway and, for bradykinin, the magnitude of the response limits the activation of extracellular-signal-regulated kinase-2 (ERK-2) [1,2]. The inhibition of adenylate cyclase, by using a P-site-specific inhibitor, deoxyadenosine, enabled bradykinin to stimulate sustained activation of ERK-2 with similar kinetics to that induced by PDGF [2]. The PKC-dependent activation of adenylate cyclase appears to be down-stream of phosphatidylcholine-specific phospholipase D (PLD) [1,2]. The role of PLD activity in early mitogenic signalling is controversial, with both supporting and conflicting evidence [3–6]. However, in airway smooth muscle, bradykinin does not support mitogenesis, but stimulates phosphatidylcholine-specific PLD activity [7–9], and this further questions the role of PLD as an early signalling event in the initiation of proliferation in this cell type.

Activation of adenylate cyclase by PKC can be amplified by constitutive activation of the stimulatory G-protein G_s [1,2]. In this regard, the co-incident PKC-sensitive signal detector type II adenylate cyclase is expressed as a 110/112 kDa protein in serum-depleted cell membranes [2]. The molecular basis for the activation of type II adenylate cyclase in intact cells has been

demonstrated by Jacobowitz and Iyengar [10] to be a consequence of its phosphorylation by PKC.

In this paper we have examined whether the detection and integration of the PKC and G_s signals by adenylate cyclase can subsequently elicit the complete attenuation of the activation of ERK-2 by bradykinin. We also tested the hypothesis that inhibition of adenylate cyclase activity can modulate the mitogenic potential of bradykinin. Additionally, we have also assessed the effect of serum on the PKC-dependent regulation of adenylate cyclase, in order to establish if cell progression into the cell cycle under growth-promoting conditions is associated with altered regulation of this isoform.

MATERIALS AND METHODS

Materials

All biochemicals were from Boehringer Mannheim (Germany), and general chemicals were from Sigma Chemical Co. (U.K.). Cyclic [3 H]AMP, [3 H]palmitate and ECL detection kits 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-ERK antibody was from Affiniti (U.K.). Horseradish-peroxidase-linked anti-mouse antibody was from the Scottish Antibody Production Unit (Carlisle, Scotland, U.K.).

Abbreviations used: PKC, protein kinase C; PLD, phospholipase D; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; ERK, extracellular-signal-regulated kinase; DME, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; DHS, donor horse serum.

* To whom correspondence should be addressed.

Cell culture

Primary cultures of guinea-pig airway smooth-muscle cells were prepared as described previously [7]. The cells were maintained in Dulbecco's modified Eagle's medium (DME) containing 10% (v/v) fetal-calf serum (FCS) and 10% (v/v) donor horse serum (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 showing the presence of α -actin using a smooth-muscle-specific mouse anti- α -actin monoclonal antibody [7].

Cyclic AMP and PLD assays

Cells were placed in fresh DME containing either 10% (v/v) FCS and 10% (v/v) DHS or 1% (v/v) FCS and 1% (v/v) DHS for 24 h before use. Cell counts were performed after this procedure, and no significant difference was observed (results not shown). For cyclic AMP assays, cells were sometimes pre-incubated for 18 h with cholera toxin (0.5 ng/ml). 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 bradykinin (1 μ M), PMA (1 μ M) or forskolin (100 μ M) for 10 min at 37 °C.

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. [11].

PLD assays were performed as described by Pyne and Pyne [7].

Thymidine incorporation

Cells (70–80% confluent) were placed in DME containing 0.1% (v/v) FCS and 0.1% (v/v) DHS for 24 h. After this time the cells were placed in serum-free DME and agonists at the indicated concentrations for a further 20 h. The cells were placed in serum-free DME plus agonists with [3H]thymidine (0.25 μ Ci/0.25 $\times 10^6$ cells) for 5 h. The incubations were terminated by washing each sample in 1 ml of ice-cold PBS, pH 7.4. The supernatant was aspirated and cells were washed with 3 $\times$ 1 ml of 10% (w/v) trichloroacetic acid. Nuclear material was dissolved in 0.25 ml of 0.1% (w/v) SDS/0.3 M NaCl, and [3H]thymidine incorporation was quantified by liquid scintillation counting.

Detection of ERK

Cells were placed in DME containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h. After stimulation with bradykinin (1 μ M), the medium was removed and samples were treated in a manner identical with that described previously [2]. In some cases the cells were pre-treated with 0.5 ng/ml cholera toxin in 1% (v/v) FCS and 1% (v/v) DHS for 24 h. Nitrocellulose sheets were blocked and probed with anti-ERK antibody (1:5000 dilution, v/v) [2]. Detection of ERK reactivity was achieved with a reporter horseradish-peroxidase-linked anti-mouse antibody (1:2000 dilution, v/v) [2]. After washing the blots, to remove excess reporter antibody, immunoreactive bands were revealed with the ECL detection kit.

RESULTS

Bradykinin- and PMA-stimulated cyclic AMP formation is amplified by coincident stimulation of G_s

PMA (1 μ M) stimulation of serum-depleted cells did not evoke a cyclic AMP signal (Table 1), despite the ability of PKC to activate adenylate cyclase activity in these cells [1]. This is probably due to a high expression of cyclic AMP phosphodiesterase activity, which can effectively down-regulate the cyclic AMP signal [12]. However, coincident activation of G_s by pre-treating cells with cholera toxin (0.5 ng/ml, 18 h) can amplify the stimulation of adenylate cyclase by PKC and enables an increase in cyclic AMP mass to be detected (Table 1). Cholera toxin routinely evokes a 3–5-fold increase in intracellular cyclic AMP formation (control, 1.1 ± 0.05 pmol/0.25 $\times 10^6$ cells; cholera toxin, 5 ± 2 pmol/0.25 $\times 10^6$ cells; $n = 3$), and therefore constitutively activates only a small proportion of the available pool of G_s . Bradykinin (1 μ M), by virtue of its ability both to stimulate adenylate cyclase via a PKC-dependent pathway and to inhibit cyclic AMP phosphodiesterase activity via a PKC-independent mechanism [1], can elicit an increase in intracellular cyclic AMP formation alone (Table 1). This response can be synergistically amplified by cholera-toxin pre-treatment of the cells (Table 1).

Detection of ERK

ERK-1 and -2 were detected as 45 and 42 kDa proteins respectively on immunoblots of electrophoresed cell lysates (Figure 1). The challenge of serum-depleted cells with maximal stimulating concentrations of bradykinin (1 μ M) provoked only the partial activation of the available pool of ERK-2 (Figure 1a). It has previously been demonstrated that phosphorylation and activation of ERK-2 results in its retarded mobility on SDS/PAGE [13–16], and this can be observed on immunoblots (Figure 1a). Surprisingly, ERK-1 did not display an electrophoretic mobility shift in response to bradykinin, even though in some cases we have detected a mobility shift in lysates isolated from cells pre-treated with PDGF (results not shown). However, our inability to observe shifts in ERK-1 consistently may be due to the experimental conditions of our SDS/PAGE.

The PKC-sensitive cyclic AMP pool can inhibit the signal pathway that leads to ERK-2 activation in response to bradykinin. This was achieved by demonstrating that the bradykinin-stimulated ERK-2 response can be completely blocked in serum-depleted cells pre-treated with cholera toxin (0.5 ng/ml, 18 h;

Table 1 Effect of PMA and bradykinin on cyclic AMP formation: integration of PKC and G_s signals

Effect of PMA (1 μ M) and bradykinin (BK, 1 μ M) challenge of cells on intracellular cyclic AMP. Results are expressed as pmol of cyclic AMP/0.25 $\times 10^6$ cells and are means $\pm$ S.D. for experiments performed in triplicate on three separate cell preparations. The basal cyclic AMP level in cholera-toxin (CT)-treated (0.5 ng/ml, 18 h) cells was 5 ± 2 pmol/0.25 $\times 10^6$ cells ($n = 3$ separate experiments). All experiments were performed in cells quiesced in 1% serum.

Treatment	Cyclic AMP (pmol/0.25 $\times 10^6$ cells)
Control	1.1 ± 0.05
BK (1 μ M)	20 ± 7
PMA (1 μ M)	1.3 ± 0.4
CT plus BK (1 μ M)	157 ± 6
CT plus PMA (1 μ M)	11.5 ± 1.3

Figure 1 Activation of ERK-2 in response to bradykinin: effect of cholera toxin

(a) Time course of bradykinin (1 μM)-stimulated ERK-2 activity; (b) time course of bradykinin (1 μM)-stimulated ERK-2 activation in cells pre-treated with cholera toxin (0.5 ng/ml, 18 h). 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 four separate cell preparations. ERK-2 is denoted by an arrow.

Table 2 Thymidine incorporation into DNA

[³H]Thymidine incorporation into DNA was determined as described in the Materials and methods section. Results are presented as means ± S.D. for *n* = 5 observations. Abbreviation: BK, bradykinin.

Treatment	[³ H]Thymidine (d.p.m.)
Control	13 937 ± 3848
BK (1 μM)	14 712 ± 2287
Deoxyadenosine (100 μM)	11 678 ± 2012
BK plus deoxyadenosine	22 000 ± 2100

Figure 1b). Under these conditions, we have demonstrated that bradykinin can elicit a substantial increase in cyclic AMP formation ([1,2]; the present work).

Thymidine incorporation

Bradykinin (1 μM) did not significantly increase [³H]thymidine incorporation into DNA. Deoxyadenosine (100 μM) pre-treatment of the cells allowed maximal concentrations of bradykinin to stimulate [³H]thymidine incorporation into nuclear DNA, with an approx. 2-fold increase being observed (Table 2). However, the increase in DNA synthesis was smaller in magnitude when compared with that produced by PDGF (results not shown) and suggests that, for this growth factor, other signal pathways are also involved in initiating mitogenesis.

Table 3 Effect of growth serum on bradykinin-stimulated intracellular cyclic AMP formation in airway smooth-muscle cells

Effect of long-term treatment with growth serum (1% FCS and 1% DHS) on the bradykinin (100 nM)-stimulated cyclic AMP accumulation: comparison with the effect of 10% FCS and 10% DHS. Results are expressed as pmol of cyclic AMP/0.25 × 10⁶ cells and are means ± S.D. for experiments performed in triplicate on three separate cell preparations.

Treatment	Cyclic AMP (pmol/0.25 × 10 ⁶ cells)
1% Serum	
Control	1.2 ± 0.16
Bradykinin (100 nM)	10.8 ± 0.8
10% Serum	
Control	1.6 ± 0.4
Bradykinin (100 nM)	2.9 ± 0.65

Table 4 Effect of growth serum on basal and forskolin-stimulated intracellular cyclic AMP formation in cholera-toxin-treated airway smooth-muscle cells

Effect of long-term treatment with growth serum on forskolin-stimulated cyclic AMP accumulation in cholera-toxin-treated cells. Results are expressed as fold increases in cyclic AMP above control levels in cholera-toxin-treated cells, and are means ± S.D. for experiments performed in triplicate and are representative of three separate cell preparations.

Treatment	Cyclic AMP (fold increase)
1% Serum	
Control	1
Forskolin (100 μM)	16.8 ± 4.2
10% Serum	
Control	1.3 ± 0.4
Forskolin (100 μM)	31 ± 6.3

Effect of serum on type II adenylate cyclase

In cells that were maintained in serum (10% FCS, 10% DHS), a partial attenuation of the ability of bradykinin to provoke cyclic AMP formation was achieved (Table 3). Whether this reflects a co-ordinate blockade or is dependent on the transition phase of the cells in the cell cycle, which renders the adenylate cyclase insensitive, remains to be determined. The lesion in adenylate cyclase regulation appears to be down-stream of the PKC that activates this enzyme, since maximal bradykinin-stimulated PLD activity was unaffected (measured as [³H]palmitate incorporation into phosphatidylbutan-1-ol. Cells in 1% serum: control, 1087 ± 71 d.p.m.; bradykinin (100 nM), 3055 ± 24 d.p.m. Cells in 10% serum: control, 1053 ± 17 d.p.m.; bradykinin (100 nM), 2875 ± 107 d.p.m.; *n* = 3}. Paradoxically, serum stimulation (10% FCS, 10% DHS) of the cells results in increased forskolin-stimulated cyclic AMP formation (approx. 2-fold increase; Table 4).

DISCUSSION

PKC-sensitive type II adenylate cyclase and mitogenesis

Bradykinin elicits a partial activation of ERK-2 in airway smooth muscle. From our previous study, we demonstrated that, in order to induce full activation of ERK-2 with bradykinin, it is necessary to obliterate the PKC-stimulated cyclic AMP signal [2]. We concluded that the cyclic AMP signal limits the magnitude of ERK-2 activation. However, the questions regarding the cyclic

AMP-sensitivity of the signal pathways that do allow bradykinin to stimulate ERK-2 partially under normal conditions remained unresolved. This is important, since multiple signal pathways can lead to the activation of ERK-2. For instance, ERK-2 can be activated down-stream of (i) other PKC isoforms, (ii) *Raf-1* kinase and (iii) MEK kinase (ERK-kinase kinase) [13–15]. Furthermore, G-protein-linked and growth factor receptors may differentially regulate these pathways. However, for bradykinin it is possible that each of these pathways contribute to the activation of ERK-2 activity. The cyclic AMP sensitivity of these pathways is only now being assessed. Indeed, cyclic AMP-dependent protein kinase can catalyse phosphorylation of *Raf-1* and prevent its association with p21^{ras} [16,17], and this leads to the down-stream blockade of ERK-2 activation. Therefore it is possible that, if one of these pathways is insensitive to cyclic AMP, it could account for the proportion of ERK-2 that is activated by bradykinin. The other pathways could then integrate at the level of ERK-2, once adenylate cyclase is blocked. However, the ability of cholera toxin to ablate the activation of ERK-2 by bradykinin, while enabling the coincident signals ($G_s\alpha$ and PKC) to be detected and integrated by adenylate cyclase, indicates that this does not appear to be the case, and supports the concept that it is the size of the cyclic AMP response that determines the magnitude and kinetics of ERK-2 activation in this case.

The effect of deoxyadenosine is to convert the non-mitogenic agonist, bradykinin, into one that can support mitogenesis. This is not immediately obvious from our previous study [2], showing that full sustained activation of ERK-2 is also achieved under these conditions, particularly as the latter requirement for mitogenesis is not unequivocally proven. Therefore, to our knowledge, this is the first demonstration describing such conversion of an agonist and indicates that its mitogenicity is determined, in part, by its ability to activate an endogenous inhibitory cyclic AMP signal pathway.

Type II adenylate cyclase regulation: effect of serum

The mechanism of blockade of PKC-sensitive activation of adenylate cyclase in serum-stimulated cells remains to be fully characterized, but may be an adaptive mechanism which enables cells to progress into the cell cycle by allowing unimpeded ERK activation. The lesion is not at the level of PLD. From our previous studies, the activation of PLD by bradykinin appears to involve a novel bradykinin receptor type, extracellular Ca^{2+} and PKC activity [7,8]. We do not know as yet whether the PKC that regulates PLD activity can also modulate adenylate cyclase,

although presumably this is not the case, given the requirement of adenylate cyclase activity for prior PLD activation. Nevertheless, none of the signal components regulating PLD appears to be altered by serum. Therefore, we speculate that the lesion occurs between adenylate cyclase and PKC. Whether, this involves the induction of a serum-factor-dependent tyrosine kinase that is capable of modifying the signal coupling remains to be determined.

Conclusion

The cyclic AMP response pathway can be negatively modulated by serum factors, although the full significance on mitogenesis requires further study. New evidence is presented, demonstrating that integration of both PKC and G_s signals by adenylate cyclase can dramatically affect ERK-2 activation kinetics and DNA synthesis, and this may have an important physiological implication for the amplification of PKC-stimulated signals by G_s -linked receptor agonists and the consequent regulation of mitogenesis.

This study was supported by the Wellcome Trust.

REFERENCES

- 1 Stevens, P., Pyne, S., Grady, M. and Pyne, N. J. (1994) *Biochem. J.* **297**, 233–239
- 2 Pyne, N. J., Moughal, N., Stevens, P. A., Tolan, D. and Pyne, S. (1994) *Biochem. J.* **304**, 611–616
- 3 Purkiss, J. R. and Boarder, M. (1992) *Biochem. J.* **287**, 31–36
- 4 McKenzie, F. R., Seuwen, K. and Pouyssegur, J. (1992) *J. Biol. Chem.* **267**, 22759–22769
- 5 Durieux, M. E. and Lynch, K. R. (1993) *Trends Pharmacol. Sci.* **14**, 249–254
- 6 Boarder, M. (1994) *Trends Pharmacol. Sci.* **15**, 57–62
- 7 Pyne, S. and Pyne, N. J. (1993) *Biochem. Pharmacol.* **45**, 593–603
- 8 Pyne, S. and Pyne, N. J. (1994) *Cell. Signalling* **6**, 269–277
- 9 Pyne, S. and Pyne, N. J. (1993) *Br. J. Pharmacol.* **110**, 477–481
- 10 Jacobowitz, O. and Iyengar, R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10630–10634
- 11 Rubin, C. S., Erlichman, J. and Rosen, O. M. (1974) *Methods Enzymol.* **38**, 308–315
- 12 Burns, F. and Pyne, N. J. (1992) *Biochem. Biophys. Res. Commun.* **186**, 1081–1088
- 13 Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. and Johnson, G. (1993) *Science* **260**, 315–319
- 14 Kolch, W., Heiddecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993) *Nature (London)* **364**, 249–252
- 15 Sozeri, O., Vollmer, K., Liyanage, M., Frith, D., Kour, G., Mark, G. E. and Stabel, S. (1992) *Oncogene* **7**, 3345–3356
- 16 Cook, S. J. and McCormick, F. (1993) *Science* **262**, 1069–1072
- 17 Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J. and Sturgill, T. W. (1993) *Science* **262**, 1065–1069