Bradykinin stimulates cAMP synthesis via mitogen-activated protein kinase-dependent regulation of cytosolic phospholipase A₂ and prostaglandin E₂ release in airway smooth muscle

Nigel J. PYNE¹, Deborah TOLAN and Susan PYNE

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

Bradykinin stimulates cAMP synthesis in cultured airway smooth muscle (ASM) cells. This occurs via a pathway that involves: (1) the protein kinase C (PKC)-dependent activation of mitogen-activated protein kinase (MAPK); (2) the MAPK-dependent phosphorylation and activation of cytosolic phospholipase A₂ (cPLA₂) and (3) the utilization of cPLA₂-derived arachidonate by the cyclo-oxygenase pathway to produce prostaglandin E₂ (PGE₂). PGE₂ is released and binds to cell surface receptors to stimulate intracellular cAMP synthesis. The signalling pathway was confirmed by the use of PD098059 [the inhibitor of MAPK kinase-1 (MEK-1) activation], AACOCF₃ (an inhibitor of cPLA₂) and indomethacin (an inhibitor of cyclo-oxygenase), which all reduced bradykinin-stimulated cAMP synthesis. Bradykinin also elicits the inhibition of approx. 60 % of the total cAMP phosphodiesterase activity in the cell [Stevens, Pyne, Grady and Pyne (1994) *Biochem. J.* **297**, 233–239]. This is likely

to decrease the rate of cAMP degradation markedly and therefore to potentiate PGE₂-stimulated cAMP synthesis. Acute treatment of ASM cells with PMA (a direct activator of PKC) also stimulated the MAPK-dependent phosphorylation of cPLA₂. However, in contrast with bradykinin, PMA did not stimulate arachidonate release, suggesting that additional signals (e.g. Ca²⁺ ions) are required for phosphorylation by MAPK to activate cPLA₂. PMA was also without effect on PGE₂ release and cAMP synthesis. Evidence that PKC can also directly regulate adenylate cyclase was obtained by using cells pretreated with cholera toxin. Under these conditions, PMA stimulated cAMP synthesis independently of arachidonate metabolites. Furthermore the combined treatment of cells with PMA (to activate PKC) and PGE₂ (to activate G_s) stimulated synergistic cAMP synthesis. This might be due to the presence of the type 2 adenylate cyclase, which is synergistically activated by G_s and PKC.

INTRODUCTION

The proliferation and formation of ‘synthetic’ airway smooth muscle (ASM) is a key event in the hyperplastic remodelling of the airway and contributes to the pathogenesis of asthma by eliciting the synthesis and release of inflammatory mediators that promote hyper-reactivity reactions. This proliferation is regulated by growth factors and G-protein linked receptor agonists that signal to the nucleus via mitogen-activated protein kinase (MAPK) to regulate gene transcription. Both growth factors and G-protein-linked receptor agonists stimulate the conversion of inactive GDP-Ras to active GTP-Ras and the activation of c-Raf, MAPK kinase (MEK) and MAPK [1]. Additional routes regulating the MAPK cascade include: (1) the stimulation of glycerophospholipid hydrolysis and the formation of diacylglycerol, an activator of protein kinase C (PKC), which phosphorylates c-Raf [2], and (2) Giβγ-mediated stimulation of Shc phosphorylation, which is upstream of the MAPK cascade [3].

MAPK can link to arachidonate metabolism and the formation of prostaglandins by its phosphorylation of cytosolic phospholipase A₂ (cPLA₂) [4]. Arachidonate is formed from the hydrolysis of phosphatidylinositol and phosphatidylcholine (reviewed in [5]).

In smooth muscle, cPLA₂ is the major rate-limiting signalling route for the synthesis and release of prostaglandin E₂ (PGE₂) [6]. Bradykinin (BK) stimulates PGE₂ synthesis and release in cultured bovine ASM cells [7]. In this regard, PGE₂ might

function as an autocrine to stimulate cAMP synthesis by binding to G_s-linked receptors coupled to adenylate cyclase [6,8,9]. This is supported by reports that show that BK stimulates cAMP formation in cultured guinea-pig ASM cells [10,11]. This response is dependent on PKC, which might link via MAPK to the phosphorylation and activation of cPLA₂ and the subsequent metabolism of arachidonate.

Adenylate cyclase is composed of at least nine isoforms (reviewed in [12]). ASM cells contain a type 2 adenylate cyclase [10]. This enzyme can be phosphorylated and activated by PKC (reviewed in [12]), which might provide an additional mechanism for the regulation of cAMP synthesis in ASM cells.

cAMP inhibits mitogen-stimulated DNA synthesis in smooth muscle [8,13]. Therefore the MAPK-dependent phosphorylation of cPLA₂ and the subsequent formation of arachidonate metabolites (e.g. PGE₂) that stimulate cAMP synthesis might represent a negative feedback pathway that can govern the efficacy of certain mitogens such as BK. We have therefore further investigated this proposed signal transduction model in cultured ASM cells.

MATERIALS AND METHODS

Materials

All biochemicals, including collagenase, elastase, soybean trypsin inhibitor, BK and PMA, were from Sigma Chemical Co. (Poole,

Abbreviations used: ASM, airway smooth muscle; BK, bradykinin; cPLA₂, cytosolic phospholipase A₂; DHS, donor horse serum; DME, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PGE₂, prostaglandin E₂; PKC, protein kinase C.

¹ To whom correspondence should be addressed.

Dorset, U.K.). [γ - ^{32}P]ATP (3000 Ci/mmol), [^3H]cAMP (24 Ci/mmol), ^{125}I -PGE₂ scintillation proximity assay system, the MAPK Biotrak assay and the enhanced chemiluminescence kits were from Amersham International (Little Chalfont, Bucks., U.K.). [^3H]Arachidonate was from NEN Dupont (Hounslow, Middx., U.K.). Cell culture supplies were from Gibco (Paisley, Scotland, U.K.). AACOCF₃ was from Affiniti (Exeter, Devon, U.K.). Reporter horseradish peroxidase-conjugated anti-mouse antibody was from the Scottish Antibody Production Unit (Carluke, Scotland, U.K.). PD098059 was from New England Biolabs Ltd. (Hitchin, Herts., U.K.). Anti-cPLA₂ antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Male Dunkin–Hartley guinea pigs (200–400 g) were used for the isolation of tracheal smooth muscle.

Cell culture

The preparation of the primary cultures of guinea-pig ASM cells was achieved as described previously [14]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) and 10% (v/v) donor horse serum (DHS) at 37 °C under air/CO₂ (19:1) and these were passaged twice with trypsin before experimentation. Cells were grown to confluence on 24-well or 6-well plates and routinely used at 15–21 days after the initial preparation. Their identity was confirmed to be smooth muscle by the presence of α -actin with a smooth-muscle-specific mouse anti- α -actin monoclonal antibody [14].

cAMP measurements

Cells in 24-well plates (typically 2.5×10^5 cells per well) were placed in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS in the presence or absence of cholera toxin (0.5 ng/ml) for 18 h at 37 °C. After this time the cells were washed and preincubated at 37 °C with gassed [O₂/CO₂ (19:1)] Krebs–Henseleit medium containing 10 mM glucose, 1% (w/v) BSA and 1.5 mM CaCl₂ for 30 min. Cells were then stimulated with either BK or PMA, in the presence or absence of inhibitors for the times indicated. Incubations were terminated by removing the medium and precipitating cell protein in 80% (v/v) ethanol. Samples were harvested and centrifuged at 14000 g and then freeze-dried for 12 h. The cAMP content in each sample was determined with a radioligand binding assay employing a bovine heart cAMP-binding protein prepared as described in [15].

Immunoblot shift assays

Cells in 6-well plates were placed in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS for 18 h at 37 °C. Cells were then stimulated with either BK or PMA in the presence or absence of PD098059 for the times indicated. The medium was then removed and boiling sample buffer [0.125 M Tris/HCl (pH 6.7)/0.5 mM Na₂P₂O₇/1.25 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, then subjected to SDS/PAGE and transferred to nitrocellulose sheets. For cPLA₂ detection, the proteins were subjected to electrophoresis until the 70 kDa protein marker reached the bottom of the gel. Nitrocellulose sheets were subsequently blocked in 3% (w/v) BSA in 10 mM Tris/HCl (pH 8)/0.15 M NaCl (TBS) for 1 h at 23 °C and then probed with anti-cPLA₂ antibody in TBS containing 1% (w/v) BSA plus 0.05% (v/v) Tween-20 for 2 h at 23 °C. After this time, the nitrocellulose sheets were washed in TBS plus 0.05% (v/v) Tween-20. Detection of immunoreactivity was

achieved by incubating nitrocellulose sheets for 1 h at 23 °C with reporter horseradish peroxidase-linked anti-mouse antibody in TBS containing 1% (w/v) BSA plus 0.05% (v/v) Tween-20. After the blots had been washed as described above, to remove excess reporter antibody, immunoreactive bands were detected with the enhanced chemiluminescence detection kit.

MAPK activity assays in isolated cell lysates

Cells in 6-well plates were placed in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS for 24 h at 37 °C. Cells were then stimulated with either BK or PMA, in the presence or absence of bisindolylmaleimide I for the times indicated. In some cases cells were treated with PMA (1 μM) for 24 h to down-regulate PKC isoforms before stimulation with agonist. MAPK activity was measured with the Biotrak MAPK assay kit. This utilizes a specific MAPK peptide substrate (epidermal growth factor receptor peptide synthesized to contain one phosphorylation site). Cell lysates were prepared by adding 0.5 ml of buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin. Cells were harvested into this buffer and homogenized by passing through a 0.24 mm gauge syringe three times to obtain a cell lysate. Each assay contained 10 μg of cell lysate protein and 0.5 μCi of [γ - ^{32}P]ATP (final concentration 0.2 mM). Assays were performed under conditions where less than 2% of the substrate was utilized and where product formation was linearly related to time. After incubation, assays were terminated by adding stopping solution. Samples were then spotted on peptide-binding papers and washed three times with 250 ml of 1% (v/v) acetic acid and twice with 250 ml of distilled water. The amount of ^{32}P -labelled peptide on each disc was quantified by liquid-scintillation counting.

Isolation of PGE₂ and assay

Cells in 24-well plates were placed in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS for 18 h at 37 °C. After this time the cells were washed and preincubated at 37 °C with gassed [O₂/CO₂ (19:1)] Krebs–Henseleit medium containing 10 mM glucose, 1% (w/v) BSA and 1.5 mM CaCl₂ for 30 min. Cells were then stimulated with either BK or PMA in the presence or absence of PD098059 for the times indicated. For each sample the medium was removed and added to 0.25 ml of water/ethanol (1:4, v/v) and 5 μl of acetic acid. These were left at 23 °C for 5 min and applied to AMprep C₁₈ (100 mg size) minicolumns that had previously been primed with two column volumes of 10% (v/v) ethanol. After applying the samples, the columns were washed with one column volume of distilled water and one column volume of hexane. PGE₂ was eluted with 2×0.75 ml of ethyl acetate. The ethyl acetate samples were then evaporated with a Univap centrifuge and taken for estimation of PGE₂ levels with the ^{125}I -PGE₂ scintillation proximity assay system. With standard PGE₂ samples the recovery from the AMprep C₁₈ (100 mg size) minicolumns was calculated to be 80%.

[^3H]Arachidonate release

Cells in 24-well plates were placed in DMEM containing 1% (v/v) FCS and 1% (v/v) DHS and 0.2 μCi of [^3H]arachidonate for 24 h at 37 °C. After this time the cells were washed and preincubated at 37 °C with gassed [O₂/CO₂ (19:1)] Krebs–Henseleit medium containing 10 mM glucose, 1% (w/v) BSA and 1.5 mM CaCl₂ for 30 min. Cells were then stimulated with

either BK or PMA for the times indicated. After incubation the medium was removed and the amount of [^3H]arachidonate released was quantified by liquid-scintillation counting.

RESULTS AND DISCUSSION

Regulation of cAMP synthesis by BK

We have confirmed our previous finding [10,11] that the incubation of ASM cells with BK for 10 min stimulated cAMP synthesis [cAMP levels expressed as pmol of cAMP per 2.5×10^5 cells (means $\pm$ S.D. for $n = 5$ cell preparations) were: control, 1.52 ± 0.1 ; BK (100 nM), 24 ± 7.5]. The pathway regulating cAMP synthesis after the binding of BK to its receptor might involve: (1) the PKC-dependent activation of MAPK, (2) the MAPK-dependent phosphorylation and activation of cPLA₂ and (3) the utilization of cPLA₂-derived arachidonate by the cyclo-oxygenase pathway to produce PGE₂. PGE₂ acts as an autocrine by binding to G_s-linked receptors to stimulate intracellular cAMP formation. The following experimental findings provide support for this model. First, BK was shown to activate MAPK. In Figure 1 we show that the incubation of cells with BK stimulated a dose-dependent increase in MAPK activity with an EC₅₀ of approx. 40 nM, which is close to the determined EC₅₀ for cAMP synthesis (approx. 60 nM) reported previously by us [11]. Maximal activation of MAPK was elicited at 1 μM BK (Figure 1). Preincubation of cells with the PKC inhibitor bisindolylmaleimide I (10 μM) for 10 min abolished the activation of MAPK in response to both BK and PMA, a direct activator of PKC (Table 1). A role for PKC was further supported by demonstrating that chronic PMA treatment (1 μM for 24 h) of cells, to down-regulate PKC isoforms, also abolished the acute stimulation of MAPK activity in response to BK and PMA (Table 1).

Secondly, BK was shown to stimulate the phosphorylation and activation of cPLA₂ via an MAPK-dependent pathway. SDS/PAGE and Western blotting with an anti-cPLA₂ antibody identified both dephosphorylated (85 kDa, fast migrating band) and phosphorylated (slowly migrating band) forms of cPLA₂ in lysates from control cells (Figure 2). The treatment of cells with BK stimulated the phosphorylation of cPLA₂, as evidenced by a shift in its mobility to the slowly migrating form. The preincubation of cells with PD098059 (50 μM) (an inhibitor of MEK-1 activation [16]) decreased the BK-stimulated phosphorylation of cPLA₂ to a level below that observed in control cells. This was detected by a decrease in the amount of cPLA₂ shifted to the slowly migrating form (Figure 2). The change in cPLA₂ phosphorylation was correlated with a similar decrease in MAPK activation (results not shown). Acute treatment of ASM cells with PMA also stimulated the phosphorylation of cPLA₂ and this was prevented by pretreating cells with PD098059 (Figure 2), supporting the idea that PKC is upstream of the MAPK-dependent phosphorylation of cPLA₂.

Although both PMA and BK stimulated the MAPK-dependent phosphorylation of cPLA₂, only BK elicited measurable [^3H]arachidonate release (Table 2). The regulation of cPLA₂ is dependent on the integration of multiple signals, such as Ca²⁺ ions, PKC and MAPK [17–20], i.e. Ca²⁺ ions might be required for phosphorylation by MAPK to activate cPLA₂. Therefore the stimulation of [^3H]arachidonate release by BK might be governed, in part, by the ability of this agonist to mobilize Ca²⁺ ions from both the extracellular medium and intracellular pools. This was supported by experiments showing that the incubation of cells in a 'calcium-free' medium decreased [^3H]arachidonate release in response to BK (Table 2). Depletion of Ca²⁺ ions from

Figure 1 Effect of BK on MAPK activity

ASM cells in 6-well plates were stimulated with various concentrations of BK (0.1 nM–10 μM) for 10 min. MAPK activity in cell lysates was measured with a specific epidermal growth factor receptor peptide assay kit. Basal MAPK activity was 115 ± 5 pmol/min per mg of protein. Results are expressed as ΔMAPK (pmol of ^{32}P incorporated into synthetic peptide/min per mg of protein; means $\pm$ S.D. for $n = 3$ cell preparations), representing the change in MAPK activity in response to BK above basal.

Table 1 Effect of PKC down-regulation and bisindolylmaleimide I on BK-stimulated MAPK activity

ASM cells in six-well plates were either treated chronically with PMA (1 μM) for 24 h or acutely with or without bisindolylmaleimide I (10 μM) for 10 min before stimulation with either BK (1 μM) or PMA (100 nM) for 10 min. In the absence of drug, cells were treated with vehicle [DMSO, final concentration 1% (v/v)] for 10 min before cell stimulation. MAPK activity in cell lysates was assayed with a specific epidermal growth factor receptor peptide assay kit. Basal MAPK activity was 123.4 ± 2.5 pmol/min per mg of protein. Results are expressed as ΔMAPK activity (pmol of ^{32}P incorporated into synthetic peptide/min per mg of protein; means $\pm$ S.D. for $n = 3$ cell preparations), representing the change in MAPK activity in response to BK and PMA above respective controls. Bisindolylmaleimide I and chronic PMA were without effect on basal MAPK activity.

Treatment	ΔMAPK activity (pmol/min per mg of protein)	
	BK	PMA
Untreated	125 ± 22	200 ± 6
Bisindolylmaleimide I (10 μM)	2.4 ± 0.5	4 ± 1
Chronic PMA (1 μM)	5.1 ± 2	4.5 ± 1

intracellular stores has also been shown to prevent the cPLA₂-derived arachidonate release from smooth muscle [21].

Thirdly, arachidonate is metabolized by the cyclo-oxygenase pathway to produce PGE₂. ASM cells release approx. 0.3 ± 0.001 ng of PGE₂ per 2.5×10^5 cells under basal conditions (results expressed as means $\pm$ S.D. for three separate cell preparations). The incubation of cells with BK (100 nM) typically stimulated a 3–4-fold increase in PGE₂ synthesis/release (Table 3). This corresponds to an extracellular concentration of 12 nM PGE₂ under the assay conditions used. The preincubation of cells with PD098059 (50 μM) decreased basal PGE₂ synthesis/release and completely abolished the stimulatory effect of BK. In contrast, PMA (100 nM) did not stimulate PGE₂ release (results

Figure 2 Effect of BK, PMA and PD098059 on the phosphorylation of cPLA₂

Immunoblot showing the effect of PD098059 on the PMA- and BK-stimulated phosphorylation of cPLA₂. ASM cells in 6-well plates were treated with or without PD098059 (50 μ M) for 10 min before stimulation with either BK (100 nM) or PMA (100 nM) for 10 min. In the absence of drug, cells were treated with vehicle [DMSO, final concentration 1% (v/v)] for 10 min before agonist stimulation. Cell lysates were immunoblotted with anti-cPLA₂ as described in the Materials and methods section. This is a representative result of an experiment performed on four separate cell preparations. The positions of cPLA₂ and phosphorylated cPLA₂ are denoted by arrows.

not shown) or cAMP synthesis under these conditions [cAMP values (pmol per 2.5×10^5 cells) expressed as means $\pm$ S.D. for $n = 5$ cell preparations: control, 1.5 ± 0.1 ; PMA (100 nM), 1.3 ± 0.2].

Fourthly, the addition of PGE₂ to ASM cells stimulated cAMP synthesis, indicating the presence of functional PGE₂ receptors. In Figure 3 we show that PGE₂ stimulated a dose-dependent increase in cAMP synthesis (approx. $EC_{50} = 0.7 \mu$ M). Maximal cAMP synthesis of 20–30 pmol of cAMP per 2.5×10^5 cells was observed at 3 μ M PGE₂. The stimulation of cAMP formation by PGE₂ was markedly amplified in cells treated with cholera toxin (0.5 ng/ml for 18 h). Under these conditions the EC_{50} for PGE₂ was considerably lower, approx. 0.05 μ M. Maximal cAMP synthesis was stimulated by 0.3 μ M PGE₂ and corresponded to the formation of 110 pmol of cAMP per 2.5×10^5 cells (Figure 3). There have been at least two previous independent studies in different cell types that replicate this effect of cholera toxin on agonist-stimulated cAMP formation [22,23]. If PGE₂ mediates the effect of BK, it follows that cAMP synthesis in response to BK should be amplified in cholera toxin-treated cells. This was shown to be so: 100 nM BK stimulated 110–120 pmol of cAMP per 2.5×10^5 cells (compared with 24 ± 7.5 pmol of cAMP per 2.5×10^5 cells in the absence of cholera toxin).

Final confirmation that BK regulates cAMP synthesis via a pathway involving the MAPK-dependent phosphorylation of cPLA₂ and the formation of arachidonate metabolites (e.g. PGE₂) was achieved with selective inhibitors. Thus pretreatment

Table 3 Effect of BK on PGE₂ release

ASM cells in 24-well plates were treated with or without PD098059 (50 μ M) for 10 min before stimulation for 10 min with BK (100 nM). In the absence of drug, cells were treated with vehicle [DMSO, final concentration 1% (v/v)] for 10 min before stimulation with agonist. PGE₂ values (means $\pm$ S.D. for $n = 3$ separate cell preparations) are expressed as ng per 2.5×10^5 cells.

Addition	PGE ₂ released (ng per 2.5×10^5 cells)
None (control)	0.3 ± 0.001
BK	1.1 ± 0.05
PD098059	0.084 ± 0.001
PD098059 + BK	0.11 ± 0.03

Figure 3 Effect of PGE₂ on cAMP synthesis

ASM cells in 24-well plates were treated with (●) or without (○) cholera toxin (0.5 ng/ml for 18 h) before stimulation with various concentrations of PGE₂ (0.001–3 μ M) for 10 min. Samples were harvested rapidly and cAMP concentration was measured in triplicate as described in the Materials and methods section. cAMP values (means $\pm$ S.D. for triplicate assays, representative result of $n = 3$ separate cell preparations) are expressed as pmol per 2.5×10^5 cells.

of cells (control and cholera toxin-treated) with either PD098059 (the inhibitor of MEK-1 activation), AACOCF₃ (a selective inhibitor of cPLA₂) or indomethacin (an inhibitor of cyclooxygenase) markedly decreased cAMP synthesis in response to

Table 2 Effect of BK and PMA on [³H]arachidonate release

ASM cells in 24-well plates were stimulated with either BK (100 nM) or PMA (100 nM) for the indicated times. In some experiments Ca²⁺ ions were excluded from the Krebs–Henseleit medium (supplemented with 2.5 mM EGTA). Cells were preincubated in this medium for 30 min before stimulation with BK. [³H]Arachidonate release (mean $\pm$ S.D. for $n = 3$ separate cell preparations) is expressed as the fold increase above that observed before stimulation. Zero time measurements were: in the presence of Ca²⁺ (+ Ca), 13226 ± 380 d.p.m. per 2.5×10^5 cells; in the absence of Ca²⁺ (– Ca), 13508 ± 366 d.p.m. per 2.5×10^5 cells.

Addition	Incubation time (min) ...	Increase in [³ H]arachidonate (fold)			
		+ Ca		– Ca	
		5	10	5	10
None (control)		1.04 ± 0.04	1.14 ± 0.06	1 ± 0.03	1.1 ± 0.09
PMA		1.04 ± 0.03	1.06 ± 0.04	–	–
BK		1.8 ± 0.05	2.41 ± 0.1	1.2 ± 0.04	1.75 ± 0.02

Table 4 Effect of PD098059, AACOCF₃ and indomethacin on BK-stimulated cAMP synthesis

ASM cells in 24-well plates were treated with or without indomethacin (10 μ M) or PD098059 (50 μ M) for 10 min or AACOCF₃ (30 μ M) for 30 min before stimulation with BK (100 nM) for 10 min. In the absence of drug, cells were treated with vehicle [DMSO, final concentration 1% (v/v)] before stimulation with agonist. DMSO was without effect on either basal or BK-stimulated cAMP formation. cAMP levels (means $\pm$ S.D. for $n = 3$ –5 separate cell preparations) are expressed as pmol per 2.5×10^5 cells.

Addition	cAMP (pmol per 2.5×10^5 cells)
None (control)	1.5 ± 0.1
BK	24 ± 7.5
PD098059	1.4 ± 0.1
PD098059 + BK	2.3 ± 0.2
AACOCF ₃	2.2 ± 0.2
AACOCF ₃ + BK	5 ± 1
Indomethacin	1 ± 0.4
Indomethacin + BK	5.8 ± 1.8

Table 5 Effect of PD098059, AACOCF₃ and indomethacin on BK- and PMA-stimulated cAMP synthesis in cholera toxin-treated cells

ASM cells in 24-well plates were treated with cholera toxin (0.5 ng/ml, 18 h) and then incubated with or without indomethacin (10 μ M) or PD098059 (50 μ M) for 10 min or AACOCF₃ (30 μ M) for 30 min before stimulation with either BK (100 nM) or PMA (100 nM) for 10 min. In the absence of drug, cells were treated with vehicle [DMSO, final concentration 1% (v/v)] before stimulation with agonist. DMSO was without effect on basal, PMA- or BK-stimulated cAMP formation. cAMP levels (means $\pm$ S.D. for $n = 3$ –5 separate cell preparations) are expressed as pmol per 2.5×10^5 cells.

Addition	cAMP (pmol per 2.5×10^5 cells)
None (control)	4.2 ± 0.3
BK	118 ± 14
PMA	13.5 ± 1.5
PD098059	6.8 ± 0.4
PD098059 + BK	22 ± 3.4
PD098059 + PMA	21 ± 1.4
AACOCF ₃	10.5 ± 1.4
AACOCF ₃ + BK	27 ± 10
AACOCF ₃ + PMA	21 ± 5
Indomethacin	1.7 ± 0.8
Indomethacin + BK	41 ± 5
Indomethacin + PMA	11 ± 1

BK (Tables 4 and 5). A decrease in cAMP formation was also observed when Ca²⁺ ions were removed from the incubation medium (results not shown).

In cholera toxin-treated cells, we noted that indomethacin decreased basal cAMP synthesis. This might be due to an inhibition of basal arachidonate metabolite release. It was also noted that AACOCF₃ stimulated a small increase in basal cAMP synthesis, possibly due to the effect of metabolites of this drug on arachidonate levels [24].

We have reported here that 100 nM BK stimulated the release of 12 nM PGE₂. The amount of cAMP measured in response to 12 nM PGE₂ (not significant above basal; Figure 3) and 100 nM BK (24 ± 7.5 pmol of cAMP per 2.5×10^5 cells) are not correlated. Therefore BK must also use additional mechanisms to regulate cAMP synthesis. We have previously obtained evidence to support this [11]. For instance, BK was shown to elicit the

Table 6 Effect of PMA and PGE₂ on cAMP synthesis

ASM cells were treated with cholera toxin (0.5 ng/ml for 18 hours) before stimulation with PMA (100 nM) and/or PGE₂ (1 nM) for 10 min. cAMP levels (means $\pm$ S.D. for $n = 3$ separate cell preparations) are expressed as pmol per 2.5×10^5 cells.

Addition	cAMP (pmol per 2.5×10^5 cells)
None (control)	3.5 ± 0.2
PGE ₂	8.3 ± 0.5
PMA	22.5 ± 0.5
PMA + PGE ₂	49 ± 6

inhibition of approx. 60% of the total cAMP phosphodiesterase activity in the cell [11]. This is likely to decrease markedly the rate of cAMP degradation and therefore to potentiate PGE₂-stimulated cAMP synthesis. BK (via the MAPK/cPLA₂ route) might also elicit the formation of additional arachidonate metabolites that stimulate cAMP synthesis.

Other studies suggest that the regulation of cAMP synthesis by arachidonate metabolites might be present in a number of mammalian cell types, thereby highlighting a wider physiological role in controlling cAMP signalling. For instance, Graves et al. [21] reported that platelet-derived growth factor (PDGF) triggers the phosphorylation of cPLA₂, the release of arachidonate/PGE₂ and the stimulation of cAMP synthesis in arterial smooth muscle cells in human newborns. The pretreatment of these cells with the cyclo-oxygenase inhibitor indomethacin abolished cAMP synthesis. Furthermore, MAPK isolated from PDGF-treated cells was shown to catalyse the phosphorylation of purified cPLA₂ *in vitro*. Rozengurt et al. [25] demonstrated that the addition of PDGF (in the presence of 3-isobutyl-1-methylxanthine) to Swiss 3T3 fibroblasts stimulated a slow sustained increase in intracellular cAMP level. This response was also inhibited by indomethacin. However, these studies did not identify a functional link between MAPK and cAMP in intact cells.

Alternative routes of cAMP synthesis

Evidence that PKC can also directly regulate adenylate cyclase was obtained by using cells pretreated with cholera toxin. Under these conditions PMA was shown to stimulate cAMP synthesis [11] (Table 5). This response was not inhibited by preincubating cells with PD098059, AACOCF₃ or indomethacin (Table 5), confirming that the stimulation of cAMP synthesis was independent of arachidonate metabolites. Furthermore the combined treatment of cells with PMA (to activate PKC) and PGE₂ (to activate G_s) elicited synergistic cAMP synthesis (Table 6). This might be due to the presence of the type 2 adenylate cyclase [10], which is synergistically activated by G_s and PKC [12].

Conclusion

We have identified a signalling pathway in ASM cells that links PKC, MAPK and Ca²⁺ ions with arachidonate metabolism, the production of PGE₂ and the subsequent activation of adenylate cyclase. Furthermore PGE₂-stimulated cAMP synthesis might be amplified by additional signalling pathways that include the inhibition of cAMP phosphodiesterases and the regulation of the type 2 adenylate cyclase by PKC.

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