Bradykinin-dependent activation of adenylate cyclase activity and cyclic AMP accumulation in tracheal smooth muscle occurs via protein kinase C-dependent and -independent pathways

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Treatment of cultured tracheal smooth-muscle cells (TSM) with phorbol 12-myristate 13-acetate (PMA) (100 nM) or bradykinin (100 nM) elicited enhanced basal and guanosine 5'-[β -imido]-triphosphate-stimulated adenylate cyclase activities in subsequently isolated membranes. Combined stimulation of cells was non-additive, indicating that both agents activate adenylate cyclase via similar routes. Both PMA (100 nM) and bradykinin (100 nM) allowed the α subunit of G_s to act as a more favourable substrate for its cholera-toxin-catalysed ADP-ribosylation *in vitro*. PMA was without effect on intracellular cyclic AMP in control cells. However, constitutive activation of G_s by treatment *in vivo* with cholera toxin (0.5 ng/ml, 18 h) sensitized the cells to PMA stimulation, resulting in a concentration-dependent increase in intracellular cyclic AMP accumulation ($EC_{50} = 7.3 \pm 2.5$ nM, $n = 5$). Bradykinin also elicited a concentration-dependent increase in intracellular cyclic AMP ($EC_{50} = 63.3 \pm 14.5$ nM, $n = 3$). Constitutive activation of G_s resulted in an increased maximal response (10-fold) and potency ($EC_{50} = 6.17 \pm 1.6$ nM, $n = 3$) to bradykinin. This response was not affected by the B_2 -receptor antagonist, NPC567 [which selectively blocks bradykinin-stimulated phospholipase C (PLC),

with minor activity against phospholipase D (PLD) activity]. Des-Arg⁹-bradykinin (a B_1 -receptor agonist) was without activity. These results suggest that the receptor sub-type capable of activating PLD may also be stimulatory for cyclic AMP accumulation. Furthermore, pre-treatment of the cells with butan-1-ol (0.3 %, v/v), which traps phosphatidate derived from PLD reactions, blocked the bradykinin-stimulated increase in intracellular cyclic AMP. These studies suggest that there may be a causal link between PLD-derived phosphatidate and the positive modulation of adenylate cyclase activity. In support of this, the concentration-dependence for bradykinin-stimulated adenylate cyclase activity was identical with that of bradykinin-stimulated phospholipase D activity ($EC_{50} = 5$ nM). Bradykinin, but not PMA, was also capable of eliciting the inhibition of cyclic AMP phosphodiesterase activity in TSM cells ($EC_{50} > 100$ nM) via an unidentified mechanism. These studies indicate that cross-regulation between the cyclic AMP pathway and phospholipid-derived second messengers in TSM cells does not occur as a consequence of PLC-catalysed PtdIns(4,5) P_2 hydrolysis, but may involve, in part, PLD-catalysed phosphatidylcholine hydrolysis.

INTRODUCTION

The 'cross-talk' regulation between receptors employing distinct signalling mechanisms serves as a means of integrating the many stimuli to which a cell is potentially exposed. There are numerous examples of perturbations of the adenylate cyclase activity by agonists that provoke activation of protein kinases C and A [1–3]. For protein kinase C, these events can occur via the phosphorylation of adenylate cyclase [4,5]. In frog erythrocytes, this event elicits activation of adenylate cyclase activity, whereas in platelets it leads to uncoupling of adenylate cyclase from the inhibitory G-protein (G_i). On the other hand, phosphorylation of G-protein α subunits has also been demonstrated both *in vitro* and *in situ* [6–13]. The protein kinase C-catalysed phosphorylation of the α subunit of G_i in intact cells leads to its inactivation [6–10]. We have also demonstrated that recombinant $G_s\alpha$ is a substrate for phosphorylation by purified protein kinases C and A [11,12]. In these cases, the guanine-nucleotide-free form of the α subunit of G_s is the preferred substrate for phosphorylation [11,12]. Recombinant $G_s\alpha$ has also been demonstrated to act as a substrate for phosphorylation by pp60^{c-src}, and this results in an

increase in the receptor-stimulated GTPase activity in reconstitution studies and an increase in guanosine 5'-[γ -³⁵S]thio]triphosphate binding [13].

Although it is appreciated that 'cross-talk' regulation exists between protein kinase C and adenylate cyclase, there is a paucity of information regarding the role of phospholipase C (PLC)- and phospholipase D (PLD)-derived phospholipid products in this molecular control mechanism. We demonstrate in this paper that the challenge of cultured tracheal smooth muscle (TSM) cells with phorbol ester (phorbol 12-myristate 13-acetate; PMA) and bradykinin leads to enhanced basal and guanine-nucleotide-stimulated adenylate cyclase activity in subsequently prepared membranes. The modulation of adenylate cyclase activity is associated with an increase in intracellular cyclic AMP accumulation, and is paralleled by an increase in the ability of $G_s\alpha$ to act as a substrate for cholera-toxin-catalysed ADP-ribosylation in subsequently isolated membranes. Bradykinin was also capable of triggering an inhibition of cyclic AMP phosphodiesterase activity. The modulation of adenylate cyclase and cyclic AMP phosphodiesterase activity by these agonists is discussed with respect to the signalling routes employed.

Abbreviations used: p[NH]ppG, guanosine 5'-[β - γ -imido]triphosphate; PMA, phorbol 12-myristate 13-acetate; TSM, tracheal smooth muscle; PLC, phospholipase C; PLD, phospholipase D; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; DHS, donor horse serum; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; DAG, diacylglycerol.

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EXPERIMENTAL

Materials

All biochemicals, such as guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG) and GTP, were from Boehringer Mannheim (Germany), and general chemicals were from Sigma Chemical Co. (U.K.). [α - 32 P]ATP, cyclic [3 H]AMP, [3 H]palmitate, [3 H]Ins(1,4,5) P_3 and cyclic [3 H]AMP-binding assay kit were from Amersham International (U.K.). [32 P]NAD $^{+}$ was from DuPont (U.S.A.). All cell-culture medium was from Gibco (U.K.) and ICN Flow. Collagenase, elastase and soya-bean trypsin inhibitor were from Sigma Chemical Co. (U.K.). Male Dunkin–Hartley guinea-pigs (200–400 g) were used for isolation of TSM.

Cell culture

Primary cultures of TSM were prepared by removing the trachea from guinea-pigs and dissecting free the cartilage and the epithelium to leave the TSM strip. This was performed under sterile conditions in a air-flow cabinet. The TSM was combined with 4 ml of Dulbecco's modified Eagle's medium (DMEM) containing collagenase (type II; 1 mg/ml), elastase (type IV; 0.2 mg/ml) and soya-bean trypsin inhibitor (50 μ g/ml) before transfer into a further 1 ml of the medium and incubation at 37 °C for 1.5–2 h until fully digested. The cell suspension was then diluted (1:5) with DMEM containing 10% (v/v) foetal-calf serum (FCS) and 10% (v/v) donor horse serum (DHS) and divided between two 25 cm 2 collagen-coated tissue flasks, which were incubated at 37 °C in air/CO $_2$ (19:1). The medium was replaced with DMEM containing 10% FCS and 10% DHS after 48 h. The cells were routinely passaged twice, by using trypsin, before experimentation. Cells were grown to confluence and routinely used 13–15 days after the initial preparation, and were confirmed as smooth muscle by the presence of α -actin by using a smooth-muscle-specific monoclonal antibody raised to α -actin [14].

Incubation of cells

Cells were placed in DMEM containing 1% FCS and 1% DHS 24 h before use. The cells were then washed twice in gassed (air/CO $_2$, 19:1) Krebs–Henseleit buffer, pH 7.4, and preincubated in Krebs–Henseleit buffer for 30 min. The cells were then separately challenged with PMA (10 pM–10 μ M), bradykinin (100 pM–10 μ M) or des-Arg 9 -bradykinin (1 μ M) for 10 min at 37 °C. Bradykinin-receptor antagonist (NPC567, 100 pM–10 μ M) was added to cells 2 min before bradykinin. In some cases the cells were given an 18 h preincubation with cholera toxin (0.5 ng/ml) in DMEM containing 1% FCS and 1% DHS. After incubation, the cells were subjected to one of two treatments. (i) They were washed twice in Krebs–Henseleit buffer, pH 7.4, before being harvested in this buffer. The cells were pelleted by centrifugation at 75 g for 2 min and membrane fractions were prepared by homogenizing the pellets in 2 ml of ice-cold buffer A, containing 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethanesulphonyl fluoride and 2 mM benzamidine. Homogenates were centrifuged at 48000 g for 20 min at 4 °C and the subsequent pellet was resuspended in buffer B (containing 10 mM Hepes, pH 7, 10 mM β -glycerophosphate, 2 mM benzamidine and 0.1 mM phenylmethanesulphonyl fluoride and taken for adenylate cyclase assay. (ii) They were precipitated in ethanol, centrifuged at 14000 g for 10 min, and cyclic AMP was extracted into ethanol and freeze-dried. Samples were taken for cyclic AMP determination by using either the cyclic [3 H]AMP-binding assay

(Amersham) or bovine heart cyclic AMP-binding protein purified by us according to [15] and cyclic [3 H]AMP.

Measurement of Ins(1,4,5) P_3

The cells were incubated with 100 μ l of Krebs–Henseleit buffer in the absence or presence of bradykinin as required. Incubations were terminated by the addition of 25 μ l of ice-cold HClO $_4$ (10%, w/v) and the samples placed on ice. Acid extracts were harvested, neutralized by addition of approx. 25 μ l of 1.5 M KOH/60 mM Hepes in the presence of a trace quantity of Universal Indicator, and the resulting supernatants were assayed for Ins(1,4,5) P_3 by using an Ins(1,4,5) P_3 -specific radioligand-binding assay [16], a crude adrenocortical microsomal fraction being used as a binding-protein preparation. A standard curve of 25 fmol–25 pmol was performed in parallel.

Incubation of cells with [3 H]palmitate; PLD and PLC assay

Confluent TSM cells were preincubated with [3 H]palmitate (2 μ Ci/ml) in DMEM containing 1% FCS and 1% DHS for 24 h. This procedure has previously been shown to result in the selective incorporation of radioactivity into phosphatidylcholine (PtdCho), which contained 82% of total radioactivity associated with major phospholipids, in comparison with only 6% associated with phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine combined [14].

Phospholipase D activity was determined using a trans-phosphatidylolation assay [14]. Briefly, [3 H]palmitate-labelled cells were washed and preincubated for 30 min with 500 μ l of Krebs–Henseleit buffer at 37 °C in air/CO $_2$ (19:1) before a further preincubation for 5 min with 250 μ l of Krebs–Henseleit buffer containing butan-1-ol (0.3%, v/v). Bradykinin was added as required, and the incubations were terminated by removal of the medium and addition of ice-cold methanol (200 μ l). Organic extracts were prepared (addition of 400 μ l of chloroform), and the non-metabolizable [3 H]phosphatidylbutanol ([3 H]PtdBut) formed was determined by its resolution on t.l.c. in a solvent of the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, by vol.) as described previously [14]. [3 H]PtdBut, which is a direct indicator of phospholipase D activity, routinely migrated with R_f = 0.35 and was quantified by excising the appropriate area from each lane and counting the associated radioactivity. [3 H]-*sn*-1,2-Diacylglycerol (DAG) levels were determined by resolving lipids by t.l.c. in hexane/diethyl ether/acetic acid (60:40:1, by vol.). A 1,2-DAG standard was co-chromatographed and detected with I_2 before sampling of [3 H]DAG.

Measurement of adenylate cyclase activity

Adenylate cyclase activity was measured as described by Salomon et al. [17]. This involved combining TSM membranes (10–20 μ g of protein) with an activation cocktail containing (final concns.) 12.5 mM Tris/HCl, pH 7.4, 2.5 mM MgSO $_4$, 0.4 mM cyclic AMP, 2.5 mM phosphocreatine, 1.5 units of creatine kinase, 100 μ M ATP, 7.5 mM KCl and 30 mM sucrose. Routinely 1000000 c.p.m. of [α - 32 P]ATP was added to the assay. In some instances a final concentration of 100 μ M p[NH]ppG was added to the reaction cocktail. The incubation was performed at 37 °C for 20 min and terminated by addition of 40 mM ATP and boiling for 2 min. Samples were then centrifuged at 14000 g for 10 minutes and the supernatants were removed. To these was added cyclic [3 H]AMP (5000 c.p.m.) in order to allow the measurement of recovered cyclic [α - 32 P]AMP from Dowex W (H $^{+}$ form) and alumina column chromatography. Recovered

cyclic [α - 32 P]AMP was quantified by liquid-scintillation counting. Experiments were performed where less than 2% of the substrate was used and under conditions of linear rate of formation of product.

Toxin-catalysed ADP-ribosylation of G_i

Cholera-toxin-catalysed ADP-ribosylation of TSM membranes was performed as described by Heyworth et al. [18]. This involved combining membranes (30 μ g of protein) with an ADP-ribosylation cocktail containing (final concns.) 150 mM potassium phosphate, pH 7.6, 3 mM dithiothreitol, 5 μ M CaCl₂, 1 mM MgCl₂ and 7.5 mM thymidine, and to this was added 2 μ Ci [32 P]NAD⁺ (20 μ M). The incubation was initiated by addition of cholera toxin (66 μ g/ml). This had been preactivated by combining it with an equal volume of 50 mM dithiothreitol and incubating for 1 h at room temperature. Ribosylation incubations were performed at 37 °C for 1 h, after which they were terminated by addition of trichloroacetic acid (final concn. 6%, w/v) and deoxycholate (final concn. 0.01%, w/v). The samples were centrifuged at 14000 *g* for 10 min at 4 °C. The resulting pellet was resuspended in 10 μ l of 1 M Tris/HCl and 20 μ l of Laemmli buffer, and the samples were boiled for 5 min before SDS/PAGE (10%-acrylamide gels). After electrophoresis, the gels were fixed in 10% trichloroacetic acid for 30 min and dried before autoradiography at -20 °C for 72 h. Location of the ADP-ribosylated peptides was followed by quantification by densitometric analysis.

Measurement of cyclic AMP phosphodiesterase activity

Cells were challenged with agonist (100 nM PMA or 1 nM–10 μ M bradykinin) and, after incubation, rinsed with and scraped into buffer A. The cells were broken by passage through a syringe needle. Phosphodiesterase activity was assayed by using 0.5 μ M cyclic [3 H]AMP as described by Thompson and Appleman [19]. In all cases, assays were performed under conditions of linear rate of information and where less than 10% of the cyclic AMP was utilized.

RESULTS

Effect of PMA and bradykinin on adenylate cyclase and PLD activity in TSM

Treatment of TSM cells with either PMA (100 nM) or bradykinin (100 nM) elicited an increase in basal and p[NH]ppG-stimu-

Table 1 Changes in adenylate cyclase activity in PMA- and bradykinin-challenged TSM cells

Adenylate cyclase activity was measured as described in the Experimental section. Cells were challenged with the indicated agonist, and subsequently membranes were prepared. This is a representative result of an experiment performed at least three times with different cell preparations, and results are expressed as means $\pm$ S.D.: * significant difference from control ($P < 0.05$ Student's *t* test); ** not significant by difference from PMA alone (Student's *t* test).

Treatment	Specific activity of adenylate cyclase (pmol/min per mg)	
	No p[NH]ppG	+ p[NH]ppG (100 μ M)
Non-challenged cells	15.0 $\pm$ 1	116.0 $\pm$ 8.2
PMA (100 nM)	22.0 $\pm$ 1.6	206.1 $\pm$ 3
Bradykinin (100 nM)	20.3 $\pm$ 0.3	141.5 $\pm$ 3*
Bradykinin plus PMA	22.4 $\pm$ 1	215.7 $\pm$ 8**

Figure 1 Effect of bradykinin on adenylate cyclase and PLD activity in TSM

(a) Bradykinin dose-response curve for the increase in p[NH]ppG-stimulated adenylate cyclase in subsequently isolated membranes; (b) bradykinin dose-response curve for [3 H]PtdBut accumulation at 10 min stimulation. The adenylate cyclase results are represented as fold activation over p[NH]ppG-stimulated activity in membranes isolated from untreated cells. PLD results are expressed as percentages of the maximal [3 H]PtdBut accumulated. Results are means $\pm$ S.D. for experiments performed in triplicate assay. This is a representative result of experiments performed on three different cell preparations.

lated adenylate cyclase activity in subsequently prepared cell membranes (Table 1). Basal adenylate cyclase activity in these membranes contains a component of GTP-stimulated activity (due to endogenous GTP), since the addition of isoprenaline (10 μ M) directly to membranes resulted in a 50% stimulation of activity (results not shown). The combination of both bradykinin and PMA resulted in an activation of adenylate cyclase activity that was quantitatively the same as that induced by PMA alone, indicating that both agents probably employ the same or similar pathways to invoke positive modulation of adenylate cyclase activity (Table 1). The effect of bradykinin on enhanced adenylate cyclase activity varied between 20 and 70% between preparations. p[NH]ppG-activated adenylate cyclase activity was en-

Figure 2 Cholera-toxin catalysed ADP-ribosylation of G_{α} in isolated TSM membranes

A typical autoradiograph after SDS/PAGE, demonstrating cholera-toxin-catalysed ADP-ribosylation of G_{α} in isolated TSM membranes. Lanes: 1, membranes isolated from unstimulated cells; 2, membranes isolated from PMA (100 nM)-stimulated cells; lane 3, membranes isolated from bradykinin (100 nM)-stimulated cells. The position of [32 P]ADP-ribosylated G_{α} is marked. Molecular-mass markers (kDa) are shown. This is a representative result of an experiment performed three times using different cell preparations.

Figure 3 Effect of PMA on intracellular cyclic AMP levels in non-treated and cholera-toxin-treated cells

A concentration-dependent accumulation of intracellular cyclic AMP occurred in response to PMA (10 pM–10 μ M) in non-treated cells (●) and cholera-toxin (0.5 ng/ml)-treated cells (□). Results are expressed as pmol of cyclic AMP/ 0.25×10^6 cells and are means $\pm$ S.D. of combined data from five experiments.

hanced by bradykinin in a concentration-dependent manner, with an EC_{50} of 5 ± 2 nM ($n = 3$; Figure 1a). Furthermore, bradykinin stimulated the activation of PLD-catalysed PtdCho hydrolysis, as evidenced by the accumulation of [3 H]PtdBut in [3 H]palmitate-labelled TSM cells [14]. Stimulation with 100 nM

Figure 4 Bradykinin-stimulated cyclic AMP accumulation in TSM

(a) Time-dependent accumulation of intracellular cyclic AMP in response to bradykinin (1 μ M) in non-treated cells; (b) time-dependent accumulation of intracellular cyclic AMP in response to bradykinin (1 μ M) in cholera-toxin (0.5 ng/ml)-treated cells; (c) bradykinin dose-response curve for the accumulation of intracellular cyclic AMP in non-treated cells; (d) bradykinin dose-response curve for the accumulation of intracellular cyclic AMP in cholera-toxin (0.5 ng/ml)-treated cells. Results are expressed as pmol of cyclic AMP/ 0.25×10^6 cells and are means $\pm$ S.D. for experiments performed in triplicate. This is a representative result of experiments performed on three different cell preparations.

bradykinin typically resulted in a maximal 2.68 ± 1.1 ($n = 25$)-fold increase in [3 H]PtdBut above basal at 10 min stimulation [14]. The bradykinin-stimulated PLD response, measured at 10 min stimulation, was concentration-dependent (Figure 1b) with an EC_{50} of 5 ± 4 nM ($n = 3$). Bradykinin-stimulated PLC activity displays an EC_{50} of approx. 30 nM [20].

Effect of PMA and bradykinin on cholera-toxin-catalysed ADP-ribosylation of G_{α}

Cholera toxin catalysed the [32 P]ADP-ribosylation of a 45 kDa polypeptide that corresponds to G_{α} (Figure 2). PMA (100 nM) and bradykinin (100 nM) challenge of TSM cells apparently allowed G_{α} to become a better substrate for cholera-toxin-catalysed ADP-ribosylation in subsequently prepared membranes, as assessed by the enhanced incorporation of [32 P]ADP-ribose into the α subunit (PMA $220 \pm 25\%$ increase; bradykinin $200 \pm 50\%$ increase in labelling; $n = 3$ different cell preparations).

Effect of PMA and bradykinin challenge on intracellular cyclic AMP

Cholera toxin (0.5 ng/ml, 18 h) pre-treatment of cells resulted in increased intracellular cyclic AMP accumulation (control, 1 ± 0.1 pmol/ 0.25×10^6 cells; cholera toxin, 5 ± 2 pmol/ 0.25×10^6 cells; $n = 6$). This response was approx. 5% of the maximal cyclic AMP response that could be obtained under certain conditions (see below). PMA (10 pM–10 μ M) was without effect on intracellular cyclic AMP in control cells (Figure 3). However, constitutive activation of G_{α} by treatment *in vivo* with

Figure 5 Effect of B_2 -receptor antagonist on bradykinin-stimulated intracellular cyclic AMP accumulation

The concentration-dependent effect of NPC567 on bradykinin (10 nM)-stimulated intracellular cyclic AMP accumulation in non-treated ($\square$) and cholera-toxin (0.5 ng/ml)-treated cells ($\bullet$) is shown. The results are expressed as pmol of cyclic AMP/ 0.25×10^6 cells and are means $\pm$ S.D. for experiments performed in triplicate. This is a representative result of experiments performed on six different cell preparations.

Figure 6 Effect of butan-1-ol on bradykinin-stimulated cyclic AMP accumulation

Effect of butan-1-ol (0.3%) on the bradykinin dose-response curve for cyclic AMP accumulation is shown. The results are expressed as pmol of cyclic AMP/ 0.25×10^6 cells and are means $\pm$ S.D. for experiments performed in triplicate assay. $\square$, Control cells; $\bullet$, butan-1-ol pre-treated cells. This is a representative result of experiments performed on three different cell preparations.

Table 2 Effect of NPC567, a B_2 -receptor antagonist, on bradykinin-stimulated $\text{Ins}(1,4,5)P_3$ and $[^3\text{H}]\text{PtdBut}$ formation

The effect of the bradykinin- B_2 -receptor antagonist NPC 567 (D-Arg-[Hyp³, D-Phe⁷]-bradykinin) on bradykinin-stimulated (10 nM) $\text{Ins}(1,4,5)P_3$ formation (10 s) and $[^3\text{H}]\text{PtdBut}$ accumulation (10 min) in cultured guinea-pig TSM cells was studied. Results are means $\pm$ S.D. ($n = 3$): * not significantly different from control (Student's t test). $\text{Ins}(1,4,5)P_3$ results are represented as pmol/ 0.25×10^6 cells, and $[^3\text{H}]\text{PtdBut}$ as d.p.m./ 0.25×10^6 cells. ND, not determined.

[Antagonist] (μM)	Control	+ Bradykinin
$\text{Ins}(1,4,5)P_3$		
0	0.92 ± 0.4	15.0 ± 3.5
1	ND	7.0 ± 0.2
10	ND	1.5 ± 0.51
$[^3\text{H}]\text{PtdBut}$		
0	736 ± 74	2517 ± 225
1	ND	$2490 \pm 139^*$
10	ND	2150 ± 171

cholera toxin (0.5 ng/ml) allowed a synergistic concentration-dependent PMA-induced increase in intracellular cyclic AMP to occur [$\text{EC}_{50} = 7.3 \pm 2.5$ nM ($n = 5$) at $t = 10$ min; Figure 3].

Bradykinin elicited a transient (Figure 4a) and concentration-dependent increase [$\text{EC}_{50} = 63.3 \pm 14.5$ nM ($n = 3$) at $t = 10$ min; Figure 4c] in intracellular cyclic AMP. The dose-response for bradykinin-stimulated intracellular cyclic AMP response was

biphasic and may reflect desensitization at higher concentrations ($> 1 \mu\text{M}$). Similarly, *in vivo* cholera-toxin-induced constitutive activation of G_s resulted in a synergistic increase in bradykinin-stimulated intracellular cyclic AMP accumulation and an increased potency for bradykinin [$\text{EC}_{50} = 6.17 \pm 1.6$ nM ($n = 3$); Figure 4d]. Cholera-toxin pre-treatment also enhanced maximal bradykinin-stimulated cyclic AMP accumulation by approx. 10-fold as compared with non-cholera-toxin-treated cells ($5\text{--}20$ pmol/ 0.25×10^6 non-treated cells and $50\text{--}200$ pmol/ 0.25×10^6 cholera-toxin-treated cells). The time course for cyclic AMP accumulation was transient in cholera-toxin-treated cells (Figure 4b).

Bradykinin-stimulated (10 nM) cyclic AMP accumulation was not affected by a B_2 -receptor antagonist (Figure 5) at concentrations that selectively blocked bradykinin-stimulated PLC activity (Table 2). For instance, complete inhibition of bradykinin-stimulated $\text{Ins}(1,4,5)P_3$ formation was achieved with $10 \mu\text{M}$ antagonist, and this concentration only inhibited bradykinin-stimulated PLD activity by $20 \pm 10\%$ (Table 2). The B_1 -receptor agonist des-Arg⁹-bradykinin (which does not stimulate PLC or PLD pathways [20]) was also ineffective at stimulating cyclic AMP accumulation: 10 pM– $10 \mu\text{M}$ elicited $< 10\%$ effect on cyclic AMP in both non-treated and cholera-toxin-treated cells (0.5 ng/ml, 18 h; $n = 3$).

Effect of 0.3% butanol on bradykinin-stimulated intracellular cyclic AMP response

We have previously demonstrated that 0.3% (v/v) butan-1-ol is saturating for the bradykinin-stimulated PLD-catalysed formation of phosphatidate [14]. Under these conditions, brady-

Figure 7 Effect of bradykinin on cyclic AMP phosphodiesterase activity in TSM

The concentration-dependent effect of bradykinin on cyclic AMP phosphodiesterase activity in homogenates isolated from treated cells is shown. Results are expressed as the percentage of activity remaining. Results are means $\pm$ S.D. for three individual experiments.

kinin-stimulated cyclic AMP accumulation in non-treated cells was almost completely attenuated (Figure 6). Butan-1-ol alone did not elicit an increase in basal intracellular cyclic AMP (Figure 6), which suggests that butan-1-ol-induced changes in membrane fluidity and adenylate cyclase activity are minimal. Butan-1-ol (0.3 %) pre-treatment for 5 min was without effect on bradykinin-stimulated [3 H]DAG levels {unstimulated [3 H]DAG, 1550 ± 300 dpm; butan-1-ol 1400 ± 30 d.p.m.; bradykinin (100 nM) 3420 ± 55 d.p.m.; bradykinin plus butan-1-ol 3400 ± 35 d.p.m. ($n = 3$)}, suggesting that the cross-regulatory effect of bradykinin on cyclic AMP accumulation is unlikely to occur via the PLC-catalysed hydrolysis of [3 H]palmitate-enriched PtdCho. The inability of butan-1-ol to modify DAG levels also indicates that PLD-derived phosphatidate is unlikely to be dephosphorylated to DAG under these stimulatory conditions.

Effect of PMA and bradykinin on cyclic AMP phosphodiesterase activity

Challenge of TSM cells with bradykinin elicited a concentration-dependent inhibition of total homogenate cyclic AMP phosphodiesterase activity with $EC_{50} > 100$ nM (Figure 7). Des-Arg⁹-bradykinin was without effect (6 ± 1 % inhibition at 1μ M agonist; $n = 3$), whereas NPC 567 failed to block the bradykinin-induced inhibition of phosphodiesterase activity [bradykinin (10 nM) elicits 21.2 ± 3.15 % inhibition; bradykinin plus 10 nM NPC567 elicits 18.3 ± 2.7 % inhibition; bradykinin plus 1μ M NPC567 elicits 16 ± 5.6 % inhibition; $n = 3$]. PMA (1 nM– 1μ M) was ineffective at modulating phosphodiesterase activity (< 10 % of activity; $n = 3$).

DISCUSSION

PMA and bradykinin treatment of TSM cells leads to enhanced basal and guanine-nucleotide-stimulated adenylate cyclase ac-

tivity and increased intracellular cyclic AMP accumulation. The effect of PMA and bradykinin together upon adenylate cyclase activity is non-additive, suggesting that both employ similar mechanisms for activation, i.e. the adenylate cyclase response is protein kinase C-dependent. This may occur via a phosphorylation mechanism which results in either an activation of adenylate cyclase activity or increased coupling between G_s and adenylate cyclase. Modification of the α subunit of G_s may occur, since cholera-toxin-catalysed ADP-ribosylation is markedly enhanced in isolated membranes. However, we cannot exclude the possibility that both bradykinin and PMA modulate the activity of the 21 kDa ADP-ribosylation factor (ARF-protein), which may facilitate an increased ADP-ribosylation of G_s *in vitro*. However, to date no evidence is available to substantiate this proposal.

The ability of PMA to elicit a cyclic AMP response in intact cells is sensitized, whereas the response to bradykinin is enhanced by the prior constitutive activation *in vivo* of G_s with cholera toxin, confirming that bradykinin elicits cross-regulation at the level of G_s /adenylate cyclase coupling. Synergism between cholera toxin and these agents may occur as a consequence of the protein kinase C-dependent activation of adenylate cyclase activity being amplified by the constitutive activation of G_s .

PtdIns(4,5) P_2 -specific PLC activity does not appear to provide the relevant source of protein kinase C activator required to facilitate cross-regulation. This is based on the observation that a B_2 -receptor antagonist, which selectively blocks the bradykinin-stimulated Ins(1,4,5) P_3 response (the present work; [20,21]) did not inhibit bradykinin-stimulated PLD activity or cyclic AMP accumulation. These studies suggest that PLD-catalysed PtdCho hydrolysis may be involved in the cross-talk regulation of adenylate cyclase. In support of this, the PLD and adenylate cyclase responses display a similar concentration-dependence for bradykinin, and addition of butan-1-ol to TSM cells, although having no apparent effect against PLC-catalysed PtdCho hydrolysis, blocked bradykinin-stimulated cyclic AMP accumulation. PLD-derived phosphatidate does not appear to be dephosphorylated to DAG in bradykinin-stimulated cells [22], and phosphatidate-stimulated phosphorylation may be a key physiological trigger. In this regard, phosphatidate has been demonstrated to activate protein kinase C- ζ *in vitro* [23] and to promote the phosphorylation of proteins in lung and brain [24].

In contrast with PMA, bradykinin induced a protein kinase C-independent inhibition of cyclic AMP phosphodiesterase activity in TSM cells. Our results suggest that, although modulation of both adenylate cyclase and phosphodiesterase activity occurs via distinct routes, it is probable that the same or a similar novel B_3 -receptor species is involved. Indeed, this novel receptor type has been proposed to mediate Ca^{2+} efflux in TSM [25].

The intracellular cyclic AMP response displays an increased potency for bradykinin in cholera-toxin-treated cells. This is probably due to an increased contribution of the adenylate cyclase pathway to the total intracellular cyclic AMP accumulated, this being a consequence of constitutive activation of G_s . Since the potency of the bradykinin-stimulated adenylate cyclase response is 20-fold higher than for phosphodiesterase inhibition, a leftward shift in the concentration-dependence plot is observed.

In summary, bradykinin can activate both PLC and PLD in TSM. The PLD-derived phosphatidate may play a role in allowing cross-regulation with adenylate cyclase. In addition, bradykinin elicits inhibition of cyclic AMP phosphodiesterase activity via an unidentified mechanism. The physiological role of this cross-regulation may be related to the proliferative potential of bradykinin in TSM since cyclic AMP is an inhibitor of mitogenesis in these cells.

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