Regulation of GH₃ pituitary tumour-cell adenylate cyclase activity by activators of protein kinase C

Lawrence A. QUILLIAM,* Pauline R. M. DOBSON† and Barry L. BROWN

Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, U.K.

The influence of protein kinase C (PKC) activation on cyclic AMP production in GH₃ cells has been studied. The stimulation of cyclic AMP accumulation induced by forskolin and cholera toxin was potentiated by 4 β -phorbol 12,13-dibutyrate (PDBu). Moreover, PDBu, which causes attenuation of the maximal response to vasoactive intestinal polypeptide (VIP), also induced a small right shift in the dose–response curve for VIP-induced cyclic AMP accumulation. PDBu-stimulated cyclic AMP accumulation was unaffected by pretreatment of cells with pertussis toxin or the inhibitory muscarinic agonist, oxotremorine. PDBu stimulation of adenylate cyclase activity required the presence of a cytosolic factor which appeared to translocate to the plasma membrane in response to the phorbol ester. The diacylglycerol-generating agents thyroliberin, bombesin and bacterial phospholipase C each stimulated cyclic AMP accumulation, but, unlike PDBu, did not attenuate the stimulation induced by VIP. These results suggest that PKC affects at least two components of the adenylate cyclase complex. Stimulation of cyclic AMP accumulation is probably due to modification of the catalytic subunit, whereas attenuation of VIP-stimulated cyclic AMP accumulation appears to be due to the phosphorylation of a different site, which may be the VIP receptor.

INTRODUCTION

Tumour-promoting phorbol esters have been shown to affect cyclic AMP metabolism in many cell types, resulting in modulation of both basal and agonist-stimulated adenylate cyclase activity (see Sibley & Lefkowitz, 1985; Nishizuka, 1986). Attenuation of agonist-stimulated adenylate cyclase activity by phorbol esters can occur as a result of receptor phosphorylation (Sibley *et al.*, 1984). In addition, modulation of adenylate cyclase activity can occur distal to the receptor, possibly at the level of the inhibitory GTP-binding regulatory protein, G_i (Jakobs *et al.*, 1985; Bell & Brunton, 1986). Indeed, the α subunit of G_i has been shown to be a substrate *in vitro* for the phorbol ester receptor protein kinase C (PKC) (Katada *et al.*, 1985). *In vivo*, PKC is activated by 1,2-*sn*-diacylglycerol, the product of agonist-induced phosphoinositide hydrolysis (see Bell, 1986; Nishizuka, 1986). Thus there is the potential for interaction between the phosphoinositide and cyclic AMP second-messenger pathways.

We have previously demonstrated stimulation of cyclic AMP accumulation in GH₃ pituitary tumour cells by 4 β -phorbol 12,13-dibutyrate (PDBu) and the phosphoinositide-hydrolysis-stimulating hormone thyroliberin (TRH) (Quilliam *et al.*, 1985). In addition, we found that the phorbol ester attenuated VIP-stimulated cyclic AMP accumulation. In the present study, we have used agents which act on different components of hormone-sensitive adenylate cyclase to try to determine the site(s) of PKC action. The results obtained suggest that PKC probably

acts at the level of the catalytic subunit and the VIP receptor to modulate GH₃-cell adenylate cyclase activity.

MATERIALS AND METHODS

Bombesin, oxotremorine sesquifumarate and bacterial phospholipase C (*Clostridium perfringens*) were from Sigma Chemical Co., Poole, Dorset, UK. Forskolin was from Calbiochem, La Jolla, CA, U.S.A. [α -³²P]ATP (10–50 Ci/mmol) and cyclic [8-³H]AMP (20–30 Ci/mmol) were from Amersham International, Amersham, Bucks., U.K. Other materials were obtained as described previously (Quilliam *et al.*, 1985).

Experiments were performed on monolayers of GH₃ cells cultured in 24-well multiwell plates, and intracellular cyclic AMP levels were determined by radioimmunoassay essentially as described previously (Quilliam *et al.*, 1985). For assay of adenylate cyclase activity, GH₃ cells grown in monolayer culture were washed and scraped into ice-cold Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954), pH 7.4. After centrifugation (5 min, 200 g), the cells were resuspended in Hepes (50 mM) containing EDTA (1 mM), pH 7.4, and allowed to swell for 20 min on ice. The cells were then homogenized with a glass Dounce homogenizer, and the homogenate was used immediately for the assay of adenylate cyclase activity. For membrane assays, the homogenate was centrifuged (40000 g, 20 min), and the pellet was washed, re-centrifuged and resuspended in homogenization buffer. For experiments requiring cytosol, 2 $\times$ 10⁷ cells were homogenized and centrifuged (20 min, 40000 g) in

Abbreviations used: ED₅₀, concentration causing half-maximal stimulation; G_s, G_i, the stimulatory and inhibitory GTP-binding proteins of adenylate cyclase, respectively; IBMX, 3-isobutyl-1-methylxanthine; PDBu, 4 β -phorbol 12,13-dibutyrate; PKC, protein kinase C; TRH, thyrotropin-releasing hormone (thyroliberin); VIP, vasoactive intestinal polypeptide.

* Present address: Research Institute of Scripps Clinic, La Jolla, CA 92037, U.S.A.

† To whom correspondence and reprint requests should be addressed.

approx. 2 ml of buffer, and 20 μ l of the supernatant was added back to the cyclase assay. Adenylate cyclase activity was measured in a total volume of 100 μ l containing MgCl_2 (5 mM), EDTA (0.25 mM), cyclic AMP (2 mM), ATP (1 mM), $(8-10) \times 10^6$ c.p.m. of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, phosphocreatine (12 mM), creatine kinase (3 units/tube), Hepes (50 mM), pH 7.4, and homogenate or membranes (25 μ l). Incubations were carried out at 37 °C for 10 min and were stopped by transferring tubes to a boiling-water bath for 3 min. Cyclic $[\text{P}^{32}]\text{AMP}$ was purified by sequential Dowex and alumina chromatography as described by Salomon *et al.* (1974). Protein was measured by the method of Bradford (1976). Significant differences were determined by analysis of variance.

RESULTS

Forskolin stimulated cyclic AMP accumulation in GH_3 cells in a dose-dependent fashion, with an ED_{50} of around 5 μM (Fig. 1). Co-incubation of cells with forskolin plus PDBu resulted in a synergistic response approx. 50% above that of forskolin alone over a wide range of concentrations of the diterpene. However, PDBu alone did not stimulate cyclic AMP accumulation, unlike in the presence of 3-isobutyl-1-methylxanthine (IBMX) (Fig. 3a).

We demonstrated previously that PDBu-stimulated cyclic AMP accumulation in GH_3 cells persisted after pretreatment of cells for 2 h with cholera toxin (Quilliam *et al.*, 1985). The degree of effect of these two agents depended on their period of co-incubation (Fig. 2). After a lag period of approx. 15 min (during which time cholera toxin enters the cells and is activated; Gill, 1977), the initial rate of accumulation of cyclic AMP induced by cholera toxin plus PDBu was greater than by cholera toxin alone. This was not the case at longer incubation times, when the two curves were parallel. This effect could not be accounted for by excretion of cyclic AMP, since total (intracellular plus extracellular) nucleotides were measured in these experiments. Treatment with cholera toxin did not alter the efficacy or the potency [$\text{ED}_{50} = 5.1 \pm 0.2 \text{ nM}$ ($n = 3$) in the presence of cholera toxin compared with $6.6 \pm 1.4 \text{ nM}$ ($n = 5$) in its absence] of PDBu action.

To examine the effects of PDBu on inhibition of adenylate cyclase, cells were pretreated with pertussis toxin, which ADP-ribosylates the α subunit of G_i and attenuates inhibition of adenylate cyclase (Wojcikiewicz *et al.*, 1984a), or with the inhibitory muscarinic cholinergic agonist, oxotremorine. Oxotremorine can stimulate phosphoinositide hydrolysis, and thus activate PKC in some cells (Blackshear *et al.*, 1987); however, it does not modulate this pathway in GH_3 cells (results not shown; R. J. H. Wojcikiewicz, unpublished work). Stimulation of cyclic AMP production by PDBu in cells pretreated with pertussis toxin (50 ng/ml; 24 h) was only slightly less than in controls, stimulation being $194.6 \pm 6.0\%$ in controls compared with $170.1 \pm 6.0\%$ in toxin-treated cells (means $\pm$ S.E.M., $n = 14$ and 5 respectively).

After stimulation of G_i by incubating cells with oxotremorine [10 μM , a dose previously shown to depress cyclic AMP levels maximally in GH_3 cells (Wojcikiewicz *et al.*, 1984a)], PDBu caused an increase in cyclic AMP accumulation similar to that in the absence of the muscarinic cholinergic agonist (Fig. 3b). Further, pretreatment of cells with PDBu did not significantly affect

Fig. 1. Effect of PDBu on forskolin-stimulated GH_3 -cell cyclic AMP accumulation

GH_3 cells were incubated with a range of concentrations of forskolin in the absence (○) or presence (●) of PDBu (250 nM) for 5 min, and cyclic AMP in the cell monolayer was determined by radioimmunoassay. Results are means $\pm$ S.E.M. of three independent experiments performed in triplicate; * ($P < 0.05$) denotes significant stimulation by PDBu plus forskolin over forskolin alone.

Fig. 2. Time course showing the effect of PDBu on cholera-toxin-stimulated cyclic AMP accumulation in GH_3 cells

GH_3 cells were incubated with cholera toxin (1 nM) and IBMX (1 mM) for the times shown in the absence (○) or presence (●) of PDBu (500 nM). The reaction was stopped by the addition of 2 vol. of ice-cold ethanol, and total cyclic AMP content was determined. The results (means $\pm$ S.D., $n = 3$) are representative of four experiments.

the ability of oxotremorine to inhibit cyclic AMP accumulation (results not shown). VIP (10 nM) stimulated cyclic AMP accumulation by 5–8-fold; however, the size of the VIP-induced response was greatly diminished after

Fig. 3. Effects of oxotremorine on VIP- and PDBu-stimulated cyclic AMP accumulation in GH₃ cells

Cells were incubated with IBMX (1 mM) in the absence or presence of oxotremorine (10 μ M) for 20 min. The medium was then changed for a solution containing, in addition to the original drugs, PDBu (250 nM), VIP (10 nM) or both agents for a further 5 min. Data are means $\pm$ S.E.M. for seven (a) and four (b) independent experiments. $\square$, Control; ▨ , +PDBu; ▩ , +oxotremorine; ▧ , +PDBu + oxotremorine.

pretreatment of cells with oxotremorine (Fig. 3b). In contrast with the attenuation of VIP-stimulated cyclic AMP accumulation by PDBu observed in the absence of oxotremorine (Quilliam *et al.*, 1985; Fig. 3b), PDBu potentiated VIP-stimulated cyclic AMP accumulation in the presence of the cholinergic agonist (Fig. 3b).

The rise in the cellular cyclic AMP concentration induced by VIP (10 nM) was normally attenuated by 40–50 % after pretreatment of cells with PDBu (Fig. 3b). In order to test whether PDBu decreased the maximal response or the potency of VIP, the effect of the phorbol ester on VIP-stimulated cyclic AMP accumulation was investigated over a range of concentrations of the peptide. As shown in Fig. 4, in addition to decreasing the maximal

Fig. 4. Effect of PDBu on VIP-stimulated cyclic AMP accumulation

GH₃ cells were incubated for 20 min with (●) or without (○) PDBu (250 nM) before addition of a range of doses of VIP for 5 min. Cumulated data (means $\pm$ S.E.M.) from three experiments are presented; * ($P < 0.001$) denotes significant inhibition by PDBu at each VIP concentration.

Fig. 5. Effect of preincubation with bacterial toxin on phorbol ester- and diacylglycerol-stimulated cyclic AMP accumulation in GH₃ cells

Cells were incubated with (a) cholera toxin (1 nM; 2 h) or (b) pertussis toxin (50 ng/ml; 24 h). Test agents [4 β -phorbol (4 β P, 500 nM); PDBu (500 nM); TRH (250 nM); bombesin (Bom, 50 nM); bacterial phospholipase C (PL-C, 0.05 unit/ml); VIP (10 nM); oxotremorine (Oxo, 10 μ M)] were then added in the presence of IBMX (1 mM) for periods of 10–30 min. Combined data (means $\pm$ S.E.M.) from (n) experiments are presented; * ($P < 0.05$) denotes significant stimulation over IBMX alone.

Table 1. Effects of cytosol and PDBu on GH₃-cell particulate adenylate cyclase activity

Membranes, prepared from cells which had been pre-incubated with 0.05 % dimethyl sulphoxide in the absence or presence of PDBu (1 μ M) for 30 min before harvesting, were incubated in the absence or presence of PDBu (500 nM) and/or the supernatant obtained from centrifugation of the GH₃-cell homogenates (see the Materials and methods section) as indicated. Results (means $\pm$ S.E.M., $n = 3$) are representative of three experiments (one for last value).

Preincubation with PDBu	Addition to assay		Cyclic AMP (pmol/min per mg of protein)
	PDBu	Supernatant	
—	—	—	81.7 $\pm$ 12.7
—	+	—	80.4 $\pm$ 8.8
—	—	+	113.7 $\pm$ 7.8
—	+	+	168.6 $\pm$ 9.8
+	—	—	262.7 $\pm$ 15.6
+	—	+	280.4 $\pm$ 6.9

response, PDBu induced a small right shift in the ED₅₀ value for VIP from 0.9 to 3.7 nM. Forskolin has been shown to prevent desensitization of VIP-stimulated cyclic AMP production in GH₃ cells (Guild & Drummond, 1984), and we have observed a similar effect (results not shown). However, preincubation of cells with forskolin (100 nM) before exposure to PDBu did not prevent attenuation of the response to VIP by the phorbol ester. The percentage attenuation of the VIP response in the presence and absence of forskolin was very similar (approx. 40 %; results not shown).

The diacylglycerol-generating agents bombesin and bacterial phospholipase C, like TRH, stimulated cyclic AMP accumulation in the presence of IBMX. These effects were non-additive with PDBu in at least three independent experiments (results not shown), and persisted after incubation of cells with cholera or pertussis toxin (Fig. 5). No significant effects were observed with the non-tumour-promoting phorbol ester 4 β -phorbol, or with VIP after cholera-toxin treatment (Fig. 5a), or with oxotremorine after pertussis-toxin treatment (Fig. 5b).

Bombesin and phospholipase C, unlike PDBu, but as previously shown for TRH (Quilliam *et al.*, 1985), did not attenuate the stimulation of cyclic AMP accumulation induced by VIP; rather, they slightly enhanced cyclic nucleotide accumulation in the presence of the peptide to 115.2 $\pm$ 4.5 % (mean $\pm$ S.E.M., $n = 3$) and 129.9 $\pm$ 6.3 % (mean $\pm$ S.E.M., $n = 5$) of control respectively.

PDBu stimulated adenylate cyclase activity in GH₃-cell homogenates. Enzyme activity in homogenates was greater after preincubation of intact cells with PDBu than upon addition of the phorbol ester directly to the assay (198.3 $\pm$ 8.0 % for addition to intact cells, compared with 136.6 $\pm$ 7.1 % for addition to GH₃-cell homogenates; means $\pm$ S.E.M., $n = 3$ and 10 respectively). NaF-stimulated adenylate cyclase activity was also elevated in homogenates from PDBu-treated cells. PDBu-mediated stimulation of adenylate cyclase activity in crude membrane preparations was very slight in the absence of pretreatment of intact cells with the phorbol ester

(Table 1). However, some phorbol ester sensitivity of the adenylate cyclase was restored after addition of the centrifuged homogenate supernatant to the cyclase assay.

DISCUSSION

As the presence of IBMX is necessary for the detection of PDBu-stimulated cyclic AMP accumulation, this suggests that modification of adenylate cyclase or associated proteins by PKC has only a small effect on cyclic AMP generation. Nonetheless, this modification appears to potentiate greatly forskolin- and cholera-toxin stimulated enzyme activity. An effect of PDBu on cyclic AMP degradation is unlikely to be responsible for the observed effects, since the phorbol ester stimulated adenylate cyclase activity in the presence of 2 mM unlabelled nucleotide. Similar findings have been reported in other cell lines (Bell *et al.*, 1985; Summers & Cronin, 1986). Moreover secretion of cyclic AMP from GH₃ cells was not blocked by PDBu treatment.

ADP-ribosylation of the α subunit of G_s by cholera toxin occurs most efficiently under conditions conducive to G-protein subunit dissociation/activation (Ribiero-Neto *et al.*, 1987). Thus, since addition of PDBu to GH₃ cells increases the initial rate of cholera-toxin-induced cyclic AMP accumulation, an effect of PKC on G_s subunit dissociation is suggested. As PDBu-stimulated cyclic AMP accumulation was not attenuated by preincubation of GH₃ cells with cholera toxin (Quilliam *et al.*, 1985), a direct effect of PKC on G_s seems unlikely. Interestingly, potentiation of cholera-toxin-stimulated adenylate cyclase activity, at both early time points and after maximal ADP-ribosylation of G_{s α , has similarly been observed with forskolin at concentrations that, alone, have no effect on cyclic AMP accumulation (Barovsky & Brooker, 1985). Forskolin has been shown to interact directly with the catalytic unit of adenylate cyclase; however, the presence of G_s appears to be required for full expression of the action of forskolin (Seamon & Daly, 1985; Yamashita *et al.*, 1986). Since PDBu and forskolin stimulated cyclic AMP accumulation in GH₃ cells in a synergistic fashion, these agents probably act at close but independent sites on adenylate cyclase.}

Studies on several cell types suggest that phorbol esters may act by modulating the inhibition of adenylate cyclase (Jakobs *et al.*, 1985; Bell & Brunton, 1986; Summers & Cronin, 1986; Johnson *et al.*, 1986). It has been demonstrated that G_i can be phosphorylated by PKC *in vitro* (Katada *et al.*, 1985). Phorbol ester-stimulated cyclic AMP accumulation was only slightly attenuated after pretreatment of GH₃ cells with pertussis toxin, under conditions which were previously shown to cause maximal ADP-ribosylation of the α subunit of G_i in these cells (Wojcikiewicz *et al.*, 1984b). Also, pretreatment of cells with PDBu or oxotremorine did not prevent the ability of these agent to stimulate or to inhibit cyclic AMP accumulation, respectively. These findings suggest that attenuation of adenylate cyclase inhibition through G_i is not the major mechanism of PKC action on cyclic AMP synthesis in GH₃ cells. One possible reason for the difference between our results and those obtained with S49 lymphoma cells (Bell & Brunton, 1986; Johnson *et al.*, 1986) is that G_i plays a lesser role in the regulation of GH₃-cell adenylate cyclase activity. Treatment with pertussis toxin results in elevation of basal and stimulated cyclic AMP formation in S49 cells

(Jakobs *et al.*, 1984; Bell & Brunton, 1986), but not in GH₃ cells (Brown *et al.*, 1984). Since ADP-ribosylation of either G_s or G_i did not greatly affect PDBu-induced cyclic AMP accumulation in GH₃ cells, but PDBu synergized with forskolin-induced nucleotide formation, it seems likely that PKC acts by phosphorylating the catalytic unit of adenylate cyclase, as previously demonstrated in frog erythrocytes (Yoshimasa *et al.*, 1987). Potentiation of the rate of cholera-toxin-induced cyclic AMP accumulation at early time points by PDBu suggests that, like forskolin (Yamashita *et al.*, 1986), the phorbol ester may act by stabilizing the G_s-activated state of adenylate cyclase.

Attenuation of VIP-stimulated cyclic AMP accumulation by PDBu was not mimicked by endogenous diacylglycerol generation, and had a slightly slower time course ($t_{1/2} = 2.5 \pm 1.2$ min; mean $\pm$ S.E.M., $n = 3$) than stimulation of basal cyclic AMP accumulation by the phorbol ester, which was maximal in less than 2 min. These data suggest that this effect may be due to a separate phosphorylation event from that required for stimulation of adenylate cyclase by PKC. Since PDBu attenuated the cyclic AMP response to VIP, but potentiated the response to NaF, which acts on G_s, an effect of PKC on the VIP receptor seems most likely. The report that phorbol myristate acetate decreased ¹²⁵I-VIP binding without changing the affinity of the peptide for its receptor or affecting G-protein-stimulated cyclic AMP accumulation in HT 29 cells (Bozou *et al.*, 1987) supports this notion.

Since the two agents known to stimulate diacylglycerol production in GH₃ cells also stimulated cyclic AMP accumulation distal to their receptors (as judged by pretreatment of cells with bacterial toxins and non-additivity with PDBu), it seems likely that TRH and bombesin as well as bacterial phospholipase C activate adenylate cyclase through PKC. Indeed, the EC₅₀ values for TRH- (6.1 ± 1.4 nM; mean $\pm$ S.E.M., $n = 4$) and bombesin- (approx. 0.6 nM) stimulated cyclic AMP accumulation were very similar to those obtained for stimulation of phosphoinositide hydrolysis in these cells (Sutton & Martin, 1982; Quilliam *et al.*, 1986). Interestingly, none of the diacylglycerol-generating agents attenuated VIP-stimulated cyclic AMP accumulation. Phorbol esters have been shown to have a different effect from hormones that stimulate phosphoinositide metabolism or from exogenously added diacylglycerol (Kiss & Luo, 1986; Ramsdell *et al.*, 1986). This may be due to the tighter interaction of PKC with membranes after activation by phorbol esters (Gopalakrishna *et al.*, 1986). Alternatively, PKC isoenzymes may have different susceptibility to stimulation by diacylglycerol and phorbol esters (Coussens *et al.*, 1986; Ono *et al.*, 1988). In the present study the apparent ability of the mediator of phorbol ester stimulation of cyclic AMP production to translocate to the particulate fraction in response to PDBu strongly suggests that PKC is responsible for regulating adenylate cyclase activity.

In conclusion, these data suggest that activation of PKC by phorbol esters stimulates basal and attenuates VIP-stimulated cyclic AMP production via an effect on multiple components of hormone-sensitive adenylate cyclase. The stimulatory effects were mimicked by agents which generate diacylglycerol. The ability of PDBu to potentiate cyclic AMP accumulation in the presence of cholera toxin, forskolin and oxotremorine may best be

explained by the phosphorylation of the catalytic unit of adenylate cyclase, resulting in increased α_s -catalytic-unit coupling.

Attenuation of VIP-stimulated cyclic AMP accumulation, however, probably reflects phosphorylation of a separate site which may be the VIP receptor. Thus there is potential for multisite regulation of GH₃-cell adenylate cyclase activity by agents which act through PKC. Such interactions between cell signalling pathways may be involved in the integrated response of cells which respond to multiple exogenous regulators.

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