Insulin and vasopressin elicit inhibition of cholera-toxin-stimulated adenylate cyclase activity in both hepatocytes and the P9 immortalized hepatocyte cell line through an action involving protein kinase C

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Incubation of hepatocytes or the SV40-DNA-immortalized hepatocyte P9 cell line with cholera toxin led to a time-dependent activation of adenylate cyclase activity, which occurred after a defined lag period. When added together with cholera toxin, each of the hormones insulin and vasopressin was capable of attenuating the maximum stimulatory effect achieved by cholera toxin over a period of 60 min through a process which could be blocked by the compounds staurosporine and chelerythrine. Attenuating effects on cholera-toxin-stimulated adenylate cyclase activity could also be elicited by using either the protein kinase C (PKC)-stimulating phorbol ester PMA (phorbol 12-myristate 13-acetate) or the protein phosphatase inhibitor okadaic acid. Alkaline phosphatase treatment of membranes reversed the inhibitory effect of PMA. Cholera toxin also stimulated the

adenylate cyclase activity of intact CHO (Chinese-hamster ovary) and NIH-3T3 cells, but this activity was insensitive to the addition of PMA. Overexpression of various PKC isoforms in CHO cell lines did not confer sensitivity to inhibition by PMA upon cholera-toxin-stimulated adenylate cyclase activity. Rather, overexpression of the γ isoform of PKC allowed PMA to stimulate adenylate cyclase activity in CHO cells. It is suggested that the PKC-mediated phosphorylation of a membrane protein attenuates cholera-toxin-stimulated adenylate cyclase activity in hepatocytes and P9 cells. The cellular selectivity of such an action may be due to the target for this inhibitory action of PKC being a particular isoform of adenylate cyclase which provides the major activity in hepatocytes and P9 cells, but not in either CHO or NIH-3T3 cells.

INTRODUCTION

The adenylate cyclase system is responsible for generating the intracellular second messenger cyclic AMP. It is now recognized that this activity is expressed by a large multi-gene family whose regulatory characteristics would seem to be remarkably different [1–6]. For example, there are distinct isoforms whose activity can be regulated by Ca^{2+} /calmodulin, phorbol esters and G-protein $\beta\gamma$ subunits. All species reported on to date, however, appear to be activated by the diterpene forskolin, which acts directly on the catalytic unit of the enzyme [7], and also by the stimulatory G-protein G_s , which mediates not only the effect of stimulatory hormones but also the action of cholera toxin [8–10]. Thus, occupied receptors interact with G_s , causing it to bind GTP and dissociate to yield a GTP-bound α -subunit (α - G_s) which serves to activate adenylate cyclase [8–10]. Cholera toxin achieves adenylate cyclase activation after a characteristic lag phase [11], which is considered [12,13] to reflect toxin entry into the cell by endocytosis, with subsequent processing to deliver the activated cholera-toxin α -subunit to the cytosol surface of the plasma membrane. This activated species then elicits the NAD^+ -dependent ADP-ribosylation of α - G_s [8–10], allowing it to dissociate in a GTP-bound state. However, ADP-ribosylated α - G_s is unable to hydrolyse GTP and undergo inactivation. Thus the entire pool of G_s becomes chronically activated by attaining the fully activated GTP-bound state, leading to the constitutive activation of adenylate cyclase [7–12].

Hepatocytes have an adenylate cyclase system that is able to be potentially activated by the hormone glucagon [14–17]. However, in intact cells, this activation is rapidly followed by a profound desensitization effect which occurs independently of any increase

in intracellular cyclic AMP concentrations [15–18]. This effect is in itself transient, with resensitization attaining near-completion some 20 min after hormone addition. The nature of this desensitization appears to take the form of an uncoupling of the ability of the glucagon receptor to stimulate G_s , with no inhibition being evident of either the catalytic activity of adenylate cyclase or G_s -adenylate cyclase coupling [15,16,19,20]. Such a process appears to be mediated through the action of protein kinase C (PKC) [16,19–21], which itself becomes activated in hepatocytes in response to glucagon [22,23]. Thus desensitization can be mimicked by hormones such as vasopressin and angiotensin, as well as the tumour-promoting phorbol ester PMA (phorbol 12-myristate 13-acetate), all of which activate PKC in hepatocytes [16,19–24] and whose abilities to elicit desensitization can be blocked by PKC inhibitors [21].

We have previously shown that cholera toxin activates hepatocyte adenylate cyclase activity after a characteristic lag time of ~ 15 min [11,12]. Additionally, we noted that the maximal degree of activation that was achieved by cholera toxin could be attenuated by the presence of the hormones glucagon and insulin [25]. These hormones did not affect the lag time for the action of cholera toxin, nor did they alter the degree of ADP-ribosylation of G_s [25].

In this study we show that a profound inhibitory action on the ability of cholera toxin to stimulate hepatocyte adenylate cyclase can be achieved through either the activation of PKC or the inhibition of protein phosphatase activity. Such inhibition appears to be regulated through the phosphorylation of a membrane protein. This target may be provided by a specific form of adenylate cyclase, for, although such inhibition could be demonstrated in hepatocytes and the immortalized hepatocyte

Abbreviations used: G_s , stimulatory G-protein acting on adenylate cyclase; G_i , inhibitory G-protein acting on adenylate cyclase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; CHO cells, Chinese-hamster ovary cells; IBMX, 3-isobutyl-1-methylxanthine.

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P9 cell line, it was not apparent in either NIH-3T3 fibroblasts or Chinese-hamster ovary (CHO) cells.

MATERIALS AND METHODS

[Arginine]-vasopressin, insulin, BSA, rat tail collagen, alkaline phosphatase, staurosporine and ATP were purchased from Sigma Chemical Co., Poole, Dorset, U.K. GTP, phosphocreatine, creatine kinase, cholera toxin, insulin (pig), cyclic AMP and all nucleotides were supplied by Boehringer Mannheim (U.K.), Lewes, East Sussex, U.K. Okadaic acid, staurosporine and chelerythrine chloride were from Calbiochem Novabiochem, Nottingham, U.K. Collagenase was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. All other biochemicals were from Sigma. Radiochemicals were obtained from Amersham International, Amersham, Bucks. U.K. All other chemicals were of AnalaR grade, from BDH Chemicals, Poole, Dorset, U.K. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxy-4-en-11-one) was from Calbiochem.

Isolation and incubation of hepatocytes

Isolated hepatocytes were prepared from fed 225–250 g male Sprague–Dawley rats and incubated as described by Heyworth and Houslay [15] by using an adaptation of previous methods [26]. Cells (3–5 mg dry wt./ml) were preincubated at 37 °C for 20 min, with constant gassing (O₂/CO₂, 19:1), before use. Ligands were added to the reaction vessel in a volume which was less than 1% of the total incubation volume. After the appropriate time interval, samples were removed either for the determination of intracellular cyclic AMP concentration [15] or for the preparation of a washed membrane fraction. The washed membrane fraction was prepared as described previously by us [11,12]; at the specified time, the cells were quenched by adding an equal volume of ice-cold 1 mM KHCO₃, pH 7.2, and then placing them on ice before disruption, centrifugation and washing of the membrane pellet. In all cases membranes were used within 2 h of preparation. Where indicated, cells were incubated with 1 μ g/ml cholera toxin.

Treatment of hepatocyte membranes with alkaline phosphatase

This was done as described previously by us in some detail [16,21] with 100 μ l incubations containing 200 μ g of membrane protein being treated for 10 min with 20 units of alkaline phosphatase. As before, membranes were harvested by centrifugation, washed and then used for assay of adenylate cyclase activity.

Culture and incubation of P9 cells

P9-cell culture, incubation, harvesting, determination of intracellular cyclic AMP levels and the preparation of a membrane fraction were done as described previously by us in some detail [27]. Where indicated, cells were incubated with 1 μ g/ml cholera toxin.

Culture and incubation of CHO and NIH-3T3 cells

Wild-type CHO cells and cells transfected so as to overexpress the α , β -II, γ and ϵ forms of PKC were generously given by Dr. Richard A. Roth, Department of Pharmacology, Stanford University, U.S.A. The generation and characterization of these cell lines, which were also transfected so as to overexpress the human insulin receptor, are described in [28].

CHO cells were cultured in Ham's F12 medium supplemented by addition of 1% penicillin/streptomycin and glutamine as well as 10% foetal-calf serum. Cells were grown in 75 cm² flasks at

37 °C in an atmosphere of air/CO₂ (19:1) in a humidified incubator. Confluency was achieved within 3–4 days, after which they were split 1:4 into 12-well plates for these experiments.

NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% penicillin/streptomycin and glutamine together with 10% foetal-calf serum. Cells were grown in 75 cm² flasks at 37 °C in an atmosphere of air/CO₂ (19:1) in a humidified incubator. Confluency was achieved within 3–4 days, after which they were split 1:4 into 12-well plates for these experiments.

Determination of ATP content of hepatocytes

The ATP content of the isolated hepatocytes was determined by the luciferase method on a neutralised HClO₄ extract as described previously [15]. Cells with an ATP concentration of ~8–10 nmol/mg dry wt. were judged to be viable, as described previously [15].

Adenylate cyclase assay

Adenylate cyclase activity was determined by a modification of an assay described previously by us [29]. Briefly, this involved using an assay mixture containing 10 μ l of membranes (30–40 mg/ml protein), 50 μ l of buffer/ATP-regenerating system, with final concentrations of 1.5 mM ATP, 5 mM MgSO₄, 10 mM theophylline, 1 mM EDTA, 1 mM dithiothreitol, 25 mM triethanolamine HCl (final pH 7.4; adjusted with KOH), phosphocreatine (7.4 mg/ml), creatine kinase (0.2 mg/ml) and BSA (0.8 mg/ml). The volume of each incubation was made up to 100 μ l with water. These were then incubated at 30 °C for 10 min before an equal volume of 4% HClO₄ was added to stop the reaction. The samples were centrifuged, the supernatants were neutralized with KOH, and the cyclic AMP content was measured as described previously by us [15], using a protein binding procedure [30].

Cyclic AMP phosphodiesterase assay

This was done with 1 μ M cyclic AMP as substrate by a modification of the two-step procedure of Thompson and Appleman [31] and Rutten et al. [32], as described previously by Marchmont and Houslay [33].

Protein determination

Protein was routinely measured by the method of Bradford [34] with BSA as a standard.

RESULTS

We have previously shown [11,12,25] that treatment of intact hepatocytes with cholera toxin led to the constitutive activation of adenylate cyclase activity after a well-defined lag period. The magnitude of this stimulatory effect of cholera toxin was shown to be profoundly attenuated if either glucagon or insulin was added to hepatocytes along with cholera toxin, without any change in the lag time for onset of activation of adenylate cyclase [25]. Similar data were obtained in this study for the action of insulin (Table 1). However, in addition to this, we show here that both the hormone vasopressin and the tumour-promoting phorbol ester PMA can both also attenuate cholera-toxin-stimulated adenylate cyclase activity (Table 1) without affecting the lag time for activation by this toxin (results not shown).

This inhibitory effect elicited by both insulin and vasopressin could, however, be blocked (Table 1) by challenge of the hepatocytes with either of the selective PKC inhibitors stauro-

Table 1 Cholera-toxin-stimulated adenylate cyclase activity of hepatocyte membranes

Hepatocytes were incubated for 1 h with cholera toxin (1 $\mu\text{g}/\text{ml}$), harvested, and a washed membrane fraction was then prepared and taken for assay of adenylate cyclase activity as described in the Materials and methods section. Where indicated, other ligands were added together with cholera toxin in the hepatocyte incubation. Insulin and vasopressin were added at final concentrations of 10 nM, and PMA was added at 100 nM. In some instances the isolated membrane fraction was subsequently treated with alkaline phosphatase activity as described previously by us [21] and defined in the Materials and methods section. Experiments were done the indicated (*n*) number of times using separate hepatocyte preparations, with errors expressed as S.D. of the given means. Data are presented relative to that activity seen in membranes from cells treated with cholera toxin alone (100%). Typically, the activity noted in the washed membranes for untreated hepatocytes ranged from 0.8 to 1.3 pmol/min per mg of protein.

Treatment	Activity (%)	(<i>n</i>)
Cholera toxin	(100)	
Cholera toxin + vasopressin	47 $\pm$ 8	6
Cholera toxin + PMA	62 $\pm$ 7	14
Cholera toxin + insulin	55 $\pm$ 4	12
Cholera toxin + staurosporine	92 $\pm$ 5	3
Cholera toxin + staurosporine + vasopressin	96 $\pm$ 4	3
Cholera toxin + staurosporine + insulin	89 $\pm$ 5	3
Cholera toxin + chelerythrine	93 $\pm$ 6	3
Cholera toxin + chelerythrine + vasopressin	88 $\pm$ 6	3
Cholera toxin + chelerythrine + insulin	87 $\pm$ 8	3
Cholera toxin + okadaic acid	49 $\pm$ 9	3
Cholera toxin, then membrane alkaline phosphatase treatment	97 $\pm$ 6	3
Cholera toxin + vasopressin, then membrane alkaline phosphatase treatment	95 $\pm$ 8	3
Cholera toxin + PMA, then membrane alkaline phosphatase treatment	88 $\pm$ 3	3
Cholera toxin + insulin, then membrane alkaline phosphatase treatment	90 $\pm$ 8	3
Cholera toxin (60 min), then cell PMA treatment (15 min)	63 $\pm$ 10	3

Table 2 Cholera-toxin-stimulated adenylate cyclase function in P9 cells

The effect of cholera toxin (1 $\mu\text{g}/\text{ml}$; 60 min) on P9-cell adenylate cyclase function was assessed either by measuring adenylate cyclase activity in an isolated membrane fraction ('Membranes') or by incubating P9 cells in the presence of the cyclic AMP phosphodiesterase inhibitor IBMX (1 mM) and measuring the intracellular concentration of cyclic AMP ('Intracellular') as described in the Materials and methods section. Resting cells had an intracellular cyclic AMP concentration of 1.5–2.6 pmol of cyclic AMP/ 10^6 cells, which was typically raised to 28–37 pmol/ 10^6 cells after 1 h treatment of the intact cells with cholera toxin (1 $\mu\text{g}/\text{ml}$) (range; *n* = 3). Membrane adenylate cyclase activity in resting cells was 2.1 ± 0.4 pmol of cyclic AMP/min per mg of membrane protein. This rose to 9.8 ± 0.6 pmol/min per mg of membrane protein after cholera-toxin treatment. Where indicated, other ligands were added together with cholera toxin in the P9-cell incubation, with insulin and vasopressin at final concentrations of 10 nM and PMA at 100 nM. Experiments were done five times with different cell preparations, and results are given as means $\pm$ S.D. Shown are changes in cholera-toxin-stimulated adenylate cyclase activity relative to that found in cells not also challenged with ligands (100%).

Treatment	Membranes (%)	Intracellular (%)
Cholera toxin	(100)	(100)
Cholera toxin + vasopressin	57 $\pm$ 6	48 $\pm$ 5
Cholera toxin + PMA	59 $\pm$ 8	68 $\pm$ 4
Cholera toxin + insulin	63 $\pm$ 6	61 $\pm$ 8

sporine or chelerythrine [35–37]. These compounds had, however, no apparent effect on the magnitude of activation of adenylate cyclase by cholera toxin achieved in the absence of these hormones (Table 1). In contrast with this, exposure of cells to the

Figure 1 Inhibition of the ability of cholera toxin to stimulate P9-cell adenylate cyclase activity by insulin and PMA

The immortalized hepatocyte P9 cell line was used in these studies. Cells were incubated with cholera toxin (1 $\mu\text{g}/\text{ml}$) for the indicated times together with the cyclic AMP phosphodiesterase inhibitor IBMX (1 mM). They were then harvested, and a washed membrane fraction was prepared for assay of adenylate cyclase activity as described in the Materials and methods section. Data are shown for treatment with either cholera toxin alone (■) or when it was added with either insulin (▲), at a final concentration of 10 nM, or PMA (●), at a final concentration of 100 nM, to the hepatocyte incubation. In the absence of cholera toxin, IBMX elicited a small [20–28% (range; *n* = 5)] increase in the intracellular level of cyclic AMP. This is consistent with similar observations made on intact hepatocytes [5], which have indicated that the basal activity of hepatocyte adenylate cyclase is very low. IBMX (1 mM) has been shown to inhibit > 94% of hepatocyte cyclic AMP phosphodiesterase activity assayed at 1 μM cyclic AMP [5], and similar observations hold for P9 cells (> 93%). The Figure shows typical data from experiments that were done on three separate occasions.

protein phosphatase inhibitor okadaic acid [38–41] elicited a profound decrease in the cholera-toxin-stimulated adenylate cyclase activity (Table 1), again with no change in lag of onset of activation by cholera toxin (results not shown).

Treatment of membranes with alkaline phosphatase (Table 1) has been used to achieve the dephosphorylation of phosphorylated cellular proteins [21,42]. Exposure of membranes obtained from hepatocytes which had been treated with cholera toxin alone did not affect the adenylate cyclase activity (Table 1). In contrast with this, a marked increase in cholera-toxin-stimulated adenylate cyclase activity (Table 1) was observed in membranes from cells which had been challenged with PMA, vasopressin or insulin (Table 1). In each of these instances, membrane treatment with alkaline phosphatase increased adenylate cyclase activity up to a level approaching that seen in membranes from cells which had only been challenged with cholera toxin (Table 1).

We have generated [27] an immortalized hepatocyte cell line, using SV40 DNA, and shown that this expresses G-protein-regulated adenylate cyclase activity and a functional high-affinity insulin response. Here we demonstrate that the adenylate cyclase activity in such cells could be markedly stimulated by cholera toxin and that this activity could be inhibited by either PMA or insulin treatment in a similar fashion to that seen with native hepatocytes (Table 2; Figure 1).

Cholera toxin was also able to achieve a marked stimulation of adenylate cyclase activity in both CHO and NIH-3T3 cells

Figure 2 Cholera toxin activates adenylate cyclase in CHO and NIH-3T3 cells in a manner which is resistant to inhibition by PMA

Cells were incubated for 2 h with cholera toxin ($1 \mu\text{g/ml}$) in the presence of the cyclic AMP phosphodiesterase inhibitor IBMX (1 mM). They were then harvested, and the intracellular concentration of cyclic AMP was determined as described in the Materials and methods section. Experiments were done with cholera toxin either alone (■, □) or together with PMA (●, ○) added to the incubations at 100 nM . The Figure shows data for wild-type CHO cells (■, ●) and wild-type NIH-3T3 cells (□, ○). Incubation of cells with IBMX alone over this period led to a small increase in intracellular cyclic AMP concentration of 22–30% (range; $n = 3$) for both cell types. This was unaltered by the presence of PMA. Data are typical of experiments done on three separate occasions, and are given as means $\pm$ S.D.

Table 3 Cholera-toxin-stimulated adenylate cyclase function in CHO cell lines and NIH-3T3 cells

The effect of cholera toxin ($1 \mu\text{g/ml}$; 120 min) on adenylate cyclase function was assessed either by measuring adenylate cyclase activity in an isolated membrane fraction or by incubating the cells in the presence of the cyclic AMP phosphodiesterase inhibitor IBMX (1 mM) and measuring the intracellular concentration of cyclic AMP. The various resting cells had intracellular cyclic AMP concentrations which ranged from 1.3 to $2.6 \text{ pmol}/10^6$ cells after 2 h with IBMX alone. The intracellular concentration of cyclic AMP is given in $\text{pmol}/10^6$ cells for cells treated for 2 h with cholera toxin ($1 \mu\text{g/ml}$) either with toxin alone (No PMA) or with PMA added to the incubations at 100 nM (Plus PMA). Control membrane adenylate cyclase activities (pmol of cyclic AMP produced/min per mg of protein) were 1.5 ± 0.1 , 1.3 ± 0.2 , 1.4 ± 0.2 and 1.7 ± 0.2 for CHO-k, CHO- α , CHO- γ and NIH-3T3 cells respectively. An indication of the effect of PMA on the cholera-toxin-stimulated adenylate cyclase function is given by expressing the levels of cyclic AMP accruing with PMA and cholera toxin added together as a percentage of those found when cholera toxin was added alone. The various CHO cell lines are denoted as: k, wild-type CHO cells; T, transfected so as to overexpress the human insulin receptor; and otherwise with Greek letters indicating the particular isoform of PKC that these cells have been transfected to overexpress. All CHO cells overexpressing PKC isoforms also overexpressed the human insulin receptor [27]. NIH-3T3 cells were wild-type cells. Experiments were done three times with different cell preparations, and results are given as means $\pm$ S.D.: –, not examined.

Cell line	Cyclic AMP accumulation			Adenylate cyclase activity		
	No PMA	Plus PMA	(%)	No PMA	Plus PMA	(%)
CHO-k	24.0 ± 2.4	22.8 ± 1.0	95	4.7 ± 0.2	4.5 ± 0.3	96
CHO-T	22.0 ± 1.5	23.4 ± 2.1	106	–	–	–
CHO- α	23.6 ± 1.5	21.6 ± 1.2	92	4.8 ± 0.3	4.6 ± 0.5	96
CHO- β II	25.2 ± 0.8	31.8 ± 2.5	126	–	–	–
CHO- ϵ	30.1 ± 0.9	30.2 ± 0.6	100	–	–	–
CHO- γ	21.1 ± 1.2	28.5 ± 1.5	135	4.5 ± 0.1	6.5 ± 0.2	144
NIH-3T3	25.4 ± 1.0	32.0 ± 1.6	126	5.4 ± 0.2	7.1 ± 0.3	132

(Figure 2; Table 3). However, in contrast with experiments done with hepatocytes and P9 cells, this activity was not attenuated by treatment with the phorbol ester PMA (Figure 2; Table 3). Indeed, treatment of NIH-3T3 cells with PMA led to an increase in the cholera-toxin-stimulated adenylate cyclase activity (Table 3; Figure 2). This was not observed in native CHO cells (Table 3). However, we also investigated the action of PMA in CHO cells which had been transfected so as to overexpress various PKC isoforms. In no instance, however, did we observe any inhibition. Interestingly, though, we noted that PMA mediated an increase in the cholera-toxin-stimulated adenylate cyclase activity of CHO cells transfected so as to overexpress PKC- γ (Table 3). These observations appeared to be independent of whether adenylate cyclase activity was gauged by direct membrane assay or through determining intracellular cyclic AMP accumulation in the presence of a phosphodiesterase inhibitor.

DISCUSSION

Treatment of intact hepatocytes with cholera toxin leads to the constitutive activation of adenylate cyclase activity after a well-defined lag period [11,12,25]. The magnitude of this stimulatory effect can be profoundly attenuated by the action of either glucagon or insulin [24]. These hormones, however, did not affect the lag time for the action of cholera toxin, nor did they alter the degree of ADP-ribosylation of G_s [25], suggesting that either the functioning of the catalytic unit of adenylate cyclase, or its ability to be stimulated by G_s , was attenuated.

A number of investigators have suggested that glucagon may exert actions which are seemingly independent of cyclic AMP, and it has been suggested that this may involve stimulation of phospholipid metabolism (see [19,20] for reviews). In this regard, glucagon has been shown to increase intracellular Ca^{2+} levels [43–45] and also to stimulate metabolism of both phosphatidylcholine [22] and inositol phospholipid [46–48], leading to the reported production of diacylglycerol [22,43,49] and activation of PKC [22,23]. Glucagon clearly cannot be exerting its inhibitory effect on the ability of cholera toxin to activate adenylate cyclase through any elevation of intracellular cyclic AMP levels, as cholera toxin itself achieves just that [11,12,24]. This suggests that such an action of glucagon might be mediated through PKC activation. We show here that PMA, which exerts its effects through a well-established direct action on PKC [35,50,51], and vasopressin, which stimulates inositol phospholipid metabolism in hepatocytes [52], as with glucagon and insulin [25], both attenuated the ability of cholera toxin to activate adenylate cyclase through an action mediated by PKC (Table 1). Consistent with this, the inclusion of the compounds staurosporine and chelerythrine, which can serve as protein kinase inhibitors with some selectivity in their action as regards the inhibition of PKC [35–37], ablated the inhibitory action of vasopressin (Table 1). Interestingly, however, they also served to ablate the inhibitory action of insulin (Table 1), implying that insulin can exert effects on adenylate cyclase activity through the action of PKC. In this regard, it has been suggested [53] that insulin might activate PKC activity in various cells. However, it is also possible that members of the protein kinase cascade known to form one branch of the pleiotropic network which comprises the insulin-receptor signalling system [54] may transduce the inhibitory effect of insulin noted here, and that such a species could be inhibited by these compounds.

We have previously suggested that, under resting conditions in hepatocytes, there is an active phosphorylation/dephosphorylation cycle involving PKC action [55,56]. As such, the level of phosphorylation of any protein which provides a target for PKC

might be expected to be amplified by the action of the protein phosphatase inhibitor okadaic acid [38–41]. Thus exposure of hepatocytes to okadaic acid has been shown to mimic the effect of PKC action in eliciting the phosphorylation of the inhibitory G-protein α -G_i-2 at the PKC phosphorylation ('C') site, Ser¹⁴⁴ [57]. Okadaic acid also appears able to mimic PKC-mediated glucagon desensitization of adenylate cyclase activity in hepatocytes [21]. In contrast, under such basal conditions no phosphorylation of the inhibitory G-protein α -G_i-2 at the protein kinase A phosphorylation ('AN') site Ser²⁰⁷ was elicited by okadaic acid [57]. That we observed here a profound attenuation of cholera-toxin-stimulated adenylate cyclase activity by okadaic acid (Table 1) would be consistent with PKC mediating this response.

Previously, however, we have shown [16,24] that treatment of hepatocytes either with the phorbol ester PMA or with vasopressin did not inhibit the activity of either the catalytic unit of adenylate cyclase, assessed by monitoring both basal activity and that amplified by forskolin, or G_s-stimulated adenylate cyclase when monitored with either NaF or non-hydrolysable GTP analogues. However, in such experiments hepatocytes were challenged with agents which activated PKC under very different conditions from those prevailing in the cells which have been exposed to cholera toxin. For, in the presence of cholera toxin not only do intracellular levels of cyclic AMP rise above the threshold for activation of protein kinase A [19,58], but adenylate cyclase is subject to chronic stimulation by α -G_s. It may be that either one or both of these conditions is a pre-requisite for PKC to be able to modify a component of the G_s-adenylate cyclase system and thus attenuate cholera-toxin-stimulated adenylate cyclase activity. The possibility that chronic elevation of intracellular cyclic AMP levels may allow for this novel action of PKC might be inferred from an earlier study by us [18]. In that, we showed that when hepatocytes were exposed to glucagon in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) a stable and profound inhibition of membrane adenylate cyclase activity ensued, rather than a transient inhibition as seen when using glucagon alone [18–20]. Indeed, in the presence of both glucagon and IBMX the levels of cyclic AMP achieved were comparable with those observed in hepatocytes treated solely with cholera toxin [18]. Such data might be taken to imply that elevated intracellular cyclic AMP levels in hepatocytes allow PKC to act at a site which can lead to the attenuation of G_s-stimulated cyclase activity.

We have generated [27] an immortalized hepatocyte cell line using SV40 DNA and have shown that this expresses G-protein-regulated adenylate cyclase activity and a functional high-affinity insulin response. Here we demonstrate (Table 2) that the adenylate cyclase activity in such cells could be markedly stimulated by cholera toxin, and that this activity could be inhibited by either PMA or insulin treatment in a similar fashion to that seen with native hepatocytes (Figure 2; Table 2). This provides further evidence that the P9 cell line should provide a useful cell line for analysing insulin action in a manner which reflects that of native hepatocytes. Intriguingly, we also observed that vasopressin could elicit an inhibition of cholera-toxin-stimulated adenylate cyclase activity comparable with that seen in native hepatocytes (Tables 1 and 2), thus, to our knowledge, providing the first immortalized hepatocyte cell line to have functional vasopressin receptors.

That an attenuated cholera toxin-stimulated adenylate cyclase activity can be observed in an isolated hepatocyte membrane preparation might imply that a component of this system is stably phosphorylated. Consistent with this, we were able to show that treatment of hepatocyte membranes with alkaline

phosphatase markedly increased cholera-toxin-stimulated adenylate cyclase activity in membranes from PMA-, vasopressin- and insulin-challenged cells, but not in membranes from unchallenged ones (Table 1). That the cholera-toxin-stimulated activity increased up to that seen in unchallenged cells shows that the inhibitory effect of these agents could be simply reversed by alkaline phosphatase action, consistent with the notion of reversible phosphorylation providing the basis of the effect.

In contrast with experiments done on hepatocytes and P9 cells, cholera-toxin-stimulated adenylate cyclase activity in both CHO and NIH-3T3 cells was not attenuated by treatment with the phorbol ester PMA (Figure 2; Table 3), implying a fundamental difference in susceptibility of these systems. One possible explanation for this might be that levels of PKC were limiting in such cells, or that a specific isoform of PKC may be required. However, this would appear to be unlikely, as PMA failed to inhibit cholera-toxin-stimulated adenylate cyclase activity in CHO cells which had been transfected so as to overexpress various PKC isoforms (Figure 2; Table 3). α -G_s and adenylate cyclase form two possible targets for the action of PKC. However, α -G_s has been shown to be a single gene product whose sequence is highly conserved between species [8–10,59]. In contrast, adenylate cyclase forms part of a large multi-gene family where differential expression in various cells is well established [1–5], and differential susceptibility to phorbol esters has been noted, with activation of the activity of certain, but not all, isoforms of adenylate cyclase occurring [3,4,6,19,60]. Thus the differences in susceptibility to PMA action in these cells may be due to the expression of different forms of adenylate cyclase. Indeed, in this regard we noted that treatment of NIH-3T3 cells with PMA led to a recognizable increase in the cholera-toxin-stimulated adenylate cyclase activity (Table 3; Figure 2). Additionally, in CHO cells transfected so as to overexpress PKC- γ , we observed a PMA-mediated increase in the cholera-toxin-stimulated adenylate cyclase activity of cells (Table 3). This may indicate a specificity for the action of particular PKC isoforms in being able to modify a particular adenylate cyclase isoform in CHO cells. It does not appear to relate to differences in the relative magnitudes of PKC activity in these transfected cell lines. For, compared with total PKC levels seen in wild-type CHO cells, Chin et al. [28] have shown that the PKC β I transfectants exhibited the highest levels of PKC expression, with levels 18-fold higher, followed by PKC- ϵ transfectants, with levels 10-fold higher, and then the PKC- α and PKC- γ transfectants, with levels 5-fold higher.

The cholera-toxin-stimulated adenylate cyclase activity of hepatocytes appears thus to become inhibited through the action of PKC, achieved either directly, by PMA, or by the hormones glucagon, insulin and vasopressin. Such an inhibitory effect can be reversed by alkaline phosphatase treatment of membranes, consistent with a phosphorylation event. That insulin and vasopressin can exert such an inhibitory action on cholera-toxin-stimulated adenylate cyclase in the P9 immortalized hepatocyte cell, indicates that these cells can provide a useful model of a hormonally responsive system. Our study thus identifies a novel point of cross-talk between the cyclic AMP, the phospholipid and the tyrosine kinase signalling pathways which involves PKC.

This work was supported by a grant from the Medical Research Council to M.D.H.

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