Co-transfection with protein kinase D confers phorbol-ester-mediated inhibition on glucagon-stimulated cAMP accumulation in COS cells transfected to overexpress glucagon receptors

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Glucagon elicited a profound increase in the intracellular cAMP concentration of COS-7 cells which had been transiently transfected with a cDNA encoding the rat glucagon receptor and under conditions where cAMP phosphodiesterase activity was fully inhibited. This was achieved in a dose-dependent fashion with an EC_{50} of 1.8 ± 0.4 nM glucagon. In contrast with previous observations made using hepatocytes [Heyworth, Whetton, Kinsella and Houslay (1984) FEBS Lett. **170**, 38–42], treatment of transfected COS-7 cells with PMA did not inhibit the ability of glucagon to increase intracellular cAMP levels. PMA-mediated inhibition was not conferred by treatment with okadaic acid, nor by co-transfecting cells with cDNAs encoding various protein kinase C isoforms (PKC-α, PKC-βII and PKC-ϵ) or with the PMA-activated G-protein-receptor kinases GRK2 and GRK3. In contrast, PMA induced the marked inhibition of glucagon-stimulated cAMP production in COS-7 cells that had been co-transfected with a cDNA encoding protein kinase D (PKD). Such inhibition was not due to an action on the catalytic unit of adenylate cyclase, as forskolin-stimulated cAMP production was unchanged by PMA treatment of COS cells that had been co-transfected with both the glucagon receptor and PKD. PKD transcripts were detected in RNA isolated from hepatocytes but not from COS-7 cells. Transcripts for GRK2 were present in hepatocytes but not in COS cells, whereas transcripts for GRK3 were not found in either cell type. It is suggested that PKD may play a role in the regulation of glucagon-stimulated adenylate cyclase.

INTRODUCTION

Glucagon, a hormone that is secreted by the pancreas in response to hypoglycaemia, is a key regulator of hepatic glucose production. The rat hepatic glucagon receptor cDNA has been isolated by both homology cloning [1,2] and expression cloning [3]. It encodes a 485-amino acid glycoprotein with the seven putative *trans*-membrane domains which are characteristic of the G-protein-coupled receptor superfamily and appears to be a member of the secretin subfamily. The receptor has also been cloned from human liver [4] and from mouse liver [5]. Glucagon binding causes its receptor to interact with the stimulatory G-protein, G_s, which subsequently activates adenylate cyclase [6–8]. The consequent elevation of intracellular cAMP leads, in liver parenchymal cells (hepatocytes), to the stimulation of both gluconeogenesis and glycogenolysis.

In isolated hepatocytes, hormonal activation of lipid signalling pathways and challenge of cells with either the tumour-promoting phorbol ester, PMA, or synthetic diacylglycerols has been demonstrated to lead to a profound reduction in the ability of glucagon to stimulate adenylate cyclase activity [9–14]. Such ligands appear to prevent the glucagon receptor from activating adenylate cyclase, as they have little, if any, effect on either the functioning of the catalytic unit of adenylate cyclase or the ability of G_s to stimulate adenylate cyclase [15]. Such observations led to the proposal that the reduction in glucagon-stimulated adenylate cyclase activity caused by challenge of hepatocytes with these various ligands may be mediated through the action of protein kinase C (PKC) [16].

In order to gain further insight into the nature of the protein kinase species that conferred phorbol-ester-mediated inhibitory effects on glucagon-stimulated adenylate cyclase activity, we have used a model system using COS-7 cells transfected so as to transiently overexpress glucagon receptors. Intriguingly, although such transfected cells showed a profound glucagon-stimulated increase in cAMP accumulation elicited through the activation of adenylate cyclase, challenge with PMA failed to elicit an inhibitory response. Sensitivity to inhibition by PMA was not restored by co-transfection with either a variety of PKC isoforms or various PMA-stimulated G-protein-coupled receptor kinases (GRKs). However, it was restored on co-transfection of the glucagon receptor with the recently identified phorbol ester and phospholipid/diacylglycerol-stimulated serine kinase, protein kinase D (PKD) [17,18].

MATERIALS AND METHODS

Materials

BSA, cAMP, isobutylmethylxanthine (IBMX), cholera toxin, PMA, DEAE-dextran, glucagon, N,N,N',N'-tetramethylethy-

Abbreviations used: GRK, G-protein-coupled receptor kinase; IBMX, isobutylmethylxanthine; PDE, cAMP phosphodiesterase; PKC, protein kinase C; PKD, protein kinase D; RT-PCR, reverse transcriptase-PCR; DMEM, Dulbecco's modified Eagle's medium; βARK, β-adrenergic receptor kinase; TBS, Tris-buffered saline.

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lenediamine, ethidium bromide and Tri Reagent™ were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). $NaHCO_3$, KCl, $MgSO_4$, KH_2PO_4, $CaCl_2$, glycerol, SDS and DMSO were from Fisons (Loughborough, Leics., U.K.). [^{3}H]cAMP, ^{125}I-glucagon and the enhanced chemiluminescence detection kit were supplied by Amersham International (Amersham, Bucks., U.K.). Tris, trypsin–EDTA, $NaHCO_3$ (7.5% solution), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin/streptomycin and foetal bovine serum were from Gibco–BRL (Paisley, Scotland, U.K.). Bradford reagent and acrylamide/bisacrylamide mix were from Bio-Rad. Nonidet P40 was from Calbiochem–Behring (La Jolla, CA, U.S.A.). Ammonium persulphate was from May and Baker (Dagenham, Essex, U.K.). Horseradish peroxidase-conjugated anti-rabbit antibody was from the Scottish Antibody Production Unit. Agarose and keyhole limpet haemocyanin were from Boehringer-Mannheim. Freund's complete adjuvant was from Difco (Detroit, MI, U.S.A.), and Freund's incomplete adjuvant was from Pierce (Rockford, IL, U.S.A.). The first-strand cDNA synthesis kit was obtained from Pharmacia (Uppsala, Sweden). Perfect Match® DNA polymerase enhancer was from Stratagene (La Jolla, CA, U.S.A.). *Taq* polymerase, dNTPs, $MgCl_2$ and restriction enzymes were purchased from Promega (Madison, WI, U.S.A.). MC1061/P3 *Escherichia coli* were purchased from Invitrogen (San Diego, CA, U.S.A.). All other chemicals were of AR grade from BDH Chemicals (Glasgow, Scotland, U.K.). We are extremely grateful to Professor M. Svoboda (University Libre de Bruxelles, Belgium), Dr. P. J. Parker (ICRF, London, U.K. and Dr. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA, U.S.A.) for gifts of the plasmids used in this study.

COS cell culture, transfection and incubation with ligands

Stock cultures of COS-7 cells were maintained in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine and 10% (v/v) foetal bovine serum in an atmosphere containing 5% CO_2 at 37 °C. Exponentially growing COS-7 cells, 40–60% confluent were transfected, where indicated, with expression vectors for various species using the DEAE-dextran method as described previously by us [19]. The species used encoded the rat hepatic glucagon receptor in the plasmid type pCDM8 [1], PKC-α in the plasmid type pMT2 [20], PKC-βII in the plasmid type pCO2 [20], PKC-ϵ in the plasmid type pMT2 [20], both β-adrenergic receptor kinase (βARK)1 and βARK2 each in the plasmid type pBC12BI [21,22] and mouse lung PKD in the plasmid type pcDNA3 [18]. The kinase-inactive form of PKD, PKDK618M, was in pcDNA3 [23]. Unless otherwise stated, cells were transfected with 5 μg of DNA/75 cm^2 flask. At 24 h after transfection, cells were divided up into six-well plates and subsequently grown to confluence in DMEM supplemented as above. When confluent, approx. 72 h after transfection, cells were incubated with serum-free DMEM containing 1 mM IBMX and ligands at the indicated concentrations. When PMA was added, it was present at a concentration of 1 μM, and, unless stated otherwise, a preincubation period of 15 min was employed.

Hepatocyte preparation

Hepatocytes were prepared by a modification of the method of Berry and Friend [24] described previously by us [25], using 220–250 g fed male Sprague–Dawley rats. Cells (10^6–10^7 cells/ml) were incubated at 37 °C in Krebs–Henseleit buffer supplemented with 2.5% (w/v) BSA, 2.5 mM $CaCl_2$ and 10 mM glucose while being gassed with O_2/CO_2 (19:1, v/v) for 30 s every 10 min. The cells were harvested by centrifugation (100 $\boldsymbol{g}$; 2 min) as described previously by us [25].

Protein determination

Protein was routinely measured by the method of Bradford [26] with BSA as standard.

Determination of intracellular cAMP

Intracellular cAMP accumulation was employed as a means of determining adenylate cyclase activity in intact COS-7 cells. cAMP phosphodiesterase (PDE) activity in these cells was inhibited more than 96% by the addition of the non-specific PDE inhibitor [27] IBMX (1 mM) to the incubation mixtures. This degree of inhibition of PDE activity was unaffected by either transfection with any of the plasmids used in this study or by treatment with the phorbol ester, PMA. IBMX in itself did not lead to any marked increase in cAMP accumulation.

Intracellular cAMP accumulation was determined using a modification of the procedures described previously by us [16]. Briefly, after the indicated period of incubation with ligands, incubations were terminated by the aspiration of the medium from the cells and the addition of 500 μl of 2% $HClO_4$. The cells were then scraped and the precipitated protein pelleted by centrifuging the samples in a Microfuge (13000 $\boldsymbol{g}_{av}$) for 2 min at room temperature. The supernatant was neutralized with 2 M KOH/0.5 M triethanolamine hydrochloride before a further centrifugation to remove the $KClO_4$ 0.5 M precipitate. The cAMP content of the supernatant was determined using a cAMP-binding protein prepared from bovine adrenal glands as described previously [28].

Western-blot analysis

COS cell extracts (100 μg of protein) were boiled in Laemmli buffer [29] for 3 min. Electrophoresis was performed on an 8% acrylamide gel (running buffer 25 mM Tris, 0.19 M glycine, 3.5 mM SDS) at 7 mA/gel overnight or 60 mA/gel for 3–4 h with cooling. Western blotting was performed essentially as described previously by us [30] using a 1 h transfer time on to nitrocellulose paper. The blots were blocked for 2 h in 5% (w/v) skimmed milk in Tris-buffered saline (TBS), followed by an incubation with the designated antiserum overnight. The blots were washed four times for 10 min each with TBS/0.05% (v/v) Nonidet P40 and then probed with horseradish peroxidase-conjugated donkey anti-rabbit serum [diluted 1:500 in 1% (w/v) skimmed milk in TBS] for 30 min, followed by another four washes for 10 min each with TBS/0.05% Nonidet P40. The blots were finally rinsed in TBS, and immunodetection was carried out using an enhanced chemiluminescence kit. The procedure was carried out as per the manufacturer's instructions. We used isoform-specific PKC antisera generated and characterized previously by us [31]. PKD-specific antiserum was raised against a synthetic peptide, EEREMKALSERVSIL, that corresponds to the extreme C-terminal region of the predicted amino acid sequence of PKD, as described previously [18].

Reverse transcriptase-PCR (RT-PCR) analysis

Total cellular RNA was extracted by the acid guanidinium thiocyanate/phenol/chloroform extraction method [32] from confluent monolayers of COS-7 cells and from isolated hepatocytes. First-strand cDNA was subsequently prepared by the use of the moloney murine leukaemia virus reverse transcriptase and oligo-dT primer, according to the instructions provided with the

Pharmacia First-strand cDNA Synthesis Kit. Briefly, 5 μg of RNA, as determined by its A_{260}, was denatured for 10 min at 65 °C and subsequently incubated with reverse transcriptase, 6 mM dithiothreitol, dNTPs and 0.2 μg of the supplied poly(dT) primer [*Not*I-d(T)$_{18}$] for 1 h at 37 °C. PCR was performed in the presence of 1 × *Taq* buffer (50 mM KCl, 20 mM Tris/HCl), 1.5 mM $MgCl_2$, 40 μM each dNTP, 1.5 μM each primer, 3 μl of first-strand reaction mixture, 5 units of *Taq* polymerase and 0.5 unit of Perfect Match® DNA polymerase enhancer in a total volume of 50 μl. Primers were designed to specifically detect transcripts of the indicated protein kinases. Some 35 thermal cycles were undertaken, each consisting of a 1 min denaturation segment at 95 °C, a 2 min annealing step at 52.5 °C and a 3 min extension time at 72 °C.

For PKD the primer pair designed was: 5′ sense oligonucleotide ET-PKD-as1, TCGTTCACTGTGACCTCAAGC; 3′ antisense oligonucleotide ET-PKD-as1, CTAGCACTCAGACTGATCAGG. This allowed the amplification of a specific 565 bp product corresponding to nucleotides 2123–2687 for the open reading frame of murine PKD [17] (accession no. Z34524). For GRK2 (βARK) the primer pair designed was: 5′ sense oligonucleotide ET-GRK2-s1, AAGCTGGAGACAGAGGAGG; 3′ antisense oligonucleotide ET-GRK2-as1, TCGTCCAGCAGGATGTTGG. This allowed the amplification of a specific 701 bp product corresponding to nucleotides 280–980 of the open reading frame of bovine GRK2 [21] (Genbank accession no. M34019). For GRK3 (βARK2) the primer pair designed was: 5′ sense oligonucleotide ET-GRK3-s1, TTCAGAGGCATCGACTGG; 3′ antisense oligonucleotide ET-GRK3-as1, CATGAATGTCTCCGTCAGC. This allowed the amplification of a specific 600 bp product corresponding to nucleotides 1357–1956 of the open reading frame of bovine GRK3 [22] (accession no. M73216). For β-actin the sense and antisense oligonucleotide primers were CATCGTCACCAACTGGGACGAC and CGTGGCCATCTCTTGCTCGAAG respectively, allowing the amplification of a specific 466 bp product corresponding to nucleotides 222–687 of the open reading frame of human β-actin [33] (accession no. M10278).

PCR products were resolved by electrophoresis on a 2% agarose gel with ethidium bromide and visualized under UV light.

PDE assay

PDE activity was determined by a modification [34] of the two-step procedure of Thompson and Appleman [35] using [^{3}H]cAMP and unlabelled cAMP to give a final concentration of 1 μM cAMP as substrate in 20 mM Tris/HCl, 10 mM $MgCl_2$ buffer (final pH 7.4). Appropriate samples of enzyme extracts, to give linear time courses, were incubated in this assay mixture for 10 min at 30 °C and the reaction was terminated by boiling for 2 min. IBMX was dissolved in DMSO so that the final DMSO concentration was 0.1%, which was shown to have no effect on PDE activity.

RESULTS AND DISCUSSION

Kidney-derived cells have been shown to express low levels of endogenous glucagon receptors and to exhibit glucagon-stimulated cAMP accumulation [36,37]. COS-7 cells are a monkey kidney epithelial cell line [38], and we show here that challenge with glucagon caused a small dose-dependent increase in the intracellular accumulation of cAMP (Figure 1, top) determined in the presence of the non-selective cAMP PDE inhibitor IBMX [27], added to inhibit degradation of cAMP. However, when COS cells were transiently transfected with a cDNA encoding the rat glucagon receptor, we observed a profound dose-dependent increase in intracellular cAMP (Figure 1, top), with an EC_{50} value of 1.8 ± 0.4 nM (mean ± S.D. from three different transfection experiments). Such a value is comparable with that observed for the native response seen in intact hepatocytes [39–41] and that observed by Jelinek et al. [3] in their studies using the cloned rat glucagon receptor. In COS-7 cells that had

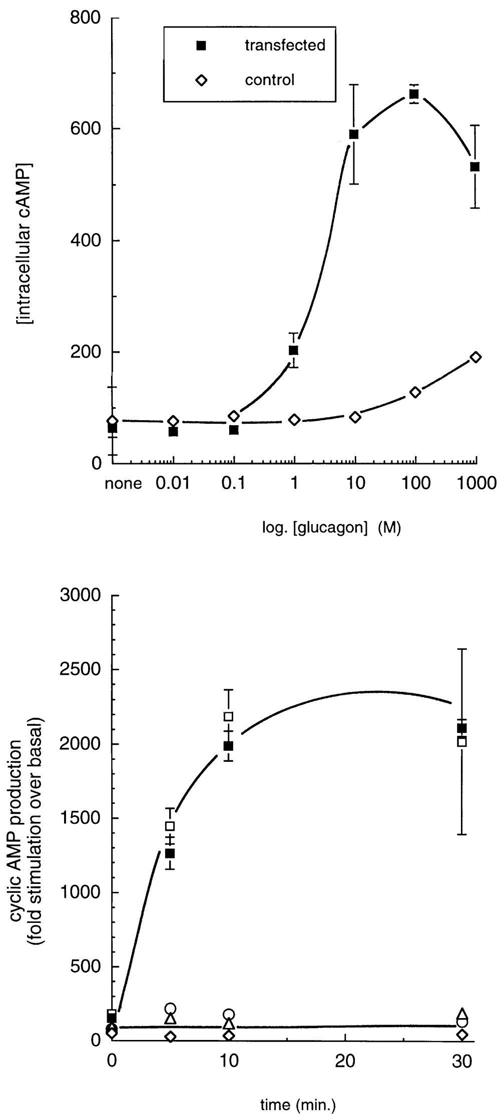

Figure 1 Dose–response curves and time courses for glucagon-stimulated cAMP accumulation in untransfected and glucagon-transfected COS cells

Top, dose–response curves for the glucagon-stimulated cAMP accumulation in intact COS cells, either control (◇) or transfected with pCDM8-GR DNA (■). Cells were stimulated for 6 min with the indicated concentrations of glucagon, in the presence of the cAMP PDE inhibitor, IBMX. Incubations were terminated by the addition of 2% $HClO_4$ and cAMP accumulation was determined by the cAMP-binding assay. Means ± S.D. from three experiments are shown. Bottom, time courses for glucagon-stimulated cAMP accumulation in glucagon-receptor-transfected COS cells in the presence and absence of IBMX. Glucagon-receptor-transfected COS cells were stimulated with 10 nM glucagon (○) or with no additions, i.e. medium alone in the presence (△) or absence (◇) of the PDE inhibitor, IBMX, at a concentration of 1 mM. The responses to glucagon were recorded in the presence of either IBMX (■) or both IBMX and 1 μM-PMA (□). Incubations were terminated after the times indicated by the addition of 2% $HClO_4$. The values shown represent means ± S.D. from three experiments. Intracellular [cAMP] is given as pmol/10^6 cells.

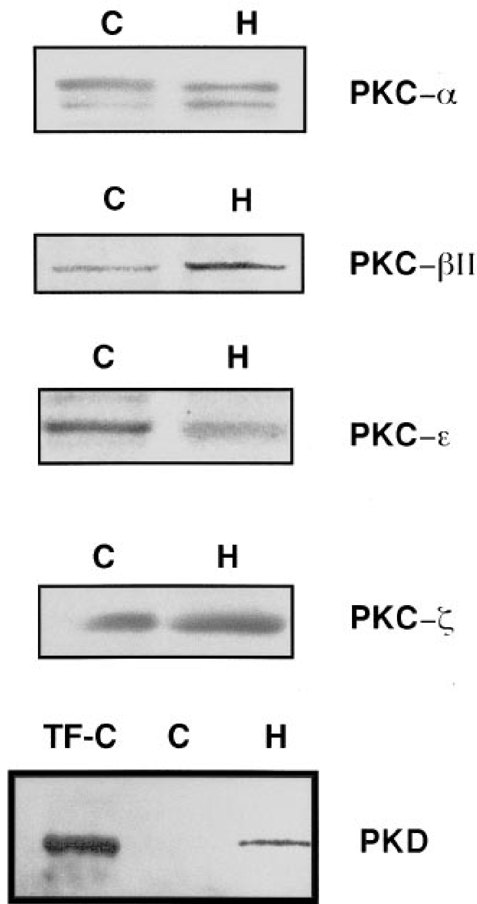

Figure 2 Immunoblots for PKC isoforms and for PKD in untransfected COS-7 cells and hepatocytes

Homogenates of both untransfected COS-7 cells (C) or rat hepatocytes (H) were analysed by SDS/PAGE with subsequent immunoblotting using the appropriate antisera specific for the indicated protein kinase species as described in the Materials and methods section. Track C reflects COS-7 cells that had been transfected with a plasmid encoding PKD and analysed subsequently by immunoblotting with anti-PKD serum. Samples each contained 100 μg of protein. The immunoreactive species were shown to correspond to the indicated isoforms and were identified using standards and peptide competition as described in detail previously by us [31]. PKC-α migrated as a doublet of 81 and 90 kDa in both COS-7 cells and hepatocytes; PKC-βII migrated as an 82 kDa species; PKC-ϵ migrated as a 95 kDa species; PKC-ζ migrated as a 79 kDa species; PKD migrated as a 110 kDa species. These data are typical of experiments performed at least three times with different cell preparations.

been transfected to express the rat glucagon receptor, challenge with glucagon caused a rapid time-dependent increase in the intracellular concentration of cAMP in cells (Figure 1, bottom). This increase in glucagon-stimulated cAMP accumulation was, however, only apparent (Figure 1, bottom) if the non-selective PDE inhibitor IBMX [27,42] was added to the assay mixtures. This presumably reflects the activity of endogenous cAMP PDEs, which have been demonstrated in these cells [43]. Maximal levels of cAMP accumulation were attained about 10 min after the challenge with glucagon, after which time intracellular cAMP remained at a stable elevated level. As IBMX is a reversible competitive inhibitor of PDE action [27], the plateau of accumulation reached presumably reflects a steady-state where the glucagon-stimulated production of cAMP matches the degradation by the residual PDE activity.

Table 1 Effect of PMA on glucagon-stimulated cAMP accumulation in co-transfected COS cells

Transfected COS cells, grown to confluence in six-well plates as described in the Materials and methods section, were preincubated for 15 min at 37 °C with serum-free DMEM containing 1 mM IBMX and either 0.1% DMSO (control) or 1 μM PMA. Glucagon was then added to a final concentration of 10 nM and the incubation was continued for another 10 min. $HClO_4$ (2%) was subsequently added, the cells were harvested and their cAMP content determined. Over a 10 min incubation with glucagon the [cAMP] in cells transfected with the glucagon receptor (GR) alone and in the absence of PMA was 921 ± 12 pmol of cAMP/10^6 cells. The results shown represent means $\pm$ S.D. from three experiments. The glucagon-stimulated cAMP production in the presence of PMA is expressed as a percentage of the control (without PMA). *Difference from control significant at $P < 0.01$ (Student's t test).

Transfection	Glucagon-stimulated cAMP production in presence of PMA (% of control response seen without PMA)
GR	101 ± 6
GR + PKC α	113 ± 6
GR + PKC βII	95 ± 5
GR + PKC ϵ	87 ± 12
GR + GRK2	108 ± 8
GR + GRK3	115 ± 2
GR + PKD	49 ± 2
GR + PKDK618M	99 ± 4

Treatment of rat hepatocytes with the phorbol ester PMA has been demonstrated to lead to a profound inhibition of glucagon-stimulated adenylate cyclase activity as determined by glucagon-stimulated cAMP accumulation in the presence of IBMX [9]. However, in contrast with this, using COS-7 cells transfected to express the rat glucagon receptor, we singularly failed to elicit any inhibition of glucagon-stimulated cAMP accumulation on challenge of these transfected cells with PMA ($< 5\%$; $n = 3$ experiments) (Figure 1, bottom). This was unlikely to be due to any potent protein phosphatase activity serving to antagonize the action of PMA-stimulated protein kinase activity, as the presence of the protein phosphatase inhibitor, okadaic acid [44,45], failed to allow PMA to exert an inhibitory effect on glucagon-stimulated cAMP accumulation in transfected COS-7 cells ($< 2\%$ inhibition in the presence of 100 nM okadaic acid and 1 μM PMA; $n = 3$).

PKC activity is provided for by a large family of isoforms [46,47]. It is possible that our inability to observe PMA-mediated inhibition of glucagon-stimulated cAMP accumulation in COS cells might have been due to inadequate PKC expression. Hepatocytes have been shown to express the PKC isoforms PKC-α, -βII, -ϵ and -ζ, but not PKC-β1, -γ, -δ or -η [31]. Immunoblotting indicated that, although PKC-α, -βII, -ϵ and -ζ were all detectable in native COS cells, the levels of PKC-βII appeared to be lower in COS cells than in hepatocytes (Figure 2). In order to increase levels of PKC isoforms, we co-transfected COS cells with the glucagon-receptor-encoding cDNA and with plasmids encoding each of the three PMA-responsive PKC isoforms present in hepatocytes (PKC-α, -βII and -ϵ). As the activity of atypical PKC-ζ is known not to be stimulated by phorbol ester [48], we did not attempt to co-transfect this isoform. Immunoblotting analyses showed that transfection of COS cells with these various PKC cDNAs led to the expression of the appropriate isoform at levels that were approx. 10–20-fold greater than in control untransfected cells (results not shown). However, in no instance was PMA-induced inhibition of

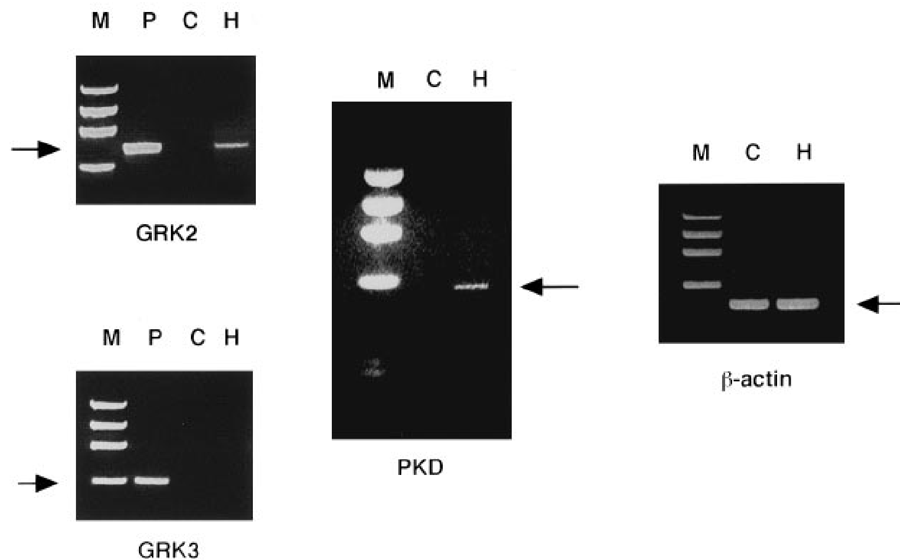

Figure 3 RT-PCR detection of GRK2, GRK3 and PKD in hepatocyte and untransfected COS cell RNA

RNA was prepared from washed hepatocytes or COS-7 cells using Tri Reagent™ as described in the Materials and methods section. First-strand cDNA synthesis was carried out using identical quantities of RNA, and was checked by PCR using oligonucleotide primers specific for β-actin. The results of experiments using primers designed to detect GRK2, GRK3, PKD and β-actin are shown. These primers were designed to amplify 701, 600, 565 and 466 bp DNA sequences respectively. PCR was carried out as described in the Materials and methods section using a template consisting of the cDNA derived from COS cells (C), hepatocytes (H) or plasmid control DNA containing the appropriate cDNA (P). Promega $\phi \times 174$/*Hae*III DNA markers were run in lanes (M) alongside the PCR products on a 2% agarose gel. The arrows indicate the position of the amplified species being analysed.

glucagon-stimulated cAMP accumulation reconstituted by co-transfection with the plasmids encoding these PKC isoforms (Table 1).

Analysis of the regulation of adrenoceptors has led to the identification of a family of GRKs [49]. Among these are GRK2 and the structurally similar GRK3, the activities of which can be regulated by PMA. These species can effect the attenuation of functioning of G_2-coupled β-adrenoceptors in a fashion that is markedly enhanced by PMA [50]. RT-PCR analysis showed that GRK2 was present in hepatocytes but not in COS cells, whereas GRK3 was not present in either cell type (Figure 3). To test the possibility that such forms may provide the basis for PMA-mediated inhibition of glucagon-stimulated adenylate cyclase activity, we co-transfected the glucagon receptor cDNA with cDNAs encoding each of these two kinases. However, this did not confer any PMA-induced inhibitory action on glucagon-stimulated cAMP accumulation (Table 1) despite our being able to detect the presence of the appropriate transcripts for these kinases in transfected COS cells by RT-PCR (results not shown).

A novel protein kinase, PKD, has been identified [17]. It has N-terminal cysteine-rich zinc-finger-like domains and is potently stimulated by both phorbol esters and diacylglycerol [51]. PKD is, however, distinguished from members of the PKC family by its catalytic domain structure and possession of a distinct substrate specificity, a putative transmembrane domain, a pleckstrin homology domain [52] and the absence of any pseudo-substrate motif [18]. Using RT-PCR analysis and immunoblotting studies we were able to show that PKD was expressed in hepatocytes but was not detectable in COS-7 cells (Figure 3). In view of this, we undertook co-transfections with cDNAs encoding the glucagon receptor and PKD. Immunoblotting analysis showed that this successfully led to the expression of PKD in these transfected cells (Figure 2). In such co-transfected cells, the level of glucagon-stimulated cAMP accumulation was about 20 % lower than that observed in cells that had been transfected with the glucagon receptor alone (665 ± 17 and 529 ± 8 pmol of cAMP/10^6 cells for glucagon receptor and glucagon receptor+PKD transfected COS-7 cells respectively challenged with 10 nM glucagon for 6 min; $n = 3$). One possible explanation for this might be the increased demands placed on the cell's finite transcriptional and translational capacity by the introduction of additional cDNA into these cells. However, we observed little difference (< 10 %) on co-transfection with plasmids encoding the various other constructs (glucagon receptor alone $= 665 \pm 17$; +PKC-$\alpha = 651 \pm 20$; +PKC-βII $= 671 \pm 15$; +PKC-$\epsilon = 641 \pm 12$; +GRK2 $= 661 \pm 18$; +GRK3 $= 655 \pm 26$ pmol of cAMP/10^6 cells in transfected COS-7 cells challenged for 6 min with 10 nM glucagon; $n = 3$). Thus the lower glucagon-stimulated cAMP accumulation seen in the cells that had been co-transfected with both the glucagon receptor and PKD may reflect a small inhibitory effect of basal PKD activity on glucagon-stimulated cAMP production. Indeed, when COS-7 cells that had been co-transfected with plasmids encoding the glucagon receptor and PKD were challenged with PMA, we observed a profound inhibition of glucagon-stimulated cAMP production (Table 1; Figure 4). This effect appeared to be directed at glucagon-stimulated adenylate cyclase activity, as analyses performed on homogenate cAMP PDE activity from cells transfected with plasmids encoding both the glucagon receptor and PKD showed no change in total PDE activity on challenge with PMA (< 5 % change; 9.2 ± 0.3 and 8.8 ± 0.5 pmol of cAMP hydrolysed/min per mg of protein for untreated and PMA-treated cells; $n = 3$). In addition, IBMX was able to inhibit a similar fraction of the total PDE activity in both treated and PMA-treated cells, namely 95.5 ± 2.5 and 96.4 ± 1.8 % respectively (means; $n = 3$). For co-transfection with PKD to confer PMA-mediated inhibition of glucagon-stimulated cAMP accumulation, an active form of PKD was needed. This was demonstrated in co-transfection studies carried out with the glucagon receptor and a kinase-inactive form of PKD, PKDK618M [23], where

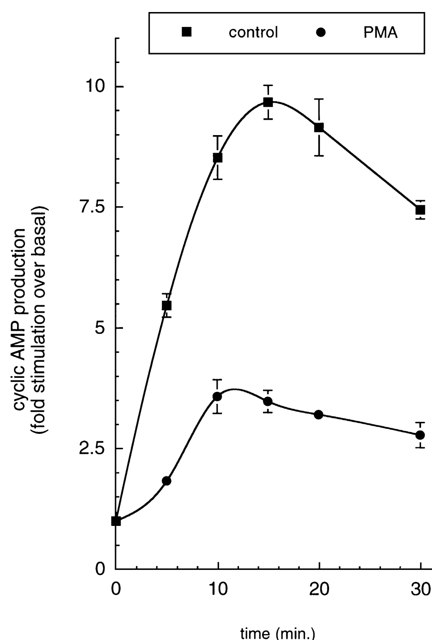

Figure 4 PMA-mediated attenuation of glucagon-stimulated cAMP accumulation in COS cells co-transfected so as to express both the glucagon receptor and PKD

After a preincubation for 15 min in either the presence (●) or absence (■) of PMA (1 μM), glucagon was added (10 nM). Incubations were terminated after the times indicated by the addition of 2% $HClO_4$. IBMX was present throughout (1 mM). The values shown are expressed as fold stimulation in cAMP accumulation over basal values and represent means ± S.D. from three separate transfection experiments.

Table 2 Effect of PMA treatment on forskolin-stimulated cAMP accumulation in control and PKD-transfected COS cells

Control or PKD-transfected COS cells, grown to confluence in six-well plates as described in the Materials and methods section, were preincubated for 15 min at 37 °C with serum-free DMEM containing 1 mM IBMX and either 0.1% DMSO (control) or 1 μM PMA. Forskolin, where indicated, was then added to a final concentration of 10 μM, and the incubation were continued for 20 min. $HClO_4$ (2%) was then added, the cells were harvested, and their cAMP contents determined. The intracellular cAMP levels under basal conditions for PKD-transfected cells were 70 ± 12 pmol/10^6 cells with control medium and 69 ± 18 pmol/10^6 cells in the presence of PMA. For untransfected cells the cAMP levels were 85 ± 16 pmol/10^6 cells and 81 ± 12 pmol/10^6 cells respectively. The results shown represent means ± S.D. from three experiments, expressed as fold stimulation over basal values.

	Control (fold change over basal)	PMA-treated (fold change over basal)
Control cells + forskolin	15 ± 3	17 ± 2
PKD-transfected cells + forskolin	14 ± 3	14 ± 3

PMA now failed to exert an inhibitory action on glucagon-stimulated cAMP accumulation in these cells (Table 1).

It is possible that the PMA-induced attenuation of glucagon-stimulated cAMP accumulation seen in these co-transfected cells was due to an effect on the catalytic unit of adenylate cyclase itself. However, using the diterpene forskolin, which activates the catalytic unit of adenylate cyclase directly [53], we were also able to show (Table 2) that transfection of COS cells with PKD did not cause PMA to inhibit forskolin-stimulated cAMP accumulation. Thus the inhibitory effect exerted by PMA on glucagon stimulation in PKD co-transfected cells cannot be due to any attenuation of the functioning of the adenylate cyclase catalytic unit.

Conclusion

Transfection of COS cells with the rat glucagon receptor has demonstrated that the PMA-mediated inhibition of glucagon-stimulated adenylate cyclase activity seen in hepatocytes is not an inherent property of the receptor itself. Rather another component is required. Considerable evidence, utilizing a variety of different approaches in analyses performed on hepatocytes, has indicated that a diacylglycerol/phorbol-ester-stimulated protein kinase mediates such an action [9–14,16]. On the basis that the first enzyme species identified that possessed such a property were PKC species, we have until now [16] considered that a member(s) of this PKC family would mediate such an action. It was thus a considerable surprise to us to find that PMA was unable to inhibit glucagon-stimulated cAMP accumulation in COS cells transfected so as to overexpress glucagon receptors, particularly when we were unable to reconstitute PMA-mediated inhibition by co-transfection with various PKC isoforms. It is thus highly intriguing that PKD, a novel diacylglycerol/phorbol-ester-stimulated protein kinase [17,18], of unknown role and which is expressed in hepatocytes but not in COS cells (Figure 3c), appears able to reconstitute in COS-7 cells the PMA-mediated inhibition of glucagon-receptor-stimulated cAMP accumulation (Figure 4, Table 2). The mechanism whereby inhibition is elicited remains to be ascertained. However, our studies indicate that it does not inhibit the functioning of the catalytic unit of adenylate cyclase but prevents its activation by glucagon, as has previously been observed by us in hepatocytes [9,10]. The interesting suggestion has been made that PKD may form part of a kinase cascade with PKC, where activation of PKC serves to stimulate PKD activity [23]. Thus at least part of the action of PMA in stimulating PKD may be exerted indirectly through PKC activation. Indeed, such an effect has been shown to occur in COS cells [23].

COS-7 cells express, albeit at much lower levels, many of the PKC isoforms found in hepatocytes [31]. However, overexpression of a range of these PKC isoforms in COS-7 cells transfected to express the glucagon receptor singularly failed to confer PMA-mediated inhibition on glucagon-stimulated cAMP production. In contrast with this, hepatocytes but not COS-7 cells express PKD, the co-transfection of which with the glucagon receptor into COS-7 cells confers PMA-mediated inhibition on glucagon-stimulated cAMP accumulation. It remains to be determined which PMA- and diacylglycerol-stimulated protein kinase serves to regulate glucagon-stimulated adenylate cyclase activity in native hepatocytes. The present study implies that PKD may be a reasonable candidate.

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