Induction of Ca^{2+} /calmodulin-stimulated cyclic AMP phosphodiesterase (PDE1) activity in Chinese hamster ovary cells (CHO) by phorbol 12-myristate 13-acetate and by the selective overexpression of protein kinase C isoforms

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The cAMP phosphodiesterase (PDE) activity of CHO cells was unaffected by the addition of Ca^{2+} +calmodulin (CaM), indicating the absence of any PDE1 (Ca^{2+} /CaM-stimulated PDE) activity. Treatment with the tumour promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) led to the rapid transient induction of PDE1 activity which attained a maximum value after about 13 h before slowly decreasing. Such induction was attenuated by actinomycin D. PCR primers were designed to hybridize with two regions identified as being characteristic of PDE1 forms found in various species and predicted to amplify a 601 bp fragment. RT-PCR using degenerate primers allowed an approx. 600 bp fragment to be amplified from RNA preparations of rat brain but not from CHO cells unless they had been treated with PMA. CHO cells transfected to overexpress protein kinase C (PKC)- α and PKC- ϵ , but not those transfected to overexpress PKC- β I or PKC- γ , exhibited a twofold higher PDE activity. They also expressed a PDE1 activity, with Ca^{2+} /CaM effecting a 1.8–2.8-fold increase in total PDE activity. RT-PCR, with PDE1-

specific primers, identified an approx. 600 bp product in CHO cells transfected to overexpress PKC- α and PKC- ϵ , but not in those overexpressing PKC- β I or PKC- γ . Treatment of PKC- α transfected cells with PMA caused a rapid, albeit transient, increase in PDE1 activity, which reached a maximum some 1 h after PMA challenge, before returning to resting levels some 2 h later. The residual isobutylmethylxanthine (IBMX)-insensitive PDE activity was dramatically reduced (approx. 4-fold) in the PKC- γ transfectants, suggesting that the activity of the cyclic AMP-specific IBMX-insensitive PDE7 activity was selectively reduced by overexpression of this particular PKC isoform. These data identify a novel point of ‘cross-talk’ between the lipid and cyclic AMP signalling systems where the action of specific PKC isoforms is shown to cause the induction of Ca^{2+} /CaM-stimulated PDE (PDE1) activity. It is suggested that this protein kinase C-mediated process might involve regulation of PDE1 gene expression by the AP-1 (*fos/jun*) system.

INTRODUCTION

The cyclic AMP (cAMP) and lipid signalling pathways provide major routes for controlling cellular function [1–3]. Over the past few years it has become well established that a complex interplay between these systems can modulate the functioning of such regulatory pathways, thereby initiating the concept of ‘cross-talk’ [4,5]. A key element involved in the regulation of cAMP signalling has been identified as protein kinase C (PKC) [4].

PKC activity is a multigene family composed of a number of distinct isoforms [6–8] which are grouped on the basis of their distinct structural and biochemical properties. Thus ‘conventional’ PKCs (α , β I, β II and γ) are sensitive to Ca^{2+} and diacylglycerol and ‘non-conventional’ PKCs (ϵ , δ , θ and η) are Ca^{2+} -insensitive. There are also atypical PKCs (ζ , μ , λ and ι), which are structurally related to the PKC family but have activities that are not regulated by either diacylglycerol or phorbol esters [9]. There is evidence suggesting that certain PKC isoforms may have particular functional roles, which is consistent with them exhibiting distinct biochemical characteristics and tissue distributions [6–11].

The action of PKC has been shown to regulate various aspects of the AMP signalling pathway [4]. Such effects appear to show distinct cell specificity, a fact that may relate to the occurrence of multiple forms of the enzymes involved. As regards the control of cAMP synthesis, modulation by PKC activity can affect, for example, the functioning of stimulatory and inhibitory receptors [4], the inhibitory G-protein G_{i2} [4,5,12,13] and adenylate cyclase itself [4,14–16]. An example of selectivity is the selective activation of a specific isoform of adenylate cyclase, namely type V, by both PKC- ζ and PKC- α [15]. In addition, the ability of PKC to attenuate the action of G_i appears to show distinct cell specificity [4,5,12,13].

The only known mechanism of inactivation of cAMP is its hydrolysis to 5'-AMP which is achieved by cAMP phosphodiesterase (PDE) activity [17–22]. It is becoming increasingly apparent that the control of cAMP degradation within cells provides a major regulatory system [18–20]. Diversity within the PDE enzyme family arises from both the presence of multiple genes and multiple splicing [21,23–25]. This yields a collection of enzymes that exhibit distinct biochemical characteristics and show selective expression in various cells [18,26–28]. These

Abbreviations used: CHO cells, Chinese hamster ovary cells; cAMP, cyclic AMP; PDE, cyclic AMP phosphodiesterase; PDE1, Ca^{2+} /calmodulin-stimulated cyclic AMP phosphodiesterase activity, also known as type-I PDE activity; CaM, calmodulin; PKC, protein kinase C; IBMX, isobutylmethylxanthine; rolipram, 4,3-(cyclopentoxyl)-4-methoxyphenylpyrrolidone; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine (also known as MEP-1); PMA, phorbol 12-myristate 13-acetate; cilostimide, 4,5-dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-5-methyl-3(2*H*)-pyrazone; G_i , inhibitory G-protein controlling adenylate cyclase activity; RT-PCR, reverse transcription PCR.

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enzymes have been divided into a number of functional classes [17]: PDE1 species with activities that are stimulated by Ca^{2+} and calmodulin (CaM); PDE2 species with activities that are stimulated by micromolar concentrations of cGMP; PDE3 species with activities that are inhibited by low (micromolar) concentrations of cGMP; PDE4 species which specifically hydrolyse cAMP in a cGMP-insensitive manner but are inhibited by the selective inhibitor rolipram. There is also a little-investigated PDE7 species which specifically hydrolyses cAMP in a manner that is insensitive not only to cGMP but also to the PDE4 selective inhibitor, rolipram, and the non-selective PDE inhibitor, isobutylmethylxanthine (IBMX) [29–31]. Lipid signalling pathways are considered to exert their prime action on cAMP degradation via the stimulation of PDE1 activity under conditions where Ca^{2+} levels are elevated [17]. However, there is evidence that suggests that the action of PKC may also regulate the degradation of cAMP in certain cell types [4,32].

Here we show that treatment of Chinese hamster ovary (CHO) cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) causes a rapid induction of novel PDE1 activity and the appearance of transcripts as detected by reverse transcription (RT)-PCR. The expression of this Ca^{2+} /CaM-stimulated PDE isoform is evident in CHO cells that have been transfected to overexpress PKC- α and PKC- ϵ isoforms, but not PKC- β I and PKC- γ isoforms.

EXPERIMENTAL

Chemicals

CHO cells that had been transfected [33] to overexpress specific PKC forms α , β I, ϵ or γ were kindly provided by Dr. Richard Roth, UCSF, San Francisco, CA, U.S.A. [^3H]cAMP was from Amersham International (Amersham, Bucks., U.K.). Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Tris, benzamidine hydrochloride, PMSF, aprotinin, pepstatin A, antipain, EDTA, cAMP, cGMP, Dowex 1 (X8-400; Cl^- form; 200–400 mesh), IBMX, snake venom (*Ophiophagus hannah*), bovine brain CaM, NaF and BSA were from Sigma Chemical Co. (Poole, Dorset, U.K.). CaCl_2 was from BDH (Glasgow, Scotland, U.K.), and all other biochemicals were from Fisons (Loughborough, Leic., U.K.). All cell culture reagents were from Gibco-BRL (Paisley, Scotland, U.K.) except hygromycin B which was from Boehringer (U.K.) Ltd. (Lewes, Sussex, U.K.). Okadaic acid came from Moana Bioproducts (Honolulu, HI, U.S.A.). Rolipram was a gift from Roche, Basle, Switzerland and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) a gift from Dr. T. Podzuweit, W. G. Kerckhoff Institute, Bad Nauheim, Germany. Tri Reagent was obtained from Molecular Research Center, Inc. First-strand cDNA kit was supplied by Pharmacia. Taq DNA polymerase was from Promega. Taq Start Antibody came from Clontech Laboratories, Palo Alto, CA, U.S.A. Perfect Match PCR additive was supplied by Stratagene, La Jolla, CA, U.S.A. Gene Jockey II was from BIOSOFT, Cambridge, U.K.

Cell culture

CHO cells were routinely grown in Ham's F-12 medium supplemented with 10% foetal calf serum [33]. PKC transfectants were selected with 400 $\mu\text{g}/\text{ml}$ G418 (Geneticin sulphate) and 150 $\mu\text{g}/\text{ml}$ hygromycin [33]. Cells were cultured in 75 cm^2 flasks at 37 °C in an atmosphere of 95% air/5% CO_2 in a humidified incubator. Confluent cells were harvested in homogenization buffer containing 10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 10 mM NaF, 30 mM sodium pyrophosphate, together with a

protease inhibitor cocktail formed of 0.1 mM PMSF, 2 mM benzamidine, 2 mM aprotinin, 2 mM pepstatin A, 2 mM leupeptin and 2 mM antipain. Okadaic acid (100 nM) was also present. After freeze-thawing, cells were homogenized by 25–30 up/down strokes in a Dounce homogenizer and used at a final protein concentration of approx. 2 mg/ml.

Protein determination

Protein content was estimated by the method of Lowry et al. [34] as modified by Peterson [35], with BSA as standard.

PDE assay

PDE activity was determined with 1 μM cAMP as substrate using a modification of the two-step procedure of Thompson and Appleman [36] and Rutter et al. [37] as described previously by Marchmont and Houslay [38]. To obviate the possibility that Ca^{2+} arising from cell homogenates might elicit full activation of any PDE1 activity without added exogenous Ca^{2+} , we also performed assays in the presence of EGTA (1 mM/2 mM) to determine whether the subsequent addition of Ca^{2+} then caused stimulation. Thus, in some instances, assays for PDE1 activity were performed in the presence of either 2.0 mM EGTA plus 2.1 mM Ca^{2+} and 10 units of CaM or with 1 mM EGTA plus 5 mM Ca^{2+} and 10 units of CaM. In no instance did we observe any difference between analyses performed in this fashion and those in which Ca^{2+} was added without EGTA. All assays were conducted at 30 °C and in all cases a freshly prepared slurry of Dowex/water/ethanol (1:1:1, by vol.) was used to determine activity. PDE inhibitors were dissolved in 100% DMSO as 10 mM stock solutions and diluted in 20 mM Tris/HCl, pH 7.4 for use in the assay. The residual levels of DMSO were shown not to affect PDE activity at the concentrations used in this study. PDE activities were determined from linear time courses.

Immunoblotting of PKC isoforms

This was performed using anti-peptide antisera generated and described previously [39] using methods described therein. They were raised against peptides corresponding to unique sequences within the different isoenzymes α , β I, γ and ϵ . Their specificity has been reported on previously [39] and is confirmed here. The antiserum used for PKC- α was based on the peptide VISPS-EDRRQPSC, PKC- β I (β 2-) with antiserum GUCSG-647 β I based on the peptide (C)-RDKRDTSNFDKEFT, PKC- γ , with antiserum GUCSG-1726 γ based on the peptide (C)-NYPLEL-YERVRTG and PKC- ϵ , with antiserum GUCSG-637 ϵ based on the peptide (C)-NQEEFKGFSYFGEDLMP. The cysteine group (C) appearing at the N-terminus of some of these peptides is not found in these PKC sequences but was used for linkage to the carrier for antiserum production.

RT-PCR

CHO cells were incubated as described above under the conditions indicated in the text before medium removal. Tri Reagent was then added to each flask (1 ml/80 cm^2 flask) and the cells were lysed by scraping. Total RNA was extracted from the recovered lysate using a modification of the protocol originally described by Chomczynski and Sacchi [40]. Total RNA was also extracted from rat brain using the same methodology [40].

Preparation of first-strand cDNA was performed as described in the instruction manual supplied with the Pharmacia First strand kit using a total reaction volume of 33 μl . Briefly, 5 μg of

RNA, as determined by its absorbance at 260 nm, was denatured at 65 °C for 10 min and then incubated with murine reverse transcriptase, 6 mM dithiothreitol, dNTPs and 0.2 µg of the supplied poly(dT) primer [*NotI*-d(T)₁₈] for 60 min at 37 °C.

Degenerate primers [GR18 (5'-RYCTYATCARCCGYTTY-AAGATTCC-3') and GR19 (5'-RAAYTCYTCCATKAGGCG-CWTGG-3')] were designed (see below) to amplify a 601 bp fragment identified in all known PDE1 species. In these primers R = A/G, Y = C/A, K = G/T and W = A/T. Amplification was performed in 1× PCR buffer (50 mM KCl, 20 mM Tris/HCl) containing 200 µM each dNTP, 1.5 µM each primer, 1.5 mM MgCl₂ and 5 units of *Taq* DNA polymerase which had first been mixed with *Taq* Start antibody for Hot-Start PCR according to the manufacturer's instructions. Then 0.5 units of the PCR additive Perfect Match was added to give a total volume of 50 µl. Mineral oil (100 µl) was overlaid to prevent evaporation. The amplification protocol consisted of 40 cycles of denaturation for 60 s at 94 °C annealing for 80 s at 50 °C and extension for 70 s at 72 °C. A 9 µl aliquot from each reaction mixture was resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

RESULTS

Induction of PDE1 activity in CHO cells by PMA

Homogenates of wild-type CHO cells (CHO-k cells) exhibited PDE1 activity (Table 1) which was insensitive to activation by the addition of Ca²⁺/CaM (50 µM; 20 ng/ml). This indicates the absence of any PDE1 activity in these cells. There is a possibility that sufficient endogenous Ca²⁺ might be carried over into the assay mixtures from the cell homogenates to stimulate any PDE1 enzyme in full. To obviate this, we added 1 mM (or 2 mM) EGTA to the assay mixtures and then determined PDE activity in both the presence and absence of 5 mM (or 2.1 mM) Ca²⁺/CaM (see the Experimental Section); no activation was elicited by the addition of Ca²⁺/CaM (< 4% change; results not shown). This was also true for a CHO cell line (CHO-t cells) that had been transfected with a plasmid, in this instance one allowing for the expression of the human insulin receptor [33]. It behaved identically with the wild-type cells (CHO-k), having a similar

total PDE activity that was insensitive to stimulation by Ca²⁺/CaM (50 µM; 20 ng/ml) (Table 1).

When CHO-k cells were challenged for 6 h with the PKC activator, PMA (10 µM), PDE1 activity was clearly apparent, the addition of Ca²⁺/CaM causing a 2.2–2.7-fold increase (range; *n* = 3) in total cellular PDE activity. This was examined in more detail in CHO-t cells in which treatment with PMA similarly led to the induction of novel PDE1 activity (Figure 1, Tables 1 and 2). Such PDE1 activity was apparent within 25 min of exposure to PMA. Stimulated activity then continued to rise for a number of hours before it began to decrease. Thus the induction of PDE1 appeared to be transient (Figure 1). This induction of PDE1 activity could be attenuated by the addition of actinomycin D (Table 2).

Detection of transcripts for PDE1

Antisera able to detect all forms of PDE1 in a species-independent fashion are not available, nor is the sequence of Chinese hamster PDE1 known. This precludes analysis of the enzyme by Western or Northern blotting. To create a probe for CHO cell PDE1 we exploited knowledge of the sequences of PDE1 forms from various mammalian sources [41–43] to try to identify stretches of sequence that would be specific for PDE1 and conserved in a species-independent fashion. We performed a multiple-gap alignment of either the complete or partial nucleic acid sequences of all the mammalian PDE1 forms listed [41–43] in GenBank [44]. As might be expected, this revealed substantial homology across the whole reading frame (52% identity among four complete sequences). However, within a central 1252 bp stretch of sequence, which is known to encompass coding sequence for the catalytic unit of the enzyme [45], identified as nucleotides 439–1691 in mouse, there were three stretches of sequence (Figure 2; regions A, B and C) that showed high homology between the PDE1 species from various sources but not with other PDE forms. These were, with numbering shown for mouse PDE1 and identity gauged from pairwise alignments: nucleotides 669–693 (region A), showing 77–96% identity, nucleotides 1243–1261 (region B), showing 88–100% identity and nucleotides 1330–1369 (region C), showing 83–98% identity. De-

Table 1 Assessment of PDE1 activities in CHO cells that overexpress specific forms of PKC

Data are given as means ± S.D. of separate experiments employing different cell preparations with the number of experiments given in parentheses. In each experiment triplicate PDE assays were performed and a mean value taken. PDE activity in homogenates is expressed as pmol of cAMP hydrolysed/min per mg of protein. Assays were performed in the presence of 1 µM cAMP. PDE1 activity was assessed by determining the effect of adding Ca²⁺ (50 µM) and CaM (10 units). This led to either no change in activity (none; < 5%) or an increase, which is expressed as the net value by which PDE activity was elevated over that in the absence of Ca²⁺/CaM. A fold stimulation of total homogenate PDE activity by Ca²⁺/CaM is given where appropriate (n/a, not appropriate). Inhibitor concentrations were 10 µM rolipram, 10 µM cilostimide, 10 µM cGMP and 100 µM IBMX. The inhibition data are expressed as the value by which PDE activity was decreased by the selective inhibitor. Thus inhibition by rolipram approximates the PDE4 activity and that by cilostimide the PDE3 activity. No significant (< 4%) change in activity was seen using EHNA (10 µM) in either the absence or presence of cGMP (10 µM), indicating the absence of PDE2 activity. On this basis the inhibition seen in assays carried out using low concentrations of added cGMP (10 µM) are likely to approximate the PDE3 activity. For IBMX, the residual uninhibited activity is shown along with data for the percentage age inhibition of total PDE activity achieved by this compound.

Cell type	Total PDE activity	Rolipram fraction	cGMP fraction	Cilostimide fraction	IBMX		Ca ²⁺ /CaM	
					Inhibition (%)	Uninhibited component	Increase	Fold stimulation
CHO-k	7.9 ± 0.5 (5)	4.9 ± 0.5 (5)	1.9 ± 0.8 (3)	1.9 ± 0.7 (3)	82	1.4 ± 0.2 (3)	None (6)	n/a
CHO-t	7.0 ± 0.2 (6)	4.0 ± 0.6 (5)	1.6 ± 0.3 (3)	1.9 ± 0.7 (3)	72	2.0 ± 0.1 (3)	None (6)	n/a
CHO-α	17.2 ± 2.5 (5)	4.2 ± 0.9 (6)	3.6 ± 0.8 (5)	3.7 ± 0.5 (5)	88	2.4 ± 0.3 (3)	18.9 ± 2.1 (4)	2.1
CHO-β1	8.1 ± 1.3 (3)	4.7 ± 0.8 (3)	2.6 ± 1.0 (3)	2.2 ± 1.6 (3)	81	1.6 ± 0.2 (3)	None (4)	n/a
CHO-ε	14.2 ± 0.9 (7)	3.9 ± 0.4 (7)	5.2 ± 1.1 (6)	2.5 ± 0.3 (3)	80	2.3 ± 0.3 (3)	10.8 ± 0.6 (4)	1.8
CHO-γ	6.0 ± 0.9 (5)	5.0 ± 0.5 (6)	2.4 ± 0.5 (3)	2.3 ± 0.5 (6)	90	0.5 ± 0.1 (3)	None (5)	n/a

Figure 1 PDE1 activity in CHO cells challenged with PMA

CHO cells were challenged for various periods of time with PMA (10 μ M) before harvesting, homogenization and assay of PDE activity as described in the Experimental section. The net effect of the addition of Ca^{2+} /CaM (50 μ M; 20 ng/ml) is shown, i.e. the Ca^{2+} /CaM-stimulated component as an index of PDE1 activity. (a) CHO-t cells; (b) CHO- α cells. Typical examples are shown of experiments performed three times using different cell preparations.

Table 2 Attenuation by actinomycin D of the PMA-mediated induction of PDE1 activity

Cells were treated with 10 μ M PMA in either the presence or absence of actinomycin D. In experiments using CHO-t cells and CHO- β I cells, PMA was present for 6 h whereas with CHO- α cells it was present for 1 h. Cells were then harvested and homogenized, and PDE activity was assayed in the presence and absence of Ca^{2+} /CaM. The increase in PDE activity achieved by Ca^{2+} /CaM is given in pmol of cAMP hydrolysed/min per mg of protein. Data are expressed as means $\pm$ S.D. of separate experiments using different cell preparations with the number of experiments given in parentheses. ne, No evident change in PDE activity ($< 4\%$) with added Ca^{2+} /CaM.

Procedure	CHO-t	CHO- α	CHO- β I
Control	ne (3)	15.5 $\pm$ 1.3 (3)	ne (3)
PMA only	9.8 $\pm$ 1.0 (3)	26.3 $\pm$ 1.5 (3)	11.3 $\pm$ 1.5 (3)
PMA + actinomycin D	3.9 $\pm$ 0.4 (3)	10.7 $\pm$ 0.9 (3)	1.3 $\pm$ 0.9 (3)

generate primers (Figure 2) were designed for the first two of these regions to try to amplify a detectable PDE1-specific fragment in a species-independent fashion. The sequences used

to form the primers were chosen so that degeneracy would be minimized and restricted mainly to the 5' end of the primer (see Figure 2). RT-PCR was performed first on rat brain RNA, as this is known to provide a good source of PDE1 [41–43]. We were thereby able to amplify a species of approx. 600 bp (Figure 3) which correlated well with the predicted size for the fragment (601 bp). In contrast, no such species could be amplified from either CHO-k or CHO-t cells unless they had been treated with PMA (6 h), whereupon a species of approx. 600 bp was clearly evident (Figure 3b).

Determination of PDE1 activity in cells transfected to overexpress various PKC isoforms

Overexpression of PKC in transfected CHO cells has previously been quantified on the basis of binding and activity studies [33]. Here, in order to ascertain that transfectants overexpressed the appropriate PKC forms and did not either alter or induce other PKC species, we used specific anti-peptide antisera [39] to detect various PKC forms (Figure 4). In CHO-t (Figure 4) and CHO-k (results not shown) cells, the presence of PKC- α was clearly demonstrated. However, we obtained no evidence to support the expression of the β I, ϵ and γ forms of PKC (Figure 4). Certainly, PKC- α appears to be found ubiquitously in mammalian cells [6–8] and its occurrence in CHO cells is consistent with the data of other investigators [33]. Analysis of PKC- α transfectants showed a marked increase in the expression of this isoform (Figure 4). Indeed, levels of PKC- α appeared to have increased some 4–7-fold (range; $n = 3$) over those found in CHO-t cells (Figure 1), which bears comparison with binding data [33]. In the various other transfectants, we were able to identify the expression of the appropriate PKC species (Figure 4). There was no apparent induction of any of the other isoforms analysed here nor any reduction in the expression of PKC- α in such transfectants (Figure 4).

Total homogenate PDE activity, determined in the presence of 1 μ M cAMP, was not significantly different between CHO-t cells and cells transfected to overexpress either the PKC- β I or PKC- γ isoforms (Table 1). However, PDE activity was markedly enhanced, by around twofold, in cells transfected to overexpress either PKC- α or PKC- ϵ (Table 1). Furthermore, whereas Ca^{2+} /CaM singularly failed to stimulate homogenate PDE activity in the PKC- β I and PKC- γ transfectants, we observed a profound stimulation of PDE activity in the PKC- α and PKC- ϵ transfectants (Table 1): 2.1- and 1.8-fold respectively. This suggests that these two isoforms of PKC are able to induce PDE1 activity selectively.

The apparent selectivity of the action of the different PKC isoforms might be accounted for by different levels of expression. However, induction of CHO cell PDE1 activity does not seem to be proportionally related to the increase in PKC levels found in these various transfected cell lines, for Chin et al. [33] have shown that the PKC- β I transfectants exhibited the highest levels of PKC expression, as determined by [^3H]phorbol dibutyrate binding, with 18-fold higher levels of PKC than those found in control CHO cells. However, we observed no induction of PDE1 activity in these cells. Chin et al. [33] also showed that [^3H]phorbol dibutyrate binding was 10-fold higher in PKC- ϵ transfectants than in CHO-t cells and 5-fold higher in cells transfected to overexpress either PKC- α or PKC- γ . Consistent with such large changes, our immunoblot analyses showed a dramatic signal for the respective PKC isoforms in the β I, ϵ - and γ -transfected cells where we were unable to detect any immunoreactivity indicative of such endogenous isoforms (Figure 4). Thus it would seem

Figure 2 Design of degenerate primers for RT-PCR detection of PDE1 transcripts

There is no published sequence for PDE1 from Chinese hamsters. Analysis of the complete and partial sequences of PDE1 enzymes in GenBank [44] from mouse, rat, human and bovine sources revealed substantial homology across a 1252 bp stretch of sequence (labelled 439–1691 in mouse) which is known to include coding sequence for the catalytic unit of this enzyme [45]. The majority of this sequence is also highly homologous to other PDE types (thin horizontal line). Within this region three stretches of sequence (hatched boxes) were found to be unique to PDE1 forms (numbering for mouse sequence). Degenerate primers were designed to anneal within two of these stretches such that they would be predicted to allow for the amplification of a 601 bp fragment from all known PDE1 forms. On this basis it would seem reasonable to predict that such primers might serve to amplify a fragment specific to PDE1 enzymes in a species-independent fashion and thus could be used to probe CHO cells for PDE1 transcripts. In the primers shown R = A/G, Y = C/A, K = G/T and W = A/T. The PDE1 forms analysed (with their GenBank [44] accession numbers in parentheses) were the bovine 61 kDa form (M90358), bovine 63 kDa form (M94867), mouse 63 kDa form (M94538), human 63 kDa form (M94539) and the rat 63 kDa form (M94537). These were compared against 15 rat and 11 mouse sequences of other PDE forms [2–5]. The accession numbers for the PDEs were: RPDE6 (L27057), RD1 (M26715), RD2 (M26717), RD3 (M26716), DPD (J04563), PDE4 (M25350), RPDE36 (L27061), PDE3.1 (M25349), PDE3.2 (U09456), PDE3.3 (U09457), RATCGSPDE (M94540), RAT CGI PDE (Z22867), RATNPHIII (D28560), RATCNPII (L16532), RATCNPF (M18630), MUSCAMPDE (M94541), MUSCNP2 (M58047), MUSCNP1 (M58046), MUSPDEB (M75166), MMPB (X60133; X57656), MMPA (X60664; X57656), MMPHDIEST (X69827), MUSCNP2 (M58045), MUSCNP4 (M31810), MMPDE (X55968), MMCGMPPD (Y00746), HUMCAMPPOS (L12052). Within this 1252 bp stretch are three regions (A, B and C) that show high homology between PDE1 enzymes from various sources but weak homology with other PDE subtypes. Region A showed 77–96% identity, region B 88–100% identity and region C 83–98% identity in pairwise alignments between different PDE1 forms. In contrast, alignment analysis of four PDE4 forms showed only about 3% identity for region A, 19% identity for region B and 49% identity for region C. Similar results were obtained for PDE2, PDE3 and PDE7 forms. We therefore focused on sequences within regions A and B for the design of probes aimed at selectively detecting PDE1 transcripts in a species-independent fashion. Although GR19 was not fully contained within the short stretch at 1243–1261, the majority of the primer was within this sequence, including the 3' end which is crucial for specificity.

probable that induction of PDE1 activity is related to the action of specific PKC isoforms rather than generic actions due to an overall increase in PKC expression.

Treatment with PMA of cells overexpressing PKC- α (Figure 1) led to a further increase in PDE1 activity. This appeared to increase at a faster rate than that seen in CHO-t cells (Figure 1b). However, as with CHO-t cells, the stimulation was transient with a maximum value attained about 1 h after challenge with PMA. This PMA-mediated increase in PDE1 activity was attenuated by actinomycin D (Table 2).

PMA was also able to induce PDE1 in the PKC- β I transfectants in a manner that was attenuated by actinomycin D (Table 2). Thus overexpression of PKC- β I, although it did not trigger PDE1 expression, did not affect the ability of PMA to induce this enzyme.

RT-PCR analysis for PDE1 in CHO cells overexpressing specific PKC isoforms

RT-PCR analysis identified an approx. 600 bp species in RNA from the PKC- α and PKC- ϵ transfectants but not from either the PKC- β I or the PKC- γ transfectants (Figure 3). However, PMA treatment of the PKC- β I transfectants led to the occurrence of this species (Figure 3).

Action of isoform-selective PDE inhibitors on CHO cell PDE activity

Compounds selective for effects on specific PDE isoforms [17–19, 22, 48] were used to identify and gauge the magnitude of the activity of various PDE isoforms in these various CHO cell lines. Thus PDE3 species (cGMP-inhibited PDE) can be selectively inhibited by low concentrations of cilostimide, exhibiting IC_{50} values of 0.1–1 μ M when assayed at 1 μ M cAMP [19,49] in contrast with other PDEs which are either insensitive to cilostimide or show IC_{50} values that are typically some 500-fold higher [19,48]. With assays performed at 1 μ M cAMP and 10 μ M cilostimide, which can be expected to inhibit all of the PDE3 activity, we have shown that PDE3 activity in CHO cells is slightly, but not significantly, increased in all the PKC transfectants (Table 1).

PDE4 species are cAMP-specific and can be inhibited selectively by rolipram [20–22,27,50–52]. They typically exhibit IC_{50} values of 0.1–1 μ M rolipram when assayed at 1 μ M cAMP, whereas other PDE species are either insensitive to rolipram or show IC_{50} values some 50–100 times higher [20,22,27,48]. Thus assays carried out in the presence of 10 μ M rolipram, with 1 μ M cAMP as substrate, can be expected to yield complete, or near complete, inactivation of PDE4 activity. Using this approach we

Figure 3 RT-PCR analysis of PDE1 transcripts in CHO cells

This was performed as described in the Experimental section using the degenerate primers designed to detect transcripts selectively for PDE1 forms in a species-independent fashion. The predicted position, from a marker ladder, of the 601 bp fragment is shown with an arrow. (a) Analysis of rat brain (Br), control blank (ctr), CHO-k cells (k), CHO-t cells (t), CHO-ε cells (ε), CHO-α cells (α), CHO-β1 cells (β1) and CHO-γ cells (γ). (b) Analysis of cells treated with PMA (10 μM) for 6 h before harvesting. Typical experiments performed at least three times using different cell preparations are shown.

Figure 4 Immunoblot analysis of CHO cells for PKC isoforms

Immunoblot analyses were carried out with antisera against the PKC isoforms as described previously [39]. In each case, samples of cell homogenate containing 100 μg of protein were analysed by SDS/PAGE and transferred to nitrocellulose, and Western blotting was performed with antisera specific for the indicated forms of PKC. Typical experiments performed three times are shown for CHO-t cells (t) and the PKC-overexpressing transfectants CHO-α cells (α), CHO-β1 (β), CHO-γ cells (γ) and CHO-ε cells (ε). For the CHO-α cells were observed a doublet. The upper band of 80 kDa reflects the specific identification of PKC-α. It is this species that was increased on transfection within PKC-α (track α) and could be displaced by treatment with a specific peptide [39] (results not shown). In track β an 80 kDa species appeared in PKC-β1-overexpressing cells only; in track γ a 78 kDa species appeared in PKC-γ-overexpressing cells only; in track ε an 85 kDa species appeared in PKC-ε-overexpressing cells only. These sizes are similar to those noted by others and which can be deduced from their sequences [7,46,47].

were able to show that PDE4 isoforms constitute a major fraction of the total PDE activity in CHO cells (Table 1). As the absolute amount of PDE activity inhibited by rolipram appeared to be remarkably similar for all the cell lines studied (Table 1), PDE4 activity appears to be unchanged by PKC overexpression.

PDE2 activity is characterized by its activation by low concentrations of cGMP [17–20,22,53]. Here, however, low (1 μM) concentrations of cGMP only inhibited the cAMP PDE activity (Table 1) in assays of homogenates of the various CHO cells. Furthermore, the magnitude of this inhibition was comparable with that observed when the PDE3-selective inhibitor, cilostimide, was used (Table 1). This suggests that cGMP was acting solely to inhibit a PDE3 activity and that little if any PDE2 activity is present in CHO cells. The compound EHNA [54] can act as a PDE2-selective inhibitor and we have been able to confirm this (A. Michie and M. D. Houslay, unpublished work). Consistent with our deduction that CHO cells lack PDE2, addition of EHNA (10 μM) to assays of cAMP hydrolysis in the presence of cGMP (10 μM) failed to elicit any inhibition (< 5% change; results not shown) of PDE activity in the various CHO cell lines.

PDE7 species specifically hydrolyse cAMP in a manner that is resistant to inhibition by the non-selective reversible PDE inhibitor, IBMX [29–31]. There is no satisfactory way of quantifying PDE7 activity. Nevertheless, one can reasonably calculate, on the basis of K_m (cAMP) and K_i (IBMX) values for various PDE species [18,45,48], that, in the absence of any PDE7 activity, CHO cell PDE activity can be predicted to be inhibited by about 90% when assayed in the presence of 100 μM IBMX and 1 μM cAMP. That a value considerably lower than this was observed (Table 1) might indicate the presence of PDE7 activity. The proportion by which the total PDE activity was inhibited by IBMX would not be expected to change between these various transfectants unless either the activity due to the PDE7 altered or there was a large change in the expression of another PDE component with kinetic constants very different from the average. Despite the dramatic increase in total PDE activity seen with the PKC-α and PKC-ε transfectants, the residual PDE activity under these conditions was remarkably similar to that found in the CHO-t cells (Table 1). This indicates that the increased PDE activity in these transfectants was not due to any increase in PDE7 activity. Intriguingly, however, the residual IBMX-insensitive PDE activity was dramatically reduced, by around 4-fold, in the PKC-γ-transfectants, suggesting that the level of PDE7 may well be reduced by overexpression of PKC-γ. Indeed, consistent with such a contention, we noted that the proportion of total PDE activity inhibited by IBMX in the PKC-γ transfectants was increased (Table 1).

DISCUSSION

There is now considerable evidence that identifies cross-talk between distinct signalling systems [4,5]. The family of isoforms that provides PDE activity in cells seems to be particularly well endowed in this regard, with many isoforms having properties that allow regulation by other signalling systems [17–22,54]. This, coupled with a breadth of adenylate cyclase isoforms having distinct properties that allow input from other signalling systems [14,58,59], indicates that the cAMP pathway may provide a key means of integrating the plethora of different signals to which any cell can be subjected. In particular, this appears to extend to the influence of lipid signalling systems where the activation of PKC and increases in intracellular Ca^{2+} have been shown to affect the functioning of various receptors (see ref. [4])

and specific adenylate cyclase isoforms [14,56,57] and can alter the degradation of cAMP by PDEs [4,32,45].

Here we show that the PMA-mediated activation of PKC in CHO cells elicits a profound increase in cAMP PDE activity. This takes the form of a specific induction of Ca^{2+} /CaM-stimulated PDE1 activity. Intriguingly, cAMP has been shown to be capable of both inhibiting and stimulating cell growth, depending on the type of cell analysed [58–60]. Undoubtedly these different effects are due to cell-specific expression of various regulatory proteins involved in controlling cAMP responses. In the case of CHO cells, elevated cAMP concentrations appear to inhibit cell growth [61,62]. As many agents that stimulate cell growth elicit activation of PKC [2,3,7], any ability to elicit the induction of PDE1 activity might be expected to facilitate their action. Furthermore, catabolism of cAMP might be expected to be amplified further as such agents often trigger the elevation of intracellular Ca^{2+} concentrations [2,3], which would activate the induced PDE1.

Our studies identify a profound selectivity of certain PKC isoforms to elicit the induction of PDE1 in CHO cells (Table 1). This action is mediated by the overexpression of either PKC- α or PKC- ϵ but not by PKC- β I or PKC- γ . Interestingly, such a functional grouping of PKC isoforms has also been noted in studies on RBL-2H3 cells, where PKC- α and PKC- ϵ effected inhibitory actions on phospholipase C activity, whereas PKC- β and PKC- γ mediated stimulatory actions on this enzyme [63]. In wild-type CHO cells, however, it is presumably the endogenous PKC- α that provides the means whereby PMA induces PDE1 activity. The rapid induction of PDE1 activity by PMA is reminiscent of the ability of phorbol esters to activate immediate early genes [64,65]. In this regard, studies on 3Y1 fibroblasts [66] have shown that overexpression of PKC- α , but not PKC- γ , leads to a profound, but transient, induction of *c-jun*. It may be that the gene for PDE1, like those for *c-jun* [62] and various other proteins [68,69] is controlled by a *cis*-acting PMA-responsive element to which the AP-1 transcription factor binds. Certainly, as shown here for PDE1 (Figure 1), PMA can, within 30 min of exposure, induce *c-fos* in CHO cells in a transient fashion [70]. The transient nature of the induction of PDE1 elicited by PMA might, however, also be due to the down-regulation of PKC activity. In this regard it has been shown [70] that 16 h of treatment of CHO-k cells with PMA can attenuate PKC activity by about 94%. We have confirmed this, with 16 h of treatment with PMA causing a 78–87% down-regulation (range; $n = 3$; immunoblot results not shown) of PKC- α in both CHO-k cells and the CHO- α transfectants. Nevertheless, when PKC- α was down-regulated in the CHO- α transfectants, its levels were still higher than those seen in the untreated CHO-k cells, suggesting that another mechanism underpins the transience of the induction of PDE1. It may also be influenced by an attenuating effect on PKC activity caused by either its translocation to a functionally inactive compartment or some covalent modification [6–8]. Whatever the mechanism may be, we can deduce from these studies that the turnover of PDE1 is relatively rapid in CHO cells.

Whether the induction of PDE1 activity mediated by specific PKC isoforms is a universal action or one that is specific to CHO cells remains to be seen. Nevertheless, it adds strength to the contention that PKC isoforms have distinct functional roles [71]. It also identifies a novel point of cross-talk between the lipid and cAMP signalling systems, namely the induction of PDE1 activity by a process that can be mediated by either PKC- α or PKC- ϵ . This may be determined by the control of PDE1 expression through the AP-1 (*fos/jun*) system. PDE1 activity may, however, also be influenced by other signalling pathways, as insulin and

epidermal growth factor receptors can, under certain conditions, elicit tyrosyl phosphorylation of calmodulin [72]. Our study may then have relevance in disease states in which immediate early genes are activated and also in diseases such as diabetes in which selective increases in the expression of PKC isoforms have been noted to occur [39,73].

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