Protein kinase C in cultured human placental trophoblasts: identification of isoforms and role in cAMP signalling

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The protein kinase C (PKC) superfamily is a family of serine/threonine kinases involved in the regulation of many cell functions. The objective of the present work was to identify the PKC isoenzymes present in human placental trophoblasts and to compare their relative responses to acute and chronic phorbol 12-myristate 13-acetate (PMA) treatment. In addition, the effect of PMA treatment on ligand-stimulated cAMP production was determined. In total extracts prepared from cultured or freshly purified trophoblasts, PKC isoforms α , ϵ and ζ were detected. Following acute treatment with PMA, PKC α and PKC ϵ were translocated from the cytosol to the particulate fraction. Prolonged treatment (up to 36 h) with PMA resulted in the temporal down-regulation of PKC α and PKC ϵ . PKC ζ did not respond to either acute or chronic treatment with PMA. An acute 10 min

treatment of the cells with PMA (10^{-10} – 10^{-6} M) enhanced isoprenaline- and adrenaline-stimulated cAMP production. An enhanced response was observed at all concentrations of isoprenaline tested (10^{-9} – 10^{-4} M), suggesting an increased capacity to respond. The acute PMA effect was evident within 2 min and near maximal by 5 min. The acute response to PMA was lost in cells where PKC was down-regulated by prior PMA treatment. Epidermal growth factor, a potential ligand for the activation of PKC in trophoblasts, enhanced isoprenaline-stimulated cAMP production. In summary, activation of PMA-responsive PKCs (PKC α or PKC ϵ) appears to enhance ligand-stimulated cAMP production in trophoblasts. This may be a physiologically important example of ‘cross-talk’ between various signalling pathways in human placental trophoblasts.

INTRODUCTION

The protein kinase C (PKC) family of serine/threonine kinases is thought to play a critical role in the regulation of cellular differentiation and proliferation in response to diverse stimuli [1,2]. PKC is comprised of several isoforms, which have been divided into three subfamilies based upon domain structure. The classical or conventional PKCs (cPKCs; isoforms α , β_1 , β_2 and γ) contain the putative Ca^{2+} -binding region C-2 and are Ca^{2+} -responsive [3]. The novel or new PKCs (nPKCs; δ , ϵ , η and θ or L) lack the Ca^{2+} -binding region [4–6]. The atypical PKCs (aPKCs; ζ and λ or ι) lack the Ca^{2+} -binding region and have only one cysteine-rich zinc-finger-like motif in the C-1 region [3,7–9]. In addition, recently described PKC μ [10] and protein kinase D [11] appear to share a high degree of identity and may constitute a separate subfamily. Activation of these enzymes can vary with the subfamily. The activators include several lipids such as *sn*-1,2-diacylglycerol, a second messenger [12] which activates cPKCs and nPKCs but not aPKC isoforms. Similarly, phorbol esters and other tumour promoters mimic natural activators of cPKCs and nPKCs, but not of aPKCs [13]. Moreover, activation of PKC may influence other signalling pathways, including cAMP production. Modification of cAMP production by PKC may involve direct action on adenylate cyclase [14,15] or on G-proteins [16,17].

PKCs have been described in many tissues, and the isoforms can vary with the tissue. The human placenta contains PKC [18] but the isoform pattern has not been characterized. The placenta is a multifunctional organ, critical to the maintenance of preg-

nancy and normal development of the fetus. In the human placenta, trophoblasts are the key cell type responsible for carrying out many of the functions normally attributed to the placenta, including production of various pregnancy hormones and transport of essential nutrients to the fetus. These functions are under the regulation of second messengers including cAMP [19]. Therefore modification of cAMP production by activation of PKC may affect trophoblast function.

The objective of the present work was to identify the PKC isoenzymes present in human placental trophoblasts and to compare the relative responses to acute or chronic phorbol 12-myristate 13-acetate (PMA) treatment. In addition, the effect of PMA treatment on ligand-stimulated cAMP production was determined.

MATERIALS AND METHODS

Materials

Anti-peptide antisera to the predicted unique sequences in the V_3 region of PKC isoforms α (AGNKVISPSEDRRQ), β (GPKTPEEKTANTISKFD), δ (SFVNPKEYEQFLE), ϵ (KGFSYFGEDLMP) and ζ (GFEYINPLLLSAEESV) were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). In addition, an anti-peptide antibody against a unique V_3 -region PKC α peptide (GNKVISPSEDRRQ) was obtained from Dr. Marie W. Wooten (Auburn University, Auburn, AL, U.S.A.), and an anti-peptide antibody against a unique V_3 -region PKC β peptide (GPKTPEEKTANTISKFD) was obtained from Dr. D. Kirk Ways (East Carolina University School of Medicine,

Abbreviations used: PKC, protein kinase C; cPKC, classical or conventional PKC; nPKC, novel or new PKC; aPKC, atypical PKC; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; IBMX, 3-isobutyl-1-methylxanthine; PVDF, poly(vinylidene difluoride).

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Greenville, NC, U.S.A.). Peroxidase-conjugated goat anti-rabbit IgG antibody was from Bio-Rad Laboratories (Melville, NY, U.S.A.). Renaissance chemiluminescence detection system was from DuPont–New England Nuclear Research Products (Boston, MA, U.S.A.). Rolipram®, a potent phosphodiesterase inhibitor, was provided by Dr. M. E. Sullivan (Berlex Laboratories, Cedar Knolls, NJ, U.S.A.). Assay kits were obtained as follows: cAMP (KAPH2) from either Diagnostic Products (Los Angeles, CA, U.S.A.) or Amersham (RPA509; Arlington Heights, IL, U.S.A.); human chorionic gonadotropin from Ciba–Corning Diagnostics (MAIA Clone, Serono; Medfield, MA, U.S.A.); epidermal growth factor (EGF) from Bachem Bioscience Inc. (King of Prussia, PA, U.S.A.). Leupeptin and aprotinin were from Calbiochem (La Jolla, CA, U.S.A.); Percoll®, adrenaline (epinephrine), isoprenaline (isoproterenol), PMA, 3-isobutyl-1-methylxanthine (IBMX) and routine chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Trophoblast culture

Purified cytotrophoblasts were diluted in Dulbecco's modified Eagle's medium containing 4 mM glutamine, 50 µg/ml gentamicin and 20% (v/v) heat-inactivated fetal calf serum. The cells were plated at a density of 1.5×10^5 cells/cm² into Falcon Primaria® culture dishes (Becton Dickinson, Lincoln Park, NJ, U.S.A.) and incubated in humidified 5% CO₂ at 37 °C. The medium was changed every 24 h. At the time of plating, all cells were mononuclear and relatively uniform in size. Previous studies had shown that the initial purification methods yield approx. 95% pure trophoblasts [20–22]. For experiments to examine PKC by Western blotting, cells were further purified by using anti-HLA A,B,C (Class I) and anti-HLA DR, DQ (Class II) antibodies attached to Dynabeads® (Dynal, Great Neck, NY, U.S.A.) to remove non-epithelial cells [23] by magnetic separation. Inclusion of this additional purification step completely eliminated non-trophoblasts, as determined by cytokeratin and vimentin immunocytochemistry. Upon inoculation, trophoblasts attached and spread within 12 h and later differentiated into multinucleated syncytiotrophoblast-like cells. Within the first 24 h period, measurable amounts of human chorionic gonadotropin appeared in the culture media. This was accompanied by uniform immunocytochemical detection of cytoplasmic human chorionic gonadotropin in all attached cells.

PMA treatment

To determine the effect of acute treatment with PMA on translocation of PKC, cultured trophoblasts were treated for 0 (control) or 10 min with 100 nM PMA dissolved in DMSO (0.025% final concn.). To determine the effect of long-term treatment with PMA (potential down-regulation of PKC), trophoblasts were treated with 1 µM PMA for up to 36 h.

Preparation of cell extracts for Western blot analysis

For extraction, either freshly purified or cultured cells were washed twice with cold PBS (pH 7.4). Whole-cell extracts were prepared by scraping cells into ice-cold lysis buffer (25 mM Tris/HCl, pH 7.4, 2.5 mM magnesium acetate, 2 mM dithiothreitol, 50 mM 2-mercaptoethanol, 50 µg/ml aprotinin, 50 µg/ml leupeptin) [24]. Following determination of protein, samples were either diluted with Laemmli sample buffer or concentrated using the deoxycholate/trichloroacetic acid precipitation method [25] prior to solubilization in Laemmli sample buffer [26]. Samples were boiled for 4 min and stored at –80 °C until subjected to SDS/PAGE. To check for any degradation of

PKCs during the extraction procedure, both freshly isolated and cultured trophoblasts were harvested by the rapid denaturation method, which solubilized cells in boiling Laemmli sample buffer [27].

To determine translocation or distribution of PKC, particulate and cytosol fractions were prepared. Cultured trophoblasts were treated with PMA and, upon termination of the experiment, cells were placed on ice, scraped into homogenization buffer (10 mM Tris/HCl, pH 7.4, 250 mM sucrose, 5 mM MgCl₂, 50 µg/ml aprotinin, 50 µg/ml leupeptin) (modified from [28,29]) and homogenized in a Dounce homogenizer with a tight-fitting pestle. Unlysed cells and nuclei were sedimented at 200 g for 3 min at 4 °C. The post-nuclear fraction was centrifuged at 100 000 g for 20 min at 4 °C. The pellet (particulate fraction) was resuspended in homogenization buffer, and the protein concentrations in the cytosol (supernatant) and particulate fractions were determined using a modified Lowry assay [30]. Samples were concentrated using the deoxycholate/trichloroacetic acid precipitation method and reconstituted in Laemmli sample buffer to yield 2 or 4 mg/ml protein.

Immunoblot analysis

To determine the presence of various PKC isoforms, 1.5 mg protein extracts from rat brain or trophoblasts (freshly purified or cultured for 3 days) were separated by SDS/PAGE (9% gel) using a Mini-Protein II system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Separated protein samples were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Polyscreen; DuPont–New England Nuclear) at 70 °C [31] in Towbin transfer buffer and the membrane was placed in a multichannel miniblitter (Immunetics, Cambridge, MA, U.S.A.) for incubation with PKC-isoform-specific primary antibodies. Anti-peptide antibodies were diluted to obtain optimal saturating concentrations (as determined in preliminary studies) and incubated with the membrane for 16 h at 4 °C. When used, antigen-adsorbed antibodies were prepared by preincubation with optimal concentrations of specific peptides. The blots were washed and then incubated with an optimal concentration (determined for each lot) of horseradish peroxidase-conjugated antibodies for detection of immunoreactive bands by chemiluminescence (Renaissance; DuPont–New England Nuclear). For other studies, an aliquot equivalent to 50–200 µg of total protein (whole-cell extracts, cytosolic or particulate fractions) was subjected to SDS/PAGE; proteins were transferred to a PVDF membrane, incubated with anti-peptide antibodies and developed as described. To control for loading variability, each sample was resolved in at least two gels. The films (Reflection autoradiography film; DuPont–New England Nuclear) were exposed for various periods of time to obtain the appropriate density of non-overlapping grains. For each film, specific bands were quantified by densitometric scanning of the X-ray film and analysis with ImageQuant software.

cAMP production

Trophoblasts were cultured in 24-well cluster plates. To determine adrenaline- or isoprenaline-induced cAMP production, the culture medium was replaced with 400 µl of isotonic buffer (25 mM Hepes containing Hanks balanced salt solution) and incubated at 37 °C for 10 min. The medium was changed to 400 µl of fresh buffer containing phosphodiesterase inhibitors (10 µM Rolipram and 50 µM IBMX) and test substance (PMA, EGF) for up to 10 min. cAMP production was stimulated by the addition of isoprenaline or adrenaline (see Figure legends for concentrations). The reaction was usually terminated after 2 min with

ice-cold 0.5 M perchloric acid (250 μ l) followed by extraction of cAMP for 30 min with agitation using an Orbit Shaker (20 rev./min; Lab-line Instruments, Inc., Melrose Park, IL, U.S.A.). The extracts were transferred into Microfuge tubes, each well was washed with 0.1 ml of 0.5 M perchloric acid and extracts were combined. The extract was neutralized with 25% KOH/30% K_2HCO_3 . Following removal of $KClO_4$, 150 μ l of the supernatant was assayed for cAMP content using a competition binding assay with kits from Diagnostic Products. Any variations in these procedures are detailed in the Figure legends. The cAMP extraction procedure was a modification [32] of a method provided by the manufacturer (Diagnostic Products), originally described by Brown et al. [33]. The extraction efficiency of this procedure was >94% based upon the recovery data from samples spiked with [3H]cAMP. In preliminary studies, using known amounts of cAMP, perchloric acid extraction did not affect ligand displacement in the competition binding assay. The range of the kit was from 0.11 to 27 pmol/tube. In most experiments, the cAMP concentration in the samples ranged from 0.5 to 10 pmol/tube. However, unstimulated basal levels of cAMP were near the low end of the detection range. Therefore, in some studies, the unstimulated basal levels were assayed following acetylation using a radioimmunoassay kit from Amersham. The range of the kit was 2–128 fmol/tube.

Data analyses

For Western blot analysis of PKC isoforms, extracts from at least three separate cell preparations were utilized. Each sample was analysed at least twice to check for loading variability. The mean of such replicates was used as a single datum point for analysis. For each cell preparation, cAMP production was determined in 3–6 separate dishes for a given condition and/or time point. The mean of such replicates was used as a single datum point for analysis, unless stated otherwise. All data are expressed as means $\pm$ S.E.M. For statistical evaluation, either the Wilcoxon non-parametric signed rank sum test or the paired-sample *t*-test was utilized to compare groups. Differences were considered significant at $P < 0.05$.

RESULTS

Identification of PKC isoforms

Antibodies against specific PKC isoforms were first tested against rat brain extracts (Figure 1A). Each antibody recognized specific band(s) which could be blocked by the presence of isoform-specific antigenic peptide during incubation. Similar specific bands were observed regardless of the source of the antibody. In the extracts prepared from cultured trophoblasts, positive signals were observed for PKC isoforms α , ϵ and ζ (Figure 1B). No specific bands were observed for PKC isoforms β , γ or δ . Similar patterns of isoforms were observed whether the extracts were prepared from freshly isolated, undifferentiated trophoblasts (results not shown) or differentiated, cultured trophoblasts (Figure 1B). In addition, samples prepared by the rapid denaturation method gave the same results (not shown). Compared with rat brain, there was a relatively low concentration of PKC α and an even lower concentration of PKC ϵ . In contrast, the concentration of PKC ζ in human trophoblasts was at least as high as that in rat brain. For most isoforms, a single specific band was observed. However, for PKC ζ , several lower-molecular-mass bands were recognized by the affinity-purified anti-peptide antibody. Similar results were obtained with the rapid denaturation method [27], making proteolysis of this isoform less

Figure 1 PKC isoforms in rat brain (A) and cultured human placental trophoblasts (B)

Whole-cell extracts were prepared as described in the Materials and methods section. Each protein extract (1.5 mg/gel) was separated by SDS/PAGE (9%), transferred to a PVDF membrane, placed in a multichannel Miniblotter 28, and probed with isoform-specific antibodies. To detect specific bands, antigen-adsorbed antibodies were also used.

Figure 2 Effect of acute PMA treatment on distribution of PKC isoforms

Trophoblasts were purified and maintained in culture for 48 h, and were treated with PMA (0.1 μ M) for 15 min. Control cells received DMSO. Trophoblasts were scraped into ice-cold lysis buffer, homogenized, centrifuged to remove unlysed cells and nuclei, and the post-nuclear fraction was centrifuged at 100 000 *g* for 20 min to obtain particulate and cytosol fractions. Protein samples (50–200 μ g/lane) were separated by SDS/PAGE and transferred to a PVDF membrane, and immunodetection was performed for PKC isoforms α , ϵ and ζ .

likely. Rather, PKC ζ may exist in several modified forms *in situ* or other proteins may be recognized by this antibody.

Effect of PMA treatment on PKC isoforms α , ϵ and ζ

Treatment of cultured trophoblasts with 100 nM PMA for 15 min resulted in increases in particulate PKC α and PKC ϵ , but not PKC ζ (Figure 2; Table 1). Corresponding decreases in the cytosolic concentrations were observed for PKC α and PKC ϵ , but not for PKC ζ (Figure 2; Table 1).

Continuous treatment with 1 μ M PMA for up to 36 h resulted in the quantitative down-regulation of the PKC α and PKC ϵ

Table 1 Effect of acute PMA treatment on distribution of PKC isoforms

Trophoblasts were purified and maintained in culture, treated with PMA (0.1 μ M) or DMSO for 15 min and processed to prepare particulate and cytosol fractions, as described in the legend to Figure 2. Equal amounts of protein samples were separated by SDS/PAGE and transferred to a PVDF membrane, and immunodetection was performed for PKC isoforms α , ϵ and ζ . Relative densitometric results were obtained from three separate trophoblast preparations. Actual estimates of each PKC isoform under basal (DMSO) condition were as follows. Particulate: PKC α , 50.6 ± 8.3 ; PKC ϵ , 20.3 ± 6.6 ; PKC ζ , 37.6 ± 13.7 ; cytosol: PKC α , 55.5 ± 17.6 ; PKC ϵ , 20.0 ± 4.3 ; PKC ζ , 44.9 ± 14.5 arbitrary densitometric units. Results are means $\pm$ S.E.M.

PKC isoform	Amount (% relative to control)	
	Particulate	Cytosol
PKC α	171.6 ± 18.8	46.7 ± 3.1
PKC ϵ	150.9 ± 37.6	31.4 ± 6.5
PKC ζ	110.1 ± 12.6	101.0 ± 11.0

Figure 3 Effect of long-term PMA treatment on PKC isoforms

Trophoblasts were purified and maintained in culture for 24 h, and were treated with PMA (1 μ M) for 0–36 h, as indicated above the lanes. Trophoblasts were scraped into ice-cold lysis buffer, homogenized and diluted into Laemmli sample buffer. Protein samples (50–200 μ g/lane) were separated by SDS/PAGE and transferred to a PVDF membrane, and immunodetection was performed for PKC isoforms α , ϵ and ζ . PKC α and PKC ϵ were down-regulated by continuous PMA treatment.

isoforms, but not of PKC ζ , in whole-cell extracts (Figure 3). Densitometric analyses showed approx. 50 % decreases in PKC α and PKC ϵ after 36 h of treatment with PMA.

Characterization of the PMA effect on cAMP production

cAMP production in trophoblasts is sensitive to various ligands. In the present study, adrenaline or isoprenaline was used to stimulate cAMP production. In preliminary studies, isoprenaline-stimulated cAMP accumulation was found to be linear for at least 2 min and reached a peak at approx. 5 min (results not shown). Inhibition of phosphodiesterases with 10 μ M Rolipram and 50 μ M IBMX increased the linear phase to approx. 4 min, but beyond this period the accumulation slowed. This was presumably due to rapid down-regulation of receptor function. Accordingly, in the present study, cAMP accumulation over the initial 2 min period was used to assess cAMP production as a function of PKC activation.

In preliminary studies, it was determined that pretreatment with PMA (1 μ M) for as little as 2 min enhanced isoprenaline-stimulated cAMP production. The PMA-dependent enhancement continued to increase up to 10 min, at which time maximal stimulation was observed (results not shown). To examine the

Figure 4 Effect of PMA concentration on isoprenaline-stimulated cAMP production

Trophoblasts were purified and maintained in culture for 48 h as described in the Materials and methods section. On the day of the experiment, cells were washed with isotonic buffer and treated with 10 μ M Rolipram, 50 μ M IBMX and PMA (10^{-10} – 10^{-6} M) for 10 min. cAMP production was stimulated by the addition of isoprenaline (10 μ M). The reaction was terminated after 2 min and cAMP (pmol/2 min per mg of protein) was determined using the binding protein displacement assay. Each experiment was carried out with four determinations. Data are means $\pm$ S.E.M. from six placental preparations (* $P < 0.05$ compared with DMSO-treated control group).

effect of PKC activation on ligand-stimulated cAMP production, trophoblasts were exposed to 10^{-10} – 10^{-6} M PMA for 10 min and stimulated with 10 μ M isoprenaline for 2 min (Figure 4). In control cells, isoprenaline (10 μ M) stimulated cAMP production by 19.3 ± 3.1 -fold over untreated cells ($n = 6$ cell preparations). Similarly, adrenaline (1 μ M) stimulated cAMP production by 19.1 ± 4.0 -fold. Exposure of trophoblasts to various concentrations of PMA for 10 min enhanced isoprenaline-stimulated cAMP production (Figure 4). Significantly increased stimulation of cAMP production occurred at PMA concentrations as low as 0.1 nM. Similar results were seen with adrenaline-stimulated cAMP production (not shown). In another experiment, acute treatment with 4 α -phorbol (1 μ M), an inactive phorbol ester, did not enhance isoprenaline-stimulated cAMP production (isoprenaline alone, 565 ± 26 ; 4 α -phorbol, 647 ± 15 ; PMA, 1052 ± 114 pmol/2 min per mg). In the absence of phosphodiesterase inhibitors, PMA pretreatment (1 μ M for 10 min) increased cAMP production in response to isoprenaline (10 μ M). The relative increase in cAMP production in the absence of phosphodiesterase inhibitors was 39 ± 6 % (control, 644 ± 46 ; PMA, 902 ± 102 pmol/2 min per mg; $n = 3$ cell preparations; $P < 0.05$). Therefore a PKC-associated increase in cAMP production may be physiologically relevant.

When trophoblasts were pretreated with a single concentration of PMA (1 μ M for 10 min) followed by stimulation with various concentrations of isoprenaline (10^{-9} – 10^{-4} M for 2 min), cAMP production was enhanced at all concentration of isoprenaline except the lowest (1 nM) (Figure 5). PMA treatment alone (10 min) had no effect on cAMP levels (control, 6.0 ± 1.0 ; PMA, 5.4 ± 0.4 pmol/mg; $n = 4$ cell preparations; $P < 0.05$). Therefore acute PMA treatment appears to increase the capacity for cAMP production in response to isoprenaline stimulation without altering basal cAMP production.

To confirm the involvement of PKC in the enhancement of cAMP production by PMA, trophoblasts were treated with

Figure 5 Effect of PMA on the dose-response for isoprenaline (isoproterenol) stimulation of cAMP production

Trophoblasts were maintained in culture for 2 days and treated with PMA (1 μ M) for 10 min, followed by treatment with isoprenaline (10^{-9} – 10^{-4} M). The reaction was terminated 2 min thereafter. ●, Control; ■, PMA-treated. Data are means $\pm$ S.E.M. from three experiments (* P < 0.05 compared with DMSO-treated control group).

Figure 6 Effect of EGF concentration on isoprenaline-stimulated cAMP production

Trophoblasts maintained in culture for 2 days were washed with isotonic buffer and treated with EGF (8×10^{-10} – 8×10^{-7} M) for 10 min. cAMP production (pmol/2 min per mg of protein) was stimulated by the addition of isoprenaline (10 μ M) and the reaction was terminated after 2 min. Data are means $\pm$ S.E.M. from four placental preparations (* P < 0.05 compared with control group).

1 μ M PMA for 24 h to down-regulate PKC. In these down-regulated cells, isoprenaline-stimulated cAMP production did not differ from that in control cells (control, 433 ± 69 ; down-regulated, 404 ± 74 pmol/2 min per mg of protein; $n = 3$ cell preparations). In addition, subsequent acute PMA (0.1 μ M) treatment in down-regulated cells did not enhance isoprenaline-stimulated cAMP production (control, 712 ± 38 ; down-regulated, 438 ± 82 pmol/2 min per mg of protein; $n = 3$). The chronic PMA effect could not be overcome at any PMA concentration up to 1 μ M (results not shown). Therefore down-regulation of PKC resulted in elimination of the acute PMA-induced enhancement of isoprenaline-stimulated cAMP production. Taken

together, these data suggest the occurrence of PMA-dependent PKC modulation of cAMP production in trophoblasts.

Effect of EGF on cAMP production

To examine the effect of an endogenous ligand known to activate PKC via a receptor [34], trophoblasts were pretreated with EGF (0.8 nM–0.8 μ M) for 10 min followed by isoprenaline (10 μ M) treatment for 2 min. As seen with acute PMA exposure, isoprenaline-stimulated cAMP production was enhanced by EGF pretreatment. The enhancement was dependent upon EGF concentration, with maximal stimulation occurring at 8 nM (Figure 6).

DISCUSSION

The placenta carries out many functions which are critical to the maintenance of pregnancy as well as the normal growth and development of the fetus. These include production of various pregnancy-related hormones and transport of essential nutrients. Such functions are subject to regulation by signal transduction mechanisms, including those which involve cAMP or PKC.

PKC is an important regulatory enzyme with multiple effects on cell function. Phorbol esters [35–38] and EGF [37], factors which activate PKC, have been shown to alter hormone production in cultured human placental trophoblasts or chorio-carcinomas. In addition, PKC activity has been partially characterized in the placenta [18,39,40]. However, identification of specific PKC isoforms and the mechanism by which PKC may affect placental function have not been described.

The results described in the present study show that human placental trophoblasts contain at least three PKC isoforms: α , ϵ and ζ . Therefore, one member from each of the PKC subfamilies (c, n and aPKCs) can be detected in trophoblasts. PKC isoforms β , γ and δ were not found in trophoblasts. Acute treatment with PMA resulted in translocation of PKC α and PKC ϵ from the cytosol to the particulate fraction. The distribution of PKC ζ was not affected by PMA. Long-term treatment with PMA down-regulated PKC α and PKC ϵ , but not PKC ζ . These findings are all consistent with observations in other cell types [41].

Moreover, acute treatment with PMA enhanced isoprenaline-stimulated cAMP production in trophoblasts. This effect was apparent in the presence or absence of phosphodiesterase inhibitors, suggesting that PKC enhances cAMP production without affecting catabolism. Therefore activation of PKC with an associated increase in cAMP production may be a physiologically relevant phenomenon. PKC activation is known to either increase [42] or decrease [16] cAMP production in a tissue/cell-specific manner. In trophoblasts, the enhancement was dependent upon increasing concentrations of PMA, with an apparent maximal effect at 0.1 μ M, although significant stimulation occurred at 0.1 nM PMA. At a single concentration of PMA (1 μ M), cAMP production was enhanced at various concentrations of isoprenaline (10^{-8} – 10^{-4} M), suggesting that PMA had increased the capacity for cAMP production in response to isoprenaline.

The exact mechanism of the effect of PKC activation is not clear. There are several possibilities, such as facilitating interactions between isoprenaline and its receptor, the receptor and a G-protein, and/or G-protein and adenylate cyclase. In addition, PKC activation may directly affect adenylate cyclase activity [15]. This would depend on the presence of specific adenylate cyclase isoforms in the trophoblast [43]. Regardless of the exact mechanism involved in this process, the PMA-dependent enhancement of cAMP production could be duplicated with low concentrations of EGF (0.8 nM–0.8 μ M). Therefore the inter-

action between PKC and cAMP production may represent a physiologically important cross-talk between signalling mechanisms. Such cross-talk to enhance cAMP production would be especially important in trophoblasts, since cAMP is known to stimulate specific hormone production [19] and amino acid transport [20] in these cells.

The identity of the PKC isoform responsible for enhancing trophoblast cAMP production is uncertain. In the present study, chronic PMA treatment down-regulated PKC α and PKC ϵ , but not PKC ζ . Moreover, down-regulation of PKC inhibited the acute PMA-dependent enhancement of isoprenaline-stimulated cAMP production. These data alone would suggest that either PKC α or PKC ϵ is mediating the effect of PMA on cAMP production.

In summary, the present results indicate that at least three PKC isoforms, α , ϵ and ζ , are present in human placental trophoblasts. Acute activation of PKC by phorbol ester leads to translocation of PKC α and PKC ϵ ; chronic treatment leads to down-regulation of these two isoforms. Thus PKC α and/or PKC ϵ may be involved in the regulation of cAMP production in human placental trophoblasts.

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