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IMMUNOCYTOCHEMICAL EXPRESSION AND LOCALIZATION OF PROTEIN KINASE C IN BOVINE AORTIC ENDOTHELIAL CELLS[1]

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Total PKC activity in BAEC incubated for 24 hrs in either 10% serum (FBS) or serum-deprived media (SDM) was similar. However, most of the activity (69%) in the FBS group was detected in the particulate fraction, while it was mainly in the cytosolic fraction (66%)in the SDM group. By confocal microscopy, there was diffuse cytoplasmic localization of the antibodies to the α and ß PKC isoforms. γ PKC was not detected. Treatment of FBS or SDM cells with a phorbol ester resulted in an increase in PKC activity with translocation to the particulate fraction. PKC α immunofluorescence redistributed to the perinuclear region whereas PKC ß staining remained mostly cytosolic. Calphostin C, a PKC inhibitor, prevented the phorbol ester-induced increase in PKC activity and translocation.

Protein kinase C (PKC) is a group of calcium- and phospholipid-dependent protein kinases which appears to be involved in the signal transduction response of many cell systems to a variety of hormones, growth factors and drugs (1). Cloning studies have demonstrated that PKC is a family of closely related genes that encode a number of isoenzymes which exhibit distinct tissue-specific patterns of expression. Initially, three different genes (α, ß, and γ) were reported; additional cDNA clones (δ, ε and ζ) have subsequently been isolated (2) and others will probably be discovered in the future. Activation of PKC is thought to occur in response to the synergistic action of diacylglycerol and calcium, both of which can be generated by signal-induced hydrolysis of membrane phospholipids. PKC can be measured in both cytosolic and particulate fractions. An indication of the activation of PKC is an increase in membrane-associated PKC activity following cell stimulation (2). This "translocation" has been noted in a variety of cell types including cultured BAEC (3). However, the individual patterns of expression of the PKC isoenzymes in BAEC have not been described.

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Using both PKC activity translocation assays and immunocytochemistry analysis with confocal microscopy, we examined the effect of calf serum on the intracellular distribution of PKC activity in confluent BAEC. By utilizing monoclonal antibodies directed against the α, ß and γ isoforms of PKC, we demonstrated a predominant cytosolic localization of the α and ß isoforms of PKC in BAEC. No staining could be observed with the antibody directed against the γ isoform of PKC. Brief stimulation of BAEC with 12-O-tetradecanoylphorbol 13-acetate (TPA), led to translocation of PKC activity to a particulate fraction. By immunofluorescent staining PKC α displayed a dense perinuclear pattern after 10 min of stimulation with TPA whereas PKC ß remained in the cytosol. PKC translocation and increase in activity was blocked by calphostin C.

METHODS

Experimental Protocol

BAEC were obtained by gentle scraping of the intima of calf thoracic aorta and maintained in DMEM F12 supplemented with 10% heat inactivated calf serum, 5 µg/ml deoxycytidine, 5 µg/ml thymidine, 1% penicillin-streptomycin and fungizone. BAEC were grown to confluence in 35 mm polystyrene wells and maintained in 10% calf serum for 24 hrs prior to experimentation (FBS) or serum-deprived for 24 hrs (SDM). BAEC were then treated with either the vehicle (0.1% DMSO) for 10 min or with 100 nM TPA dissolved in 0.1% DMSO for 10 min. In other studies, 0.05 µM calphostin C (Kamiya, Mountain View, CA) was activated by light (4) and added 30 min prior to the addition of TPA. Experiments were performed in quadruplicate.

Cytosol and Membrane Fractionation

BAEC were washed twice with cold PBS and with buffer A (20 mM tris-HCl, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF, 25 µg/ml leupeptin, and 0.33 M sucrose) and scraped from the wells into 4 ml of buffer A. The suspension was homogenized and centrifuged at 1500 g for 15 min. Completeness of cell disruption was confirmed by light microscopic evaluation of an aliquot of the suspension. The supernatant (cytosolic fraction) was saved and the pellet (membrane fraction) was washed in 5 ml of buffer B (20 mM tris-HCL, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF), centrifuged and resuspended in 4 ml of buffer B. Protein determinations were performed by the method of Bradford (5). To solubilize PKC associated with the membrane fraction, 1% NP-40 in buffer B was added and then centrifuged to obtain detergent-solubilized PKC from the membrane fraction. Both soluble and detergent-solubilized particulate fractions were applied to a DE-52 cellulose (Whatman) column polypropylene columns (Macalester Bicknell, New Haven, CT), equilibrated with buffer consisting of 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, and 5 mM ß-mercaptoethanol (pH 7.5). After the columns were washed, PKC was eluted with equilibration buffer containing 150 mM NaCl.

Protein Kinase C Assay

Calcium- and phospholipid dependent PKC activities were measured by determining ^{32}P transferred from [γ-^{32}P]-ATP (specific activity 10-25 Ci/mmol, New England Nuclear, Boston, MA) to histone III-S as described by Kraft et al (6). In brief, 1-3 µg of protein from the membrane or cytosolic fractions of BAEC were assayed in a reaction mixture (200 µl) containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 µg/ml 1,2-diolein, 200 µg/ml histone III-S, in the absence or presence of 0.5 mM $CaCl_2$ and 25 µg/ml phosphatidylserine. The reaction was initiated by addition of 10 µl of 0.4 mM [γ-^{32}P]ATP (250-500 cpm/pmol), for 10 min at 30°C. Twenty five µl aliquots were transferred to 1.5 cm^2 Whatman P-81 phosphocellulose paper, the paper washed three times in 75 mM phosphoric acid, and ^{32}P determined by standard liquid scintillation techniques. PKC activity was determined by subtracting the amount of ^{32}P incorporated into histone in the absence of calcium and phospholipid from the amount of ^{32}P incorporated into histone in the presence of calcium and phospholipid and was expressed as nmoles of ^{32}P incorporated/mg of protein/min.

Immunocytofluorescence of PKC Isoenzymes

Immunocytochemistry of PKC isoenzymes was examined by slight modifications of previously reported methods (7,8). We used monoclonal antibodies directed against rabbit brain-PKC that react specifically with the α (MC-3a), ß (MC-2a) and γ (MC-1a) subspecies of PKC (Seikagaku Kogyo Co., Ltd, Tokyo, Japan). The specificity of these antibodies for the different PKC-isoenzymes has been assessed by immunocytochemical analysis of the rabbit cerebellum (7).

BAEC were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 30 min and incubated with 50 mM ammonium chloride for 15 min. After incubation with 1% bovine serum albumin in phosphate buffer solution (BSA/PBS) for 60 min, the preparation was incubated for 2 hrs at room temperature with the PKC antibodies diluted in 1% BSA/PBS (1:20), washed three times with 1% BSA/PBS and subsequently exposed to the rhodamine-conjugated secondary antibody (1:100, 1% BSA/PBS) for 60 min. The preparation was then mounted with 50% glycerol and observed with a 40x or 63x objective on a Zeiss Axiovert microscope using a BioRad MRC-600 Confocal Imaging System. Aperture, gain and black level for imaging acquisition were maintained constant. No background or autofluorescence was visible at the chosen settings.

Statistical Analysis

Results are expressed as mean ± standard deviation. The effect of different incubation media and TPA on PKC activity was analyzed by the unpaired Students' t test. Differences were considered significant at $p < 0.05$ level.

RESULTS

PKC Activity in Cytosolic and Particulate Fractions of BAEC

Total PKC activity in unstimulated confluent SDM BAEC was 0.9±0.1 nmoles/mg/min. Figure 1 shows that 34% of the total activity was in the membrane fraction and 66% of the activity in the cytosolic fraction. After exposure of BAEC to 100 nM TPA for 10 min, total PKC activity increased to 1.5±0.2 nmoles/mg/min ($p=0.005$). The contribution of the particulate fraction to the total activity increased to 91%, while there was a commensurate drop in the contribution of the cytosolic fraction to 9%. Pre-treatment for 30 min with 0.05 μM calphostin C prior to TPA stimulation, resulted in a significant drop in total PKC activity to 0.5±0.1 nmoles/mg/min ($p=0.001$) with 60% of the total activity in the cytosolic fraction and 40% in the particulate fraction.

Total PKC activity in unstimulated confluent FBS BAEC was 1.3±0.2 nmoles/mg/min, which was higher than for unstimulated SDM BAEC ($p<0.05$). In contrast to SDM cells, Figure 1 demonstrates that the majority of the PKC activity in FBS BAEC was located in the particulate fraction (69%), with only 31% of activity arising from the cytosol. 100 nM TPA for 10 minutes also caused a significant rise in total PKC activity to 1.6 nmole/mg/min ($p<0.05$) with translocation of PKC activity to the particulate fraction (97%) associated with a reduction in cytosolic activity (3%). Pre-treatment with 0.05 μM calphostin C led to a significant decrease in total PKC activity to 0.6±0.1 nmoles/mg/min ($p<0.005$) with equal contributions from the cytosolic and particulate fractions.

Localization of PKC Isoenzymes in BAEC

Figure 2 demonstrates that SDM BAEC expressed the α and ß isoforms of PKC. Both subspecies were localized predominantly in the cytosol, extending from the

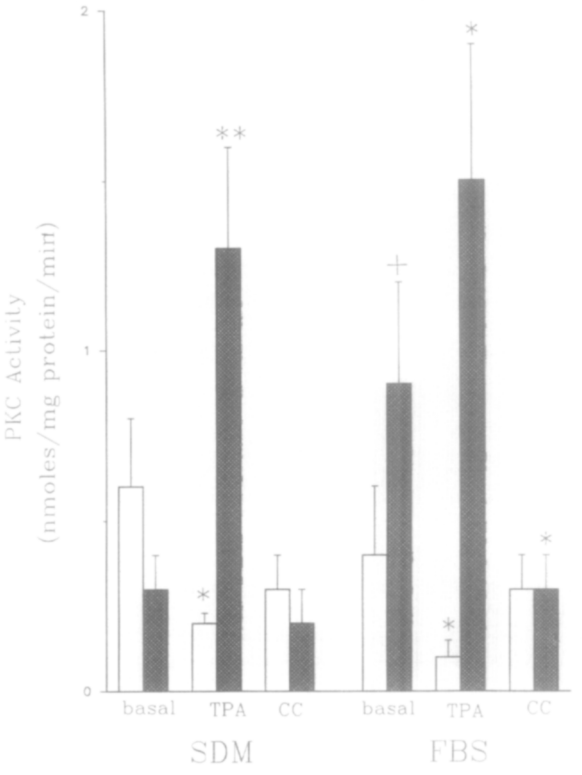

Figure 1.
Effect of TPA on intracellular partitioning of protein kinase C. Confluent BAEC in either serum-derived media (SDM) or in media containing 10% FBS were exposed to either 0.1% DMSO (Basal) or 100 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) in 0.1% DMSO for 10 minutes. In some experiments 0.05 μM calphostin c (CC) was added for 30 min prior to stimulation with TPA. After homogenization and high speed centrifugation, PKC activities were measured in the cytosolic (clear bars) and membrane (hatched bars) fractions (nmoles/mg protein/min, mean ± SD). *p<0.05 **p<0.01 compared to basal; +p<0.01 compared to SDM.

plasma membrane to the nuclear membrane with no significant intranuclear staining observed. No staining was observed utilizing the antibody directed against PKC γ or in preparations that did not include primary antibodies to the α or ß isoforms.

Incubation of SDM BAEC with 100 nM TPA for 10 min caused a marked redistribution in PKC α immunofluorescence and less significant changes in the stain pattern of PKC ß. PKC α fluorescence was most intense in the perinuclear region, with some intranuclear staining, and very faint in the periphery of the cytosol and around the plasma membrane. The fluorescent pattern of PKC ß, although remaining predominantly cytosolic, revealed mild perinuclear staining. Pre-incubation with 0.05 μM calphostin C prior to TPA stimulation resulted in diffuse cytoplasmic staining of both α and ß isoforms of PKC.

Similar results were obtained when the experiments were performed in the presence of calf serum, although the translocation of PKC α immunofluorescence

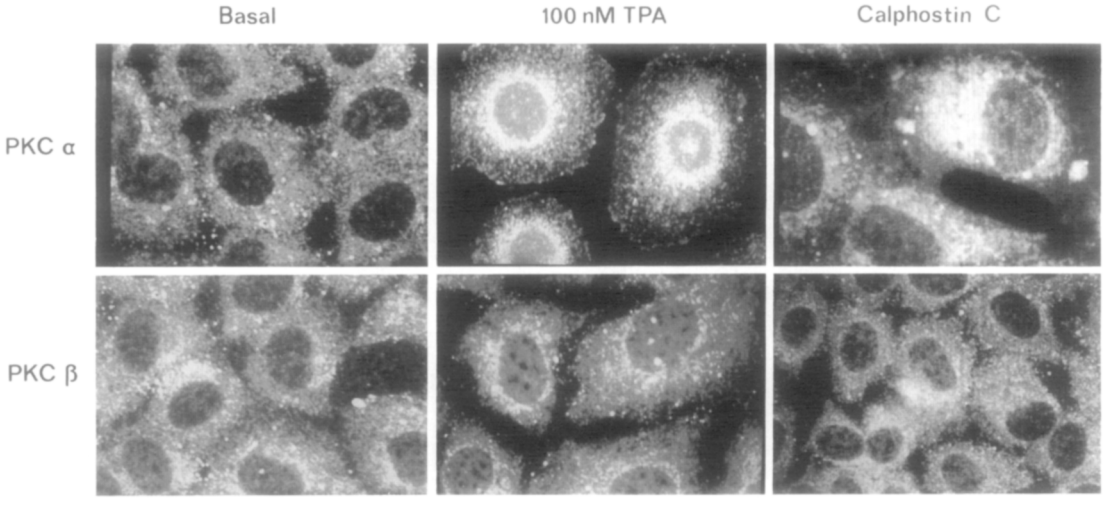

Figure 2.
Confocal fluorescence microscopy of protein kinase C localization in BAEC. Images were focussed at the middle portion of the nucleus.

(Left panel) BAEC were exposed to 0.1% DMSO for 10 min, fixed, and incubated with antibodies against PKC α, ß, or γ for 2 hrs, and subsequently exposed to the rhodamine-labelled anti-mouse IgG for 60 minutes (n=6). BAEC incubated without primary antibody or with PKC γ failed to display immunofluorescence.

(Middle panel) Exposure of BAEC to 100 nM TPA in 0.1% DMSO for 10 min, resulted in a shift of PKC α to the perinuclear region, whereas the fluorescent pattern of PKC ß remained predominantly cytosolic (n=6).

(Right panel) 0.05 μM calphostin C was added to BAEC for 30 min prior to exposure to 100 nM TPA in 0.1% DMSO for 10 min. There was diffuse cytoplasmic staining of both α and ß isoforms of PKC.

was not as striking since under basal conditions the majority of PKC activity was already localized to the particulate component (Figure 1).

DISCUSSION

Immunohistochemistry with confocal fluorescence microscopy offers distinct advantages over conventional fluorescence microscopy including higher resolution reconstruction of structures which may suffer distortion upon fixing and a more accurate assessment of stain or fluorescence distribution (9). In resting serum-deprived EC, PKC α and ß immunofluorescence is similarly expressed and is localized to the cytosol. No staining was observed in BAEC incubated with our antibody to PKC γ even under a variety of primary and secondary antibody dilutions. The antibody to PKC ß cross-reacts with both the ß1 and ß2 PKC subspecies, and hence we cannot distinguish between these two isoenzymes. Similarly, in view of the current unavailability of antibodies directed against the δ, ε and ζ isozymes of PKC, the expression of these subspecies in BAEC is still unknown.

The ratio of the membrane to soluble PKC activity in resting cells varies according to cell type, degree of confluence and differentiation and incubation

media. In this study, the activity ratio in confluent-serum deprived BAEC was about 0.5. This observation is in agreement with our immunocytochemical evidence in the same cells, in which PKC immunofluorescence is mainly expressed in the cytosol. Similar activity ratios have been reported in BALB/c-3T3 and NIH-3T3 cells (10) and in sparse, quiescent porcine aortic EC (11). In contrast, cells actively growing or incubated in serum-rich media display a higher membrane to cytosol ratio. For instance, Myers et al (3) reported that 67% of the total PKC activity in confluent bovine pulmonary arterial endothelial cells incubated in 10% FBS was membrane associated. We found a similar percentage in confluent BAEC maintained in 10% FBS.

As expected from previous studies in many cell types, including EC, phorbol ester treatment of EC causes a redistribution of PKC activity rather than in a quantitative change in total PKC activity (3,12). Translocation of PKC activity from cytosol to membrane occurred, such that >91% of PKC activity was detected in the particulate fraction in both the serum deprived and the serum fed BAEC. Recent reports with other cell systems have demonstrated that activation of PKC results not only in binding to plasma membrane lipids (12,13), but also in translocation to cytoskeletal elements (14,15) and nuclear structures (6,17). Translocation of PKC isoenzymes to different cellular elements may explain how activation of this enzyme results in phosphorylation of proteins throughout the cell and regulation of multiple and diverse biological effects (1). One of the limitations of our study is the absence of subcellular fractionation to assay for PKC activity or PKC isozymes by Western blot. Fixation may not completely prevent loss of PKC isozymes from other putative cellular compartments or may mask the eptiope recognized by one monoclonal antibody at one site but not another. Nonetheless, our immunofluorescent staining studies suggest that treatment with TPA has different effects on patterns of PKC isotype expression in BAEC. 100 nM TPA for 10 minutes caused an apparent and intense relocation of PKC α to the perinuclear and intranuclear regions while PKC ß remained mostly cytosolic and faintly stained the perinuclear region.

Evidence for or against nuclear PKC localization has been sought in several different cell types and tissues. The results of these studies have been diverse, perhaps reflecting the wide range of cell types and experimental protocols used which include both reconstituted systems as well as intact cells. Hocevar et al (16) reported that treatment of HL60 cells with bryostatin 1, and not phorbol dibutyrate, caused the activation and translocation of a PKC-like activity to the nuclear membrane. Leach et al (17) examined the immunocytochemical localization of PKC in NIH 3T3 cells using antibodies directed against PKC α. In control cells, PKC α localized in a diffuse cytoplasmic pattern, while the nuclei were unstained. Treatment of cells with phorbol 12-myristate 13-acetate (PMA) resulted in a redistribution of PKC with a specific increase in nuclear PKC. In contrast to these studies, Halsey et al (10) also showed increased perinuclear staining

in PMA-treated 3T3-L1 cells using a polyclonal antibody to PKC. However, PKC activity was associated only with nonnuclear membranes and not with the nuclear fraction.

The exact role of PKC in the nucleus is not yet known. Phorbol esters affect the transcription of many genes, including c-fos, plasminogen activator and interferon (17). PMA-stimulated phosphorylation of a variety of nuclear proteins has been reported, including lamin B (16), histones (18), matrix proteins and DNA topoisomerase II (19). Most of these studies used reconstitution systems, and only some of these proteins, such as lamin B, are located in the nuclear envelope. Thus, the physiological PKC nuclear substrates have not been identified.

In summary, in the present study we provide immunocytochemical evidence that BAEC express PKC α and ß isotypes, but not γ. Although phorbol ester treatment of EC results in translocation of PKC activity from the cytosol to membrane fractions, the α and ß isoforms of PKC appear to shift to different areas. Further understanding of the intracellular location of the different isoenzymes in response to different agonists and antagonists may contribute insights to the multiple regulatory effects of PKC.

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