Communication

Differential Activation of Protein Kinase C α Is Associated with Arachidonate Release in Madin-Darby Canine Kidney Cells*

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The heterogeneity of the protein kinase C (PKC) gene family strongly suggests that different isoforms may have distinct functions in mediating signal transduction. However, there is very little direct evidence for this. PKC has been implicated in arachidonate (AA) release in many cell types. We sought to investigate whether bradykinin- and phorbol ester-stimulated AA release in Madin-Darby canine kidney (MDCK) cells was correlated with differential activation of PKC isoforms. Using phorbol esters to (i) activate the enzyme and (ii) to down-regulate it, we report that differential activation (translocation) of PKC α is associated with AA release in MDCK cells and that specific down-regulation of PKC α is associated with a loss of AA release in response to stimulation with diacylglycerol and phorbol ester. We also demonstrate that bradykinin-stimulated AA release was associated with differential activation of PKC α and was inhibited in PKC α down-regulated cells. Thus, we conclude that the PKC α isoform is likely to be responsible for mediating AA release in these cells.

Protein kinase C (PKC) is a major component of transmembrane signaling systems (for review see Ref. 1). Agonist-stimulated phospholipid hydrolysis produces diacylglycerol, which activates the enzyme by promoting its association with the cell membrane. Tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate and phorbol 11,12-dibutyrate (PMA and PDBu, respectively) can substitute for diacylglycerol as activators of the enzyme. The cellular response to such phorbol esters is biphasic. The initial response involves translocation and activation of protein kinase C (2); however, prolonged activation of the enzyme results in its down-regulation and proteolysis (3). Both activation and down-regulation by phorbol esters are widely used experimental paradigms for implicating the enzyme in biological responses. It has recently become apparent that several subspecies of PKC exist. Types α, β, and γ (III, II, and I, respectively) show different tissue and subcellular distribution and vary in their diacylglycerol, fatty acid, and calcium requirement (reviewed in Refs. 4 and 5). Additionally, differential susceptibility of the isoforms to proteolysis and to phorbol ester-induced down-regulation has been demonstrated in purified preparations of the enzymes (6, 7) and in several cell types including hasphophilic leukemia cells (6), KM3 cells (8), and BCC31-1 myocytes (9). PKC α, β, and γ are the products of distinct genes, and subspecies of PKC αβ(α) and γβ(γ) are derived from alternative splicing of a single gene (see Refs. 4 and 5 for reviews). The heterogeneity of the protein kinase C gene family suggests that different isoforms may have distinct functions within a given tissue or subcellular compartment.

In these experiments we sought to identify the isoforms of PKC present in the clonally derived renal epithelial cell line MDCK-D1 (10, 11) and to determine their relative susceptibility to down-regulation and their role in mediating a cellular response, namely arachidonate release. PKC-stimulated arachidonate release has been demonstrated in a variety of cell types (12-15) including MDCK cells (16-20) in response to both phorbol ester and hormonal stimulation. Here we report that (i) PKC α and PKC β are present in MDCK-D1 cells, (ii) both bradykinin (and PMA) preferentially activate PKC α and that this activation is correlated with arachidonate release, and (iii) the specific down-regulation of PKC α results in the loss of the ability of PMA, bradykinin, and the cell-permeant diacylglycerol analog dioctanoylglycerol (diC8) to stimulate arachidonate release. Thus a specific role for PKC α in arachidonate release is implied.

EXPERIMENTAL PROCEDURES

Materials— [3H]Arachidonic acid (100 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) and [3H]Protein A (8.71 Ci/mg) were purchased from Du Pont-New England Nuclear. Histone III-S, diolein (1,2-di oleoyl-sn-glycerol C18:1 cis-9), PMA, PDBu, 4α-phorbol didecanoate (4α-PDD), and BK were purchased from Sigma. Phosphatidylserine and diC8 were obtained from Avanti Polar Lipids Inc. DEAE-Sepharose was obtained from Pharmacia LKB Biotechnology Inc. Antipeptide antibodies generated to sequences unique to PKC α or β or γ were a gift from Dr. Ora Rosen (Memorial Sloane Kettering Cancer Center, NY) (21). Purified PKC was a gift from Dr. Gordon Gill (University of California, San Diego).

Cell Culture—Cell culture procedures have previously been described (18, 22). Briefly, MDCK-D1 cells were grown in DMEM supplemented with 10% heat-inactivated serum (75% horse serum, 25% fetal calf serum), 15 mm HEPES, and 3.7 g/liter sodium bicarbonate, pH 7.4. Subconfluent cells were subcultured every 3 days with a trypsin/EDTA solution. For experimental purposes cells were used on day 3 of culture.

Evaluation of PKC—Cells (in 150-mm dishes) were washed twice with DMEM, 20 mm HEPES, 0.05% bovine serum albumin (incubation medium) and then treated with 100 nm PMA, 100 µM diC8, or vehicle (0.1% ethanol) in incubation medium for 30 min at 37°C. For bradykinin-stimulated translocation experiments the washed monolayers were treated with 1 µM BK for various periods between 30 s and 5 min. After these times the monolayers were washed twice with 5 ml of ice-cold 20 mm Tris, 2 mm EDTA, 10 mm EGTA, 2 mm DTT, pH 7.5 (lysis buffer, LB), and scraped into 5 ml of ice-cold LB. The
cell suspension was homogenized with 20 strokes of a Dounce homogenizer. The homogenates were centrifuged at 35,000 x g for 35 min (4 °C) to separate soluble and particulate fractions. The membrane pellet was homogenized in LB containing 1% Nonidet P-40. The subcellular fractions were applied to DEAE Sephadex column (2 ml bed volume) previously equilibrated with 20 mM Tris, 2 mM EDTA, 2 mM DTT, pH 7.5 (column buffer). After washing with 10 ml of column buffer, PKC was eluted with 5 ml of column buffer containing 0.15 M NaCl. The eluate was used for assay of PKC activity and identification of the isoforms present in the cytosolic and membrane fractions.

Down-regulation of PKC by PDBu Treatment—Cell monolayers (150-mm dishes) were washed twice with serum-free culture medium and then incubated overnight with 100 nM PDBu (or an equivalent concentration of vehicle) in this medium. Cells were harvested and homogenized as described above in LB containing 1% Nonidet P-40. The homogenates were centrifuged at 35,000 x g for 60 min (4 °C). The supernatants were collected and DEAE-Sephacel chromatographed as described above. For arachidonate release and lysophospholipid production experiments cells in 35-mm dishes were treated with 100 nM PDBu (or an equivalent concentration of vehicle) in culture medium containing 0.5% fetal calf serum.

Electrophoresis and Immunoblotting—Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel (approximately 150 μg of protein were applied per lane) and transferred to nitrocellulose (23). Non-specific binding was blocked by incubating the nitrocellulose membrane with 5% bovine serum albumin in PBS for 60 min. The membranes were incubated with antisera (1:100 dilution in 1% bovine serum albumin, 0.01% Tween 20, 0.5% sodium azide in PBS) for 60 min/ml phosphatidylserine, 6 μg/ml diolein, and 50 μg/ml [32P]ATP (approximately 1000 cpm/ml). Phosphorylation was initiated by the addition of 40 μl of column eluate to 30 μl of phosphorylation mixture. The reaction proceeded for 5 min at 30 °C and was stopped by adding the reaction mixture to phosphocellulose PS1 paper (Whatman) as previously described (25). All determinations were performed in triplicate. PKC activity was defined as the difference between 32P incorporation in the presence or absence of phosphatidylserine and diolein and is expressed as picomoles of 32P incorporated per min per μg of protein.

Protein Determination—Protein concentration in the DEAE-Sephacel column eluates was determined by the method of Lowry et al. (26) following precipitation of the proteins with trichloroacetic acid.

Arachidonate Release Experiments—[3H]Arachidonate release was measured as previously described (18, 19). Briefly, cells were labeled in medium containing 0.5 μCi/ml [3H]arachidonic acid in culture medium containing 0.5% fetal calf serum. After labeling for 3–5 h the monolayers were washed four times with incubation medium before treatment with PMA, bradykinin, or diC8. [3H] Released into the extracellular medium was determined by liquid scintillation counting.

Lysophospholipid Production—Lysophospholipid production was determined as described previously (18, 19). Briefly, cells were labeled for 18–24 h with 1 μCi/ml [3H]ethanolamine in DMEM containing 0.5% serum. Cells were stimulated with phosphor ester or bradykinin as described for arachidonate release experiments. Lys-PE production in response to PMA and to bradykinin was measured by scraping once into 0.5 ml of ice-cold methanol and once into 0.5 ml of ice-cold H2O. The extracts were scraped into test tubes, and 2 ml of CHCl3 and 50 μl of unlabeled lys-PE (50 μg/tube) were added. The extracts were centrifuged at 30 x g for 10 min. The lower phase was dried down under nitrogen and chromatographed on Silica Gel G plates in CHCl3:MeOH:NH4OH:H2O, 70:30:5:2.

Lysophospholipid Phosphorus Determination—Lysophospholipid phosphorus was determined by a modification of the procedure of Goppelt and Resch (27). Phospholipids were extracted as previously described (22) and assayed spectrophotometrically relative to a NaH2PO4 standard curve.

**RESULTS AND DISCUSSION**

Activation of PKC is associated with translocation of the enzyme from cytosolic to membrane compartments; such translocation occurs in response to agonist-stimulated diacylglycerol production, and this activation process can be mimicked by tumor-promoting phorbol esters and cell permentant diacylglycerol analogues such as diC8. When we treated MDCK cells with 100 nM PMA or 200 μM diC8 for 30 min we observed increased activity of the enzyme in the particulate fraction (Fig. 1), and accompanying this activation of the enzyme was an enhancement in arachidonate release (see control values, Fig. 2A). Treatment of the cells with 1 μM BK caused a transient increase in PKC activity in the particulate fraction; this was detectable as early as 30 s after the addition of BK and had declined to basal levels by 5 min (data not shown). The kinetics of this translocation were similar to those observed for α1-adrenergic activation of PKC in these cells (24).

To determine the relative involvement of PKC isoforms in these processes we used isoform-specific antibodies in immunoblotting procedures. Using anti-α, β, and γ antibodies we identified PKC types α and β in MDCK cells; no γ isoform was detectable.2 These observations are consistent with the known tissue-specific distribution of the isoforms (4, 5), the γ form being found almost exclusively in the nervous system and the α and β forms being found in peripheral tissues in varying amounts. The specificity of the isoform-specific antisera used in these experiments has previously been documented (21). Treatment of the cells with PMA caused selective translocation of PKC α, while the relative distribution of PKC β was unchanged by PMA treatment (Fig. 3A). BK treatment of the cells also stimulated PKC α translocation after 1 min (Fig. 3B); in parallel experiments translocation of PKC β0, the predominant β isofrom in these cells, was not observed. Treatment of the cells with diC8 also resulted in translocation of PKC α (data not shown). A causative relationship between PKC α activation and arachidonate release is suggested by the fact that PKC α translocation is observed prior to or concomitant with phosphor ester (18) and bradykinin (18, 19) stimulated arachidonate release. Selective translocation of PKC isoforms in response to PMA has recently been demonstrated in other cell types; in platelets (28) and in

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2 Further analysis with βγ and βδ-specific antibodies has shown the presence of both subspecies in these cells, the δ form being much more abundant than the δ form (C. Codron, unpublished observation).
Arachidonate Release Mediated by Protein Kinase C

Fig. 2. [3H]AA release stimulated by PMA, diC8, and BK in PKC down-regulated cells. A, [3H]AA release stimulated by PMA treatment for 60 min following overnight treatment with 0.1% ethanol (control, open symbols) or 100 nM PDBu (closed symbols). Results shown are mean ± S.E. for four experiments done in triplicate. Basal (unstimulated) AA release has been subtracted from each data point. Basal values for control and down-regulated conditions respectively were: 134 ± 72, 714 ± 274. B, fold stimulation of AA release by 200 nM diC8 for 60 min following overnight treatment with 0.1% ethanol (control, open bar), 100 nM 4α-phorbol (striped bar), or 100 nM PDBu (hatched bar). Results shown are mean ± S.E. for three experiments done in triplicate. The results are normalized per nanomole of phospholipid phosphorus. Basal release was 239 ± 106, 2329 ± 109, and 5146 ± 434 cpm/dish for control, 100 nM 4α-phorbol, and 100 nM PDBu, respectively. C, [3H]AA release stimulated by BK (1 μM) treatment for 4 min following overnight treatment with 0.1% ethanol (control, open symbols) or 100 nM PDBu (closed symbols). Results shown are mean ± S.E. for four experiments done in triplicate. Basal (unstimulated) AA release has been subtracted from each data point.

hepatocytes (29) PKC α is translocated preferentially whereas in KM-3 cells PKC β is translocated (7).

The selective translocation of PKC α concomitant with AA release suggested a role for PKC α in this process. To further investigate this possibility we determined the effect of long-term treatment with phorbol ester (100 nM PDBu, 18 h) on arachidonate release in response to bradykinin, PMA, and diC8 stimulation. Consistent with the data showing selective translocation of PKC α in response to short-term PMA treatment, we observed that 18-h treatment of the cells with 100 nM PDBu resulted in selective down-regulation of PKC α (Fig. 4). Moreover, down-regulation of PKC in response to prolonged stimulation with phorbol ester resulted in inhibition of arachidonate release stimulated by PMA, BK, and diC8 (Fig. 2). In rat basophilic leukemia cells (8) and in KM-3 cells (7) PKC β has been shown to be most susceptible to proteolysis whereas in BC3H-1 myocytes PKC α is more susceptible (9).

To verify that the decrease in immunoreactive PKC α which we observed is associated with decreased activity of the enzyme was assayed PKC activity in lysates of cells treated with 100 nM PDBu or 100 nM 4α-PDD (an inactive phorbol ester) or 0.1% ethanol (control). Overnight treatment with PDBu resulted in a decrease of PKC activity from 1.58 ± 0.3 to 0.73 ± 0.2 pmol of 32P incorporated per μg of protein per min (mean ± S.E. of four independent experiments). Overnight treatment with 4α-PDD did not significantly alter PKC activity in the lysates. To eliminate the possibility that the presence of PDBu in down-regulated cell lysates might compromise the ability of the added diolein to stimulate PKC down-regulated PKC α in response to PMA is typical of results obtained from three separate experiments. B, translocation of PKC α in response to BK stimulation. Lanes 2–4 contained soluble (S) and particulate (P) fractions from BK-stimulated and control cells as indicated. Lane 5 contained purified PKC. The position of a 79-kDa molecular mass standard is indicated at the right-hand side of the blot. Lanes 1–4 contained soluble (S) and particulate (P) fractions from PMA-treated and control cells as indicated (150 μg of protein/lane). Lanes 5 and 10 contained purified PKC. Lanes 1–5 were probed with antisera specific for PKC α and lanes 6–10 with antisera specific for PKC β. This immunoblot showing differential translocation of PKC α in response to PMA is typical of results obtained from three separate experiments. In parallel experiments using anti-PKC β antisera translocation of this isoform in response to BK was not observed.

Fig. 3. Differential translocation of PKC α in response to PMA stimulation. MDCK-D1 cells were treated with (A) 100 nM PMA or 0.1% ethanol (control) for 30 min, or (B) 1 μM BK or DMEM (control) for 1 min. Cell lysates were resolved into soluble and particulate fractions and immunoblotted with PKC α and PKC β specific antisera as described under "Experimental Procedures" using 125I-Protein A for detection. The position of a 79-kDa molecular mass standard is indicated at the right-hand side of the blot. A, differential translocation of PKC α in response to PMA stimulation: lanes 1–4 and 6–9 contain soluble (S) or particulate (P) fractions from PMA-treated and control cells as indicated (150 μg of protein/lane). Lanes 5 and 10 contained purified PKC. Lanes 1–5 were probed with antisera specific for PKC α and lanes 6–10 with antisera specific for PKC β. This immunoblot showing differential translocation of PKC α in response to BK is typical of results obtained from three separate experiments. In parallel experiments using anti-PKC β antisera translocation of this isoform in response to BK was not observed.

Fig. 4. Differential down-regulation of PKC α following overnight treatment with phorbol ester. Cells were treated overnight with 100 nM PDBu and the cell lysates immunoblotted with PKC α and PKC β specific antisera as described under "Experimental Procedures." Lanes 1 and 2 and 4 and 5 contained cell lysates from 100 nM PDBu or 0.1% ethanol (control)-treated cells as indicated (150 μg of protein/lane). Lanes 3 and 6 contained purified PKC. Lanes 1–3 were probed with PKC β-specific antisera, and lanes 4–5 were probed with PKC α-specific antisera. The position of a 79-kDa molecular mass standard is indicated at the right-hand side of the blot. This immunoblot showing differential down-regulation of PKC α is typical of results obtained from three separate experiments.

FIG. 2. [3H]AA release stimulated by PMA, diC8, and BK in PKC down-regulated cells. A, [3H]AA release stimulated by PMA treatment for 60 min following overnight treatment with 0.1% ethanol (control, open symbols) or 100 nM PDBu (closed symbols). Results shown are mean ± S.E. for four experiments done in triplicate. Basal (unstimulated) AA release has been subtracted from each data point. Basal values for control and down-regulated conditions respectively were: 134 ± 72, 714 ± 274. B, fold stimulation of AA release by 200 nM diC8 for 60 min following overnight treatment with 0.1% ethanol (control, open bar), 100 nM 4α-phorbol (striped bar), or 100 nM PDBu (hatched bar). Results shown are mean ± S.E. for three experiments done in triplicate. The results are normalized per nanomole of phospholipid phosphorus. Basal release was 239 ± 106, 2329 ± 109, and 5146 ± 434 cpm/dish for control, 100 nM 4α-phorbol, and 100 nM PDBu, respectively. C, [3H]AA release stimulated by BK (1 μM) treatment for 4 min following overnight treatment with 0.1% ethanol (control, open symbols) or 100 nM PDBu (closed symbols). Results shown are mean ± S.E. for three experiments done in triplicate. Basal (unstimulated) AA release has been subtracted from each data point.

To verify that the decrease in immunoreactive PKC α which
equivalent concentrations of PDBu were added to the phospholipid mixture of lysates from vehicle treated cells. The appropriate concentration of PDBu was calculated by incubating the cells with 100 nM [3H]PDBu (approximately 100,000 cpm/dish) and determining the recovery of label from DEAE-Sephacel eluates. These experiments indicated that residual PDBu from the down-regulation procedure did not compromise stimulation of the enzyme with the added diolein.

The heterogeneity of the PKC gene family strongly suggests that different isoforms may have specific physiological roles but to date there has been a paucity of data supporting this. Altered levels of expression of various isoforms have suggested their importance in growth (30), differentiation (21), neuronal plasticity (31), and exocytosis (32). We present data that indicates that arachidonate release from MDCK cells is associated with activation of PKC α but not PKC β. A role for PKC in arachidonate release has been previously demonstrated in a variety of cell types including pinealocytes (12), neutrophil (14), platelet (15) and MDCK cells (16, 29).

There are three principal mechanisms whereby arachidonate release from membrane phospholipids may be affected by PKC: (i) activation of phospholipase A2; (ii) stimulation of di- and/or monoacylglycerol lipase activity subsequent to phospholipase C activation; and (iii) inhibition of lysophosphatidate acetyltransferase. Phospholipase A2 activation results in release of lysophospholipids in a 1:1 ratio with arachidonic acid; lysophospholipid production is thus an indicator of phospholipase A2 activity (19). To determine whether PKC α plays a role in regulating phospholipase A2 in MDCK-D1 cells we investigated whether PMA- and BK-stimulated lys-PE formation was altered by overnight treatment with 100 nM PDBu. In control cells acute treatment with 100 nM PMA resulted in stimulation of lys-PE production 1.6 ± 0.2 fold over basal whereas in cells depleted of PKC α this effect was completely abolished (four independent experiments). BK stimulated lys-PE formation 1.4 ± 0.2 fold over basal in control cells; this stimulation was reduced to 38% of control value in the down-regulated cells (three independent experiments). This suggests that PKC α is involved in activation of phospholipase A2 by both phorbol esters and bradykinin.

Previous work from this laboratory has suggested that PMA, bradykinin, and epinephrine all promote deacylation of membrane phospholipids by phospholipase A2 (18, 19). The mechanism whereby PKC α activates phospholipase A2 might involve activation of the enzyme by phosphorylation (13, 17), regulation of PLA2 inhibitory proteins such as the lipocortins (33), or stimulatory proteins such as phospholipase A2 activating protein (34). Alteration of transmembrane calcium flux may also be involved (35). The inability of down-regulation of PKC α to completely abolish lys-PE production stimulated by bradykinin suggests the possibility that BK may modulate phospholipase A2 through additional mechanisms other than PKC activation. Possible mechanisms are direct activation of phospholipase A2 consequent to bradykinin-induced calcium influx (36) or coupling of bradykinin receptors to phospholipase A2 through a guanine nucleotide binding protein (37).

In conclusion, the data presented here provide the first evidence of a role for PKC α but not β in phorbol ester-stimulated arachidonate release as under acute conditions where: (i) PMA stimulates arachidonate release in these cells PKC α but not PKC β is activated (translocated); (ii) BK stimulates arachidonate release in these cells PKC α but not PKC β is translocated; and (iii) down-regulation of PKC α but not PKC β completely inhibits arachidonate release stimulated by PMA, bradykinin, and the diacylglycerol analog diolein.
Differential activation of protein kinase C alpha is associated with arachidonate release in Madin-Darby canine kidney cells.

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