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Stimulation of Phospholipase D via α_1-Adrenergic Receptors in Madin-Darby Canine Kidney Cells is Independent of PKCα and -ϵ Activation

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ABSTRACT

We have demonstrated previously that protein kinase C_α (PKC_α) plays a key role in regulating phospholipase D (PLD) activation by nucleotides and the phorbol ester phorbol-12-myristate-13-acetate in Madin-Darby canine kidney (MDCK-D1) cells. In the current work, we investigated PLD activation in MDCK-D1 cells triggered by the adrenergic receptor agonist epinephrine and its mechanism of activation. Epinephrine, acting through the α_1-adrenergic receptor subtype, promoted transient translocation of PKC_α and more prolonged translocation of PKC_ϵ to the membrane fraction, indicating activation of these two isoforms. In addition, epinephrine promoted activation of PLD, as shown by a sustained accumulation of phosphatidylethanol. All of these events were blocked by pretreatment of cells with the α_1-adrenergic antagonist prazosin. D609, an inhibitor of phosphatidylcholine hydrolysis, blocked translocation of PKC_α and PKC_ϵ but did not inhibit PLD activation. Unlike results with PMA, or with the P_2 purinergic receptor agonist ATP, epinephrine-stimulated PLD activity was not inhibited in MDCK-D1 cells in which PKC_α expression is attenuated by an antisense cDNA construct or in cells in which PKC activity was inhibited by 1 μM GF 109203X. However, PLD activation by epinephrine was abolished by concomitant incubation of cells with the calcium chelator EGTA. These data, together with previous results, are consistent with the hypothesis that in MDCK-D1 cells, epinephrine acting on α_1-adrenergic receptors, promotes a rapid increase in cytosolic Ca^{2+} that promotes activation of PLD through an as-yet poorly defined mechanism. The data demonstrate that different types of G protein-linked receptors that activate PLD can mediate this activation in either a PKC activation-dependent or -independent manner within a single cell type.

PLD activity is found in a variety of cells and tissues and seems to play a key role in cellular signaling (Liscovitch and Chalifa, 1994). PLD catalyzes the hydrolysis of the terminal phosphodiester bond of phospholipids, yielding phosphatidic acid and a free polar headgroup. Phosphatidic acid may act directly as a signal molecule, or can be metabolized to form diacylglycerol by phosphatidate phosphohydrolase (Martin *et al.*, 1994). The latter can function as an activator of PKC, a key enzyme in cellular signaling (Nishizuka, 1995)

Substantial evidence indicates that PKC activation can lead to PLD activation in response to receptor agonists (Liscovitch and Chalifa, 1994). We have demonstrated the importance of PKC, in particular the α isoform, in regulating PLD activation in MDCK-D1 cells responding to nucleotides or phorbol ester (Balboa *et al.*, 1994). Using different approaches, other researchers have also highlighted the importance of PKC_α in regulating cellular PLD activity (Eldar *et al.*, 1993; Conricode *et al.*, 1994; Lopez *et al.*, 1995).

In the current study, we examined the activation of PLD in MDCK-D1 cells stimulated by another stimulus, the adrenergic receptor agonist epinephrine. Norepinephrine and epinephrine, acting via α_1-adrenergic receptors, have been reported to stimulate PLD in a variety of tissues, including cerebral cortex, parotid gland, aorta, tail artery, hepatocytes, and cardiac myocytes (Gu *et al.*, 1992; Llahi and Fain, 1992; Pittner and Fain, 1992; Jones *et al.*, 1993; Guillemain and Rossignol, 1994; Ye *et al.*, 1994; LaBelle *et al.*, 1996). Although the mechanism involved in this activation is poorly understood, in several of those systems, a role for PKC has been inferred. Because of the previously demonstrated role for PKC_α in PLD activation in MDCK-D1 cells (Balboa *et al.*, 1994), we hypothesized that activation of PKC_α would be required for activation of PLD by epinephrine in MDCK-D1 cells. However, we now report that, although epinephrine

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ABBREVIATIONS: PLD, phospholipase D; MDCK, Madin-Darby canine kidney; PC, phosphatidylcholine; PEt, phosphatidylethanol; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

activates PKC_α, this activation was not associated with an activation of PLD. We also find that epinephrine activates PKC_ϵ, although this isoenzyme also does not seem to be involved in PLD activation. On the other hand, we find that Ca^{2+} is very important for PEt production by epinephrine. Therefore, the current study, along with our previous data (Slivka and Insel, 1987; Slivka *et al.*, 1988), demonstrates that different mechanisms can be utilized by different types of G protein-linked receptors to activate PLD in a single cell type.

Experimental Procedures

Materials. [^{3}H]Palmitic acid (specific activity, 54 Ci/mmol) was purchased from New England Nuclear (Boston, MA). PMA and ATP were from Sigma (St. Louis, MO). Phosphatidylethanol (PEt) and D609 were obtained from Biomol (Plymouth Meeting, PA). PKC antibodies against α and β isoforms were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PKC isoforms δ, ϵ, and ζ, and the PKC inhibitor GF 109203X were purchased from Calbiochem (San Diego, CA). G-60 thin layer chromatography plates were purchased from Whatman (Clifton, NJ). The organic solvents were from Fisher (Pittsburgh, PA).

PLD activation. MDCK-D1 were labeled the day before to 80% confluency with [^{3}H]palmitic acid (2.5 μCi/ml). After removal of labeling medium, cultures were rinsed twice with phosphate-buffered saline (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.6) and equilibrated with serum-free medium (Dulbecco's modified Eagle's medium) containing 1 mg/ml bovine serum albumin at 37° for 3 hr. Cells were incubated with specified concentration of epinephrine for the indicated times (see figure legends) in the presence of 1% ethanol. After stopping the reactions, the lipids were extracted and PEt was separated by thin layer chromatography as described previously (Balboa *et al.*, 1994).

Separation of cytosolic and membrane fractions. Cells were washed twice with Dulbecco's modified Eagle's medium, 20 mM HEPES, 0.05% bovine serum albumin and then treated with 50 μM epinephrine, 300 μM ATP, or 80 nM PMA for the indicated time (see figure legends) at 37°. The cells were washed twice with 4 ml of ice-cold 20 mM Tris, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, 0.1% 2-mercaptoethanol, pH 7.5, and scraped into 4 ml of that buffer. The cell suspension was homogenized with 25 strokes of a Dounce homogenizer. The homogenates were centrifuged at 500 $\times$ *g* for 5 min (4°) to separate nuclei, and the remaining supernatant was centrifuged at 35,000 $\times$ *g* for 35 min to separate soluble and particulate fractions (Slivka *et al.*, 1988). After protein quantification, Western blot analyses were performed on these two fractions.

Western blot analyses. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel (75 μg of protein/lane) and transferred to Immobilon-P (Millipore, Bedford, MA). Nonspecific binding was blocked by incubating the membranes with 5% nonfat milk in phosphate-buffered saline for 60 min. Membranes were incubated with specific antisera against PKC_α or PKC_ϵ isoform for 30 min, and then treated with horseradish peroxidase-conjugated protein A (Amersham, Arlington Heights, IL). Bands were detected by enhanced chemiluminescence (Amersham).

Results

PLD activation has been shown previously to be activated after stimulation of MDCK-D1 cells by P_2-purinergic receptors and phorbol esters (Balboa *et al.*, 1994). In the current study, we investigated whether α-adrenergic receptors on MDCK-D1 cells are also coupled to PLD activation. To this end, [^{3}H]palmitic acid-labeled cells were used and PLD activation was monitored by taking advantage of the unique ability of PLD to catalyze a transphosphatidylation reaction in the presence of ethanol.

Incubation of [^{3}H]palmitate-labeled MDCK-D1 cells with the adrenergic receptor agonist epinephrine in the presence of 1% ethanol resulted in a time-dependent generation of [^{3}H]PEt (Fig. 1A). PEt production increased linearly up to 5 min, and then ceased rapidly. The kinetics of epinephrine-induced PEt accumulation are similar to those observed previously when P_2-purinergic receptor agonists were used to elicit PLD activation (Balboa *et al.*, 1994) and suggest a rapid desensitization of PLD activity by both types of agonists.

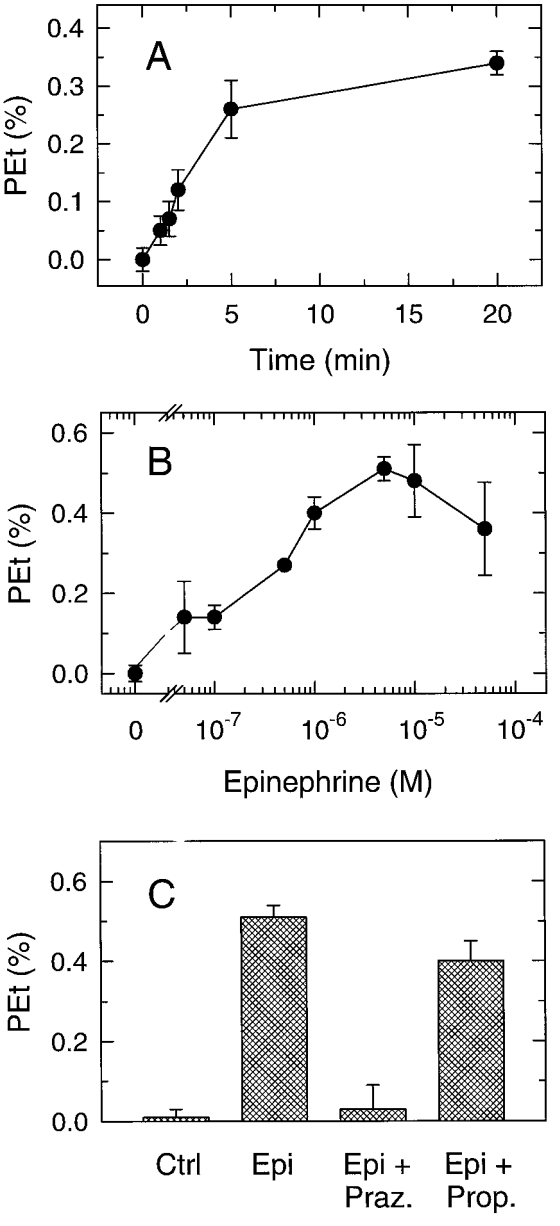

Fig. 1. Characterization of PLD activation by epinephrine in MDCK-D1 cells. A, Time-course of PEt accumulation in response to 5 μM epinephrine. B, Dose-response curve (15-min incubation). C, Effect of the adrenergic antagonists prazosin (*Praz.*) (5 μM; 5-min preincubation before agonist addition) and propranolol (*Prop.*) (5 μM; 5-min preincubation before agonist addition) on epinephrine-induced PEt accumulation (*Epi*) (5 μM). Data are presented as mean $\pm$ standard error of experiments carried out in triplicate and are representative of at least three independent experiments.

PEt accumulation in response to epinephrine was also concentration-dependent, with an EC_{50} value of ~0.5 μM and maximal response at concentrations above 1 μM (Fig. 1B). These concentrations are similar to those observed previously for epinephrine-promoted phosphoinositide hydrolysis, arachidonic acid release, and prostaglandin E_2 formation in MDCK-D1 cells (Slivka and Insel, 1987; Weiss *et al.*, 1989; Howard and Insel, 1990; Weiss and Insel, 1991). To determine which adrenergic receptor subtype was linked to PLD activation, we studied the effects of prazosin, an α-adrenergic antagonist, and propranolol, a β-adrenergic antagonist. Complete inhibition of epinephrine-induced PEt production was found in the presence of prazosin, but not propranolol (Fig. 1*C*). Thus, PLD activation in response to epinephrine is caused by α_1-adrenergic receptor occupancy.

We know that stimulation of α_1-adrenergic receptors increases phospholipase C-mediated phosphoinositide hydrolysis in different cell types, including MDCK-D1 cells (Slivka and Insel, 1987; Howard and Insel, 1990; Weiss and Insel, 1991). To study the relationship, if any, between phosphoinositide hydrolysis and PLD activation in response to epinephrine, we used the aminoglycoside antibiotic neomycin. Neomycin inhibits phosphoinositide-specific phospholipase C activation (Lipsky and Leitman, 1982; Schwertz *et al.*, 1984) and has been used previously in our laboratory to ascertain the relationship between phosphoinositide hydrolysis and PLD activation in response to purinergic stimulation and phosphoinositide hydrolysis and arachidonic acid release in response to other agonists (Slivka and Insel, 1987; Slivka and Insel, 1988). At concentrations found previously to inhibit phosphoinositide-specific phospholipase C in MDCK-D1 cells (Balboa *et al.*, 1994), we failed to detect a significant decrease in epinephrine-stimulated PLD activity by neomycin (Fig. 2A).

We have shown previously that epinephrine increases Ca^{2+} accumulation in MDCK-D1 cells, partly from entry of Ca^{2+} from the extracellular medium (Weiss and Insel, 1991). Therefore, we assessed a role for Ca^{2+} in PLD activation by employing the Ca^{2+} chelator EGTA. Addition of 2 mM EGTA to the incubation medium for 5 min before epinephrine stimulation, markedly inhibited PLD activation (Fig. 2*B*). These data, which are similar to those observed for P_2-purinergic receptor-activated MDCK-D1 cells (Balboa *et al.* 1994), suggest that Ca^{2+} is involved in PLD activation by α_1-adrenergic receptor stimulation (Huang *et al.*, 1992; Balboa *et al.*, 1994). Ca^{2+} is also necessary for observing PLD activity in homogenates from MDCK-D1 cells (Balboa *et al.*, 1995; Balboa and Insel, 1995).

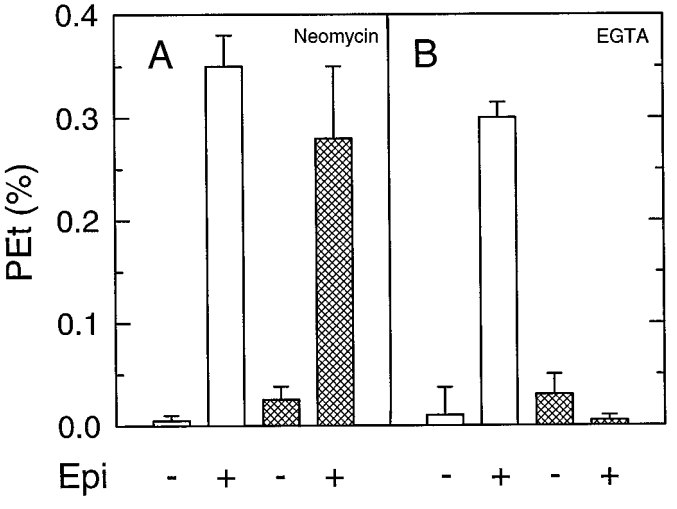

Fig. 2. Effect of neomycin and EGTA on PLD activation by epinephrine in MDCK-D1 cells. [^{3}H]Palmitic acid-labeled cells were preincubated with (▨) or without (□) 1 mM neomycin (A) or 2 mM EGTA (B) for 5 min before a 15-min incubation with 5 μM epinephrine (see *abscissa*). Data are presented as mean ± standard error of three independent experiments carried out in triplicate *Epi*, epinephrine.

Our previous studies (Balboa *et al.*, 1994; Balboa *et al.*, 1995; Balboa and Insel, 1995) have demonstrated an essential role for PKC_α (and Ca^{2+}) in P_2-purinergic stimulation of PLD in MDCK-D1 cells. To investigate whether activation of PKC_α or other PKC isoforms plays a similar regulatory role in α_1-adrenergic stimulation of MDCK-D1 cells, we analyzed the kinetics of translocation of the PKC isoforms present in these cells (α, β, δ, ϵ, and ζ) from the cytosol to membrane fractions in response to epinephrine. The data in Fig. 3 show that addition of epinephrine to the cells resulted in activation of PKC_α, leading to its translocation to the membrane fraction. Translocation of PKC_α was very transient, reaching a maximum 5 sec after addition of epinephrine and disappearing from the membrane after 30 sec of stimulation. (Fig. 3, A and B).

In comparison with results obtained with epinephrine, the pattern of PKC_α translocation in response to treatment of MDCK-D1 cells with the P_2-purinergic agonist ATP was more delayed and sustained (Fig. 3). Maximal translocation of PKC_α to the membrane fraction was found between 30 and 60 sec after addition of ATP, with a slow subsequent decay in translocated enzyme. The behavior of PKC_α in response to ATP showed a good correspondence with the kinetics of PEt accumulation induced by ATP in MDCK-D1 cells (see Fig. 3B in), lending additional evidence in favor of the modulatory role that PKC_α plays in PLD activation under these experimental conditions (Balboa *et al.*, 1994).

PKC_ϵ was also translocated to the membrane fraction in response to epinephrine (Fig. 3, E and F), reaching a maximum at 30 sec and decreasing slowly thereafter. We could not detect translocation of PKC isoforms β, δ, or ζ after epinephrine stimulation (data not shown).

To determine whether translocation of PKC_α and PKC_ϵ by epinephrine was caused by α_1-adrenergic receptors, we performed experiments in the presence of prazosin. As shown in Fig. 4, changes in PKC_α and PKC_ϵ content in the membrane fraction were prevented by pretreatment with 5 μM prazosin, indicating the involvement of α_1-adrenergic receptors.

The very transient nature of PKC_α translocation in response to epinephrine makes it difficult to envision a role for PKC_α activity in epinephrine-mediated activation of PLD. Accordingly, we found a complete dissociation of PKC_α translocation and PLD activation by epinephrine using D609, a purported inhibitor of phospholipase C-mediated breakdown of phosphatidylcholine (Muller-Decker, 1989; Schütze *et al.*, 1992). D609 blocked translocation of PKC_α and PKC_ϵ to the membrane fraction in response to epinephrine (Fig. 5A), but did not substantially inhibit epinephrine-induced PLD activation (Fig. 5B).

Further evidence for the lack of involvement of PKC_α in PLD activation by epinephrine was obtained with the use of cells in which PKC_α levels were attenuated by means of an

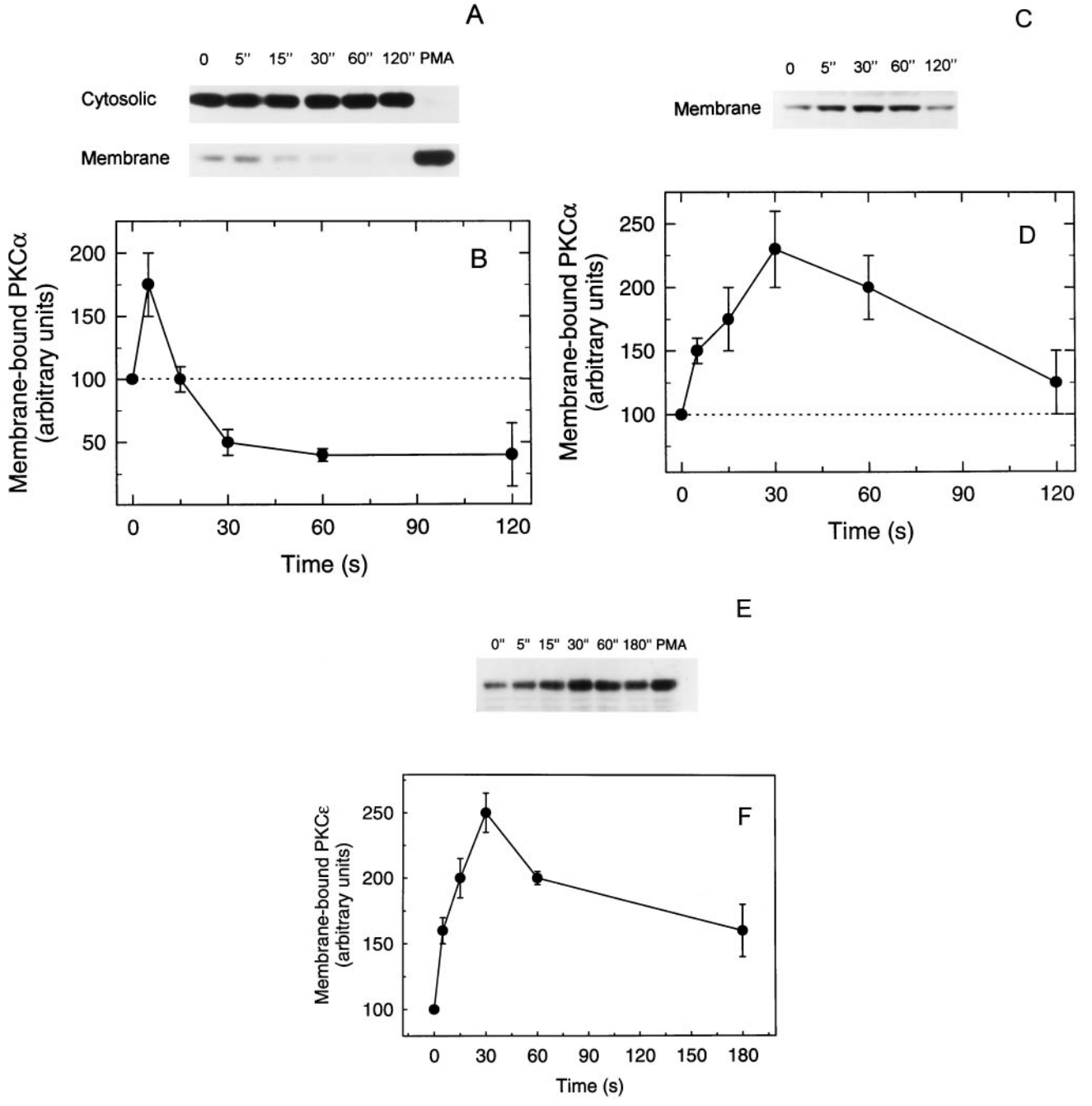

Fig. 3. Effect of epinephrine and ATP on PKC_α and PKC_ϵ translocation to the membrane fraction of MDCK-D1 cells. Cells were treated with 5 μM epinephrine (A, B, E, and F) or 300 μM ATP (C and D) for the indicated times or with 32 nM PMA for 30 min, washed and fractionated as indicated in Experimental Procedures. Seventy-five micrograms of protein from cytosolic or membrane fractions were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels, followed by Western blotting with anti-PKC_α (A, B, C, and D) or anti-PKC_ϵ (E and F) antibodies. A, C, and E, Western blots. B, D, and F, Densitometric quantifications (mean ± standard error from three different blots).

antisense technique (Godson *et al.*, 1993). Using these cells, we have previously demonstrated decreased PLD activation in response to PMA and ATP (Balboa *et al.*, 1994); in contrast, however, the extent of epinephrine-induced activation of PLD in PKC_α depleted cells was similar to that found in wild-type cells (Fig. 6).

We extended our data by using PKC inhibitors. As shown in Fig. 7, 1 μM GF 109203X (a PKC inhibitor able to inhibit PKC_α and PKC_ϵ isoforms) (Martiny-Baron *et al.*, 1993), substantially blunted PEt production in MDCK-D1 cells stimulated by PMA and ATP. However, epinephrine-induced PEt accumulation was not affected by GF 109203X, suggesting a PKC activity independent mechanism of PLD activation.

Discussion

PKC activation constitutes one of the most widespread mechanisms for regulating PLD in eukaryotic cells (Liscovitch and Chalifa, 1994). PKC comprises a family of related enzymes that are differentially expressed in a variety of tissues and cell types (Nishizuka, 1995). At least 10 PKC isoforms have been described; recently, much effort has been

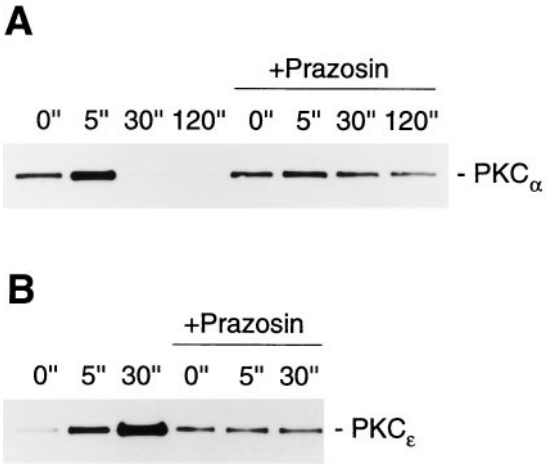

Fig. 4. Effect of prazosin on PKCα and PKCϵ translocation to the membrane fraction by epinephrine in MDCK-D1 cells. Cells were pretreated with 5 μM prazosin 5 min before addition of 5 μM epinephrine for the indicated periods of time. After homogenization and cellular fractionation, 75 μg of protein from membrane fractions were electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels, followed by Western blotting using anti-PKC$_\alpha$ antibodies (A) or anti-PKC$_\epsilon$ antibodies (B).

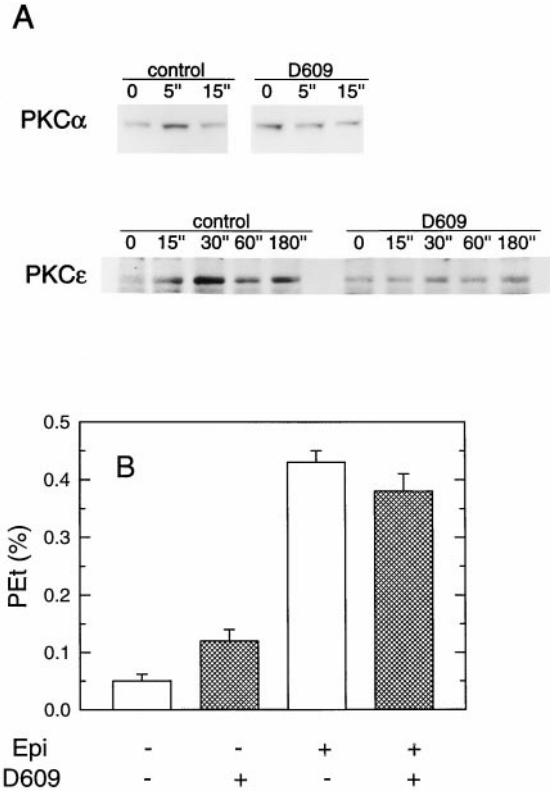

Fig. 5. Effects of D609 on PKC$_\alpha$ PKC$_\epsilon$ translocation and PEt production in epinephrine-stimulated MDCK-D1 cells. Cells were incubated with 50 μg/ml of. D609 for 1 hr before and during stimulation of the cells with 5 μM epinephrine for the indicated periods of time. Afterward, PKC$_\alpha$ and PKC$_\epsilon$ translocation to the membrane fraction was analyzed by Western blot (A). PEt accumulation was also measured (B); results are from triplicate determinations in a representative of two different experiments. Epinephrine-stimulated values were not significantly different between D609-treated and untreated samples ($p > 0.05$ by Student's t test). *Epi*, epinephrine.

directed at defining the role of PKC isoforms in the regulation of various biological processes, including activation of PLD. By reducing the levels of PKC$_\alpha$ by an antisense method,

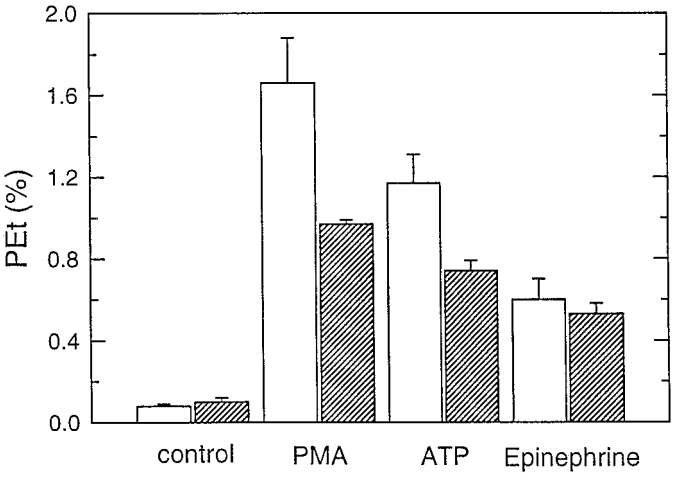

Fig. 6. PEt production in stably-transfected MDCK-D1 cells. Stably transfected cells with antisense oligonucleotides against PKCα (▨) or control cells (□) (transfected with the control vector only) were prelabeled with [^{3}H]palmitic acid and stimulated with 32 nM PMA, 300 μM ATP, 5 μM epinephrine, or medium alone (*control*) in the presence of 1% ethanol for 15 min. PEt accumulation was determined and the results are shown as mean ± standard error of triplicate determinations.

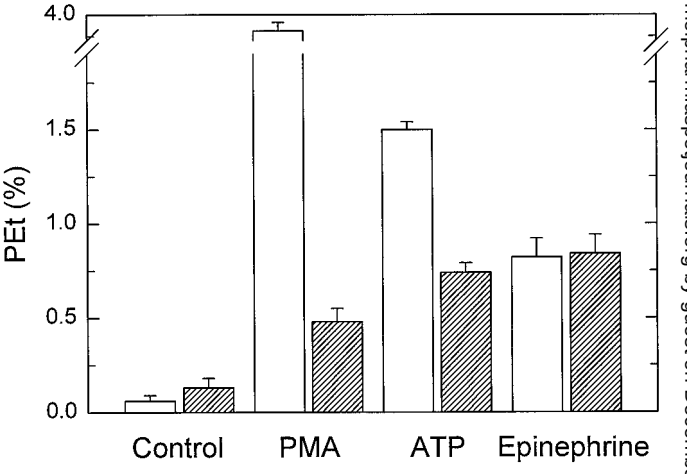

Fig. 7. Effect of GF 109203X on PLD activation by epinephrine in MDCK-D1 cells. [^{3}H]Palmitic acid-labeled cells were preincubated with (▨) or without (□) 1 μM GF 109203X for 30 min before a 15-min incubation with 32 nM PMA, 300 μM ATP, or 5 μM epinephrine (see *abscissa*). PEt accumulation was measured. Data are presented as mean ± standard error of two experiments carried out in triplicate.

we have demonstrated previously that PKC$_\alpha$ regulates P_2-purinergic activation of PLD in MDCK-D1 cells (Balboa *et al.*, 1994). Data in this study have extended this observation by showing a good correspondence between the time-course of translocation of PKC$_\alpha$ to membranes in response to ATP and the time-course of activation of PLD in response to this agonist. However, additional new data implicate a different mechanism for PLD activation in epinephrine-treated cells.

We have reorted previously that epinephrine increases cytosolic Ca^{2+} levels in MDCK-D_1 cells, a response that seems to be highly dependent on extracellular Ca^{2+} (Weiss and Insel, 1991). In this study, we observed that PLD activation by epinephrine is completely abolished by treatment of cells with 2 mM EGTA, suggesting an important role for Ca^{2+} in the response. Ca^{2+} ionophores increase PLD activity in many cellular systems (Billah *et al.*, 1989; Gustavsson *et al.*, 1994; Liscovitch and Chalifa, 1994), including MDCK-D_1

cells (Balboa MA, unpublished observations, 1997), but the mechanism for this increase is uncertain. A highly regulated PLD (PLD_1) was cloned by Hammond *et al.* (1995). The sequence reveals no Ca^{2+}-binding sites, making it difficult to explain a direct role for Ca^{2+} in PLD activation. However, it is possible that a Ca^{2+}-regulated PLD isoform, perhaps present in MDCK-D_1 cells (Huang *et al.*, 1992), has not yet been cloned and sequenced.

Some investigators have implicated Ca^{2+} mobilization in PKC activation, which could subsequently regulate PLD activity (Liscovitch and Chalifa, 1994). Nevertheless, other data have implicated a phosphorylation-independent PKC-mediated activation mechanism of PLD (Conricode *et al.*, 1992; Singer *et al.*, 1996). Such data included studies with PKC inhibitors and the fact that the PKC_α regulatory domain, but not the active kinase domain, was an effective activator for PLD (Singer *et al.*, 1996). In this scenario, Ca^{2+} plays such an important role that, in the absence of Ca^{2+}, PLD is not activated by PKC_α, whereas the presence of Ca^{2+} allows stimulation by PKC_α alone and synergy with low-molecular-weight G proteins, such as ADP-ribosylation factor (Singer *et al.*, 1996). The precise mechanism by which Ca^{2+} promotes PLD activation by α_1-adrenergic receptors in MDCK-D_1 cells is uncertain and will require future study.

MDCK-D $_1$ cells express PKC_α, $_\beta$, $_\epsilon$, $_\delta$ and $_\zeta$ (Martiny-Baron *et al.*, 1993; Balboa MA, unpublished observations, 1997). We have studied PKC translocation as an indication of PKC activation in epinephrine-treated MDCK-D_1 cells. The results show that only PKC_α and PKC_ϵ are translocated to the membrane fraction (Fig. 3). PKC_α is very transiently recruited, and after 15 sec of stimulation, it disappears rapidly from the membrane fraction (Fig. 3). Movement of PKC isoforms off of the membrane to the cytosol fraction has been described before. For example, ceramide has been shown to induce the cytosolic localization of PKC_δ and PKC_ϵ in several human leukemia cell lines; this localization seems to play an important role in ceramide-mediated apoptosis (Sawai *et al.*, 1997). It is possible, therefore, that movement of membrane-bound PKCs to the cytosol may contribute to signal transduction, albeit via mechanisms that are as yet poorly defined.

To further study the relationship between PKC activation and PLD activity, we used the PKC inhibitor GF 109203X (Martiny-Baron *et al.*, 1993). In these studies, GF 109203X, at concentrations that greatly diminished PMA and ATP-mediated activation of PLD, did not decrease epinephrine-promoted accumulation of PEt. From these experiments, along with the studies involving antisense PKC-treated cells, we conclude that PKC activity is not necessary for epinephrine-promoted activation of PLD in MDCK-D_1 cells. Many investigators have used protocols in which many hours of cell treatment with phorbol esters depletes phorbol ester-activatable PKC isoenzymes. Indeed, we used this experimental approach to implicate a role for PKC in PLD activation (Balboa *et al.*, 1994). Unfortunately, because 18-hr treatment of cells with PMA markedly decreases the number of α_1-adrenergic receptors (Yang, M, Buscher R, Taguchi K, Insel PA, and Michel MC. Agonist-induced down-regulation of α_1-adrenergic receptors in MDCK cells, submitted for publication), such a protocol is unsuitable for assessment of the role of PKC in mediating α_1-adrenergic actions in MDCK-D1 cells.

Stimulation of MDCK-D1 cells with epinephrine leads to a very rapid generation of diacylglycerol, the physiological activator of PKC (Slivka *et al.*, 1988). Such a rapid generation of diacylglycerol could account for the rapid translocation of PKC_α and PKC_ϵ to the membrane fraction. Phosphoinositide turnover via phospholipase C is not detectable in epinephrine-stimulated MDCK-D1 cells at early time points (<3 min), but increases in phosphorylcholine levels are readily detectable during the first minutes of cell activation with epinephrine (Slivka *et al.*, 1988). Collectively, these data suggest that shortly after receptor occupancy, epinephrine-promoted PC hydrolysis leads to a rapid burst of diacylglycerol formation that, in turn, promotes PKC translocation to the membrane. D609 has been reported to block PC breakdown (Muller-Decker, 1989; Schütze *et al.*, 1992). D609 was able to completely suppress PKC_α and PKC_ϵ translocation to the membrane. Treatment of cells with D609 also dissociated PKC_α and PKC_ϵ translocation from PLD activation, because the inhibitor did not significantly reduce epinephrine-stimulated PEt accumulation. This result, together with the data obtained with MDCK-D1 cells in which PKC_α expression is inhibited and the studies with the PKC inhibitor GF109203X, provides strong evidence that PLD activation by epinephrine is not linked to activation of PKC_α or PKC_ϵ.

In summary, the current results, along with our previous data (Weiss and Insel, 1991; Llahi and Fain, 1992; Balboa *et al.*, 1994) lend support to a model whereby occupancy of α_1-adrenergic receptors on the surface of MDCK-D1 leads to an early accumulation of PC-derived diacylglycerol, which leads in turn to PKC_α and PKC_ϵ activation. This sequence of events, however, is unrelated to or insufficient to promote activation of PLD activity in epinephrine-stimulated cells. By contrast, in ATP-stimulated or phorbol ester-stimulated cells, PLD activation is downstream of activation of PKC_α. Taken together, the data thus indicate that activation of PLD, in a cellular system, can occur independently of activation of PKC (perhaps by a mechanism involving Ca^{2+} mobilization) and that different types of G protein-linked receptors expressed in a single cell type can activate PLD via both PKC_α activation-dependent and -independent mechanisms.

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