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A Phospholipase D-mediated Pathway for Generating Diacylglycerol in Nuclei from Madin-Darby Canine Kidney Cells*

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Many receptors, in response to their specific ligands, trigger activation of phospholipase D (PLD), resulting in the production of phosphatidic acid which, in turn, is acted upon by a specific phosphatase, phosphatidate phosphohydrolase, to produce diacylglycerol. We report here that isolated nuclei from Madin-Darby canine kidneys (MDCK)-D1 cells exhibit a PLD activity that is enhanced by the presence of ATP. PLD activity was measured in the presence of ethanol, by quantitating the production of phosphatidylethanol. Non-phosphorylating ATP analogs were unable to substitute for ATP in activating PLD, indicating that ATP acts as a phosphoryl group donor in a kinase-mediated phosphorylation reaction. The protein kinase C inhibitors chelerythrine and calphostin completely suppressed the ATP-induced nuclear PLD, implicating protein kinase C as the kinase involved in ATP-dependent PLD activity in nuclei from MDCK-D1 cells. In the absence of ethanol, phosphatidic acid was detected in ATP-treated nuclei. Accumulation of phosphatidic acid preceded or closely paralleled that of diacylglycerol, suggesting a precursor-product relationship. Consistent with those results, we detected phosphatidate phosphohydrolase activity in MDCK-D1 cell nuclei. Measurements of phosphatidic acid and diacylglycerol levels at increasing amounts of ethanol demonstrated that PLD and phosphatidate phosphohydrolase are responsible for generating the majority of the diacylglycerol accumulating in MDCK-D1 cell nuclei. The ability of nuclei to generate diacylglycerol from the concerted action of those two enzymes provides a means to regulate nuclear lipid synthesis as well as protein kinase C activity.

Signal transduction processes are often initiated by the hydrolysis of phospholipids catalyzed by phospholipases at the plasma membrane generating lipid second messengers (1). Activation of phospholipase D (PLD)[1] results in the production of phosphatidic acid (PA), which in turn is acted upon by PA phosphohydrolase to produce diacylglycerol (DAG) (1, 2). Both of these products can serve second messenger functions and the sustained activation of PLD is believed to be the major route for generation of these two lipid messengers in many cells (1–5). DAG through its activation of protein kinase C (PKC) regulates functions in the cell nucleus, in which PKC mediates phosphorylation of regulatory and structural proteins. Moreover, nuclear accumulation of DAG has been observed in a variety of cell systems (6, 7). In addition, PA and its lyso-derivative lysoPA modulate nuclear events, including DNA synthesis (8). It is unclear whether PKC-mediated events in the nucleus result from generation of DAG at the plasma membrane. In the current work, we have tested and confirmed the hypothesis that cell nuclei possess the ability to generate DAG and PA through a PLD and PA phosphohydrolase pathway. These two enzymes, working in concert, are responsible for generating the majority of the nuclear DAG production and therefore probably contribute to regulation of nuclear PKC.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Palmitic acid (specific activity 54 Ci/mmol) and 1,2-dipalmitoyl-[U-^{14}C]glycero-3-phosphate (specific activity 144 mCi/mmol) were obtained from DuPont NEN. Phorbol 12-myristate 13-acetate (PMA), ATP, AMP-PNP, AMP-PCP, herbimycin, and H89 were from Sigma. Chelerythrine was obtained from LC Services (Woburn, MA). Calphostin was from Calbiochem. G-60 thin layer chromatography plates were obtained from either Whatman or Analtech (Newark, DE). The organic solvents were from Fisher.

Growth and Treatments of MDCK-D1 Cells—MDCK-D1 cells were grown as described elsewhere (9). The cells were labeled the day before confluence with [^{3}H]palmitic acid (3 μCi/ml) in Dulbecco's modified Eagle's medium. After removal of labeling medium, cultures were equilibrated with serum-free medium containing 1 mg/ml bovine serum albumin at 37 °C for 45 min. Cells were then incubated at 37 °C with PMA (80 nM) or ATP (300 μM) for 30 min in the presence of 1% ethanol. Reactions were stopped by aspirating the media and adding cold hypotonic buffer (see below). Nuclei were separated at 4 °C as described below, and nuclear PEt content was determined by thin-layer chromatography after extraction of total nuclear lipids (9).

Preparation of Nuclei from MDCK-D1 Cells—The method of York and Majerus (10) was used with slight modification to obtain purified nuclei from MDCK-D1 cells. Cells were labeled with 3 μCi/ml [^{3}H]palmitic acid for 20 h. After this time, the cells were washed twice with cold phosphate-buffered saline, overlaid with a hypotonic buffer consisting of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μM benzamidine, and 10 μM aprotinin (buffer A), and scraped from the plate. Cells were subjected to 15 passes in a Potter-type Teflon-on-glass homogenizer and spun at 500 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in buffer A. The resulting nuclear suspension was layered onto a 200-μl sucrose cushion (50% (w/v)) in buffer A and spun at 15,000 × *g* for 1 min in an Eppendorf centrifuge. The nuclei pelleted through the cushion were resuspended in buffer A. Purity of these nuclei was assessed by electron microscopy and by measuring enzyme marker activities. These nuclei contained less than 7% of the total cellular endoplasmic reticulum enzyme NADPH cytochrome *c* reductase, less than 4% of the total

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[1] The abbreviations used are: PLD, phospholipase D; DAG, diacylglycerol; MDCK, Madin-Darby canine kidney; PA, phosphatidic acid; PEt, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; AMP-PNP, adenosine 5′-*O*-(β,γ-imino)triphosphate; AMP-PCP, adenosine 5′-*O*-(β,γ-methylene)triphosphate.

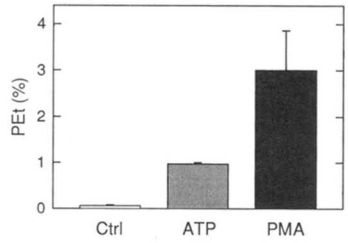

FIG. 1. **Accumulation of PEt in isolated nuclei from MDCK-D1 cells.** Intact MDCK-D1 cells were stimulated with ATP (300 μM) (*gray bar*), PMA (80 nM) (*black bar*), or neither (*white bar*) for 30 min in the presence of 1% ethanol. After extraction, PEt was separated by thin-layer chromatography. Results are shown as means ± S.E. from three different experiments with triplicate determinations and are expressed as a percentage of radioactivity in PEt relative to total radioactivity in nuclear phospholipids.

cellular cytosolic marker lactate dehydrogenase, and less than 1% and 3% of the total cellular light membrane (including plasma membrane) markers 5′-nucleotidase and alkaline phosphatase, respectively. All of these enzyme markers were assayed as described elsewhere (11). Enzyme marker activities were also assayed in nuclear preparations from activated cells (80 nM PMA, or 300 μM ATP for 30 min), and the values obtained were similar to those observed for nuclei from unstimulated cells.

Recovery of marker enzyme activities in nuclear fractions was substantially less than was observed for PLD activity (12.0 ± 1.5% of the total cellular activity), assessed using [^{14}C]phosphatidylcholine as exogenous substrate under the conditions described by Huang *et al.* (12). This indicates that nuclear-associated PLD activity does not appear to arise from contamination from other cellular compartments.

Phospholipids comprised 63 ± 4% of the ^{3}H radioactivity present in nuclear lipids, the remaining being incorporated into neutral lipids. ^{3}H radioactivity in phospholipids was distributed among major phospholipid classes as follows: phosphatidylcholine, 37 ± 1%; phosphatidylethanolamine, 18 ± 1%; phosphatidylinositol/phosphatidylserine, 5 ± 1.

Phospholipase D Activity Assay—Nuclear fractions (up to 50 μg of protein) were incubated in Olson's buffer (25 mM Hepes, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 μM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 μM benzamidine, 10 μM aprotinin, pH 7.4) (13) at 37 °C along with the indicated ATP concentration and 1.5% ethanol (285 mM). Final volume was 200 μl. Total lipids were extracted as described previously (14), and PEt was resolved by thin layer chromatography on Silica Gel G plates using the upper phase of a system consisting of ethyl acetate/isooctane/acetic acid/water (130:20:30:100, by volume) (9). When inhibitors were used, they were added at the indicated concentrations for 30 min prior to and during the incubations. PEt production is expressed as the percentage of radioactivity in PEt compared with the total radioactivity in nuclear phospholipids.

PA Phosphohydrolase Activity Assay—PA phosphohydrolase activity was determined as described by Day and Yeaman (15) with slight modification. The substrate [^{14}C]glycerol-labeled PA was delivered as mixed micelles with Triton X-100 at a detergent/phospholipid mole ratio of 10:1. Assays were conducted at 37 °C. The incubation mixture contained in a final volume of 0.1 ml: 100 μM [^{14}C]PA substrate (0.025 μCi/assay), 1 mM Triton X-100, 50 mM Tris-HCl (pH 7.1), 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and the indicated amount of nuclear protein. After the indicated times the reaction was stopped and [^{14}C]PA and [^{14}C]DAG were separated by thin layer chromatography as described elsewhere (13).

RESULTS AND DISCUSSION

The widely used method for detection of PLD is based on the formation of PEt, a product that is generated from PLD by a transphosphatidylation reaction when ethanol is present (16). By measuring PEt, we have recently characterized the activation of PLD by P_2 purinergic receptors and by phorbol ester in MDCK-D1 cells (9). In the course of assessing the intracellular distribution of PEt generated upon stimulation of MDCK-D1 cells with PMA or ATP, we have found accumulation of PEt in isolated nuclei from activated cells, but not in nuclei from unstimulated cells (Fig. 1). Cellular stimulation by phorbol ester and ATP caused respectively a 50- and 16-fold increase in

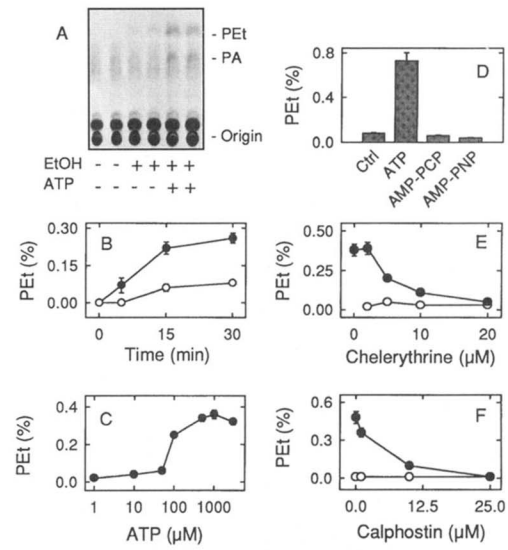

FIG. 2. **ATP-regulated PEt production in nuclei from MDCK-D1 cells.** *A*, autoradiography of phospholipids from MDCK-D1 cell nuclei after TLC separation. *B*, time course of PEt accumulation in the presence (●) or absence (○) of 500 μM ATP. *C*, concentration response of the ATP effect measured at 30 min. *D*, effect of non-hydrolyzable ATP analogs on PEt production. Effect of chelerythrine (*E*) or calphostin (*F*) on PEt production in the absence (○) or presence (●) of 500 μM ATP. Results shown are given as means ± S.E. from triplicate determinations in representative experiments. Each set of experiments was repeated at least three different times with similar results.

nuclear PEt levels as compared to unstimulated cells. Nuclear PEt production represented 19 ± 5% and 17 ± 4% of total cellular PEt production in response to PMA and ATP, respectively.

Inasmuch as PEt appears to be a metabolically inert phospholipid and it is the exclusive product of a PLD activity, its accumulation in a given compartment would suggest the presence of a PLD in such a compartment. Therefore, preparations of isolated nuclei were tested for PLD activity. Nuclei were isolated without using detergents, in order to achieve nuclear envelope integrity, which, in turn, would serve as a source for labeled phospholipid substrate in our assay. When nuclei from [^{3}H]palmitate-labeled cells were incubated at 37 °C in the presence of 1.5% ethanol, a product co-migrating with authentic PEt was formed (Fig. 2*A*). These results demonstrate the presence of a PLD activity associated with the isolated nuclei. Importantly, substantial PEt production could only be measured if ATP was present in the incubation medium. ATP promoted accumulation of nuclear PEt in a time- and concentration-dependent manner (Fig. 2, *B* and *C*). The non-phosphorylating adenine trinucleotides AMP-PNP and AMP-PCP were unable to substitute for ATP in activating PLD (Fig. 2*D*), thus strongly suggesting that ATP acts as a phosphoryl group donor in a kinase-mediated phosphorylation reaction.

In searching for the kinase involved in ATP-dependent PLD activation in MDCK-D1 nuclei, we employed a number of well established kinase inhibitors, including chelerythrine and calphostin, herbimycin, and H89, selective inhibitors of PKC, protein tyrosine kinase, and cAMP-dependent protein kinase, respectively. The two PKC inhibitors, chelerythrine and calphostin, were able to completely suppress the ATP-induced nuclear PLD (Fig. 2, *E* and *F*), whereas herbimycin and H89 were ineffective (data not shown). These results implicate PKC as the kinase involved in ATP-dependent PLD activity in nuclei from MDCK-D1 cells. In keeping with this finding, Western blot analyses of nuclear preparations revealed the presence of the PKC isoforms α, β, and ϵ associated with nuclei from MDCK-D1 cells (data not shown). PKCα has been demon-

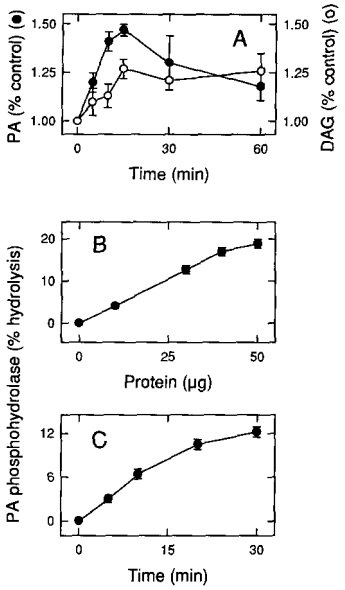

FIG. 3. **Dynamics of PA and DAG production in nuclei.** *A*, time course of PA (●) and DAG (○) accumulation in ATP-treated nuclei. *B*, effect of protein concentration (10-min incubation), and *C*, effect of time (10 μg of protein), on PA phosphohydrolase activity in nuclei. Results are given as means ± S.E. from triplicate determinations in single representative experiments. Each set of experiments was repeated at least three times with similar results.

strated to play a critical role in phorbol ester- and receptor-promoted activation of PLD in MDCK-D1 cells (9). Further proof for the involvement of PKC in regulating nuclear PLD was obtained by directly adding PMA to the nuclear preparations. Inclusion of PMA (80–250 nM) increased by 1.6 ± 0.2-fold the ATP-activated PLD activity of MDCK-D1 cell nuclei.

It has recently been suggested that ATP potentiates PLD activity in permeabilized cells (17, 18) because it is required for phosphoinositide kinase to synthesize phosphatidylinositol 4,5-bisphosphate, a phospholipid that has been demonstrated to increase PLD activity in cell-free systems (17, 19). However, the fact that the specific PKC inhibitors calphostin and chelerythrine completely suppress the ATP-dependent PLD activity of preparations of MDCK-D1 nuclei argues strongly against the possibility that increase in synthesis of phosphatidylinositol 4,5-bisphosphate is the mechanism whereby ATP promotes PLD activation in MDCK-D1 cells. Instead, our data support a model for nuclear PLD activation whereby the major ATP-requiring step is nuclear-associated PKC.

In the absence of ethanol, PA was detected in ATP-treated nuclei with a time dependence of accumulation similar to that seen for PEt (Fig. 3*A*). Accumulation of PA preceded or closely paralleled that of DAG, suggesting a precursor-product relationship. This could occur if MDCK-D1 cell nuclei contained a PA phosphohydrolase, the enzyme activity that converts PA into DAG. Aliquots of the nuclear fraction were assayed for their ability to convert exogenous PA into DAG. Fig. 3 (*B* and *C*) shows that this conversion occurred in a time- and protein concentration-dependent manner, demonstrating association of PA phosphohydrolase with MDCK-D1 cell nuclei.

In order to quantitate how much of the nuclear DAG accumulation was derived directly from the PA produced by the ATP-activated PLD, measurements were conducted in the presence of increasing amounts of ethanol, since ethanol diverts PLD activity from PA formation. Consequently, in the presence of ethanol, DAG levels derived from PLD action should decrease. As shown in Fig. 4, increasing concentrations of ethanol increased formation of PEt in parallel with decreases

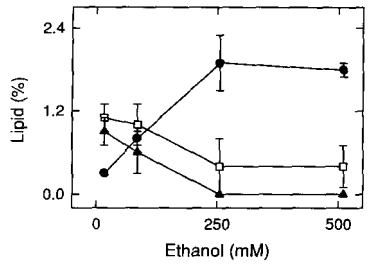

FIG. 4. **Accumulation of PEt (●), PA (□), and DAG (▲) in the presence of increasing ethanol concentrations.** Nuclei from [^{3}H]palmitic acid-labeled MDCK-D1 cells were incubated as described under "Experimental Procedures" in the presence of the indicated ethanol concentrations. Results are given as means ± S.E. from triplicate determinations in a single experiment, which is representative of three different ones.

in PA and DAG. At saturating concentrations of ethanol, at which PEt formation reaches a plateau, DAG production was completely abolished. This result is consistent with the conclusion that the majority of DAG accumulating in nuclei derives from the ATP-regulated PLD activity.

In recent years, increasing evidence has accumulated to indicate that nuclei have very active lipid metabolism which may play a crucial role in nuclear function (reviewed in Ref. 20). A nuclear phosphoinositide cycle entirely separate from that operating in the plasma membrane has been recently elucidated (21, 22). Activation of this cycle by plasma membrane receptors leads to transient increases in nuclear inositide-derived DAG (21, 22). Our data suggest an alternative and novel mechanism for increases in nuclear DAG: the sequential actions of PLD and PA phosphohydrolase endogenous to or closely associated with the cell nucleus. The ability of PKC to activate nuclear PLD suggests the existence of a signaling pathway whereby plasma membrane receptors activate one or more forms of PLC (or PLD) thereby promoting activation of PKC, and PKC, in turn, promoting activation of nuclear-associated PLD. This novel signaling pathway located in the nucleus appears to be responsible for the major portion of the DAG generated by nuclei. This DAG could be utilized both for activation of PKC as well as for synthesis of lipids, assuming accessibility to other enzymes required for lipid synthesis

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