Nuclear Phospholipase D in Madin-Darby Canine Kidney Cells

GUANOSINE 5'-O-(THIOTRIPHOSPHATE)-STIMULATED ACTIVATION IS MEDIATED BY RhoA AND IS DOWNSTREAM OF PROTEIN KINASE C*

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We have recently demonstrated the existence of an ATP-activated phospholipase D (PLD) in the nuclei of MDCK-D1 cells (Balboa, M. A., Balsinde, J., Dennis, E. A., and Insel, P. A. (1995) J. Biol. Chem. 270, 11738–11740). We have now found that nuclear PLD is synergistically activated by guanosine 5'-O-(thiotriphosphate) (GTPγS) and ATP in a time- and concentration-dependent manner, but these compounds do not alter the sensitivity of the enzyme to activation by Ca2+. The synergistic stimulation of PLD activity could be blocked by addition of the protein kinase C inhibitors chelerythrine and calphostin C. Stimulation by GTPγS was ablated by guanosine 5'-O-(2-thiodiphosphate). Incubation of isolated nuclei with Clostridium botulinum C3 exoenzyme inhibited the potentiating effect of GTPγS on ATP-dependent nuclear PLD activity. Moreover, use of the Rho GDP dissociation inhibitor to extract Rho family G proteins from cell nuclei also inhibited PLD activity. Western blot analyses of isolated nuclei revealed the presence of the small G protein RhoA, but not of RhoB or the ADP-ribosylation factor. GTPγS-stimulated ATP-dependent PLD activity could be reconstituted in Rho GDP dissociation inhibitor-washed nuclei by addition of recombinant prenylated RhoA, but not by addition of non-prenylated RhoA. Taken together, these results indicate that nuclear PLD activity is modulated via a RhoA-dependent activation that occurs downstream of protein kinase C. Nuclear PLD, which appears to be a previously unrecognized effector regulated by protein kinase C and G proteins, may be involved in the regulation of nuclear function or structure.

The detailed mechanisms by which agonists that bind to cell-surface receptors regulate nuclear events are inadequately understood. A number of studies have provided compelling evidence for a regulatory role of protein kinase C (PKC) in agonist-regulated effects on nuclear function (1, 2). One mechanism for PKC action involves translocation of PKC to the nucleus triggered by increased accumulation of nuclear diacylglycerol (DAG) (1, 3). The elucidation of a phosphoinositide cycle located at the nucleus has suggested a means by which extracellular stimuli might elicit nuclear responses via generation of DAG (reviewed in Ref. 4). In addition, nuclear DAG may arise from phospholipids other than the phosphoinositides, e.g. phosphatidylcholine (5).

Phospholipase D (PLD) catalyzes the hydrolysis of cellular phospholipids, particularly phosphatidylcholine, in response to a variety of hormones, neurotransmitters, and growth factors (6). Phosphatidic acid (PA), the primary lipid product of PLD, appears to possess growth factor-like properties and can act as a second messenger in certain cell types (7, 8). Since PA can be dephosphorylated via PA phosphohydrolase to produce DAG, this represents an alternative pathway to that initiated by phosphoinositide-specific phospholipase C for increasing cellular DAG levels (9). While DAG production by phosphoinositide hydrolysis occurs in an early and transient manner, DAG production through the PLD pathway can be delayed and prolonged, thereby allowing a sustained activation of PKC (10, 11). PLD activity can be regulated by multiple types of signals (6). Both heterotrimeric and low molecular weight G proteins have been implicated in PLD activation. Recent evidence suggests a key role for the involvement of the low molecular weight G proteins ADP-ribosylation factor (ARF) and RhoA in agonist-induced PLD activation in certain cell types (12–15). Evidence for the involvement of heterotrimeric G proteins is less well established and relies primarily on the inhibition of PLD activation produced by treatment of several types of cells with pertussis toxin (16, 17).

We have recently described that nuclei from MDCK-D1 cells possess a PKC-regulated PLD activity that seems to account for the bulk of DAG generated in the nucleus (18). In the present work, we demonstrate that nuclear PLD activity is enhanced by nonhydrolyzable GTP analogs, in particular by GTPγS, and that this response to GTPγS is mediated by the low molecular weight G protein RhoA. Our data suggest a novel mechanism for regulation of nuclear PLD that involves the sequential actions of protein kinase C and RhoA.

EXPERIMENTAL PROCEDURES

Materials—[3H]Palmitic acid (specific activity of 54 Ci/mmol) was obtained from DuPont NEN. ATP, GTPγS, and GDPβS were from Sigma. Chelerythrine was obtained from LC Services (Woburn, MA). Calphostin C was from Calbiochem. Rho antisera were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ARF monoclonal antibody was generously provided by Dr. J. Moss (National Institutes of Health, Bethesda, MD). RhoGDI was kindly provided by Dr. G. Bokoch (The Scripps Institute, La Jolla, CA). Bacterially expressed recombinant RhoA (non-prenylated) and S9 fraction-expressed RhoA (prenylated) were a generous gift from Drs. I. López and D. Lambeth (Emory University, Atlanta, GA). G-60 thin-layer chromatography plates were obtained from either Whatman or Analtech Inc. (Newark, DE). The organic solvents were from Fisher.

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Preparation of Nuclei from MDCK-D1 Cells—Our standard procedure for preparing nuclear fractions from MDCK-D1 cells has previously been published in detail (18). Briefly, [3H]palmitic acid-labeled cells (3 \muCi/ml; 20 h) were washed twice with cold phosphate-buffered saline and then placed in a hypotonic buffer consisting of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 \muM benzamidine, and 10 \muM 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 \times 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-\mul sucrose cushion (50%, w/v) in buffer A and spun at 35,000 \times g for 1 min in an Eppendorf centrifuge. The nuclei pelleted through the cushion were resuspended in buffer A. Nuclei prepared in this manner were immediately used for assay of PLD activity as described below.

Phospholipase D Activity Assay—Nuclear fractions (up to 50 \mug of protein) were incubated in Olson’s buffer (25 mM HEPES, 100 mM KCl, 3 mM NaCl, 5 mM MgCl_2, 1 mM CaCl_2, 1 mM phenylmethylsulfonyl fluoride, 10 \muM benzamidine, 10 \muM aprotinin, pH 7.4) (19) at 37 °C along with the indicated ATP and/or GTP-S concentrations and 1.5% ethanol for 30 min. The final volume was 200 \muL. Total lipids were extracted, and phosphatidylethanol (PEt) was resolved from cellular lipids 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). When inhibitors were used, they were added at the indicated concentrations for 30 min prior to and during the incubations. For experiments where Clostridium botulinum C3 exoenzyme was used, the nuclear preparations (0.5 mg of nuclear protein/ml) were treated with 10 \mug/ml C3 exoenzyme (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) for 30 min at 37 °C (20) in a buffer consisting of 20 mM HEPES, pH 8, 1 mM MgCl_2, 0.5 mM NAD^+, and 0.5 mM thymidine, and toxin-incubated nuclei were used in subsequent experiments. Free Ca^{2+} concentrations in the assays were calculated with the computer program Free Ca (21).

RhoGDI-treated nuclei were prepared as described previously (15). Isolated nuclei were incubated with the indicated concentration of RhoGDI for 15–20 min at room temperature. The nuclei were centrifuged at 12,000 rpm for 1 min at 4 °C, and supernatants were kept free for further analysis. When RhoA proteins were included, as indicated, nuclei were incubated for 5 min before initiation of the assay.

Western Blot Analysis—Samples were separated by SDS-15% polyacrylamide gel electrophoresis (150 \mug of protein/lane) and transferred to Immobilon-P (Millipore Corp., Bedford, MA). Non-specific binding was blocked by incubating the membranes with 5% nonfat milk in phosphate-buffered saline for 60 min. Membranes were incubated with antisera or monoclonal antibodies for 30 min and then treated with horseradish peroxidase-conjugated protein A or mouse antiserum (Amersham Corp.). Bands were detected by enhanced chemiluminescence (Amersham Corp.).

Statistical Analysis—Student’s paired t test was used as statistical analysis, and significance was considered as p < 0.05.

RESULTS

GTP-S Potentiates ATP-stimulated Nuclear PLD Activity—We have previously demonstrated the existence of a PKC-regulated PLD activity in nuclei from MDCK-D1 cells (18). As an initial approach to determine the possible regulation of nuclear PLD activity by G proteins, we studied the effects of the nonhydrolyzable guanine nucleotide GTP-S on this activity. Nuclear fractions from MDCK-D1 cells were incubated with GTP-S, ATP, or both in the presence of 1.5% ethanol. Under these conditions, detection of PLD is based on the formation of PEt, a product that is generated from PLD by a transphosphatidyltransferase reaction when ethanol is present (6). Consistent with our previous data (18), addition of ATP (500 \muM) increased PLD activity by 10-fold as compared with incubations in the absence of ATP. When GTP-S was added together with ATP, PEt production was further increased to a level ∼20–30-fold above controls (Fig. 1). In contrast, GTP-S alone, in the absence of ATP, was nearly ineffective (Figs. 1 and 2). Thus, ATP and GTP-S gave a synergistic enhancement of nuclear PLD activity. The effect was also observed when we used Gpp(NH)p, another nonhydrolyzable GTP analog, instead of GTP-S (Table I). Moreover, GDP⋅PS (100 \muM), a well recognized inhibitor of GTP-mediated events on G proteins, abolished the synergistic effect of GTP-S on ATP-induced PEt accumulation (Fig. 1). Together, these data strongly suggest the involvement of G proteins in the regulation of nuclear PLD activity.

A further characterization of the potentiating effect of GTP-S on ATP-induced PLD activation was carried out, and the results are shown in Fig. 2. When the ATP concentration was held constant (500 \muM), addition of GTP-S resulted in a concentration-dependent increase in PEt levels up to 1 \muM, with half-maximal effects at ∼30 nm GTP-S (Fig. 2A). The effect of varying the ATP concentration at a constant GTP-S concentration (10 \muM) is shown in Fig. 2B. Optimal PLD activation under these conditions was achieved at ATP concentrations above 100 \muM. GTP-S appeared to increase the apparent affinity of ATP for activation of PLD since at an ATP concentration of 50 \muM, which was by itself nonstimulatory, the presence of GTP-S induced substantial PEt production (Fig. 2B).

Ca^{2+} has been reported to be required for G protein-dependent activation of PLD (13, 19, 22). As shown in Fig. 2D, increasing the free Ca^{2+} concentration in the PLD assay from levels found in resting cells (0.1 \muM) to those attained in stimulated cells (1 \muM) (23) increased PLD activity under all experimental
Ca<sup>2+</sup>-conditions, of ATP or GTP potentiating effect of GTP of nuclear PLD activity to Ca<sup>2+</sup> presence of 1 mM EDTA. Increasing the Mg<sup>2+</sup> effect of varying the free Ca<sup>2+</sup> in unstimulated cells. Addition of ATP alone or together with 

### Determinations of nuclear PLD activity

Experiments were carried out as explained in the legend to Fig. 1. A, concentration response of the GTPγS effect in the presence (●) or absence (○) of 500 μM ATP; B, concentration response of the ATP effect in the presence (●) or absence (○) of 10 μM GTPγS; C, time course of nuclear PET accumulation; D, effect of varying the free Ca<sup>2+</sup> concentrations on nuclear PET production in the absence (○) or presence of 10 μM GTPγS (●), 500 μM ATP (△), or 10 μM GTPγS plus 500 μM ATP (▲). The incubations in the absence of added Ca<sup>2+</sup> received 2 mM EGTA. [3H]PET formation is expressed as percentage of radioactivity in PET with respect to total nuclear phospholipids in each point. Data 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.

**Fig. 2. Characterization of the GTPγS effect on PET production in nuclei from MDCK-D1 cells.**

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**Fig. 3. Effect of PKC inhibitors on PET production by MDCK-D1 cell nuclei.**

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Rho proteins can be ADP-ribosylated and inhibited by the C3 exoenzyme of C. botulinum (24). To address the possible role of Rho proteins in the regulation of nuclear PLD activity, we treated MDCK-D1 cell nuclei with C. botulinum C3 exoenzyme (10 μg/ml; 30 min). Such a treatment resulted in ~75% inhibition of PET production by GTPγS plus ATP, suggesting a role for Rho in regulating nuclear PLD activation (Fig. 4B). To obtain further evidence for a role of Rho in the regulation of nuclear PLD activity, we treated MDCK-D1 cell nuclei with RhoGDI, a protein that interacts with post-translationally modified Rho proteins and extracts them from membranes (25, 26). When nuclei were treated with increasing concentrations of RhoGDI and subsequently washed, nuclear PLD became progressively less responsive to GTPγS (Fig. 5A). Western blot analysis confirmed that RhoA was extracted from nuclei and appeared in the wash after RhoGDI treatment (Fig. 5B). Thus, the inhibition of GTPγS-stimulated nuclear PLD activity by RhoGDI was associated with a decrease in RhoA in the nuclear preparations (Fig. 5, A and B).

In the next series of experiments, we used recombinant RhoA to investigate whether addition of this protein to the assay mixture could restore GTPγS-stimulated PLD in RhoGDI-treated nuclei. When prenylated RhoA was added to RhoGDI-treated nuclei, PET production in response to GTPγS was almost fully restored (Fig. 6). As a control, we also used non-prenylated RhoA, which was not able to reconstitute the response (Fig. 6). This finding is consistent with the notion that small G proteins from the ras family that are not modified post-translationally do not interact efficiently with GDP/GTP exchange factors (27).

### Identification of the G Protein Regulating Nuclear PLD Activity

Low molecular weight G proteins of the ras superfamily, namely members of Rho (RhoA) and ARF subfamilies, have been implicated in PLD activation (12–14). Analysis by Western blotting revealed the presence of RhoA, but not ARF or RhoB, in nuclear preparations from MDCK-D1 cells. However, all of these proteins could be detected in cell homogenates (Fig. 4A).
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DISCUSSION

G proteins are widely recognized to play an essential role in signal transduction from many types of cell-surface receptors to effector proteins in the plasma membrane (23). Evidence is emerging to suggest that G proteins may also play a similar role in transducing signals in the nucleus, perhaps at the nuclear membrane. In fact, both heterotrimeric and low molecular weight G proteins have been demonstrated to be localized in the nucleus (Fig. 5B) (28). We have recently demonstrated the existence of a PKC-regulated PLD activity in nuclei from MDCK-D1 cells (18). In the present work, we have demonstrated that activation of a G protein provides another means of regulating nuclear PLD activity.

The nuclear PLD activity found in MDCK cells differs appreciably from other PLDs on the basis of its activation characteristics. Several authors have described PLD activities in cell-free systems that are not stimulated by ATP unless phorbol 12-myristate 13-acetate or GTP-γ-S is also present in the incubation buffer (19, 29). This is different from what we observed with nuclear PLD activity from MDCK-D1 cells, in which ATP can activate enzyme activity by acting as a phosphoryl donor apparently by a PKC-driven phosphorylation reaction (18). The data in the present study suggest that activation by PKC is required for GTP-γ-S to increase nuclear PLD activity (Fig. 3). This implies that in nuclei from MDCK-D1 cells, PLD regulation by G proteins must follow protein phosphorylation by PKC.

In resting cells, PKC is present in the cytosol, and after stimulation of cells, PKC translocates to the particulate fraction. However, it has been observed that individual PKC isoenzymes appear to be restricted to particular intracellular loci before stimulation. Thus, colocalization of some PKC isoenzymes with their substrates might serve to ensure preferential and rapid phosphorylation of these substrates after PKC activation. It has been suggested that isoenzymes of PKC bind to specific anchoring proteins, collectively termed receptors for activated protein kinase C, located at various subcellular sites (30). It is possible that these proteins are involved in targeting PKC to the nucleus. The precise substrate(s) of phosphorylation by PKC in the cascade of events leading to activation of nuclear PLD is unclear. Possible substrate(s) include G proteins that directly interact with PLD (perhaps increasing interaction of the G proteins with the lipase and/or increasing the rate of GDP/GTP exchange (31)), an intermediate protein factor such as a GTase-activating protein (32) or a GDP dissociation factor (33), and/or PLD itself (perhaps allowing interaction
with G proteins.

Based on the inhibitory effects that C. botulinum C3 exoenzyme and RhoGDI exert on activation of ATP-dependent PLD by GTPγS, our results support the involvement of a member of the Rho family of small G proteins in the regulation of nuclear PLD. More direct evidence in favor of this notion was obtained with the experiments in which addition of recombinant RhoA to RhoGDI-treated nuclei restored GTPγS-activated PLD. A Rho protein has also been implicated in PLD regulation in the studies by Bowman et al. (14) in neutrophils and by Malcolm et al. (15) in rat liver. However, in neither of these studies could the authors show PLD inhibition by C. botulinum C3 exoenzyme. The Rho family of low molecular weight G proteins is composed of three variants of Rho proteins (A, B, and C) and two forms of Rac proteins (Rac1 and Rac2) (34). C. botulinum C3 exoenzyme specifically inhibits the Rho proteins, but not the Rac proteins. This fact led Bowman et al. (14) to exclude Rho as a candidate activator of neutrophil PLD, but the possibility that in those studies, C. botulinum C3 exoenzyme did not completely inhibit Rho or that the modified form still remained active was not ruled out. Moreover, even though C. botulinum C3 exoenzyme failed to inhibit PLD activity in liver plasma membranes, Malcolm et al. (15) also achieved reconstitution of GTPγS-activated PLD activity by RhoA, thereby suggesting that RhoA regulates PLD activity in liver.

Other studies have implicated ARF as a low molecular weight G protein that regulates PLD activity (12, 14). Since ARF appears to be absent from MDCK-D1 cell nuclear preparations (but is present in homogenates), we believe it is unlikely that this G protein is a regulator of nuclear PLD activity in these cells. However, it is possible that other PLD activities of MDCK-D1 cells, distinct from that present in nuclei, might be regulated by ARF, especially since MDCK cells, as well as other cell types, demonstrate multiple PLD activities (35–37).

Collectively, the present results, along with our previous data (18), indicate that nuclear PLD may be regulated via multiple cellular constituents, acting in sequence. The data strongly suggest that a PKC-driven phosphorylation reaction is required for the subsequent ability of RhoA to activate nuclear PLD. PA, the primary product of PLD action on phospholipids, has been shown to be mitogenic for certain cell types (8). An intriguing but speculative idea is that products of nuclear PLD may help regulate binding of certain PKC isotypes to the nuclear membrane. In this regard, PA has been observed to specifically bind and activate PKC-ζ, a PKC isotype suggested to play a key role in mitogenesis and a number of nuclear events (38, 39). This raises the possibility that PA generated by PLD in the nucleus increases PKC-ζ binding and function in this cellular compartment. In addition, it is also possible that activation of nuclear PLD alters lipid composition and lipid-dependent functional activities in the nucleus.

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