Nuclear Translocation of RhoA Mediates the Mitogen-induced Activation of Phospholipase D Involved in Nuclear Envelope Signal Transduction*

(Received for publication, August 21, 1996, and in revised form, December 4, 1996)

Joseph J. Baldassare†, Matt B. Jarpe‡§, Lisa Alferes§, and Daniel M. Raben¶

From the †Department of Pharmacology and Physiological Science, St. Louis University School of Medicine, St. Louis, Missouri 63104 and the ‡Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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In this paper we demonstrate for the first time a mitogen-induced activation of a nuclear acting phosphatidylcholine-phospholipase D (PLD) which is mediated, at least in part, by the translocation of RhoA to the nucleus. Addition of α-thrombin to quiescent IIC9 cells results in an increase in PLD activity in IIC9 nuclei. This is indicated by an increase in the α-thrombin-induced production of nuclear phosphatidylethanolamine in quiescent cells incubated in the presence of ethanol as well as an increase in PLD activity in isolated nuclei. Consistent with our previous report (Wright, T. M., Willenberger, S., and Raben, D. M. (1992) Biochem. J. 285, 395–400), the presence of ethanol decreases the α-thrombin-induced production of phosphatidic acid without affecting the induced increase in nuclear diglyceride, indicating that the increase in nuclear PLD activity is responsible for the effect on phosphatidic acid, but not that on diglyceride. Our data further demonstrate that RhoA mediates the activation of nuclear PLD. RhoA translocates to the nucleus in response to α-thrombin. Additionally, PLD activity in nuclei isolated from α-thrombin-treated cells is reduced in a concentration-dependent fashion by incubation with RhoGDI and restored by the addition of prenylated RhoA in the presence of guanosine 5′-3-O-(thio)triphosphate. Western blot analysis indicates that this RhoGDI treatment results in the extraction of RhoA from the nuclear envelope. These data support a role for a RhoA-mediated activation of PLD in our recently described hypothesis, which proposes that a signal transduction cascade exists in the nuclear envelope and represents a novel signal transduction cascade that we have termed NEST (nuclear envelope signal transduction).

It is now clear that a PLD1 is activated as a component of a number of signal transduction pathways (2–7). Cleavage of PC by a PLD results in the production of a free, water-soluble choline head group, and PA. Although in some systems this PA is the source of increased DG levels generated via PA phosphohydrolase, it is now becoming clear that PA itself plays important signaling roles (3, 7–14). There is evidence, for example, implicating the PLD-mediated production of PA as an important component of the mitogenic cascade (3, 9, 10).

We recently advanced the hypothesis that a novel nuclear lipid metabolism is a component of unique nuclear signaling cascades that we defined as nuclear envelope signal transduction (NEST) (15, 16). The canonical model of lipid-mediated signal transduction assumes that all induced lipid metabolism occurs at the plasma membrane and that the nuclear envelope is a passive participant in the transduction cascade. In the NEST hypothesis, just as the plasma membrane serves as the communication link between the extracellular environment and the cytoplasm, the nuclear envelope mediates the transmission of cytosolic signals to the nucleoplasm. Recently, our laboratory and others have presented compelling data supporting this hypothesis (15–21).

Previous work from our laboratory demonstrated that PC metabolism is a component of NEST (15, 18). One of the PC-hydrolyzing enzymes, PLD, has been identified in the nucleus of Madin-Darby canine kidney cells (19–21), and further studies indicated that this activity may be modulated by RhoA (21). These data suggest that a nuclear PLD is present in these cells, and its activity can be modulated by known signal transduction components. Clearly, a central tenet of the NEST hypothesis is that the enzymatic activities involved in this cascade are induced in an agonist-dependent manner. Such an agonist-induced nuclear activity has not been demonstrated. The data in this report are the first to demonstrate an agonist-induced increase in a nuclear PLD activity. This activity contributed to the production of nuclear PA but does not affect the level of nuclear DG generated in response to α-thrombin. RhoA translocates to the nucleus in response to α-thrombin, and removal of this GTP-binding protein with a RhoGDI results in a dose-dependent decrease in nuclear PLD activity. Taken together, the data demonstrate that the addition of α-thrombin to quiescent fibroblasts leads to the translocation of RhoA to the nucleus, which mediates the activation of a nuclear PLD.

**EXPERIMENTAL PROCEDURES**

*Materials—Cell culture media were from Life Technologies, Inc. Tissue culture dishes were from Falcon. Bovine serum albumin, highly purified human α-thrombin, butylated hydroxytoluene, EGTA, EDTA, quinacrine, 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate, tetraphenylboron, and Trizma base (Tri) were obtained from Sigma. Human transferrin was from Calbiochem. Phospholipase C (Bacillus cereus), aprotinin, and leupeptin were from Boehringer Mannheim. Silica Gel G TLC plates were from Analtech. DG standards were generated by PC-PLC (B. cereus)-mediated hydrolysis of commercial PC, PA, or PE.**
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(22, 23), which were purchased from Avanti Polar Lipids. Aconitirile (high performance liquid chromatography grade) was from J. T. Baker. Isopropyl ether was from Aldrich. Diethyl ether (high purity) and chloroform, methanol, acetone, and hexane (all GC) were from Burdick and Jackson. All organic solvents contained 50 μg/ml butyrolactone. RhoGDI synthesized as a GST fusion protein (plasmid a generous gift from Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA) in an Escherichia coli expression system was isolated using a glutathione column (24). Palmitoylated RhoA containing a histidine tag (plasmid a generous gift from Dr. Alan Hall, MRC Laboratory of Cellular and Molecular Biology, University College London, London WC1E 6AX, U.K.) was expressed in SB cells and purified using a nickel affinity column (25). GTP-SyG was from Boehringer Mannheim. Anti-RhoA antibodies were purchased from Santa Cruz (SC-179G). Radiolabels were purchased from Amersham Corp.

Cells and Cell Culture—IIC9 cells, a subclone of CHEF18, were grown and serum deprived as described previously (1, 15, 17, 18, 22, 23, 26–29). Briefly, IIC9 cells were grown in 150-mm dishes for 3 days in α-MEM/Ham’s F-12 containing 5% fetal calf serum. The medium was removed and replaced with serum-free Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin and supplemented with 5 μg/ml human transferrin (serum-free medium). The cells were serum deprived for 2 days and then washed twice in serum-free medium. They were incubated at 37 °C in the fresh serum-free medium for at least 30 min before beginning the experiment. For each experiment, cells were then incubated at 37 °C in serum-free medium either alone or containing 1 NIH unit/ml α-thrombin in the presence or absence of ethanol as indicated in the figure legends.

Isolation of IIC9 Nuclei Lipid Analysis—Nuclei were isolated essentially as described previously (15, 18). Briefly, incubations were terminated by removal of medium, transferring the dishes immediately to an ice bath and adding 4 ml of ice-cold fractionation buffer (buffer B: 10 mM Tris, 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, 20 μM quinacrine, and 200 μM 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate, pH 7.5 at 4 °C). The cells were scraped from the dishes and subjected to 15 passes in a Potter type Teflon on glass homogenizer. Homogenates from four dishes were used for quantification of DG levels. Homogenation and subsequent steps were carried out at 4 °C.

Nuclei were isolated by centrifugation of the homogenate at 2,000 rpm (700 × g) for 7 min in an RT6000B centrifuge with a swinging bucket rotor. The pellet was dispersed in 5 ml of fractionation buffer and homogenized using a Dounce-type homogenizer with a tight fitting (type B) pestle for 20 passes and layered over a 5-ml cushion of 45% sucrose in fractionation buffer, followed by centrifugation at 2,800 rpm (1,660 × g) for 30 min. The pellet was resuspended in 0.8 ml of buffer B, and a small aliquot was assessed quickly for gross contamination by whole cells and other large debris by light microscopy.

For nuclear lipid analysis, isolated nuclei (typically 50 μg of nuclear protein) were suspended in 0.8 ml of water and transferred into 1 ml of chloroform. The centrifuge tube was washed twice with 1 ml of methanol, and the wash was added to the water and chloroform. Nuclear lipids were extracted (30) and dried under a stream of dry nitrogen.

All other assays, including in vivo assay for PLD, analysis of PA levels, analysis of DG levels, in vitro assay for PLD, treatment of nuclei with RhodDI, Western blot analysis, were performed as described in the figure legends. Protein was quantified as described by Bradford (33).

RESULTS

α-Thrombin-induced Activation of Nuclear PLD—As shown in Fig. 1, PEt in nuclei from cells exposed to α-thrombin in the presence of ethanol was approximately a 2-fold higher than it was in nuclei isolated from cells exposed to either alone. These data are consistent with the notion that α-thrombin induced the activation of a PLD, which catalyzes the hydrolysis of nuclear PC to PA.

The above data demonstrating an activation of PLD acting on the nucleus implies that α-thrombin-induced increase in nuclear PA should be blunted in the presence of ethanol. Indeed, α-thrombin induced an increase in nuclear PA. Radiolabeled PA as a percentage of total labeled nuclear lipid was 0.233 ± 0.072 in quiescent cells and 0.427 ± 0.034, n = 4, after incubation of cells for 15 min with α-thrombin (1 NIH unit/ml). In the presence of 1% ethanol, the increase in PA induced by α-thrombin was only 49%, significantly less than the 82% increase induced by α-thrombin without ethanol. These data are consistent with the data presented in Fig. 1 demonstrating the activation of a PLD, which acts on the nuclear membrane, and they indicate that this PLD is responsible for most of the PA generated in the nucleus in response to α-thrombin.

Nuclear PLD Is Not Involved in the Induced Production of Nuclear DGs—In previous reports, we demonstrated in whole cells that although α-thrombin induced the activation of a PLD resulting in the formation of PA, a PC-PLC was responsible for the generation of PC-derived DGs (1). We also demonstrated that α-thrombin induced an increase in nuclear DGs in IIC9 cells and that these DGs are derived from PC (1, 15, 17, 18). Because the presence of ethanol inhibited the formation of PA but not DGs even though approximately 50% of the whole-cell DGs induced by α-thrombin reside in the nucleus (15), it is unlikely that the nuclear DGs are derived by a PLD/PA-phosphohydrolase pathway.

To test this directly, we evaluated the effect of ethanol on the generation of nuclear DGs in response to α-thrombin. As shown in Fig. 2, the presence of ethanol does not significantly affect the production of α-thrombin-induced nuclear DGs generated in radiolabeled cells. Similar results have been obtained when the nuclear DG mass is quantified using the DG kinase assay (29 and data not shown). These data demonstrate that the induced nuclear PLD is not involved in the generation of nuclear DGs. In view of previously published data demonstrating that these DGs are derived from PC (15, 18), the present data implicate a PC-PLC in the production of these lipids.

α-Thrombin-modulated Nuclear Acting PLD Is Associated with the Nucleus—In view of the above, it was important to determine if the PLD activated in response to α-thrombin was a membrane-associated enzyme. We examined, therefore, the PLD activity in nuclei isolated from quiescent and α-thrombin-induced cells. As shown in Fig. 3, PLD activity was increased maximally in the nuclei isolated from α-thrombin-stimulated cells after a 15- and 20-min exposure to α-thrombin. The data
are consistent with the notion that this enzyme is not involved in the production of nuclear DGs as the PLD activity was elevated well after the major increase in nuclear DGs occurred (15, 18). These data demonstrate that a PLD activated in response to α-thrombin is associated with the nucleus.

α-Thrombin Induces the Translocation of RhoA to the Nucleus—There is now strong evidence to suggest that small molecular weight GTP-binding proteins, RhoA in particular, are involved in activating PLD (21). RhoA-mediated PLD activity requires that RhoA be constitutively present in nuclei or translocate to the nucleus in an agonist-induced manner. Western blot analysis of proteins in nuclei isolated from quiescent and α-thrombin-induced cultures demonstrated that RhoA translocates to the nucleus in response to α-thrombin (Fig. 4).

Extraction of RhoA from Nuclei Decreases Induced Nuclear PLD Activity—To investigate further the role of RhoA, nuclei isolated from α-thrombin-induced cultures were treated with RhoGDI, and the level of PLD activity was quantified. As shown in Fig. 5, treatment of these nuclei with RhoGDI resulted in a concentration-dependent decrease in nuclear PLD activity. Because this GDI can interact with several members of the Rho family, released protein was examined by Western blot analysis. Only RhoA was found to be released (data not shown). Addition of recombiant, prenylated RhoA, in the presence of GTPγS, restored the activity in the RhoGDI-treated membranes (Fig. 6). Interestingly, this RhoA also activates a PLD activity in nuclei isolated from quiescent cells (Fig. 6), suggesting that the enzyme resides in the nucleus. These data provide strong evidence indicating a role for RhoA in the α-thrombin-induced activation of a nuclear PLD.

DISCUSSION

The canonical model of signal transduction cascades involves the initiation of signals at the plasma membrane which stimulate specific cascades leading to the stimulation of activities in target organelles such as the nucleus. For some time it has been assumed that the nuclear envelope played a passive role in these signal transduction cascades. It is becoming increasingly clear, however, that the nuclear envelope is an active participant in signaling cascades, a process we have termed NEST, and that a major component of these cascades is the induction of specific nuclear lipid metabolism (15–21).

In this report we present the first evidence for the involvement of a PC-PLD in NEST and identify one of the components involved in coupling the canonical plasma membrane signaling cascades to this novel pathway in the nuclear envelope. Our data demonstrate that the addition of a potent mitogen, α-thrombin, to quiescent IIC9 cells results in increased nuclear PLD activity. This is evidenced by the α-thrombin-induced increase in PET (Fig. 1) and the increased nuclear PLD activity in nuclei isolated from α-thrombin-induced cultures (Fig. 3). We further demonstrate that RhoA is at least one of the factors involved in this activation. RhoA translocates to the membrane in response to α-thrombin, and treating nuclei isolated from α-thrombin-induced cultures with RhoGDI results in a dose-dependent decrease in PLD activity (Figs. 4 and 5). Taken together, these data suggest that the addition of α-thrombin to quiescent IIC9 cells induces the translocation of RhoA to the nucleus resulting in the stimulation of nuclear PLD.

FIG. 2. Effect of ethanol on α-thrombin-induced nuclear DGs. Cells were grown, serum starved, radiolabeled with [3H]myristate, incubated with α-thrombin and ethanol, and nuclei were isolated as described in under "Experimental Procedures" (15, 18) and resuspended in the reaction mixture (total reaction volume of 500 μl) was incubated at 37°C for 1 h, and the released water-soluble headgroups were separated from vanadate) at 4°C. 400 Ci/m mole DG were isolated by TLC and quantified by liquid scintillation counting (1). DG radioactivity was expressed as percentage of that in total in nuclear lipids, which was 8.7 ± 0.8 × 10^6 cpm, 10.0 ± 0.9 × 10^6 cpm, 9.4 ± 0.8 × 10^6 cpm, and 8.2 ± 0.9 × 10^6 cpm for control, ethanol, and thrombin plus ethanol, respectively. Data are means ± S.E. from at least six experiments.

FIG. 3. PLD activity in isolated nuclei. Quiescent cells (open symbols) or cells treated with 1 NIH unit/ml α-thrombin (closed symbols) were incubated for the indicated times at 37°C. Nuclei were isolated as described in under "Experimental Procedures" (15, 18) and resuspended in assay buffer (50 mM HEPES, pH 7.2, 2 mM EDTA, 0.5 mM EGTA, 5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM orthovanadate) at 4°C. 400 μl of nuclei (0.14 mg of protein) was incubated with 100 μl of a Triton X-100 (6.25 mM), phosphatidyl[methyl-3H]choline (2.25 μM at 25 μCi/μmol) mixed micelle (3.1, Triton X-100:PC). The reaction mixture (total reaction volume of 500 μl) was incubated at 37°C for 1 h, and the released water-soluble headgroups were separated by ion pairing with tetraphenylboron (31, 32) and quantified by liquid scintillation counting. Data are means ± S.E. from at least three experiments.

FIG. 4. α-Thrombin-induced translocation of RhoA to the nucleus. Growth-arrested IIC9 cells were incubated in serum-free medium with or without α-thrombin (1 NIH unit/ml). After 15 min at 37°C, the nuclei were isolated as described in under "Experimental Procedures." Isolated nuclei were resuspended in sodium dodecyl sulfate-sample buffer and protein (50 μg) separated by electrophoresis in 9% polyacrylamide gels (35) and transferred to Immobilon-P by electroblotting. The blot was incubated overnight in wash buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.01% Tween 20) containing 5% dry milk as described (36) followed by washing and incubation for 1 h at room temperature with anti-RhoA antibodies. After washing and incubation for 0.5 h at room temperature with anti-IgG-horseradish peroxidase conjugate, the blot was then developed using chemiluminescence detection (Amersham). Band intensities were quantified by scanning laser densitometry using a Molecular Dynamics laser densitometer (34). The data are representative of at least three experiments. C, cytosol; N, nuclei.
by α-thrombin, which hydrolyzes PC, resulting in the production of PA in the nuclear envelope. Our data also support the notion that a PC-PLC is involved in the generation of nuclear DGs (15). If a PC cycle were operating in the nucleus, enzymes involved in the biosynthesis would be expected to be present in the nucleus. Indeed, one of the enzymes involved in PC biosynthesis, CTP:phosphocholine cytidylyltransferase, has also been localized in the nucleus (40–42). This enzyme is particularly interesting as it often serves as the regulatory enzyme in PC biosynthesis, and its activity is regulated by diacylglycerol (43, 44). Taken together, these data provide strong support for the hypothesis that mitogens activate a PC cycle in the nuclear envelope as a component of NEST.

**Acknowledgments**—We thank Dr. Carolyn Machamer for helpful discussions and critically reading this manuscript.

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