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Novel Function of Phosphatidylinositol 4,5-Bisphosphate as a Cofactor for Brain Membrane Phospholipase D*

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The activation of phospholipase D (PLD) is a receptor-mediated event that has been implicated in signal transduction and membrane traffic in eukaryotic cells. Little is known about the biochemical and molecular properties of signal-activated PLDs, and none has been isolated. Here we report that phosphatidylinositol 4,5-bisphosphate (PIP2) potently stimulates brain membrane PLD activity in vitro in a highly specific manner. PIP2 increases 10-fold the maximal activity of a partially purified PLD with an EC50 of <0.5 mol %. Other acidic phospholipids, including phosphatidylinositol 4-phosphate, phosphatidylinositol, phosphatidylserine, and phosphatidic acid, are completely or nearly ineffective. Neomycin, a high affinity ligand of PIP2, inhibits membrane-bound PLD but has no effect on the activity of a detergent-solubilized or partially purified enzyme. The addition of PIP2 restores the sensitivity of partially purified PLD to neomycin inhibition, indicating that neomycin blocks membrane PLD activity by binding to endogenous PIP2. These results define a novel function of PIP2 as a cofactor for brain membrane PLD and suggest that PIP2 synthesis and hydrolysis could be important determinants in regulating PLD activity in signal transduction and membrane transport.

Phosphatidylinositol 4,5-bisphosphate (PIP2) is a quantitatively minor, highly acidic phospholipid that serves as a precursor for three second messengers: inositol 1,4,5-trisphosphate (1) and diacylglycerol (2), both produced by phosphoinositide-specific phospholipases C (3), and phosphatidylinositol 3,4,5-trisphosphate, generated by a phosphoinositide 3-kinase (4). The mobilization of these signaling pathways by cell surface receptors is often accompanied by activation of phospholipase D (PLD). PLD activation has been implicated in a novel signal transduction pathway employed by a wide variety of extracellular signal molecules in eukaryotic cells (5, 6). The activation of PLD is regulated by G protein(s) and protein kinase(s). Recent results have implicated PLD activation also in intracellular membrane traffic (7, 8). However, there is little information on the biochemical and molecular properties of signal-activated PLDs, and, to date, none of the mammalian PLDs has been isolated or cloned (reviewed in Ref. 6).

We have previously shown that neomycin and other aminoglycosides inhibit brain membrane PLD activity, as well as GTPγS-induced activation of PLD in permeabilized neural derived NG108-15 cells (9). Because neomycin binds with high affinity to negatively charged phospholipids such as phosphoinositides, we suggested that acidic phospholipids may act as essential cofactors for PLD activity (9). The evidence provided herein indicates that PIP2 functions as such a cofactor.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylinositol 4,5-bisphosphate was obtained from U. S. Biochemical Corp. and Sigma. 1-Phosphatidyl-2-[3H,N-7-nitrobenzo-2-oxa-1,3-diazol-4-yl]aminocaproyl-phosphatidylcholine (C12-NBD-PC) was from Avanti Polar Lipids. The chemical synthesis of dipalmitoylphosphatidylinositol 3,4,5-trisphosphate will be described in detail elsewhere. Other phospholipids, sodium oleate, and neomycin were from Sigma.

Purification and Assay of Rat Brain Membrane PLD—Rat brain membranes were prepared and treated with a high salt concentration (to remove peripheral membrane proteins) according to Danin et al. (10). These membranes were then utilized in measurements of membrane-bound PLD activity and for solubilization and purification of PLD. PLD activity was solubilized as described previously (10), except that Triton X-100 (1%, w/v) was utilized as the solubilizing detergent. The solubilized PLD activity was further purified by chromatography on Q-Sepharose, reactive dye-agarose, and hydroxyapatite columns. Details of the purification procedure will be published elsewhere. The activity of both membrane-bound and partially purified PLD was assayed utilizing C12-NBD-PC as a fluorescent substrate. The basic PLD reaction mixture (125 μl) contained 50 mM Na-Hepes, pH 7.2, and C12-NBD-PC (0.3 μM). Membrane-bound PLD activity was determined by measuring C12-NBD-phosphatidylpropanol (C12-NBD-PPr) production (via trans-phosphatidylation) in the presence of 1-propanol (1%, v/v) as a substrate, sodium oleate (0.45 μM, 0.5 mol %) as an activator, and 12.5 μg of membrane protein. Partially purified PLD activity was determined by measuring either C12-NBD-phosphaticid acid (C12-NBD-PA) or C12-NBD-PPr production, in the presence of Triton X-100 (1%) and 2.5 μg of partially purified PLD. PIP2 other activators, and neomycin were added to the reaction mixtures as indicated in the figures. Reactions were carried out for 10 min at 37°C. Termination and TLC separation of the products were performed essentially as described (10). The PLD products were visualized on a UV transilluminator, and a video image was captured on a UV Gel Documentation System. The bands corresponding to C12-NBD-PA and/or C12-NBD-PPr were scraped from the plates and extracted with methanol. The fluorescence of the methanol extracts was determined in a Hitachi F3000 spectrofluorometer (excitation at 485 nm and emission at 520 nm). Unless otherwise indicated, PLD activity was expressed as the amount (in fluorescence units) of C12-NBD-PA or C12-NBD-PPr produced in 10 min.

β,γ-imino)triphosphate; AMP-PCP, adenosine 5'-O-(β,γ-methylene)-triphosphate.

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Neomycin is a cationic aminoglycoside antibiotic that exhibits high binding affinity for inositol phospholipids but not for choline phospholipids (12-14). Yet, neomycin and related aminoglycosides potently inhibit brain membrane PC-specific PLD activity assayed in vitro in the presence of a detergent activator (sodium oleate) as well as G protein-mediated activation of PLD in permeabilized NG108-15 cells (9). We have therefore postulated that neomycin inhibits PLD activity by interaction with an essential membrane cofactor for PLD. The high affinity of aminoglycosides for inositol lipids and, specifically, for PIP<sub>2</sub> raised the possibility that the postulated membrane cofactor is PIP<sub>2</sub>. This hypothesis was examined by testing the effects of PIP<sub>2</sub> on the activity of a partially purified PLD in comparison with other acidic phospholipids. PIP<sub>2</sub> dramatically stimulated PLD activity, causing a 6- and 8-fold increase in PLD hydrolytic and trans-phosphatidylation activity, respectively (Fig. 1). In contrast, phosphatidylinositol 4-phosphate, phosphatidylinositol, phosphatidylserine, and PA were completely or nearly ineffective, indicating that the action of PIP<sub>2</sub> on PLD is highly specific.

The dependence of PLD on PIP<sub>2</sub> concentration was examined in mixed micellar systems containing either C<sub>6</sub>-NBD-PC/Triton X-100 (65.35 mol/mol) or Triton X-100/C<sub>6</sub>-NBD-PC (77.23 mol/mol), with increasing concentrations of PIP<sub>2</sub>. Fig. 2 shows the results obtained with C<sub>6</sub>-NBD-PC/Triton X-100 (65.35 mol/mol). The activity of PLD was stimulated by PIP<sub>2</sub> in a dose-dependent manner, with a half-maximal effect at a concentration (EC<sub>50</sub>) of 0.2-0.4 mol % and a maximal 10-fold stimulation at a PIP<sub>2</sub> concentration of 6 mol %. At a higher concentration (18 mol %) the stimulatory effect of PIP<sub>2</sub> was somewhat diminished. Essentially identical results were obtained with the standard system of Triton X-100/C<sub>6</sub>-NBD-PC (77.23, mol/mol) (Fig. 3), indicating that the effect of PIP<sub>2</sub> is independent of the physical nature of the micellar system. Fig. 3 also shows that phosphatidylinositol 3,4,5-trisphosphate, which is produced from PIP<sub>2</sub> by a phosphoinositide 3-kinase (4), can activate PLD with about the same potency as PIP<sub>2</sub> but with somewhat lower efficacy. Since phosphatidylinositol 3,4,5-trisphosphate is more negatively charged than PIP<sub>2</sub>, the results suggest that the activation of PLD by PIP<sub>2</sub> is not a simple charge effect. Given that the levels of phosphatidylinositol 3,4,5-trisphosphate in membranes are substantially lower than those of its precursor, PIP<sub>2</sub>, the possibility that PLD is activated by phosphatidylinositol 3,4,5-trisphosphate, subsequent to activation of phosphoinositide 3-kinase, seems unlikely.

Neomycin (1 μM) inhibits the activity of the membrane-bound PLD (assayed in the presence of sodium oleate, without exogenously added PIP<sub>2</sub>) by ~75% (Fig. 4A). This result confirms our previous observations obtained with [3H]IP<sub>3</sub> as a PLD substrate. However, as shown in Fig. 4, B and C, solubilization and further purification of brain membrane PLD result in a nearly complete loss of the inhibitory effect of neomycin on both basal (Fig. 4B) and oleate-activated PLD activity (Fig. 4C). This observation is consistent with our previous suggestion that neomycin does not interact with PLD directly (9). Rather, it indicates that neomycin interacts with a membrane cofactor that is essential for optimal PLD activity and that has been removed during the purification process. The inhibitory effect of neomycin, lost upon the solubilization and purification of PLD, can be restored if PLD activity is assayed in the presence of PIP<sub>2</sub>. As shown in Fig. 4D, neomycin inhibits PIP<sub>2</sub>-stimulated PLD activity in a dose-dependent manner, causing 50% inhibition at a concentration (IC<sub>50</sub>) of 90 μM, in very good agreement with its IC<sub>50</sub> on PLD in membrane preparations (65 μM) (9). This result indicates that neomycin inhibits PLD activity in membranes and permeabilized cells by binding to endogenous membrane PIP<sub>2</sub>.

The present data demonstrate that exogenous PIP<sub>2</sub> is a specific and potent activator of PLD. The specificity of PIP<sub>2</sub> action is evidenced by the ineffectiveness of other acidic phospholipids, including the closely related phosphatidylinositol and phosphatidylinositol 4-phosphate. Furthermore, the action of PIP<sub>2</sub> is evident at physiological concentrations. Sulpice et al. (15)
have shown recently that the concentration of PIP₂ in erythrocyte membranes is ~1% of total phospholipids. Assuming that PIP₂ is asymmetrically distributed and is present predominantly in the inner leaflet of the membrane, the surface concentration of PIP₂ in that leaflet will approach 2 mol%. At this concentration the activation of PLD by PIP₂ was nearly maximal. Thus, the concentration-response relationship for activation of PLD by PIP₂ is in excellent agreement with the proposal that PIP₂ acts as a PLD cofactor.

The fact that exogenous PIP₂ stimulates PLD activity does not in itself prove that endogenous PIP₂ serves as a cofactor for PLD. Evidence that this is indeed the case is provided by the experiments with neomycin. Whereas the activity of the membrane-bound PLD is inhibited by neomycin, the solubilized and partially purified enzyme is virtually insensitive to the aminoglycoside. This result shows that neomycin does not directly interact with PLD but, rather, with a cofactor. The cofactor must be (i) membrane-associated; (ii) dissociable following solubilization with a non-ionic detergent; (iii) a high affinity ligand of neomycin; and (iv) required for optimal PLD activity. PIP₂ satisfies these criteria. Further evidence that neomycin inhibits membrane-bound PLD by interaction with endogenous membrane PIP₂ is provided by the fact that it inhibits with a very similar potency the membrane-bound PLD and the purified PLD stimulated with exogenous PIP₂ (Ref. 9 and Fig. 4D, respectively). Moreover, the IC₅₀ values for neomycin inhibition of PLD (65 and 90 μM) are comparable with that obtained for inhibition of PIP₂ hydrolysis by phosphoinositide-specific phospholipase C (150 μM; Ref. 16) and are in excellent agreement with the binding affinity of neomycin to PIP₂ (11–46 μM; Ref. 17). We conclude that PIP₂ acts as a cofactor that is required for maximal PLD activity. It should be noted that, under in vitro conditions, PIP₂ is not absolutely required for detecting PLD activity (e.g. Fig. 4, B and C). Presumably the presence of detergents (Triton X-100, sodium oleate) can substitute in part for the PIP₂ requirement. In addition, while PIP₂ would normally act as a cofactor, it may also assume the role of a PLD activator under specific conditions (see below).

The present results shed new light on the effect of MgATP on PLD activation in cell-free systems. Although MgATP is not absolutely required, it greatly potentiates the activation of PLD by GTP-S in a variety of experimental systems (18–24). Significantly, the non-phosphorylating analogs of ATP, AMP-PNP and AMP-PCP, are incapable of potentiating PLD activation, suggesting that MgATP acts as a phosphoryl group donor in a kinase-mediated phosphorylation reaction. The kinase involved in MgATP action has not been identified. Protein kinase C (21), tyrosine kinase(s) (23, 24), and a Ca²⁺-calmodulin-dependent kinase (22) were proposed as mediators of this effect. The results presented herein raise the possibility that MgATP potentiates PLD activation in permeabilized cells, because it is required for the action of phosphoinositide kinases, i.e. phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase, in maintaining sufficient PIP₂ for optimal PLD activity. This possibility is consistent with the above evidence, indicating that PIP₂ acts as a cofactor for brain membrane PLD and as our own, previous, observation that MgATP inhibits GTP-S-induced activation of PLD in permeabilized cells (9). It is also consistent with recent results showing that inclusion of PIP₂ in mixed PC/phosphatidylethanolamine liposomes greatly potentiates the activation of granulocyte PLD by the small G protein ADP-ribosylation factor (ARF) (25). Studies indicating that on-going PIP₂ synthesis is indeed critical for PLD activation in permeabilized cells are currently in progress.²

ARF is involved in multiple membrane transport processes, where it promotes vesicle coat protein assembly and vesicle budding from donor membranes (see Ref. 7 for review). The identification of ARF as an activator of PLD in myeloid cells (25, 26) strongly implicates PLD and its biologically active product, PA, in vesicular trafficking. The function(s) of PLD and PA in vesicular traffic are, however, currently unknown. Intriguingly, evidence that polyphosphoinositide synthesis may be important for membrane transport events is also emerging (27). A phosphatidylinositol transfer protein (the SEC14 gene product) is required for constitutive secretion in yeast (28, 29), as well as for neurotransmitter release in PC12 cells (30). Another yeast membrane traffic mutant, VPS34, involved in protein sorting to vacuoles, was identified recently as a phosphatidylinositol 3-kinase (31). In addition, phosphatidylinositol 4-kinase activity is localized in secretory granules from adrenal chromaffin cells (32, 33), in coated vesicles (34), and in glucose

transporter-carrying (GLUT 4) vesicles (35). Thus, the activation of PLD by ARF and the biosynthesis of PIP2 may act in concert in a general mechanism for membrane vesiculation and/or fusion. This view is supported by recent studies showing that PA can dramatically stimulate the activity of the type I phosphatidylinositol 4-phosphate 5-kinase, an enzyme that produces PIP2 (36, 37).

The ability of PIP2 to activate PLD and the ability of PA to activate phosphatidylinositol 4-phosphate 5-kinase suggest a model in which the formation of PA and PIP2 by PLD and phosphatidylinositol 4-phosphate 5-kinase, respectively, participates in a positive feedback loop that may play an important role in vesicle fusion with acceptor membranes (Fig. 5). According to this model, the GTP-bound form of ARF induces the assembly of coated vesicles on donor membranes and subsequent fusion (38). Transport vesicles are likely to be enriched with phosphatidylinositol 4-phosphate because they carry phosphatidylinositol 4-phosphate 5-kinase activity (32–35). The interaction of coated vesicles bearing ARF-GTP with acceptor membranes will activate PLD associated with these membranes, producing PA (25, 26). The activity of phosphatidylinositol 4-phosphate 5-kinase, which is hypothesized to be located at acceptor membranes, will be stimulated by PA resulting in massive synthesis of PIP2 from vesicular phosphatidylinositol 4-phosphate. This, in turn, will cause further stimulation of PLD activity. Such a positive feedback loop will effect a very rapid and profound change in the lipid composition of the vesicular membranes, leading to the formation of microdomains that are depleted of PC and phosphatidylinositol and are greatly enriched in PA and PIP2. A positive feedback loop such as the one proposed here must be controlled tightly by shut-off mechanisms. ARF GTPase-activating protein (ARF GAP) is likely to subserve this function. The activity of ARF GAP is stimulated dramatically and synergistically by PIP2 and PA (39). Thus, the interaction of ARF GAP with the PIP2/PA-rich vesicle membranes will cause its activation, stimulation of the GTPase activity of ARF, and the conversion of active ARF-GTP to inactive ARF-GDP. This will shut off PLD activity, thus halting the positive feedback loop, and initiate the disassembly of the coated vesicle (40), allowing its subsequent fusion with acceptor membranes. According to this model ARF plays a role in initiating both vesicle budding and vesicle fusion. Indeed, an ARF N-terminal peptide inhibits catecholamine release in adrenal chromaffin cells (41), and several lines of evidence suggest that PLD activation is involved in exocytosis (reviewed in Ref. 6). The fusion of vesicles that are enriched with the negatively charged phospholipids PIP2 and PA is likely to be greatly facilitated by Ca2+ ions (42).

The present study defines a novel function of PIP2 as a cofactor for brain membrane PLD. The requirement of PIP2 for maximal PLD activity suggests that PIP2 synthesis and breakdown regulate PLD activation. Possible physiological functions in which this interaction is likely to be critical are delineated, and a testable hypothetical model for its role in vesicular traffic is proposed. Further studies aimed at the characterization and kinetic analysis of the interaction between PIP2 and PLD and the role of PIP2 biosynthesis in PLD activation and membrane transport are currently in progress.

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