Regulation of Phospholipase D2: Selective Inhibition of Mammalian Phospholipase D Isoenzymes by α- and β-Synucleins†

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Received November 11, 1997; Revised Manuscript Received January 8, 1998

ABSTRACT: Two widely expressed mammalian phosphatidylcholine (PC)-specific phospholipases D (PLD), PLD1 and PLD2, have been identified. Recombinantly expressed PLD2 has high basal activity and is insensitive to GTP-binding protein activators of PLD1 [Colley, W. C., et al. (1997) *Curr. Biol. 7*, 191−201]. To investigate the regulation of PLD2 we isolated PLD2, from mouse brain by immunoaffinity chromatography. The native and recombinant proteins have indistinguishable properties: PLD2 is potently activated by phosphoinositides with a vicinal 4,5-phosphate pair but is not stimulated by guanosine 5′-*O*-(3-thio triphosphate)-activated ADP-ribosylation factor-1, Rho family GTP-binding proteins, or protein kinases C-α, or -β_1. We used recombinant PLD2 in a reconstitution assay to search for regulators in cell and tissue extracts. Bovine brain contains a heat-stable protein factor that inhibits PLD2 activity in vitro. This factor was purified to homogeneity and identified as a mixture of α- and β-synucleins by microsequencing and Western blotting. Recombinantly expressed α- and β-synucleins inhibit PLD2 activity in vitro ($K_{0.5}$ 10 nM). Inhibition is not overcome by the protein or lipid activators of PLD1. Synucleins have been implicated in Parkinson's and Alzheimer's diseases. Our findings suggest that inhibition of PLD2 may be a function of synucleins. Modulation of PLD2 activity by synucleins may play a role in some aspects of the pathophysiologies that characterize these neurodegenerative diseases.

Phospholipases D (PLD)[1] initiate the formation of lipid-derived intra- and intercellular mediators by the hydrolysis of phosphatidylcholine (PC) (*1*, *2*). The primary lipid product of these enzymes, phosphatidic acid (PA), exhibits a number of biological activities in vitro and may be an important mediator of processes controlling vesicular transport and changes in cell morphology (*3*−*8*). Further metabolism of PA by phosphohydrolase and phospholipase A_2 activities is clearly important in the generation of diacylglycerol (DG) for activation of protein kinase C (PKC) isoenzymes and in the formation of lysoPA and arachidonic acid metabolites which are active at cell-surface receptors (*9*−*11*).

PLD activity is widely expressed, and biochemical studies suggest that mammalian systems contain two classes of these enzymes. One of these is stimulated by phosphoinositides, and the other is insensitive to these lipids but stimulated by fatty acids. Phosphoinositide-dependent PLD activities in brain and HL-60 cells can be increased by ARF and Rho family GTP-binding proteins and by protein kinase C (*12*−*17*). cDNAs encoding PLD1 and PLD2, two members of the phosphoinositide-dependent class of PLD enzymes, have been cloned, expressed in insect cells, and purified to homogeneity. PLD1 has low basal catalytic activity and can be activated by monomeric GTP-binding proteins of the ARF and Rho families and by protein kinase C in vitro (*18*, *19*). In contrast, when expressed and purified, PLD2 is considerably more active than PLD1 and appears to be insensitive to further stimulation by the known activators of PLD1 (*20*). PLD2 has also been expressed in yeast and reported to be insensitive to activation by ARF and Rho GTP-binding proteins (*21*). These findings were surprising since, although PLD2 RNA can be readily detected in many tissues and cell lines (*22*, *23*), reports from a number of different laboratories had described only PLD1-like activities in a variety of systems. These findings raised the possibility that the properties of PLD2 expressed and purified from sf9 cells were aberrant or that PLD2 activity in cell and tissue extracts was attenuated by an unidentified inhibitory factor. Lending weight to the latter possibility, several laboratories have described protein inhibitors of both crude PLD activities and recombinantly expressed PLD1 (*24*−*27*). To address the first possibility, we purified native PLD2 from mouse brain and compared the properties of the native and recombinant proteins. To address the second possibility, we used a reconstitution assay to search for modulators of recombinantly expressed PLD2 and report the purification of a protein that selectively inhibits PLD2 activity. Sequencing and immunological analysis identified this inhibitor as a mixture of α- and β-synucleins. Recombinantly expressed human α- and β-synucleins are potent inhibitors of PLD2

† Supported by NIH Grants GM 50388 and GM54641.

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[1] Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; DG, diacylglycerol; PLD, phospholipase D; PLC, inositol lipid-specific phospholipase C; PKC, protein kinase C; ARF, ADP-ribosylation factor; PE, phosphatidylethanolamine; PI, phosphatidylinositol; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $GPI(4,5)P_2$, glycerophosphoinositol 4,5-bisphosphate; GTPγS, guanosine 5′-*O*-thiotriphosphate); PMSF, phenylmethanesulfonyl fluoride; PMA, phorbol myrisate acetate.

S0006-2960(97)02776-1 CCC: $15.00
Published on Web 03/17/1998

activity in vitro. Although the cellular function of synucleins is not known, genetic and histological evidence implicates these proteins in familial Parkinson's and Alzheimer's diseases (*28*−*34*). Our findings raise the possibility that inhibition of PLD2 may be a normal function of synucleins and that regulation of PLD2 plays a role in the pathophysiology that accompanies these neurodegenerative diseases.

EXPERIMENAL PROCEDURES

Expression and Purification of PLD1 and PLD2. PLD1 and PLD2 were expressed in Sf9 cells and purified by immunoaffinity chromatography using minor adaptations of previously described methods (*19*, *20*). Briefly, 5×10^7 cells were plated into 300 cm^2 flasks and infected with the appropriate baculovirus at a multiplicity of infection of 5. Cells were incubated with virus for 1 h, and then cultured in complete Grace's medium supplemented with lactalbumin and yeast hydrosylate containing 10% fetal bovine serum for 60 h at 27 °C. Medium was removed, and the cells were washed once in phosphate-buffered saline and then lysed by addition of ice-cold buffer (buffer A) containing 25 mM Tris, pH 7.5, 5 mM EGTA, 1% NP-40, 1 mM benzamidine, and protease inhibitors (1 mM phenylmethanesulfonyl fluoride). After incubation at 4 °C with constant mixing, unsolubilized material was removed from the lysates by centrifugation (100000*g* for 30 min), and the supernatants were incubated for 1 h with 0.4 mL of the appropriate immunoaffinity resin (Protein A−Sepharose 4BCL cross-linked to PLD1- or PLD2-selective antipeptide antisera). The resin was washed 3 times with 10 volumes of buffer A and then transferred to a 10 mL BioRad Econo column. The resin was then washed 3 times with 10 volumes of 10 mM sodium phosphate, pH 6.8, and bound proteins were eluted by batchwise application of three 0.5 mL aliquots of 100 mM glycine buffer (pH 3.0). Each aliquot was collected on ice into a tube containing 150 μL of 1 M sodium phosphate (pH 8.0). Aliquots of the purified proteins were stored on ice and generally used within 48 h of preparation.

Purification of PLD2 from Mouse Brain. Two fresh mouse brains were homogenized in buffer A containing protease inhibitors, and proteins were solubilized at 4 °C for 30 min with constant agitation. The extract was centrifuged at 100000*g* for 30 min and the supernatant obtained added to 0.5 mL of PLD2-selective immunoaffinity resin and the resultant slurry placed at 4 °C with constant mixing for 1 h. The resin was recovered by gentle centrifugation and washed 5 times with ice-cold buffer A containing protease inhibitors and then transferred to a small column for final washing and elution using the procedures described above for the recombinant enzyme.

Phosphoinositides and Inositol Phosphates. PI4P, PI(4,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3 were purified from bovine brain (*35*) or synthesized by Glenn Prestwich, Department of Medicinal Chemistry, University of Utah (*19*). Inositol phosphates were obtained from Calbiochem. GPI(4,5)P_2 was prepared by methylamine deacylation of PI(4,5)P_2.

Mammalian PLC Enzymes. Mammalian PLC-β isoenzymes were expressed in insect cells using baculovirus vectors and purified as described (*36*). PLC-δ_1 was provided by Mario Rebbechi (Department of Anaesthesiology, SUNY−Stony Brook).

Plant and Bacterial PLD Enzymes. A cabbage PLD preparation (type I), a peanut PLD preparation (type II), and a *Streptomyces chromofuscus* PLD preparation (type VI) were obtained from Sigma Chemical Co. The lyophilized enzyme preparations were reconstituted in buffer containing 25 mM Tris, pH 7.5, 1 mM EDTA and stored frozen in aliquots. Analysis of all three preparations by SDS−PAGE revealed multiple proteins (not shown).

SDS−PAGE and Western Blotting. Proteins were analyzed by SDS−PAGE with detection by staining with silver or Coomassie blue as described previously (*20*). Western blotting was performed using anti-synuclein polyclonal antiserum (*30*) and previously described methods with detection using an alkaline phosphatase-conjugated secondary antibody (*20*).

PLD Assay. PLD activity was determined using [^{3}H]PC and a basic assay buffer that was described previously (*20*). GTP-binding proteins and protein kinase C were expressed in insect cells using baculovirus vectors and purified using previously described procedures (*20*). For measurements of PLD activity during purification of the PLD2 inhibitor, activity was measured by adding samples of the column fractions (generally 5 μL) to the assays. In some experiments, the PE:PIP_2:PC ratio was held at 16:1.4:1, and the total lipid concentration was varied. In other cases, the PE and PC concentrations were held at 87 and 5.6 μM, respectively, and the PIP_2 concentration was varied as indicated. Plant PLD enzymes were assayed in the presence of 1 mM Ca^{2+} which was achieved by addition of $CaCl_2$ to the assay buffer. Under these assay conditions, PLD activity is linear with time to approximately 40% substrate hydrolysis. In general, we measured PLD activity as initial rates of PC hydrolysis (less than 10% substrate conversion). Some experiments employed a more detailed kinetic analysis as noted.

Purification of a PLD2 Inhibitor from Bovine Brain. One frozen bovine brain (Pel-Freez Inc.) was thawed in 500 mL of TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) for 1 h at room temperature. Thawing buffer was poured off, and the brain was homogenized in 500 mL of fresh ice-cold TED buffer containing protease inhibitors (1 mM PMSF, 1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL pepstatin A) using a Waring blender. The homogenate was centrifuged for 45 min at 20000*g* to pellet most of the membrane fraction. The supernatant was harvested and centrifuged at 100000*g* for 1 h to pellet out any remaining particulate material. The clarified supernatant was harvested and then incubated at 70 °C for 45 min with periodic mixing. Denatured proteins were removed by centrifugation for 30 min at 100000*g*, and the supernatant was concentrated to approximately 20 mL by pressure filtration using a PM10 membrane (Amicon Inc.). The concentrate was applied to a 470 mL Sephacryl S-300 HR gel filtration column (90 × 2.6 cm) equilibrated with TED buffer. The column was eluted at a flow rate of 2 mL/min, and collected as 6 mL fractions after the void volume (150 mL). The fractions were assayed for inhibition of PLD2 activity, and material eluting in fractions 27−38 was selected for further purification These fractions were pooled and applied to a 17 mL Source 15Q (Pharmacia) column (8.6 × 1.6 cm) equilibrated with TED buffer and eluted with a 100 mL linear gradient of 0−400 mM NaCl at a flow rate of 1 mL/min. The eluant was

Table 1: Effects of GTP-Binding Proteins, PKC Isoenzymes, and Phosphoinositides on Native and Recombinant PLD2 Activity[a]

	PLD activity (pmol of choline/30 min)								
		ARF		Rho		Rac		cdc42	
	no addition	−GTPγS	+GTPγS	−GTPγS	+GTPγS	−GTPγS	+GTPγS	−GTPγS	+GTPγS
rPLD2	30.1	31.5	32.1	35.2	33.1	33.5	37.6	36.4	38.1
nPLD2	26.7	25.2	23.4	21.4	22.5	24.1	25.9	27.8	27.0

	PKC-α			PKC-β_1		
	no addition	+PMA	+PMA +ATP	no addition	+PMA	+PMA +ATP
rPLD2	45.6	45.0	34.2	39.9	39.1	36.4
nPLD2	16.7	17.1	13.2	17.8	15.7	16.3

	no addition	$PI(4,5)P_2$	$PI(3,4,5)P_3$	$PI(3,4)P_2$	PI4P	PI	$Ins(1,4,5)P_3$	$Ins(1,3,4,5)P_4$	$GroPIns(4,5)P_2$
rPLD2	0.4	34.5	21.3	0.5	0.8	0.3	0.5	0.4	0.4
nPLD2	0.2	13.2	ND	ND	ND	ND	ND	ND	ND

[a] Activities of native and recombinant PLD2 (nPLD2 and rPLD2) were determined in the presence of the indicated GTP-binding proteins at final concentrations of 4 μM ARF, 1.8 μM RhoA, 2.0 μM Rac1, and 2.1 μM cdc42. In some cases, the GTP-binding proteins were preactivated with GTPγS. PKCs-α and -b_1 were included in the incubations at final concentrations of 0.1 μM with 0.1 mM ATP and 0.2 μM PMA. Inositol lipids were included in the substrate-containing lipid vesicles at concentrations of 7.5 μM. Soluble inositol phosphates and glycerophosphates were added to the incubations at 7.5 μM. The data shown are means of triplicate determinations. Standard errors were within 15% of the mean.

collected as 2 mL fractions, and these were assayed for inhibition of PLD2. Fractions 28−35 were selected for further analysis. This material was pooled and exchanged into HED buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT) by pressure concentration. The concentrated material was applied to a 5 mL heparin−Sepharose 4BCL (Pharmacia) column (4.5 × 1.1 cm). The column was eluted with a 50 mL linear gradient of 0−500 mM NaCl at a flow rate of 1 mL/min; 1 mL fractions were collected and assayed for inhibition of PLD2. Fractions 9−20 contained an activity which strongly inhibited PLD2. This material was pooled, concentrated as described above, and stored at −80 °C.

Protein Microsequencing. Purified proteins were subjected to SDS−PAGE and transferred to a PVDF membrane and identified by staining with Ponceau S. The 19 kDa protein was excised, and microsequencing was performed by the Edman degradation procedure using an Applied Biosystems Model 470A protein sequencer (Center for Synthesis and Analysis of Macromolecules, SUNY−Stony Brook).

Expression and Purification of Human α- and β-Synucleins. Human α- and β-synucleins were expressed in *E. coli* using vectors generously provided by Ross Jakes, MRC Laboratory of Molecular Biology, Cambridge, U.K. (*30*, *33*). Cells were harvested and disrupted by sonication, and soluble material was recovered after centrifugation at 100000*g*. This material was heated at 70 °C, denatured proteins were removed by centrifugation, and the synucleins were purified by chromatography on a 5 mL Source 15Q anion exchange column as described above. The proteins prepared in this way were homogeneous as judged by SDS−PAGE and staining with coomassie blue.

RESULTS

Isolation of PLD2 from Mouse Brain and Baculovirus-Infected sf9 Cells. PLD2 was purified from detergent extracts of either baculovirus-infected sf9 cells or mouse brain using immunoaffinity chromatography. The resin was washed extensively, and bound proteins eluted at low pH followed by immediate neutralization. We estimate that approximately 35% of PLD activity in the sf9 cell detergent extract are approximately 10% of activity in the mouse brain detergent extract is recovered in the eluate from the immunoaffinity resin. The purified protein preparations were analyzed by SDS−PAGE and Western blotting. Both preparations contained a prominent protein of approximate molecular mass 110 kDa detected by silver staining that reacted specifically with a mixture of PLD2-selective anti-peptide antibodies on Western blotting (data not shown).

Properties of Native and Recombinant PLD2. Both the native and recombinant PLD2 preparations hydrolyzed PC substrate when presented in sonicated dispersions containing PE and $PI(4,5)P_2$. We calculated the specific activity of the recombinant enzyme to be 5.5 μmol min^{-1} mg^{-1} while the specific activity of native PLD2 was calculated to be a comparable value of 3.0 μmol min^{-1} mg^{-1}. PLD2 could also catalyze a transphosphatidylation reaction in the presence of primary alcohols, forming phosphatidyl alcohols, or in the presence of diacylglycerol, forming bisphosphatidic acid (not shown).

PLD1 is highly dependent on $PI(4,5)P_2$ for activity. We examined the effects of a variety of phosphoinositides on the catalytic activity of recombinant PLD2. Inclusion of 7.5 μM $PI(4,5)P_2$ in the substrate-containing lipid vesicles resulted in a dramatic increase in activity of both native and recombinant PLD2 (66- and 85-fold, respectively, Table 1). We examined the effects of a number of other inositol lipids and phosphates on the activity of recombinant PLD2. $PI(3,4,5)P_3$ also stimulated PLD2 activity. PLD2 activity was unchanged when assayed in the presence of $PI(3,4)P_2$, PI4P, or PI. Inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$], inositol 1,3,4,5-tetrakisphosphate [$Ins(1,3,4,5)P_4$], and glycerophosphoinositol 4,5-bisphosphate [$GPI(4,5)P_2$] did not stimulate PLD2 activity.

We compared the activation of native and recombinant PLD2 by ARF and Rho family GTP-binding proteins and by protein kinase C. The GTP-binding proteins were included in the assays with or without preactivation using GTPγS. PKCs-α and -β_1 were included in the assays in the presence or absence of 10 nM phorbol dibutyrate or 100 μM ATP. Under the assay conditions used, the ARF and Rho family GTP-binding proteins activated purified recombinant

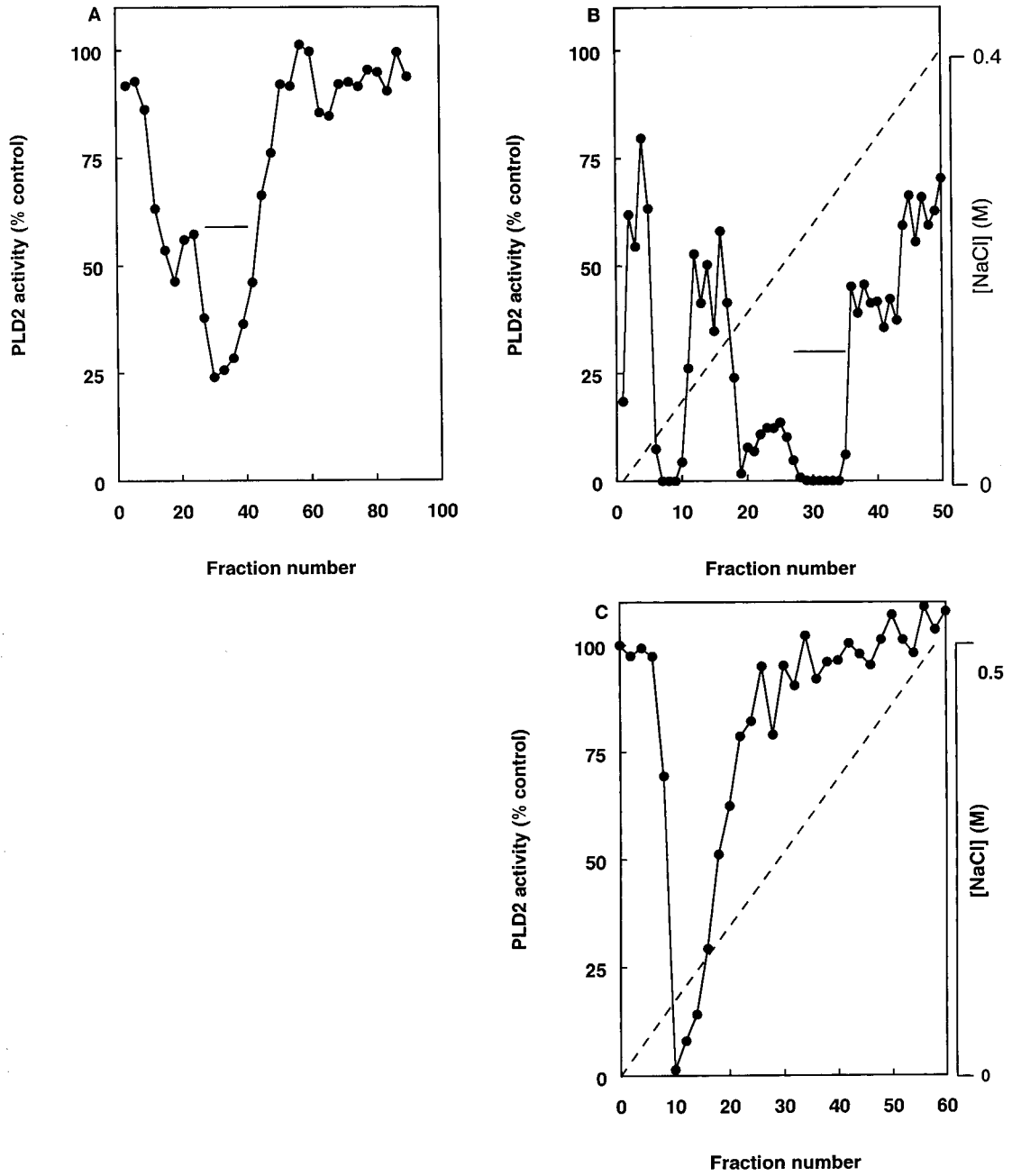

FIGURE 1: Purification of heat-stable PLD2 inhibitor from bovine brain. Proteins were separated by column chromatography and the effects of fractions obtained on PLD2 activity determined as described in the text. (A) Gel filtration chromatography. Proteins were fractionated on a Sephacryl S-300 HR gel filtration column eluted as described in the text. Pooled fractions are marked by a horizontal bar. (B) Source 15 Q anion-exchange chromatography. Pooled fractions from the gel filtration column were applied to a Source 15Q column and eluted with a gradient of NaCl as described in the text. Pooled fractions are marked by a horizontal bar. (C) Heparin−Sepharose chromatography. Pooled fractions from the Source 15 Q column were applied to a heparin−Sepharose 4BCL column and eluted with a gradient of NaCl as described in the text. Pooled fractions are marked by a horizontal bar.

PLD1. PKC-α and -β_1 also stimulated PLD1 in a manner that was increased by PMA but insensitive to inclusion of ATP in the assay (not shown, see *19* for details). The results obtained are presented in Table 1. The ARF and Rho family GTP-binding proteins and PKC isoenzymes did not increase PLD2 activity under any of the conditions tested.

Purification of a PLD2-Selective Inhibitor from Bovine Brain. The unexpected properties of PLD2 led us to search for regulators of this enzyme in cell and tissue extracts. Recombinant PLD2 activity was determined under standard conditions, and fractions of interest were included in these assays and their effects on PLD2 activity determined. Because we were particularly interested in the possibility that unidentified GTP-binding proteins or protein kinases were regulators of PLD2 activity, 10 μM GTPγS and 100 μM ATP were included in some of these experiments. Under no circumstances did we observe increases in PLD2 activity. We did, however, consistently find inhibition of PLD2 activity when soluble extracts from mouse and bovine brain were added to PLD2 assays. Further experiments were undertaken to investigate the nature of the inhibitory activity directed against PLD2 from bovine brain extracts and to explore the selectivity of this inhibitor for other PLD enzymes. We found that PLD2 was inhibited by a low

molecular weight cytosolic factor that was stable to heating at 70 °C. This factor appeared to be a protein because treatment with lysozyme or trypsin abolished the inhibitory activity. We also noted that this factor displayed a pronounced selectivity for inhibition of PLD2 over PLD1. There are many potential explanations for the inhibition of PLD2 observed. Some of these would include degradation of the PLD protein, phosphatase- or phospholipase-catalyzed degradation of the PI(4,5)P_2 cofactor, detergent-like disruption of the substrate-containing aggregates used to determine enzyme activity, or binding to these aggregates in a manner that prevented PLD activity. However, our previous studies and those presented above indicate that the basic catalytic properties of PLD1 and PLD2 are very similar. Because the heat-stable protein inhibitor appeared to show selectivity for PLD2 over PLD1, we were encouraged to investigate this observation further and initiated efforts to purify the protein responsible for this activity.

Purification of a Heat-Stable PLD2 Inhibitor from Bovine Brain. A bovine brain soluble extract was prepared and subjected to heat denaturation at 70 °C for 45 min and denatured protein removed by centrifugation. After pilot experiments, we devised a purification strategy which entailed sequential, pressure concentration, gel filtration, anion exchange, and heparin-affinity chromatography. Figure 1A shows the profile of inhibitory activity observed when fractions obtained from the gel-filtration column were included in the PLD assays. We consistently observed one major trough of inhibitory activity at this stage. We noted a less active trough of earlier eluting material that eluted close to the void volume of the column. We were concerned that this might represent aggregated material and also noted that upon further analysis the later-eluting material displayed a more pronounced specificity for inhibition of PLD2 compared to PLD1. These later-eluting fractions were applied to an anion exchange column, and the inhibitory activity further resolved itself into a broad trough with three apparently distinct activities (Figure 1B). Fractions containing the later-eluting and quantitatively major inhibitory activity were pooled and further purified by heparin-affinity chromatography, yielding a single sharp trough of inhibitory activity that showed a strong selectivity for PLD2 (Figure 1C).

SDS−PAGE and Sequence Analysis of the PLD Inhibitor Preparation. Fractions containing PLD2 inhibitory activity from the heparin−sepharose column were analyzed by SDS−PAGE. Inhibitory activity correlated with a single identified protein of estimated molecular mass 20 kDa (Figure 2A). This material was transferred to a PVDF membrane and subjected to microsequencing by a modified Edman degradation procedure. We obtained 20 amino acids of N-terminal sequence from this protein. The overall efficiency of the sequencing reaction (moles of amino acid released per cycle per mole of starting protein) was determined to be 10.6%.

Identification of the PLD2 Inhibitor as a Synuclein. A blast search of GenBank revealed that the sequence obtained corresponds to amino acids 5−25 of α- and β-synucleins (Figure 3). We obtained antibodies against these closely related proteins and analyzed fractions from the heparin−sepharose column by Western blotting. The 20 kDa protein that copurified with the PLD2 inhibitory activity was strongly recognized by these antibodies (Figure 2B).

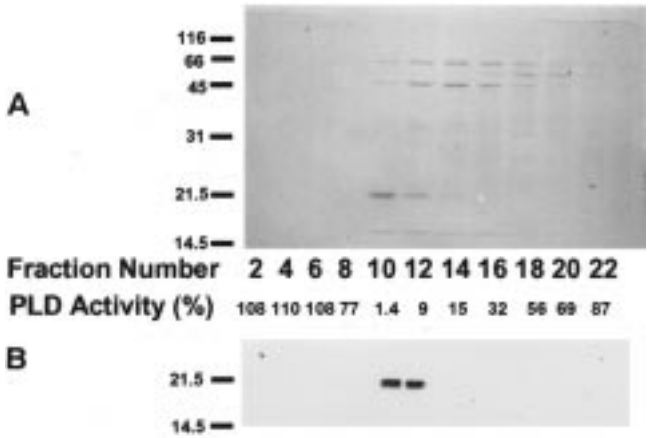

FIGURE 2: SDS−PAGE and Western blot analysis of purified PLD2 inhibitor. (A) Coomassie blue-stained SDS gel. Fractions from the heparin−sepharose column were analyzed by SDS−PAGE on a 12.5% gel. Proteins were visualized by staining with Coomassie blue, and the positions of molecular mass markers are shown. Inhibition of PLD2 activity was determined as described in the text. (B) Anti-synuclein Western blot. Fractions from the heparin−Sepharose column were analyzed by Western blotting with an α- and β-synuclein-selective polyclonal antiserum. Immunoreactive proteins were detected with an alkaline phosphatase-conjugated secondary antibody. Positions of molecular mass markers are shown.

Selective Inhibition of PLD2 by Recombinantly Expressed α- and β-Synucleins. We expressed α- and β-synucleins in bacteria and purified the recombinant proteins to homogeneity using adaptations of previously published procedures (*30*). SDS−PAGE analysis revealed that both recombinant proteins have very similar mobilities to the native preparation isolated from bovine brain. PLD1 and PLD2 activity was determined in the presence of increasing concentrations of recombinant α-synuclein. PLD1 activity was determined in the presence of activated ARF, while PLD2 was assayed alone. Recombinant α-synuclein is a potent inhibitor of PLD2 with a K_i of approximately 10 nM. The K_i for inhibition of PLD1 was estimated to be >50 μM (Figure 4A). We observed a comparable selectivity for inhibition of PLD2 by four separate preparations of synuclein isolated from bovine brain. PLD1 was similarly insensitive to inhibition by synuclein when activated by Rho family GTP-binding proteins or PKC (not shown). We examined the effect of a fixed concentration of 100 nM α- or β-synuclein on the activity of PLD1, PLD2, and a number of different plant and bacterial PLD enzymes and mammalian PLCs-β_1 and -δ_1. The α- and β-synucleins were equally effective inhibitors of PLD2. The synucleins did not inhibit the plant or bacterial PLD enzymes and exhibited very modest inhibitory effects on the activity of the PLC isoenzymes (Figure 4B).

Effects of PI(4,5)P2, Small G Proteins, and PKC on Inhibition of PLD2 by Synucleins. As noted above, PI(4,5)P_2 is a potent activator of PLD2. Certain other proteins identified as inhibitors of crude PLD activities appear to work by binding to or degradation of this activator. We examined the effect of synuclein on the ability of PI(4,5)P_2 to increase PLD2 activity. Inhibition of PLD2 activity by synuclein could not be overcome by increasing concentrations of PI(4,5)P_2. Instead, the concentration dependence with which this lipid activated the PLD enzyme was very similar in the presence or absence of synuclein, but the magnitude of PLD activity was decreased in the presence of the inhibitor (Figure

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N-term    MKGLSKAKEGV VAAAEKTKQ
      1          15 16          30 31          45 46          60 61          75 76          90
alpha MDVFMKGLSKAKEGV VAAAEKTKQGVAEAA GKTKEGVLYVGSKTK EGVVHGVATVAEKTK EQVTNVGGAVVTGVT AVAQKTVEGAGSIAA   90
beta  MDVFMKGLSMAKEGV VAAAEKTKQGVTEAA EKTKEGVLYVGSKTR EGVVQGVASVAEKTK EQASHLGGAVFS--- ---------GAGNIAA   79
slp   MDVFKKGFSIAREGV VGAVEKTTQGVTEAA EKTKEGVMYVGAKTK GERGTSVTSVAEKTK EQANAVSEAVVSSVN TVATETVEEAENIVV   90

      91         105 106         120 121         135 136         150
alpha ATGFVKKDQLGKNEE G------APQEGILE DMPVDPDNEAYEMPS EEGYQDYEPEA 140
beta  ATGLVKREEFPTDLK PEEVAQEAAEEPLIE PLME-PEGESYEDPP QEEYQEYEPEA 134
slp   TTGVVRKE------- ----------DLE PPAQ--DQEAKEQ-- EEGEEAKSGGD 123
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FIGURE 3: Sequence analysis of purified PLD2 inhibitor. The N-terminal sequence obtained from the 19 kDa protein is shown aligned with the sequences of human α- and β-synucleins and rat synuclein-like protein (SLP). The conserved N-terminal repeat sequence of the proteins is boxed and the central hydrophobic region underlined.

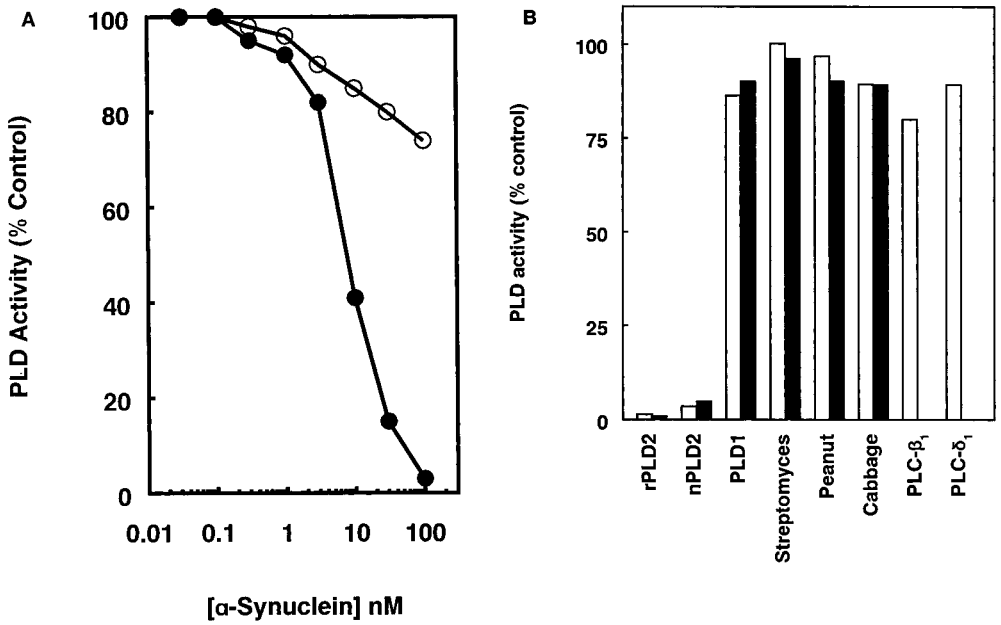

FIGURE 4: Inhibition of PLD and PLC enzymes by α- and β-synucleins. (A) Concentration-dependent inhibition of PLD2 by α-synuclein. The activity of ARF-activated PLD1 (open symbols) or PLD2 (closed symbols) was determined as the concentration of α-synuclein was varied. Assays contained approximately 2−10 ng of purified PLD enzymes. Data shown are means of triplicate determinations. Standard errors were within 10% of the mean. (B) Effect of synucleins on activity of mammalian, plant, and bacterial PLC and PLD enzymes. Activities of recombinant PLD2 (rPLD2), native PLD2 (nPLD2), *Streptomyces chromofuscus* PLD, cabbage PLD, peanut PLD, and PLC-β_1 and -δ_1 were determined in the presence of 100 nM α-synuclein (black bars) or β-synuclein (white bars). Assays contained 5−10 μg of the bacterial or plant PLD preparations and 1−5 ng of the purified PLC enzymes. Data shown are means of triplicate determinations. Standard errors were within 15% of the mean.

5A). Small G proteins and PKC are responsible for the activation of PLD1, so we tested the possibility that inhibition of PLD2 by synuclein might be overcome by these regulators. Figure 5B shows that synuclein inhibition of PLD2 is not overcome by addition of PLD1 activators such as ARF, Rac, RhoA (each activated by GTPγS), and PKC (activated by PMA) at concentrations that would produce a maximal activation of PLD1.

Phospholipid Concentration Dependence of Synuclein Inhibition of PLD2. Both synucleins and PLD enzymes (including PLD2) appear to be membrane-bound in cells, and, like other phospholipases, the activity of PLD2 is likely to display "surface dilution" kinetics in which the activity of the enzyme depends on both the bulk and surface concentration of the PC substrate. Detailed kinetic studies of lipases are best performed using mixed lipid and detergent micelles, but unfortunately activity of PLD2 is not supported by ionic or nonionic detergents. We examined the dependence of PLD2 activity on increasing concentrations of substrate-containing lipid vesicles containing a fixed surface concentration of PC substrate and PI(4,5)P_2. PLD2 activity increased with increasing lipid concentration (half-maximal effect at 50 μM) in an apparently saturable manner. Inclusion of 100 nM synuclein produced a 90% inhibition of PLD2 activity at our standard total lipid concentration of 100 μM, but we found that this inhibitory effect could be overcome by increasing the total lipid concentration in the assay further (Figure 6). Some potential explanations for this finding are discussed below.

DISCUSSION

The aim of the studies presented in this paper was to investigate the regulatory mechanisms controlling activity of PLD2. Recombinantly expressed PLD2 is highly active in comparison to PLD1 which is the only other mammalian phospholipase D isoform that has been purified to homogeneity (*19*, *20*). We isolated a native PLD2 preparation by immunoaffinity chromatography and report that this native form of the enzyme is also highly active. Both preparations of PLD2 were not further activated by the GTP-binding proteins and protein kinase C isoforms that increase PLD1 activity (*19*).

Like PLD1, PLD2 activity is strongly and selectively dependent on PI(4,5)P_2 or PI(3,4,5)P_3 (*19*). Soluble Ins-

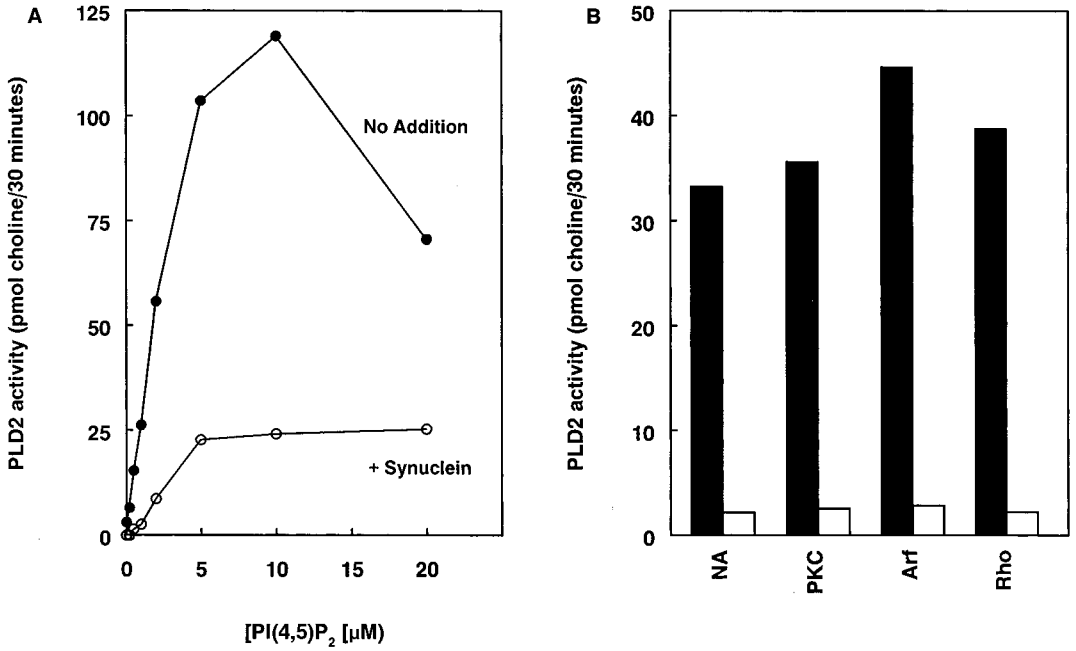

FIGURE 5: Effects of PI(4,5)P2, GTP-binding proteins, and PKC on synuclein inhibition of PLD2. (A) Effect of $PI(4,5)P_2$ on inhibition of PLD2 by α-synuclein. PLD2 activity was determined as the concentration of PI(4,5)P2 in the assays was increased as indicated. Incubations contained either no α-synuclein (closed symbols) or 100 nM α-synuclein (open symbols). Data shown are means of triplicate determinations. Standard errors were within 10% of the mean. (B) Effect of GTP-binding proteins and PKC on inhibition of PLD2 by α-synuclein. PLD2 activity was determined in the absence (black bars) or presence (white bars) of the indicated proteins. Assays contained 20 ng of PKC-α and 10 nM PMA, 4 μM GTPγS-activated ARF, or 1.8 μM GTPγS-activated RhoA. Data shown are means of triplicate determinations. Standard errors were within 10% of the mean.

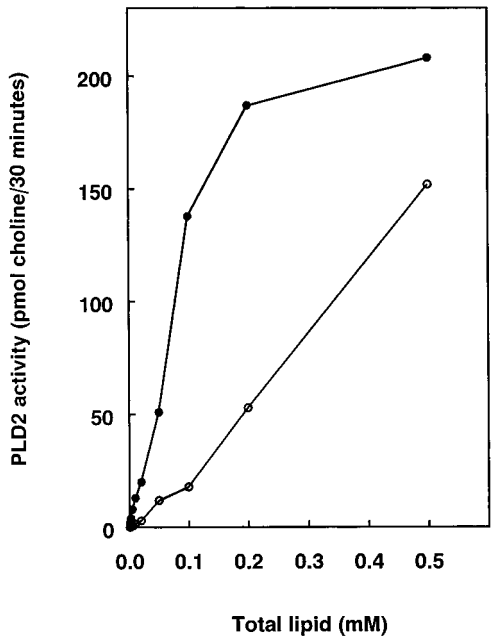

FIGURE 6: Effect of bulk lipid concentration on inhibition of PLD2 by α-synuclein. PLD2 activity was determined as the total lipid concentration in the assay was varied as indicated. Incubations contained either no added synuclein (closed symbols) or 100 nM synuclein (open symbols). Data shown are means of triplicate determinations. Standard errors were within 10% of the mean.

$(1,4,5)P_3$ or $GPI(4,5)P_2$ did not activate PLD2, so either the acyl chains of the lipids are also important in recognition of the lipid or activation requires the phosphoinositides to be contained in the same phospholipid surface as the PC substrate. As has been found for the PLC-δ class of inositol lipid-specific PLC enzymes, binding to phosphoinositides may activate the enzymes by anchoring them to a substrate-containing phospholipid surface (*37*). Phosphoinositides also activate Spo14p and *Ricinius* PLD-β (*38*, *39*). None of the phosphoinositide-responsive PLD enzymes contain any regions of obvious sequence similarity to other phosphoinositide or inositol phosphate binding proteins. The high degree of specificity with which phosphoinositides activate these enzymes suggests that they contain a novel headgroup-selective inositol lipid binding motif.

We used a reconstitution assay to identify a PLD2-specific inhibitor in bovine brain extracts. This protein was purified to homogeneity and identified as a mixture of α- and β-synucleins by Western blotting and microsequencing. The sequence obtained corresponded to an internal fragment of both α- and β-synucleins. These proteins have been previously reported to have a blocked N-terminus. It is likely that we obtained sequence from a fragment of the proteins that copurified with the intact protein. We subsequently found that recombinantly expressed α- and β-synucleins are potent and selective inhibitors of native and recombinant PLD2 having little effect on the activity of PLD1 or of plant and bacterial PLD enzymes or PLCs-β_1 and -δ_1.

Genes encoding three members of the syncuclein protein family have been identified in a number of different species. α- and β-synucleins are expressed primarily in the brain where the proteins appear to localize to presynaptic nerve terminals. Histochemical studies have shown synucleins to be associated with presynaptic vesicles in the hippocampus, cerebral cortex, olfactory tract, and dentate gyrus, suggesting a role in some aspect of synaptic transmission (*28*−*34*). The synucleins share a similar domain structure with a conserved amino terminus containing 6 or 7 conserved repeats of 11 amino acids (boxed in Figure 3) with a consensus core

sequence KTKEGV and a carboxy terminus enriched in acidic amino acids linked by a central hydrophobic section. Biophysical studies suggest that the synucleins have a "natively-unfolded" structure, and, as noted below, members of this class of proteins often appear to participate in or facilitate protein−protein interactions (*31*). It is worth noting that other heat-stable "natively-unfolded" proteins appear to play important roles in cell regulation. These include tau and MAP2 which bind to and influence the polymerization of actin and tubulin as well as chromogrannin and caldesmon which bind to calmodulin and regulate its interaction with protein kinases (*40*, *41*). The protein serine/threonine protein phosphatase inhibitor 1 and a protein kinase inhibitor also belong to this class of proteins (*42*, *43*). Tau has also been found to inhibit PLC-γ, and arachidonic acid specifically activates this inhibited form of the enzyme (*44*).

A number of identified and unidentified proteins have been reported to inhibit the activity of crude and purified PLD enzymes. The polyphosphoinositide 5-phosphatase synaptojanin inhibits the activity of phosphoinositide-dependent PLD enzymes including PLD1 in vitro by hydrolysis of the activator PI(4,5)P_2 (*27*). Fodrin (a nonerythroid form of spectrin) was reported to inhibit ARF-activated PLD in permeabilized HL-60 cells (*24*). The mechanism and specificity of this inhibition were not investigated further. It is possible that the PH domain of fodrin inhibits PLD activity by binding PI(4,5)P_2. Finally the clathrin assembly protein AP3 has been reported to interact with and inhibit PLD1 (*26*). The present work adds the synucleins to this group of PLD inhibitory proteins. Although we do not have a detailed understanding of the mechanism by which synuclein inhibits PLD2 activity, this effect is clearly independent of the activating effect of PI(4,5)P_2. We also note that although PLD1 and PLD2 exhibit an identical dependence on PI(4,5)-P_2, synuclein exhibits a dramatic selectivity for inhibition of PLD2. The simplest explanation for this finding is that inhibition results from a direct interaction between PLD2 and synuclein. Our finding that inhibition of PLD2 by synuclein was dependent on the bulk lipid concentration suggests that this interaction occurs at the membrane surface. Further work will be required to test this possibility and to examine the role of synucleins in PLD2 regulation in vivo. If synucleins are physiologically important regulators of PLD2 activity, it is logical to postulate that some mechanism for derepression of this inhibition exists. We do not find that the activators of PLD1 relieve inhibition of PLD2 by synucleins. α-Synuclein has been reported to be a phosphoprotein in vivo and an in vitro substrate for phosphorylation by Ca^{2+}−calmodulin-dependent protein kinase (*32*). We note that there are several currently unexplained reports of PLD activation in cells by manipulations that increase cytoplasmic Ca^{2+} (*45*). Perhaps Ca^{2+}-dependent phosphorylation relieves synuclein inhibition of PLD2.

Converging lines of evidence point to a role for α-synuclein in the pathophysiology of two neurodegenerative disorders, Alzheimer's disease and familial form of Parkinson's disease. The non-β amyloid component of some types of β-amyloid deposits found in Alzheimer's diseased brains is a fragment of α-synuclein (*29*, *33*). More recently, an alanine to threonine substitution mutation at position 53 of α-synuclein (the A53T mutation) has been associated with a familial form of Parkinson's disease (*34*). These findings focus attention on the cellular function of the synucleins in general and α-synuclein in particular. Clusters of charged amino acids such as those within the amino terminus and carboxy termini of the synucleins are often involved in protein−protein interactions, and native−unfolded proteins have been found to potentiate protein−protein interactions, assuming structure when bound to other proteins. One possibility is that α-synuclein binds to an as yet unidentified partner and that the proteolysis and sequestration of synuclein associated with Alzheimer's disease or the A53T mutation found in familial Parkinson's disease abrogates or otherwise modifies this interaction. At present, cellular functions are not known for any of the synucleins, and their putative binding partners have not been identified. Our results suggest that regulation of PLD2 may be one function of synucleins. In neuronal cells, PLD activation has been linked to pathways involved in cell growth, differentiation, and neurotransmitter release (*46*). A broader series of studies suggest that PLD (most notably ARF-activated PLD1) plays a role in control of vesicular transport processes (*2*, *7*, *14*, *15*). Since synucleins are components of synaptic vesicles, it is possible that PLD2 is involved in synaptic vesicle cycling and that the synucleins are important regulatory components of this process.

ACKNOWLEDGMENT

We thank Ross Jakes for providing some of the reagents used in this research and helpful advice. Michael Frohman and members of his laboratory made many valuble contribuitions to this work.

REFERENCES

1. Exton, J. H. (1997) *J. Biol. Chem. 272*, 15579−15582.
2. Morris, A. J., Engebrecht, J. E., and Frohman, M. A. (1996) *Trends Pharm. Sci. 201*, 182−185.
3. Tsai, M. H., Yu, C. L., Wei, F. S., and Stacey, D. W. (1989) *Science 243*, 522−256.
4. Bauldry, S. A., Elsey, K. L., and Bass, D. A. (1992) *J. Biol. Chem. 267*, 25141−25142.
5. Zhao, Z., Shen, S. H., and Fischer, E. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4251−4255.
6. Bocckino, S. B., Wilson, P. B., and Exton, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6210−6213.
7. Ktistakis, N. T., Brown, H. A., Sternweis, P. C., and Roth, M. G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4952−4956.
8. Cross, M. J., Roberts, S., Ridley, A. J., Hodgkin, M. N., Stewart, A., Claesson-Welsh, L., and Wakelam, M. J. O. (1996) *Curr. Biol. 6*, 588−597.
9. Nishizuka, Y. (1995) *FASEB J. 9*, 484−496.
10. Moolenaar, W. H. (1995) *J. Biol. Chem. 270*, 12949−12952.
11. Dennis, E. A. (1997) *Trends Biochem. Sci. 22*, 1−2.
12. Massenburg, D., Han, J. S., Liyanage, M., Patton, W. A., Rhee, S. G., Moss, J., and Vaughan M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11718−11722.
13. Okamura, S., and Yamashita, S. (1995). *J. Biol. Chem. 269*, 31207−31213.
14. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) *Cell 75*, 1137−1144.
15. Cockcroft, S., Thomas, G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) *Science 263*, 523−526.
16. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) *J. Biol. Chem. 268*, 21509−21512.
17. Malcolm, K. C., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1994) *J. Biol. Chem. 269*, 25951−25954.
18. Hammond, S. M., Alutschuller, Y. M., Tsung, T.-C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) *J. Biol. Chem. 270*, 29640−29643.

19. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. *J. Biol. Chem. 272*, 3860−3868.
20. Colley, W. C., Sung, T.-C., Roll, R., Jenco, J., Hammond, S. M., Alutschuller, Y., Bar-sagi, D., Morris, A. J., and Frohman, M. A. (1997) *Curr. Biol. 7*, 191−201.
21. Kodaki, T., and Yamashita, S. (1997) *J. Biol. Chem. 272*, 11408−11413.
22. Colley, W. C., Altschuller, Y. M., Sue-Ling, C. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Branch, K. D., Tsirka, S. E., Bollag, R. J., Bolag, W. B., and Frohman, M. A. (1997) *Biochem. J. 326*, 745−753.
23. Yoshimura, S., Sakai, H., Ohguchi, K., Nakashima, S., Banno, Y., Nishimura, Y., Sakai, N., and Nozawa, Y. (1997) *J. Neurochem. 69*, 713−720.
24. Lukowski, S., Lecomte, M.-C., Mira, J.-P., Marin, P., Gautero, H., Russo-Marie, F., and Geny, B. (1996) *J. Biol. Chem. 271*, 24164−24171.
25. Kim, J. H., Suh, Y. J., Lee, T. G., Kim, Y., Bae, S. S., Kim, M. J., Lambeth, J. D., Suh, P.-G., and Ryu, S.-H. (1996) *J. Biol. Chem. 271*, 25213−25219.
26. Lee, C., Kang, H.-S., Chung, J.-K., Sekiya, F., Kim, J.-R., Han, J.-S., Kim, S. R., Bae, Y. S., Morris, A. J., and Rhee, S. G. (1997) *J. Biol. Chem. 272*, 15986−15992.
27. Chung, J.-K., Sekiya, F., Kang, H.-S., Lee, C., Han, J.-S., Kim, S. R., Bae, Y. S., Morris, A. J., and Rhee, S. G. (1997) *J. Biol. Chem. 272*, 15980−15985.
28. Maroteaux, L., Campanelli, J. T., and Scheller, R. H. (1988) *J. Neurosci. 8*, 2804−2815.
29. Masliah, E., Iwai, A., Mallory, M., Ueda, K., and Saitoh, T. (1996) *Am. J. Pathol. 148*, 201−210.
30. Jakes, R., Spillantini, M. G., and Goedert, M. (1994) *FEBS Lett. 345*, 27−32.
31. Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996) *Biochemistry 35*, 13709−13715.
32. Nakajo, S., Tsukada, K., Omata, K., Nakamura, Y., and Nakaya, K. (1993) *Eur. J. Biochem. 217*, 1057−1063.
33. Jensen, P. H., Hojrup, P., Hager, H., Nielsen, M. S., Jacobsen, L., Olesen, O. F., Gliemann, J., and Jakes R. (1997) *Biochem. J. 323*, 539−546.
34. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) *Science 276*, 2045−2047.
35. Morris, A. J., Rudge, S. A., Mahlum, C. E., and Jenco, J. M. (1995) *Mol. Pharmacol. 48*, 532−539.
36. Jenco, J. M., Becker, K. P., and Morris, A. J. (1997) *Biochem J. 327*, 431−437.
37. Rebecchi, M., Peterson, A., and McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
38. Rose, K., Rudge, S. A., Frohman, M. A., Morris, A. J. and Engebrecht, J. *Proc. Natl. Acad. Sci. U.S.A. 92*, 12151−12155.
39. Pappan, K., Qin, W., Dyer, J. H., Zheng, L., and Wang, X. *J. Biol. Chem. 272*, 7055−7061.
40. Yoo, S. H., and Albanesi, J. P. (1990) *J. Biol. Chem. 265*, 14414−14421.
41. Lynch, W. P., Riseman, V. M., and Bretscher, A. (1997) *J. Biol. Chem. 262*, 7429−7437.
42. Hemmings, H. C., Nairn, A. C., Aswad, D. W., and Greengard, P (1984*) J. Neurosci. 4*, 99−110.
43. Nimmo, G. A., and Cohen, P. (1978) *Eur. J. Biochem.* 87, 341−351.
44. Hwang, S. C., Jhon, D. Y., Bae, Y. S., Kim, J. H., and Rhee, S. G. (1997) *J. Biol. Chem. 271*, 18342−18349.
45. Kanaho, Y., Nishida, A., and Nozawa, Y. *J. Immunol. 149*(2), 622−688.
46. Klein, J., Chalifa, V., Liscovitch, M., and Loffelholz, K. (1995) *J. Neurochem. 65*(4), 1445−1455.

BI972776R