Inhibition of Phospholipase D by Amphiphysins*

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Two distinct proteins inhibiting phospholipase D (PLD) activity in rat brain cytosol were previously purified and identified as synaptojanin and AP180, which are specific to nerve terminals and associate with the clathrin coat. Two additional PLD-inhibitory proteins have now been purified and identified as the amphiphysins I and II, which forms a heterodimer that also associates with the clathrin coat. Bacterially expressed recombinant amphiphysins inhibited both PLD1 and PLD2 isozymes in vitro with a potency similar to that of brain amphiphysin (median inhibitory concentration of ~15 nM). Expressions of either amphiphysin in COS-7 cells reduced activity of endogenous PLD as well as exogenously expressed PLD1 and PLD2. Coprecipitation experiments suggested that the inhibitory effect of amphiphysins results from their direct interaction with PLDs. The NH2 terminus of amphiphysin I was critical for both inhibition of and binding to PLD. Phosphatidic acid formed by signal-induced PLD is thought to be required for the assembly of clathrin-coated vesicles during endocytosis. Thus, the inhibition of PLD by amphiphysins, synaptojanin, and AP180 might play an important role in synaptic vesicle trafficking.

Phosphatidylcholine (PC)1-specific phospholipase D (PLD) catalyzes the hydrolysis of PC to produce choline and phosphatidic acid (PA) (1). PA produced by PLD as a result of signaling activity is thought to play many roles as an intracellular messenger, and one of its functions is promoting formation of the clathrin coat in Golgi and endoplasmic reticulum, Golgi, trans-Golgi network, and lysosomes (2–6). PA can be further hydrolyzed to diacylglycerol by a specific phosphatase or to lysophosphatidic acid by phospholipase A2. Diacylglycerol activates protein kinase C, and lysophosphatidic acid is a potent mitogen (7). Although the basal activity of PLD in mammalian cells is low, the enzyme is activated in a variety of cells by a wide range of stimuli, including hormones, growth factors, neurotransmitters, and cytokines (1, 8). Activation of PLD is thought to occur through its interaction with small GTP-binding proteins such as ADP-ribosylation factor (ARF) and Rho as well as through protein kinase C (9).

PLD has been cloned from a wide variety of species ranging from bacteria to humans. Two distinct mammalian PLD genes have been identified in humans (10–12), mice (13, 14), and rat (15, 16), and the encoded proteins, PLD1 and PLD2, share ~50% amino acid sequence identity. Purified PLD1 exhibits a low basal activity but is markedly stimulated in a synergistic manner by protein kinase C-α, ARF, or Rho in the presence of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), a cofactor that is required for PLD1 activation (1, 17). PLD2 is also dependent on PI(4,5)P2 but differs from PLD1 in that it exhibits constitutively high activity; it also appears to be weakly activated by ARF (12, 18). PLD1 has been reported to be localized in endoplasmic reticulum, secretory granules, and lysosomes and PLD2 in plasma membrane (13, 19). However, PLD1 upon stimulation was found to translocate to plasma membrane in RBL-2H3 cells (19).

We have previously shown that rat brain cytosol contains several distinct proteins that inhibit PLD activity as measured in the presence of PI(4,5)P2 and ARF (20). Two such inhibitory proteins were purified to homogeneity and identified as synaptojanin and clathrin assembly protein 180 (AP180), previously named as clathrin assembly protein 3 or AP3 (21, 22). The inhibition of PLD by synaptojanin was attributed to its ability to dephosphorylate PI(4,5)P2 (21, 23), whereas the inhibitory effect of AP180 is a result of its direct interaction with PLD (22). We have now purified a third type of PLD inhibitor from rat brain and have shown that it comprises amphiphysin I (AmphI) and AmphII, both of which are nerve-terminal proteins that are important for clathrin-mediated endocytosis (24, 25).

EXPERIMENTAL PROCEDURES

Materials—Rat brains were obtained from Pel-Freez Biologicals (Rogers, AR); bovine brain PC and phosphatidylethanolamine (PE) were from Avanti Polar Lipids (Alabaster, AL); PI(4,5)P2 was from Roche Molecular Biochemicals; [choline-methyl-3H]dipalmityl-PC [(pam)2PC] (50 Ci/mol) was from DuPont; and n-octyl-p-glucopyranoside was from Calbiochem. ARF was purified from rat brain as described (20). Rabbit antibodies generated in response to the COOH-terminal 12 amino acids of human PLD1, but which also recognize mouse PLD2, were kindly provided by Sung Ho Ryu (Postech, Pohang, Korea).

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1 The abbreviations used are: PC, phosphatidylcholine; PLD, phospholipase D; PA, phosphatidic acid; ARF, ADP-ribosylation factor; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; AP180, clathrin assembly protein 180; Amph, amphiphysin; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HA, hemagglutinin epitope; PMA, phorbol 12-myristate 13-acetate; BHK, baby hamster kidney; SH3, Src homology 3; PE, phosphatidylethanolamine; (pam)2PC, dipalmitoyl-PC; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GTP-S, guanosine 5′-O-(3′-thiotriphosphate); PI, phosphatidylinositol; PI(4)P 5-kinase, phosphatidylinositol 4-phosphate 5-kinase.
Recombinant PLD1 and PLD2—Human PLD1 and mouse PLD2 were isolated from SF9 insect cells that had been infected with a recombinant baculovirus containing PLD1 or PLD2 cDNA (10, 14). Partially purified PLD1 was obtained by fractionating detergent-solubilized SF9 cell membrane proteins on a heparin-5PW column as described (21). PLD2 was purified from SF9 cells in a similar fashion with the exception of containing 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM MgCl2, and 0.1 mM diethiothreitol, and 0.1 mM 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride. The lysate was then centrifuged at 50,000 × g for 30 min, and the resulting supernatant was used as the source of PLD2.

In vitro Assay of PLD Activity—ARF-dependent PLD activity was assayed by measuring the generation of 3H-labeled choline from [3H]choline-(pam)2PC as described (21). Briefly, 25 μl of lipid vesicles containing PE, PI(4,5)P2, and PC (with 200,000 cpm of [3H]choline—methyl-3H(pam)2PC) were added to 125 μl of a mixture containing partially purified PLD1, 100 mM ARF, 5 mM guanosine 5'-O-(3-thiotriphosphate), 50 mM Hepes-NaOH (pH 7.5), 3 mM EGTA, 25 mM KCl, 2.5 mM MgCl2, and 2 mM CaCl2. The final concentration of the assay was made up to 300 μM. The assay procedure for PLD2 was identical to that for PLD1 with the exception that ARF and GTP-S were omitted from the reaction mixture. The reactions were stopped by adding 1 ml of a mixture of chloroform and methanol (1:1, v/v) followed by 350 μl of 1 M HCl. The resulting upper layer (500 μl) containing the released [3H]choline was subjected to liquid scintillation spectrometry.

Purification of PLD Inhibitors—Fractions containing PLD inhibitory activity were obtained by subjecting rat brain cytosol to chromatography on a column of DEAE-Sepharose as described (20). Fractions corresponding to the second peak (II) of inhibitory activity (see Fig. 1 of Ref. 20) were pooled, dialyzed against purification buffer (20 mM Tris-HCl (pH 7.5), 1 mM diethiothreitol, leupeptin (1 μg/ml), and aprotinin (1 μg/ml)), and centrifuged to remove insoluble particles. The resulting supernatant (220 μg of protein) was applied to a TSK-gel DEAE-5PW column (21.5 × 150 mm) that had been equilibrated with purification buffer. After washing the column with purification buffer, proteins were eluted at a flow rate of 5 ml/min with a 400-ml linear gradient of 0–1.0 M NaCl in purification buffer. Fractions (5 ml) were collected, and those corresponding to the peak of PLD inhibitory activity were pooled, dialyzed against purification buffer, and centrifuged. The resulting supernatant (10 mg of protein) was applied to a TSK-gel phenyl-5PW column (21.5 × 75 mm) that had been equilibrated with purification buffer. After washing the column with purification buffer containing 0.8 M ammonium sulfate, proteins were eluted at a flow rate of 5 ml/min with a decreasing linear gradient of ammonium sulfate from 0.8–0 M in purification buffer. Fractions (5 ml) were collected, and those corresponding to the peak of PLD inhibitory activity were pooled, dialyzed against purification buffer, and centrifuged. The resulting supernatant (10 mg of protein) was applied to a TSK-gel phenyl-5PW column (7.5 × 75 mm) that had been equilibrated with purification buffer. Proteins were eluted at a flow rate of 5 ml/min with a 150-ml linear gradient of NaCl from 0 to 1.0 M in 150 mM Tris-HCl buffer (pH 7.4), 1 mM MgCl2, and 1 mM EGTA. After washing the column with purification buffer, proteins were eluted at a flow rate of 1 ml/min with a 60-ml linear gradient containing 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM MgCl2, and 0.1 mM diethiothreitol, and 0.1 mM 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride. The lysate was then centrifuged at 50,000 × g for 30 min, and the resulting supernatant was used as the source of PLD2.

Peptide Purification—Proteins in fraction 28 of the heparin-5PW column (Fig. 1A) were reductively denatured by treatment with 6 M guanidine hydrochloride and 2 mM diethiothreitol in 50 mM Tris-HCl (pH 8.0), after which sulfhydryl groups were labeled with 10 mM iodoacetamide. The labeled proteins were digested with V8 protease, and the resulting peptides were applied to a C8 hydrophobic column (4.6 × 250 mm; Vydac) that had been equilibrated with 0.1% trifluoroacetic acid and 0.1% acetonitrile and eluted at a flow rate of 1 ml/min with a stepwise linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Peptides were detected by measuring absorbance at 215 nm. Fractions corresponding to the highest four peaks were separately collected, dried, and subjected to digestion with trypsin, and the resulting peptides were purified on the Vydac C8 column by applying the same gradient as before. The 85-kDa protein from fraction 23 of the heparin-5PW column (Fig. 1B) was electroeluted from gel bands and digested with Lys-C protease. The resulting peptides were purified on the Vydac C18 column as described above. Purified peptides were subjected to amino acid sequence analysis.

Preparation of GST-Amph Fusion Proteins—A cDNA (bAmpy-Z1A) encoding full-length (695-residue) human Amph was described (26), and a plasmid (pHloxf–1+) that encodes a 434-residue variant of mouse AmphII was kindly provided by Dr. B. Kay (University of Wisconsin). The Amph and AmphII cDNAs were separately ligated into the 5' BamHI and 3' EcoRI sites of the pGEX4T1 vector (Amersham Pharmacia Biotech) by standard techniques of subcloning and the resulting expression vectors (pGEX-Amph and pGEX-AmphII) encoding the respective GST fusion proteins. Similarly, truncated cDNAs encoding the NH2-terminal 373 residues or COOH-terminal 404 residues of AmphII were cloned into the 5' BamHI and 3' EcoRI sites of pGEX4T1 to produce pGEX-N-Amph and pGEX-C-Amph. Escherichia coli BL21 cells were transfected with the various expression vectors encoding the GST fusion proteins and grown at 37 °C until the absorbance at 600 nm of the culture reached 0.5–0.6. Expression of GST-Amph proteins was then induced by incubation of the cells for 3 h in the presence of 100 μM isopropyl-β-D-thiogalactosidase. Cells were collected, washed with phosphate-buffered saline (PBS), sonicated in PBS containing 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, N-tosyl-l-phenylalanine chloromethyl ketone (PMSF), and aprotinin, and centrifuged at 25,000 × g for 30 min. GST fusion proteins were purified from the resulting supernatant with the use of glutathione-Sepharose 4B (Amersham Pharmacia Biotech). The concentration of purified proteins was determined spectrophotometrically with extinction coefficients at 280 nm of 39,970 for GST, 97,870 for GST-Amph, 81,700 for GST-AmphII, 70,140 for GST-N-Amph, and 69,370 for GST-C-Amph.

Antibodies to Amph Proteins—Rabbit monospecific antibodies to Amph were described previously (26). Rabbit antisera to N-Amph or to C-Amph were prepared by injection of recombinant proteins obtained by removal of the GST moiety from the corresponding GST fusion proteins with the use of thrombin.

Expression of Recombinant PLD and Amphiphysins in Mammalian Cells—pCGN expression vectors containing cDNAs for full-length PLD1 and PLD2 have been described previously (10, 14). pCGN also encodes an influenza virus HA tag that becomes expressed in-frame at the NH2 terminus of the PLD proteins. The XbaI-SmaI fragments of the pCGN vectors were subcloned into the mammalian expression plasmid pCDNA3.1 (Invitrogen), yielding pCDNA-PLD1 and pCDNA-PLD2. For preparation of Amph expression vectors, the NcoI-EcoRI fragments from pGEX-AmphI and pGEX-AmphII were inserted into pCDNA3.1, yielding pCDNA-AmphI and pCDNA-AmphII, respectively.

Subconfluent COS-7 cells were harvested by exposure to trypsin, centrifuged at 700 × g for 5 min, and washed once with ice-cold transfection buffer (10 mM sodium phosphate buffer (pH 7.4), 1 mM MgCl2, 250 mM sucrose) (27). Cells (4 × 105 in 450 μl) were placed in a 0.4-mm diameter glass capillary (300 μl) containing (two 90-μs pulses at 850 V) in the presence of the indicated plasmids with a T820 electroporator (BTX, San Diego, CA). The cells were maintained on ice for 10 min and then mixed with 9 μl of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A retroviral vector for the expression of Amph (pSinRep5-Amph) was constructed by inserting the NheI-EcoRI IV fragment of pCDNA-AmphI into a pSinRep5 virus vector (Invitrogen) that had been digested with XbaI and StulI. By using the empty pSinRep5 vector (Invitrogen) and the Amph-expressing vectors, control and Amph-expressing virus were generated with the Sindbis Expression System according to manufacturer’s guide, and their titers were determined by measuring relative lucinescent unit in BEHK-LUc cells (ATCC). The volumes of virus stocks were adjusted to equalize titers.

Measurement of PLD Activity in Intact Cells—PLD activity in cells was assayed by measuring the accumulation of [3H]-labeled phosphatidylethanol. Transfected COS-7 cells (7 × 105 per well) were labeled by incubation for 18 h in 2 ml of DMEM containing 10 μCi of [3H]myristic acid (ICN) and 1% FBS. The labeled cells were washed with DMEM, kept in 2 ml of DMEM containing 0.5% ethanol for 5 min, and then incubated in the same buffer plus 100 μM PMA at 37 °C. The medium was then removed and replaced with ice-cold PBS to terminate cell stimulation. The cells were scraped into an ice-cold vial and then extracted with a mixture of chloroform and methanol (2:1). The organic phase was dried, redissolved with an equal mixture of chloroform and methanol, and spotted onto Silica Gel 60 thin layer chromatography plates (Merck). The labeled compounds were separated with the use of...
the organic phase of a mixture of ethyl acetate:n-octylbutane:acetic acid:methanol:water (13:2:3:10:10) (28). The phosphatidylethanol spots were detected by staining with iodine vapor, scraped into a vial, and sonicated in scintillation mixture. The amount of radioactivity associated with these spots was normalized on the basis of the radioactivity incorporated into total lipids of the cells and was then used as the measure of PLD activity.

RESULTS

Purification and Identification of PLD-inhibitory Proteins—We previously showed that chromatography of rat brain cytosol on a DEAE-Sephacel column (peak II from Fig. 1 in Ref. 20), followed by sequential HPLC on DEAE-5PW and phenyl-5PW columns, were further subjected to HPLC on a heparin-5PW column, as described under “Experimental Procedures.” B, the indicated fractions from the heparin-5PW column were subjected to SDS-PAGE on an 8% gel; the separated proteins were then either visualized by silver staining (upper panel) or transferred to a nitrocellulose sheet and subjected to immunoblot analysis with rabbit polyclonal antibodies to Amph1 and horseradish peroxidase-conjugated antibodies to rabbit immunoglobulin G (lower panel). The positions of molecular size standards (in kilodaltons) are shown on the right. C, fractions 23 (open squares) and 28 (closed squares) from the heparin-5PW column (at the indicated amounts of protein) were assayed for their inhibitory effect on PLD1 activity. Data are expressed as a percentage of the PLD activity apparent in the absence of test protein and are means of two separate assays. D, a pool of fractions 22–25 of the heparin-5PW column was further separated by Superose gel column for the micropurification as described under “Experimental Procedures.” Each fraction was tested for the inhibitory activity. Inset is SDS-PAGE analysis on 8% gel that was visualized by the silver staining method. E, fraction 25 from the Superose gel column was subjected to PAGE on a nondenaturing 6% gel (left panel). The protein corresponding to the major (240 kDa) band was eluted from the native gel and analyzed by SDS-PAGE on an 8% gel (right panel). The positions of molecular size standards are indicated on the right of each panel.

Fig. 1. Purification of PLD-inhibitory proteins from rat brain cytosol. A, PLD-inhibitory proteins that had been partially purified from rat brain by chromatography on a DEAE-Sephacel column (peak II from Fig. 1 in Ref. 20), followed by sequential HPLC on DEAE-5PW and phenyl-5PW columns, were further subjected to HPLC on a heparin-5PW column, as described under “Experimental Procedures.” B, the indicated fractions from the heparin-5PW column were subjected to SDS-PAGE on an 8% gel; the separated proteins were then either visualized by silver staining (upper panel) or transferred to a nitrocellulose sheet and subjected to immunoblot analysis with rabbit polyclonal antibodies to Amph1 and horseradish peroxidase-conjugated antibodies to rabbit immunoglobulin G (lower panel). The positions of molecular size standards (in kilodaltons) are shown on the right. C, fractions 23 (open squares) and 28 (closed squares) from the heparin-5PW column (at the indicated amounts of protein) were assayed for their inhibitory effect on PLD1 activity. Data are expressed as a percentage of the PLD activity apparent in the absence of test protein and are means of two separate assays. D, a pool of fractions 22–25 of the heparin-5PW column was further separated by Superose gel column for the micropurification as described under “Experimental Procedures.” Each fraction was tested for the inhibitory activity. Inset is SDS-PAGE analysis on 8% gel that was visualized by the silver staining method. E, fraction 25 from the Superose gel column was subjected to PAGE on a nondenaturing 6% gel (left panel). The protein corresponding to the major (240 kDa) band was eluted from the native gel and analyzed by SDS-PAGE on an 8% gel (right panel). The positions of molecular size standards are indicated on the right of each panel.
fractions corresponding to each of the four major peaks obtained each appeared to contain more than one peptide; these fractions were therefore incubated with trypsin and again subjected to chromatography on the same C18 column. Edman degradation of the resulting four peptides yielded sequences that matched exactly stretches of amino acids present in human Amph as follows: AFTIQ (positions 242–246), TPSIPPE (positions 261–266), KGPP (positions 299–302), and ISVTT (positions 332–336).

The human Amph cDNA encodes a protein of 695 amino acids with a calculated molecular mass of 76,256 (26). The mobility of human Amph on SDS-PAGE was previously shown to be less than that expected from its predicted size, which is likely a result of the acidic nature of the protein (isoelectric point, 4.50) (26). Identification of the 125-kDa protein in fractions 20–28 from the heparin-5PW column as Amph thus was further confirmed by immunoblot analysis with monospecific antibodies to Amph (Fig. 1E). The broadness of the Amph bands in Fig. 1B is likely attributable to the fact that Amph is constitutively phosphorylated at multiple sites (29).

The potencies of fractions 23 and 28 from the heparin-5PW column with regard to inhibition of PLD1 were similar (Fig. 1C), suggesting that both the 83- and 125-kDa proteins in fraction 23 were able to inhibit PLD1 activity. However, neither antibodies to the NH2-terminal 373 residues of Amph nor those to the COOH-terminal 404 residues of Amph recognized the 83-kDa protein (data not shown), suggesting that the latter was not derived from Amph. To separate further these two bands, a pool of fraction 22–25 from the heparin-5PW column was subjected to micropurification by Superose gel column, and each fraction was visualized by silver staining 8% on gel (Fig. 1D). Because the staining intensities of the 125- and 83-kDa proteins in the fractions from the gel column showed equal ratio (Fig. 1D, inset), we investigated whether the two polypeptides exist as a complex. PAGE analysis of fraction 25 on a nondenaturing gel yielded one major band corresponding to an apparent molecular mass of 240 kDa (Fig. 1E). When the 240-kDa protein was electroeluted from the native gel and then subjected to SDS-PAGE, two bands of 125 and 83 kDa were apparent (Fig. 1E), suggesting that the 125- and 83-kDa proteins indeed form a complex. The 83-kDa protein was then electroeluted from an SDS-PAGE gel and digested with Lys-C, and the resulting fragments were separated by HPLC on a C18 column. The sequence of one purified peptide (SPSPPPP-DGSPAATPEIRV) matched perfectly that of residues 296–313 of rat AmphII (30). At least 10 splice variants of rat AmphII have been identified, with the largest such variant consisting of 588 amino acids. Unlike Amph, AmphII proteins are not acidic and do not migrate abnormally on SDS-PAGE.

Inhibition of PLD1 and PLD2 by GST Fusion Proteins of Amph and AmphII—We prepared glutathione S-transferase (GST) fusion proteins containing full-length (695 residues) human Amph (GST-AmphI), the NH2-terminal 373 amino acids (GST-N-AmphI), or the COOH-terminal 404 amino acids (GST-C-AmphI) of Amph, or a full-length 434-residue variant of mouse AmphII (GST-AmphII) and then tested their abilities to inhibit PLD1 and PLD2. The bacterially expressed GST fusion proteins were isolated with the use of glutathione-Sepharose beads, and their purity was verified by SDS-PAGE (Fig. 2A). Both GST-AmphI and GST-AmphII inhibited PLD1 activity in a concentration-dependent manner which was measured in the presence of ARF and PI(4,5)P2 (Fig. 2B); the two fusion proteins showed similar potencies, with half-maximal inhibition apparent at 10–15 nM, similar to the value of 5 nM calculated from the data in Fig. 1C for Amph proteins purified from rat brain. GST alone had no effect on PLD1 activity. The inhibitory potency and efficacy of GST-N-AmphI were similar to those of GST-AmphI, whereas GST-C-AmphI had virtually no effect on PLD1 activity (Fig. 2B). The NH2-terminal 373 residues, but not the COOH-terminal 404 residues, of Amph thus appear critical for inhibition of PLD1. PLD2 activity measured in the presence of PI(4,5)P2 was also inhibited by GST-AmphI and by
GST-AmphII with similar potencies (Fig. 2B).

We also investigated the possibility that the Amph-dependent inhibition of PLD was attributable to blockage of the access of PLD to PI(4,5)P2. The ARF-dependent activity of PLD1 in the absence of PI(4,5)P2 is about 5% of that measured in the presence of PI(4,5)P2 (21). Inhibition of PLD1 by various concentrations of GST-AmphII was measured with substrate vesicles containing or not containing PI(4,5)P2, with the amount of PLD1 used in the absence of PI(4,5)P2 being 20 times that used in its presence. The concentration dependence of inhibition of PLD1 by GST-AmphII was similar in the absence or presence of PI(4,5)P2 (Fig. 3), indicating that AmphII does not inhibit PLD activity by interfering with the interaction of the enzyme with PI(4,5)P2. The observation that GST-AmphII potently inhibited the activity of PLD2 measured in the absence of ARF also suggests that the inhibition of PLD1 activity is not due to interference with ARF binding.

Physical Association of Amphiphysins with PLD—We next investigated whether the amphiphysin proteins physically interact with PLD enzymes. GST-AmphII or GST-AmphII was mixed with partially purified PLD1 or PLD2, and the GST fusion proteins were then precipitated with glutathione-Sepharose 4B. The resulting precipitates and supernatants were subjected to immunoblot analysis with antibodies that recognize both PLD1 and PLD2. The positions of PLD1 and PLD2 are indicated.

Inhibition of PLD by Amphiphysins in Intact Cells—The effects of amphiphysins on PLD activity in intact cells were investigated by transient expression of AmphI or AmphII in COS-7 cells with the use of pCDNA3.1 vector. Because endogenous PLD activity in unstimulated COS-7 cells is too low for reliable measurement, activity was measured after stimulation of the cells with phorbol 12-myristate 13-acetate (PMA). The PMA-stimulated PLD activity was reduced by ~25% in cells expressing AmphI or AmphII (Fig. 6), suggesting that the endogenous PLD activity was inhibited by the exogenous amphiphysins. In Fig. 6, inset, the AmphI blot was shown but not AmphII because the antibody was not available. This relatively low percentage of inhibition, given the marked inhibitory efficacy and potency of AmphI and AmphII apparent in vitro (Fig. 2B), is likely attributable to the fact that only a fraction (usually 20–40%) of the cells actually became transfected, and the PLD activity of the nontransfected cells thus gives rise to a high background.

In another experiment, COS-7 cells were transiently cotransfected with vectors encoding AmphI and either hemagglutinin epitope (HA)-tagged PLD1 or HA-tagged PLD2. Cell lysates were subjected to immunoprecipitation with a monoclonal antibody to HA, and the resulting precipitates were subjected to immunoblot analysis with antibodies to AmphI. AmphI was detected in both the PLD1 and PLD2 immunoprecipitates; AmphII immunoreactivity was not apparent in precipitates prepared from control cells not expressing either recombinant AmphI or HA-tagged PLD (Fig. 5). These results demonstrate that AmphI tightly associates with PLD1 and PLD2 in intact cells.
Redundant Inhibition of PLD by Nerve-terminal Proteins

Fig. 5. Association of AmphI with HA-PLD1 and HA-PLD2 in COS-7 cells. COS-7 cells (4 × 10⁶) were transfected by electroporation with the indicated combinations of empty vector (control), pCGN vectors encoding HA-PLD1 and HA-PLD2, or pCDNA vector containing AmphI. The total amount of vector DNA per transfection was 20 µg, and the amounts of each vector used are indicated (in micrograms) at the top. Transfected cells were plated in 225-cm² dishes and cultured for 36 h in 30 ml of DMEM supplemented with 10% FBS. They were then rinsed three times with ice-cold PBS and scraped into Eppendorf microcentrifuge tubes. The cells were isolated by a brief centrifugation and sonicated in the presence of 1 ml of lysis buffer (1% n-octyl-β-D-glucopyranoside, 150 mM NaCl, 100 mM Tris-HCl (pH 8.5), 1 mM EGTA, 1 mM dithiothreitol, leupeptin (1 µg/ml), aprotonin (1 µg/ml), and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at maximum speed for 20 min, and the resulting supernatants were then subjected to immunoprecipitation (IP) with a monoclonal antibody to HA (αHA) and protein A. Immune complexes were collected by a brief centrifugation, fractionated by SDS-PAGE on an 8% gel, and subjected to immunoblot (IB) analysis with rabbit polyclonal antibodies to AmphI and horseradish peroxidase-conjugated antibodies to mouse immunoglobulin G (upper panel). The same membrane was subsequently also probed with the monoclonal antibody to HA and horseradish peroxidase-conjugated antibodies to mouse immunoglobulin G (middle panel). Cell lysates were also subjected to direct immunoblot analysis with antibodies to AmphI (lower panel). The position of an 83-kDa molecular size standard is indicated.

Fig. 6. Effects of transient expression of AmphI or AmphII on endogenous PMA-stimulated PLD activity in COS-7 cells. Cells (4 × 10⁶) were transfected by electroporation with 20 µg of empty pCDNA3.1 (control), pCDNA-AmphI, or pCDNA-AmphII, transferred to 6-well plates (7 × 10⁶ cells per well), labeled with [3H]myristic acid, incubated for 30 min with 0.5% ethanol in the absence or presence of 100 nM PMA, and assayed for PLD activity, as described under “Experimental Procedures.” The PMA-induced increase in PLD activity was calculated, and data (means ± S.E. of values from five independent experiments) are expressed as a percentage of this value for control cells. Inset, immunoblot analysis of the cell lysates derived from control (lane 1) and AmphI-expressing cells (lane 2) with antibodies against AmphI.

Amphiphysins are implicated in clathrin-mediated endocytosis in nerve terminals, and AmphII is also implicated in endocytosis outside synapses (24, 25). Assembly of clathrin coats is thought to be initiated by binding of the AP2 adapter protein complex to membrane receptors. Through its interaction with the AP2 complex, clathrin assembles and polymerizes to form a polygonal lattice, eventually leading to the formation of a coated bud. Dynamin is then recruited to the neck of the invaginating clathrin-coated vesicle, possibly to effect vesicle closure by activating its downstream effector, endophilin I which has lysophosphatidic acid acyl transferase activity to form PA around neck squeezed by dynamin. PA thus produced facilitates a drastic change in the membrane curvature (37). It is important to note that PA derived by the action of endophilin I was proposed to contain much higher content of arachidonic acid compared with PA derived by the action of PLD. The AmphI-AmphII heterodimer is thought to be responsible for dynamin recruitment as a result of its ability to bind dynamin and AP2 simultaneously. The heterodimer interacts with multiple dynamin molecules through its two SH3 domains and simultaneously binds to AP2 through a region distinct from its SH3 domains. Amphiphysins also bind through their SH3 domains to synaptic proteins. The protein-protein interaction regulates the recruitment of dynamin by competing with it for binding to the SH3 domains of amphiphysins as well as by hydrolyzing PI(4,5)P₂, which serves as a binding site for the pleckstrin homology domain of dynamin (23, 36, 38). Clathrin-mediated endocytosis in nerve terminals requires many other protein components, including AP180, which promote coat assembly by
Repetitive Inhibition of PLD by Nerve-terminal Proteins

We have previously shown that synaptotagmin and AP180 each inhibit PLD activity (21, 22). We have now demonstrated that AmphI and AmphII each inhibit the activities of both PLD1 and PLD2 as a result of direct interaction with these enzymes. Our observation that the COOH-terminal 404 residues of AmphI were not required for inhibition of PLD indicates that the SH3 domain of AmphI does not contribute to this effect. Although the NH2 terminus of AP-180 and AmphI showed PLD inhibition, their NH2 termini did not show strong common denominators. Similar to our results, GST fusion proteins of full-length and NH2-terminal AmphI showed tubulation of liposomes, but the COOH-terminal ones could not (39).

Recent evidence suggests that PA and PI(4,5)P2 also play pivotal roles in the initiation of vesicle complex formation in several types of coated membrane (20, 40), probably by interacting with AP2, AP180, dynamin, and synaptotagmin (41–45). The production of PA on the lysosomal membrane induced by treatment with bacterial PLD was reported to be sufficient to trigger AP2 translocation and limited coat assembly (6). An additional role of PA was proposed to change the membrane curvature of neck already constricted by dynamin and to accelerate vesicle fission (37). PIP2 formed by PI(4)P 5-kinase was shown to be necessary for Ca2+-activated secretion in neuroendocrine cells (46). The formation of constitutive secretory vesicles from trans-Golgi network was activated by PI transport protein (47). In yeast enzymes related to PI metabolism were demonstrated to be critical in the vesicular transportation (48, 49). The production of these two acidic lipids is closely linked. Thus, PI(4,5)P2 is a cofactor for PLD and is generated by the sequential action of PI 4-kinase and PI(4)P 5-kinase. PA, produced by the action of PLD, is a potent activator of PI(4)P 5-kinase (50–52) and thereby increases the synthesis of PI(4,5)P2, which, in turn, leads to further stimulation of PLD activity. This positive feedback loop would be expected to result in a rapid increase in the cellular concentrations of PA and PI(4,5)P2 as well as a consequent marked change in the microdomain architecture of the lipid bilayer that would facilitate coat assembly (21, 22, 53, 54).

According to this model, it would be necessary to halt the feedback loop before initiation of the disassembly of the coated vesicle and its subsequent fusion with the target membrane. We propose that inhibition of PLD by amphiphysins and AP180, together with the hydrolysis of PI(4,5)P2 by synaptotagmin, provides a mechanism to break the feedback loop. Given that AmphI, AP180, and synaptotagmin are all nerve terminal-specific proteins, such a mechanism would be specific to synaptic vesicles. Such redundant inhibition of PLD by multiple proteins might be necessary during synaptic transmission to ensure a rapid response; the rate of response is likely less critical for endocytotic events in other cell types, where AmphII might suffice by itself. Finally, it is important that PLD inhibition occurs after coat assembly to prevent further hydrolysis of PC. Such timing might be related to the facts that synaptotagmin and dynamin compete for binding to the SH3 domains of amphiphysins and that AmphI, AP180, synaptotagmin, and dy

AmphI

PLD1

PLD2

AmphII

Fig. 7. Effects of transient expression of AmphI or AmphII on PMA-induced PLD activity in COS-7 cells also expressing recombiant PLD1 or PLD2. Cells were transfected by electroporation with the indicated combinations (amounts shown in micrograms) of empty pCDNA3.1(control) and vectors encoding PLD1, PLD2, AmphI, or AmphII. The cells were labeled with [3H]myristic acid, and PMA-induced PLD activity was measured as described in Fig. 6. Data are expressed as a percentage of PMA-induced PLD activity in cells transfected with the control vector and are means of values from two independent experiments. Insets, immunoblot analysis of cell lysates with antibodies against AmphI (upper panel) or to PLD (lower panel). The positions of AmphI, PLD1, and PLD2 are indicated. The order of immunoblot samples is the same as that for PLD activity measurement.

![Fig. 7. Effects of transient expression of AmphI or AmphII on PMA-induced PLD activity in COS-7 cells also expressing recombinant PLD1 or PLD2. Cells were transfected by electroporation with the indicated combinations (amounts shown in micrograms) of empty pCDNA3.1(control) and vectors encoding PLD1, PLD2, AmphI, or AmphII. The cells were labeled with [3H]myristic acid, and PMA-induced PLD activity was measured as described in Fig. 6. Data are expressed as a percentage of PMA-induced PLD activity in cells transfected with the control vector and are means of values from two independent experiments. Insets, immunoblot analysis of cell lysates with antibodies against AmphI (upper panel) or to PLD (lower panel). The positions of AmphI, PLD1, and PLD2 are indicated. The order of immunoblot samples is the same as that for PLD activity measurement.](http://www.jbc.org/)

![Fig. 8. Effects of virally expressed AmphI on PMA-stimulated PLD activity in BHK cells. BHK cells (5 x 105) that had been grown in 6-well plate with α-minimum Eagle’s medium containing 5% FBS were infected by incubation for 12 h at 37 °C in 2 ml of the same medium containing 0.5% FBS and the indicated volumes (in microliters) of control and AmphI viruses; the cells were labeled for the same period by inclusion of 10 μCi of [3H]myristic acid in the infection mixture. PMA-stimulated PLD activity was then measured as described in Fig. 6. Data are expressed as a percentage of PMA-stimulated PLD activity measured in cells infected with the control virus alone and are means of two independent experiments. Inset, immunoblot analysis of cell lysates with antibodies against AmphI. The order of three immunoblot samples is the same as that for PLD activity measurement.](http://www.jbc.org/)
Inhibition of Phospholipase D by Amphiphysins
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