Synaptojanin Inhibition of Phospholipase D Activity by Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate*

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A 150-kDa protein that inhibits phospholipase D (PLD) activity stimulated by ADP-ribosylation factor and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) was previously purified from rat brain. The sequences of peptides derived from the purified PLD inhibitor now identify it as synaptojanin, a nerve terminal protein that has been implicated in the endocytosis of fused synaptic vesicles and shown to be a member of the inositol polyphosphate 5-phosphatase family. Further characterization of the enzymatic properties of synaptojanin now shows that it hydrolyzes only the 5-phosphate from inositol 1,4,5-trisphosphate (I(1,4,5)P3) and that it does not catalyze the dephosphorylation of either I(1,3,4)P3 or inositol 1,4-bisphosphate. However, synaptojanin hydrolyzes both the 4- and 5-phosphates of PI(4,5)P2 and the 4-phosphate of phosphatidylinositol 4-phosphate, converting both compounds to phosphatidylinositol. Magnesium is required for the hydrolysis of I(1,4,5)P3 but not for that of phosphoinositides, by synaptojanin. The inhibition of PLD by synaptojanin is attributable to its ability to hydrolyze PI(4,5)P2. Synaptojanin did not inhibit PLD in the absence of PI(4,5)P2, and the extent of PLD inhibition was related to the extent of PI(4,5)P2 hydrolysis in substrate vesicles. It has been proposed that the biosynthesis of PI(4,5)P2 and the activation of PLD by ADP-ribosylation factor constitute a positive loop to increase rapidly the concentrations of PI(4,5)P2 and phosphatidic acid (PA) during membrane vesiculation. The PA thus produced, probably together with PI(4,5)P2, facilitates vesicle coat assembly. The hydrolysis of PI(4,5)P2, and consequent inhibition of PLD, by synaptojanin might therefore constitute a mechanism to halt the positive loop connecting PI(4,5)P2 and PA during the endocytic cycle of synaptic vesicles and serve as a signal for uncoating.

The hydrolysis of phosphatidylcholine (PC)† to phosphatidic acid (PA) and choline catalyzed by phospholipase D (PLD) appears to be a crucial step for transmembrane signaling by many cell surface receptors as well as for membrane trafficking (1, 2). PA functions as an intracellular messenger and can be further hydrolyzed by a PA-specific phosphatase to diacylglycerol, an activator of protein kinase C. Mammalian PLD appears to exist in multiple isoforms (3), the activation of some of which requires a member of the family of small GTP-binding proteins, ADP-ribosylation factor (ARF) (4, 5). The activity of such PLD isoforms is further enhanced by the presence of PI(4,5)P2, but not by the presence of PI or PI(4)P in the substrate vesicles (4, 6). Human PLD1 (hPLD1), one of the PLD isoforms activated by ARF and PI(4,5)P2, has been cloned (3).

We recently identified multiple proteins that inhibit the activity of ARF- and PI(4,5)P2-stimulated PLD (7). One of these inhibitors was purified to homogeneity to yield a 150-kDa protein that was tentatively named inhibitor IA. Sequences of two tryptic peptides derived from IA protein were determined (7), but at the time they did not match any protein sequences in the data bases. However, a subsequent search revealed that the two sequences, EANAPAFD and GSVPLFWE, match exactly those of residues 276–283 and residues 251–258, respectively, of synaptojanin, a protein that is enriched in nerve terminals and was recently cloned (8). In the accompanying paper (9), we purified another inhibitor termed IB and identified it as clathrin assembly protein 3 (AP-3).

The central region of synaptojanin is highly homologous to a family of inositol polyphosphate 5-phosphatases (IP 5-Pases) that catalyzes the dephosphorylation of inositol polyphosphates or phosphoinositides at the 5′-position of the inositol ring (8). Indeed, synaptojanin was shown to possess IP 5-Pase activity (8). Here, we confirm that inhibitor IA is indeed synaptojanin, characterize its catalytic properties, and show that it inhibits PLD by hydrolyzing PI(4,5)P2.

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); GTP•S and PI(4,5)P2 were from Boehringer Mannheim; [choline- methyl-3H](dipalmitoyl PC (pam), PC (pam), PC) (50 Ci/mmol), [2-palmitolyl-9,10-O]-H](pam), PC (89 Ci/mmol), [inositol-2-H]PI(4,5)P2 (7 Ci/mmol), and [2-3H]inositol 1,4,5-trisphos-
Phospholipase D Inhibitor

Purification of hPLD1—hPLD1 was partially purified as described (10).

FIG. 1. Effects of synaptojanin on ARF-dependent hPLD1 activity in the absence or presence of PI(4,5)P$_2$. hPLD1 (11 ng for lanes 2 and 3, and 216 ng for lanes 5 and 6) was incubated with phospholipid substrate vesicles in a reaction mixture containing 0.67% (v/v) ethanol in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 8 nM synaptojanin. The substrate vesicles comprised either PE, PI(4,5)P$_2$, and [2-palmitoyl-9,10-$^3$H](pam)$_2$PC in a molar ratio of 16:1:4 (lanes 3 and 6) or PE and [2-palmitoyl-9,10-$^3$H](pam)$_2$PC in a molar ratio of 16:1 (lanes 4–6). The final concentration and specific radiactivity of PE were identical in the two substrate systems. After incubation for 1 h at 37 °C, the lipid products were analyzed by TLC and visualized by autoradiography. The positions of PC, PA, and PEtOH are indicated. Lanes 1 and 4 represent analysis of the reaction mixture before the addition of hPLD1.

RESULTS

We purified three additional tryptic peptides of inhibitor IA; their sequences (QQNWDLSIAGDLQNK, SAEDDLNLAS, and EVIADQGFPD) matched exactly those of synaptojanin residues 748–764, 820–830, and 884–893, respectively. Furthermore, rabbit antiserum prepared against inhibitor IA recognized a 150-kDa protein in rat brain homogenate but not in the homogenates of other rat tissues including liver, kidney, lung, heart, and spleen (data not shown). This tissue distribution is consistent with the nerve terminal–specific expression of synaptojanin (12). These results confirm that inhibitor IA is synaptojanin.

Given that synaptojanin is an IP 5-Phase, we investigated the possibility that synaptojanin inhibits PLD by hydrolyzing the activator PI(4,5)P$_2$. First, the inhibitory activity of synaptojanin was evaluated in the absence of PI(4,5)P$_2$. The ARF-dependent activity of hPLD1 in the absence of PI(4,5)P$_2$ is ~5% of that in its presence, so the amount of hPLD1 used to measure activity in the absence of PI(4,5)P$_2$ was 20 times that used in the presence of the activator. Because this increased amount of partially purified hPLD1 might introduce enzymes that cause nonspecific hydrolysis of PC, we measured the production of PEtOH, as well as that of PA, from [2-palmitoyl-9,10-$^3$H](pam)$_2$PC in the presence of ethanol. PLD attacks PC to form a transient phosphatidyl-PLD intermediate. With water as an acceptor, PA is the product; however, in the presence of ethanol, the phosphatidyl moiety is also transferred to the alcohol, generating PEtOH, in a reaction specific to PLD (11). Whereas synaptojanin at 8 nM completely inhibited the formation of both PA and PEtOH in the presence of PI(4,5)P$_2$, it had no effect on the formation of either product in the absence of PI(4,5)P$_2$ (Fig. 1).

We next incubated substrate vesicles containing PE, PI(4,5)P$_2$, and [choline-methyl-$^3$H](pam)$_2$PC in a molar ratio of 16:1:4 with synaptojanin for various times before the addition of hPLD1. The activity of hPLD1 decreased as the time of preincubation of vesicles with synaptojanin increased (Fig. 2). The extent of PI(4,5)P$_2$ hydrolysis during the preincubation with synaptojanin was measured with substrate vesicles with a lipid composition identical to that for the PLD inhibition assay with the exception that the $^3$H-labeled lipid was PI(4,5)P$_2$ instead of PC. Under this assay condition, only ~20% of total PI(4,5)P$_2$ appeared to be available for hydrolysis by synaptojanin (Figs. 2 and 3), probably because the lipid substrate consisted predominantly of multimellar vesicles with only 20% of their PI(4,5)P$_2$ exposed to the enzyme. Nevertheless, the extent of PI(4,5)P$_2$ hydrolysis correlated qualitatively with the extent of PLD inhibition (Fig. 2).

The products of PI(4,5)P$_2$ hydrolysis by synaptojanin were analyzed with the use of substrate vesicles containing PE, PI(4,5)P$_2$, and PC (Fig. 3). The major product was PA rather than PEtOH; the amount of PIP produced varied from negligible (Fig. 3A) to ~25% of that of PIP (see Fig. 5). However, with a micellar substrate containing 0.1% Triton X-100 and PI(4,5)P$_2$, synaptojanin generated PIP and PI in a 3:1 ratio (data not
I(1,4,5)P₃ was converted to IP₂ in a synaptojanin concentration-
relative rates of hydrolysis of PI(4,5)P₂ and PI(4)P by synapto-
Janin were added as carriers (400 mM anol, and concentrated HCl as described for the PLD assay, and

The reaction was stopped, and lipids were extracted and sepa-
rated by TLC (Silica gel 60, Merck), which had been treated with oxalate, using a solvent system of n-
propanol, ammonium hydroxide, and water (65:20:15, v/v). The amount of radioactivity associated with PI and PIP were added to calculate the

The origin is indicated, as are the positions of PI(4,5)P₂, PI(4)P, and PI, after incubation of lipid vesicles in the absence
(lane 1) or presence of synaptojanin at 1 nM (lane 2) or 10 nM (lane 3).

The origin is indicated, as are the positions of PI(4,5)P₂, PIP, and PI, and an unidentified impurity, B, the amounts of PIP, PI, and the sum of PIP
plus PI generated in the presence of various concentrations of synap-
tojanin, expressed as a percentage of total PI(4,5)P₂. Each point represents the mean (± S.E.) of triplicate determinations from one of three similar experiments.

![Figure 2](image1.png)

**Fig. 2.** Relation between PI(4,5)P₂ dephosphorylation and PLD inhibition by synaptojanin. Lipid vesicles comprising PE, PI(4,5)P₂, and choline-methyl-[³H]PI(4,5)P₂ PC in a molar ratio of 16:1:4:1 were prepared in a buffer containing 50 mM Hepes-NaOH (pH 7.5), 3 mM EGTA, and 80 mM KCl. The vesicles (25 µl) were then added to 125 µl of a solution containing 0.5 nm synaptojanin, 100 nM ARF, 5 µM GTP/S, 50 mM Hepes-NaOH (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl₂, and 2 mM CaCl₂. The final concentration of PC was 3.4 µM, with 

The hydrolysis of PI(4)P was demonstrated directly with vesicles containing PE, PI(4)P, and PC in a molar ratio of 16:1:4:1; the rate of PI(4)P hydrolysis was ½ to 1.5 times that of PI(4,5)P₂ hydrolysis measured with vesicles of identical lipid composition with the exception that PI(4)P was replaced by PI(4,5)P₂ (data not shown). These results suggest that the relative rates of hydrolysis of PI(4,5)P₂ and PI(4)P by synapto-
janin depend on the physical nature of the substrate. Because PI(4)P and PI(5)P cannot be distinguished by TLC, we do not

We tested the phosphatase activity of synaptojanin with I(1,4,5)P₃, I(1,3,4)P₃, and I(1,4)P₂. As previously shown (9), I(1,4,5)P₃ was converted to IP₂ by synaptojanin concentration-
dependent manner, and no further dephosphorylation to IP was observed (Fig. 4A). Hydrolysis of I(1,3,4)P₃ (Fig. 4B) or I(1,4)P₂ (Fig. 4C) was not apparent, even in the presence of high concentrations of synaptojanin. Thus, unlike the situation with phosphoinositides, the hydrolysis of inositol polyphosphates by synaptojanin appears specific to the 5-phosphate.

Furthermore, whereas the hydrolysis of PI(4,5)P₂ by synaptojanin did not require Mg²⁺ (the rate was slightly faster in the absence of Mg²⁺), the synaptojanin-catalyzed hydrolysis of

I(1,4,5)P₃ was absolutely dependent on the presence of Mg²⁺ (Fig. 5).

We previously showed that inhibitor IA (synaptojanin) did not inhibit the PI(4,5)P₂-hydrolyzing activity of phospholipase C-γ1 (PLC-γ1) when measured with substrate vesicles containing PE, PI(4,5)P₂, and PC in a molar ratio of 16:1:4:1 (7). Inhibition was not apparent probably because PLC activity was assayed by measuring the release of water-soluble [³H]-labeled inositol phosphates from [inositol-²H]PI(4,5)P₂ and because PI and PIP derived from dephosphorylation of PI(4,5)P₂ by synaptojanin are also substrates for PLC-γ1, PLC-δ1, PLC-β1, PLC-β1, and PLC-β1 all hydrolyze PI, PIP, and PI(4,5)P₂ but the selectivity for PI(4,5)P₂ over PI decreases in the rank order PLC-β1 > PLC-δ1 > PLC-γ1 (13). We therefore studied the effect of synaptojanin on the [inositol-²H]PI(4,5)P₂-hydrolyzing activity of the three PLC isozymes (Fig. 6). Whereas the activity of PLC-γ1 was not affected, the activities of PLC-β1 and PLC-β1 were inhibited by synaptojanin in a concentration-dependent manner. The rank order of the extent of inhibition, PLC-β1 > PLC-δ1 > PLC-γ1, is consistent with the selectivity of the PLC isozymes for PI(4,5)P₂ over PI.

**DISCUSSION**

IP 5 Pases vary markedly in both molecular size and amino acid sequence. However, they contain two short motifs (six to nine amino acids) that are separated by fairly constant num-

![Figure 3](image2.png)

**Fig. 3.** Dephosphorylation of PI(4,5)P₂ by synaptojanin. Lipid vesicles containing PE, [inositol-²H]PI(4,5)P₂, and PC were incubated for 5 min at 37 °C with various concentrations of synaptojanin as described for the measurement of PI(4,5)P₂ hydrolysis in the legend to Fig. 2. The reaction was stopped, and lipids were extracted and sepa-
rated by TLC. A, autoradiogram showing separation of [³H]-labeled PI(4,5)P₂, PIP, and PI, after incubation of lipid vesicles in the absence
(lane 1) or presence of synaptojanin at 1 nM (lane 2) or 10 nM (lane 3). The origin is indicated, as are the positions of PI(4,5)P₂, PIP, PI, and an unidentified impurity, B, the amounts of PIP, PI, and the sum of PIP
plus PI generated in the presence of various concentrations of synap-
tojanin, expressed as a percentage of total PI(4,5)P₂. Each point represents the mean (± S.E.) of triplicate determinations from one of three similar experiments.
bers of amino acids (14–16). The enzymes have been classified according to their substrate specificity (15): Group 1 enzymes hydrolyze I(1,4,5)P3 and I(1,3,4,5)P4, but not lipid substrates such as PI(4,5)P2 and PI(3,4,5)P3, group 2 enzymes hydrolyze the two inositol phosphate substrates I(1,4,5)P3 and I(1,3,4,5)P4 as well as the two lipid substrates PI(4,5)P2 and PI(3,4,5)P3, and group 3 enzymes hydrolyze only substrates containing a 3-phosphate [PI(3,4,5)P3 and I(1,3,4,5)P4].

The central region of synaptojanin contains the two IP 5-Pase-specific motifs and was previously shown, in the presence of Mg2+ ions, to hydrolyze I(1,4,5)P3 and I(1,3,4,5)P4, but not I(1,3,4)P3, and was reported to convert PI(4,5)P2 to an unspecific PIP (8). Synaptojanin was, therefore, considered a group 2 IP 5-Pase (15). The 75-kDa human platelet IP 5-Pase, a group 2 member, also requires Mg2+ ions for activity (17).

We have now further characterized the catalytic properties of synaptojanin with a homogeneous preparation. The synaptojanin used in the previous study of McPherson et al. (8) was either immunoprecipitated with antibodies to synaptojanin or had been exposed to a denaturing condition during purification. Our data suggest that synaptojanin specifically hydrolyzes the 5-phosphate in inositol polyphosphates. However, it dephosphorylates both the 5- and 4-positions, showing a preference for the 4-phosphate over the 5-phosphate under certain conditions, of lipid substrates. Furthermore, Mg2+ was not required for the activity toward lipid substrates. Synaptojanin thus resembles the 90-kDa polyphosphoinositide phosphatase purified from rat brain, which hydrolyzes phosphate from the 4- and 5-positions of PI(4,5)P2 to yield PI in the absence of Mg2+ (18). However, the 90-kDa enzyme is specific for phosphoinositides and does not act on I(1,4,5)P3 (18).

The characteristics of the NH2- and COOH-terminal regions of synaptojanin suggest a possible role in membrane trafficking. The NH2-terminal region is similar to the cytosolic domain of yeast Sac1 protein (8). The sac1 gene was identified as a...
suppressor of the sec14 mutant, which is defective in post-Golgi membrane trafficking (19); Sec14 protein is a PI transfer protein. The proline-rich COOH terminus of synaptojanin interacts with the Sro homology 3 (SH3) domains of amphiphysin (8) and Grb-2 (12), which also bind to the proline-rich domain of dynamin. Dynamin is a GTPase that colocalizes with synaptojanin in nerve terminals and functions in the closure of coated vesicles budding from the plasma membrane, including nerve terminal clathrin-coated vesicles that participate in the recycling of synaptic vesicle membranes after exocytosis (12, 20). Amphiphysin also interacts with the clathrin adapter protein AP-2 through a region distinct from its SH3 domain and may therefore help concentrate dynamin and synaptojanin at clathrin-coated pits (20, 21). Amphiphysin contains regions that are similar to the yeast proteins Rvs161p and Rvs167p, mutations in which result in defects in endocytosis (22–24). On the basis of these observations, synaptojanin was suggested to participate with dynamin and amphiphysin in the endocytic pathway of synaptic vesicles (12, 20, 21).

A growing body of evidence indicates that phosphoinositide metabolism is critical for the regulation of vesicular traffic (20). Studies on Ca^{2+}-dependent neurotransmitter release from permeabilized PC12 cells indicate that PI transfer protein and PIP 5-kinase are cytosolic factors required for exocytosis (25), probably because the generation of PI(4,5)P_{2} is a critical event in priming. It is thought that PI transfer protein presents PI to PI 4-kinase associated with secretory vesicles; PI is thus phosphorylated to PI(4)P, which is further converted to PI(4,5)P_{2} by PI P 5-kinase. PI(4,5)P_{2} binds AP-2 and dynamin, both of which function in clathrin-mediated endocytosis. Therefore, the generation of PI(4,5)P_{2} appears to be important for not only exocytosis but also subsequent budding of the discharged vesicles.

Studies on the formation of coatomer-coated Golgi vesicles also suggest a role for PI(4,5)P_{2} in bud formation. Cytosolic ARF has been proposed to exchange bound GDP for GTP at the Golgi surface; the resulting Golgi-bound ARF-GTP then recruits coatomer from the cytosol to assemble the bud (26). PI(4,5)P_{2} stimulates the ARF GTP-GDP exchange reaction (27), and might thereby contribute to coat assembly. PI(4,5)P_{2}, together with the GTP-bound ARF, also activates PLD, with the resulting generation of PA (6). PA stimulates the activity of PI(4)P 5-kinase (28), resulting in increased synthesis of PI(4,5)P_{2}, which, in turn, would cause further stimulation of ARF and PLD activity, leading to more production of PA and PI(4,5)P_{2}. This positive feedback loop would be expected to cause a rapid increase in PA and PI(4,5)P_{2} and result in a substantial change in the microdomain architecture of the lipid bilayer that would facilitate coat assembly (29). The hydrolysis of ARF-bound GTP stimulated by an ARF-specific GTPase-activating protein has been proposed as a signal for terminating the positive feedback loop (29) and serving as a switch for uncoating (26). Consistent with this model, loading ARF with GTPγS was shown to prevent uncoating and induce an accumulation of coated vesicles (26).

Recent studies by Ktistakis et al. (2) indicate that the dual actions of ARF, activation of PLD and induction of coatomer binding, are related events. ARF-dependent PLD is abundant in Golgi membranes (30). When Golgi membranes were prepared from cell lines with high constitutive PLD activity, cytosolic ARF was not necessary for initiating coat assembly. Furthermore, the generation of PA in Golgi membranes by an exogenous bacterial PLD was shown to induce coatomer binding. Purified coatomer also bound selectively to artificial lipid vesicles that contained PA and PI(4,5)P_{2}. These observations led Ktistakis et al. to propose that the main role of activated ARF is to activate PLD, and that both the PA produced by the activated PLD and PI(4,5)P_{2} are responsible for stable binding of coatomer (2).

The observations of Ktistakis et al. (2) also raise a question with regard to the role of the hydrolysis of ARF-bound GTP as the signal for uncoating and the halt of the positive loop between PA and PI(4,5)P_{2} generation. One can hypothesize that hydrolysis of PI(4,5)P_{2} by a specific phosphatase might instead constitute such a signal; the resulting decrease in PI(4,5)P_{2} concentration would result in the deactivation of PLD as well as in the termination of the PI(4,5)P_{2}-dependent GTP-GDP exchange on ARF. In support of this hypothesis, an IP 5-Pase, termed OCRL, has been specifically localized to the Golgi complex (31). Mutations in the OCRL gene are responsible for the oculocerebrorenal syndrome of Lowe (OCRL), which is characterized by mental retardation, cataracts, and defects in renal tubular absorption (32). We have recently shown that OCRL protein inhibits ARF-activated PLD (2).

PLD activity has not been detected in association with synaptic vesicles. However, by analogy with Golgi vesicular traffic, a similar positive interaction between PI(4,5)P_{2} formation and PLD activation may play a regulatory role in clathrin coating. Furthermore, the hydrolysis of PI(4,5)P_{2} by synaptojanin may serve to interrupt the positive loop and to act as the signal for uncoating. In such a model, it would be important that synaptojanin acts on PI(4,5)P_{2} after coating. Such timing might be achieved through the SH3-dependent interaction with amphiphysin or phosphorylation of synaptojanin. Synaptojanin, like dynamin, is a phosphoprotein that undergoes dephosphorylation in response to nerve terminal depolarization (12).

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