We have designed a novel approach for studying the specificity of neutral phospholipase D from rat brain synaptic plasma membranes for endogenous phospholipid substrates in native membranes. A procedure was established that provides synaptic membranes labeled in selected phospholipids. This labeling procedure exploits the presence of endogenous acyl-coenzyme A synthetase and acyl-coenzyme A:lyso phospholipid acyltransferase in synaptosomes for acylating various lysophospholipid acceptors with radioactive fatty acid. With \(^{3}H\)arachidonate for acylation and optimal concentrations of the respective lysophospholipids, membranes were labeled in either of the following phospholipids: phosphatidylycholine (93% of total label in phospholipids), 1-O-alkyl-phosphatidylcholine (87%), phosphatidylglycerol (90%), phosphatidylethanolamine (85%), phosphatidylethanolamine-plasmalogen (81%) or phosphatidylserine (59%). These membranes were employed to study the substrate specificity of the neutral, oleate-activated rat brain phospholipase D. This phospholipase exhibited almost absolute specificity for the choline-phospholipids phosphatidylcholine and 1-O-alkyl-phosphatidylcholine: 0.34% of the former labeled substrate were transphosphatidylated to phosphatidylpropanol during the assay and 0.28% of the latter. Activity toward other phospholipids was barely detectable and could largely be accounted for by utilization of residual labeled phosphatidylcholine present in those preparations. The phospholipase D exhibited some preference for fatty acids in the C-2 position of phosphatidylcholine in the following order: 2-oleoyl-phosphatidylcholine (0.67% of this labeled phosphatidylcholine were converted to phosphatidylpropanol), 2-myristoyl-phosphatidylcholine (0.60%), 2-palmitoyl-phosphatidylcholine (0.46%) and 2-arachidonoyl-phosphatidylcholine (0.34%). The present approach of labeling membrane phospholipids \textit{in vitro} could be useful in studies of phospholipase specificity as an alternative to the use of sonicated vesicles or mixed detergent-phospholipid micellar systems.

Receptor-mediated activation of mammalian phospholipase D (EC 3.1.4.4., PLD)\(^{1}\) by various hormones, neurotransmitters, or growth factors has emerged as a novel putative signal transduction pathway (for review see Refs. 1–3). Whereas evidence for the signal-dependent stimulation of PLD activity in intact cells has accumulated rapidly, there is little information on the molecular and biochemical properties of the enzyme molecule itself. Nevertheless, current evidence suggests that mammalian PLD exists in several forms, which might be distinctly regulated and/or utilize different phospholipid substrates.

Determination of PLD substrate specificity \textit{in vitro} is usually carried out by comparing various classes of exogenous phospholipids, added in the form of sonicated vesicles, as substrates. With this method, Saito and Kanfer (4) first identified mammalian PLD, an acid-active enzyme (pH optimum 6.0–6.5) from rat brain membranes which catalyzes the hydrolysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), requiring unsaturated fatty acids or bile salts as activators (5). Other mammalian PLD activities have subsequently been reported to preferentially utilize the following substrates: platelet-activating factor and PC (6), PC (7, 8), PE (9, 10), N-acyl-PE (11, 12), phosphatidylglycerol (13–15), or PI-glycan (16–18). Recently, we identified and partially characterized in \textit{vitro} a highly active neutral PLD (pH optimum 7.2) from rat brain synaptic plasma membranes (SPM) (19). This enzyme requires the presence of Na-oleate for activity in \textit{vitro} and is stimulated by Mg\(^{2+}\). In that study (19), exogenous sonicated \(^{3}H\)dipalmitoyl-PC was supplied as a substrate, but the substrate specificity of the enzyme was not further characterized.

The use of exogenous phospholipids in studies of phospholipase substrate specificity can be problematic, as different phospholipids aggregate in different forms according to their physicochemical properties when sonicated in aqueous solutions (20). The poorly defined physical state of the substrate may have considerable influence on the accessibility of the various phospholipids to the enzyme, and hence, on the apparent substrate preference exhibited by the enzyme. Mixed detergent-phospholipid micellar systems have been introduced to solve this problem (21–23). However, in such systems the substrate is not present in a bilayer and, moreover, such

\footnote{The abbreviations used are: PLD, phospholipase D; alkyl-PC, 1-O-alkyl-2-acyl-phosphatidylcholine; BSA, bovine serum albumin; Hepes, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; PA, phosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE-plasmalogen, 1-O-alkyl-2-acyl-phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PPr, phosphatidylpropanol; PS, phosphatidylserine; SPM, synaptic plasma membranes; TLC, thin layer chromatography; GTP\(_{y}\)S, guanosine 5’-3-O-(thio)-triphosphate.}

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\footnote{Recipient of an Yigal Allon Fellowship.}

\footnote{Recipient of an Yigal Allon Fellowship and the incumbent of the Shlomo and Michla Tomarin Career Development Chair in Membrane Physiology. To whom correspondence should be addressed: Dept. of Hormone Research, P. O. Box 26, Rehovot 76100, Israel. Tel.: 972-8-342773; Fax: 972-8-344116.}

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we have developed an alternative and complementary approach designed to study the specificity of phospholipases for their phospholipid substrates in native membranes. An in vitro procedure was established for labeling selected phospholipids in synaptic membranes. The procedure is based on the presence of endogenous acyl-CoA synthetase and acyl-CoA:lyso phospholipid acyltransferase in synaptosomes (24) and their ability to radioactively label phospholipids by catalyzing a given lysophospholipid with a radioactive fatty acid. Membranes labeled in different phospholipids were obtained and were utilized to study the substrate specificity of the neutral rat brain SPM-PLD.

**EXPERIMENTAL PROCEDURES**

**Materials**—[5,6,8,9,11,12,14,15-3H]Arachidonic acid, [9,10-3H]myristic acid, [9,10-3H]oleic acid, and EN3HANCE spray were purchased from Du Pont-New England Nuclear. [9,10-3H]Palmitic acid was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Scintillation fluid (Lumax) was from Lumac (Landgraaf, The Netherlands). Glass-backed silica gel LK6 thin layer chromatography (TLC) plates were purchased from Whatman International (Maidstone, England). Aluminum oxide thin layer sheets and a centrifuge for filtration were from Merck (Darmstadt, Federal Republic of Germany). Egg lecithin was obtained from Fluka (Buchs, Switzerland). L-α-Lyso phosphatidycholine (egg yolk), 1-α-alkyl-α,γ-glycerol-3-phosphocholine (lyso-platelet-activating factor), L-α-lyso phosphatidylserine (bovine), L-α-lyso phosphatidyl ethanolamine (egg yolk), ethanolamine-phosphatidyl ethanolamine and phoshatidylserine (PS), PI, PE, and PE-plasmalogen, 0.51; L-a-lysophosphatidylserine (PS), PI, PE, and PE-plasmalogen, 0.51; 

**RESULTS**

**Selective Labeling of PC**—The labeling conditions were developed from a procedure which we had utilized previously to investigate the activation of neutral SPM-PLD on endogenous phospholipids in vitro (19). The phospholipid labeling obtained with that procedure resulted from acylation of intrinsic membrane lysophospholipids and was therefore rather nonspecific: 15% of the [3H]oleate incorporated into phospholipids, of that ~46% labeled PC, 4% labeled PI, and the rest labeled unidentified lipids. Addition of exogenous lyso-PC to the nonselective labeling system significantly increased the incorporation of [3H]fatty acid into PC (Fig. 1A). For acylation with [3H]oleate and [3H]arachidonate, the maximal labeling of PC was observed at lyso-PC concentrations of 50 and 100 μM, respectively (Fig. 1A). The labeling of other phospholipids decreased concomitantly (not shown). A rate-limiting factor for the acylating enzymes was free fatty acid. In the presence of 50 μM lyso-PC, addition of up to 50 μM oleate (of which labeled oleate contributed 1 μM) further increased the acylation of lyso-PC to PC (Fig. 1B). Unlabeled oleate, at a concentration of 50 μM, was therefore included in the experiments to optimize the labeling assay. Lyso-PC and oleate were inhibitory at superoptimal concentrations, probably due to their detergent properties. In the labeling experiments, incubations were generally carried out for 15 min at 37°C. Time course experiments showed the labeling reaction to progress linearly for at least 30 min (Fig. 2A). Formation of the intermediate product oleyl-CoA seemed to

**PLD Assay with Endogenously Labeled SPM**—Unless otherwise indicated, 30 μg of labeled SPM were incubated for 10 min at 37°C in the presence of 50 mM Na-Hepes, pH 7.2, 1 mM MgCl₂, 100 mM 1-propanol, and 0.3 mM oleate, in a final volume of 120 ml. Termination and determination of the products PF₆ and PA were carried out as described before (19).

**Protein Determination**—Protein was determined by the method of Bradford (28) with a k-band of BSA (1 mg/ml) as a standard. Membrane samples and standards were dissolved in 0.1 M NaOH.

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like that shown in Fig. 1. Incorporation of [3H]oleate and [3H]arachidonate into PC. A, rat brain synaptic membranes were incubated with increasing concentrations of lyso-PC as indicated, with 1 μCi/tube of either [3H]oleate at 1 μM (solid circles) or [3H]arachidonate at 0.05 μM (open circles), and production of [3H]PC was determined as detailed under "Experimental Procedures." B, synaptic membranes were incubated with 1 μM [3H]oleate (1 μCi/tube) and increasing concentrations of unlabeled oleate as indicated, in the presence of 50 μM lyso-PC. Other conditions were as described under "Experimental Procedures." [3H]PC production was measured.

be the rate-limiting step, since hardly any oleoyl-CoA accumulated under these conditions (Fig. 2A). Protein concentrations up to 0.3 mg/ml were within the linear range (Fig. 2B). Optimal MgATP and coenzyme A concentrations were 2.5 mM and 25 μM, respectively (Fig. 3, A and B). Under overall optimal conditions, usually 90% of the [3H]oleate-derived radioactivity present in phospholipids was found in PC and the total radioactivity in PC was 20-fold higher than under the nonselective labeling conditions. Typically, the labeling activity was about 300 pmol/mg/min; the variation between membranes from different preparations was within the range of 150-450 pmol/mg/min.

Labeling of Selected Phosphoglycerides—After establishing the conditions for the selective labeling of PC, we modified the assay in order to achieve maximally high specific radioactivity in any one chosen phosphoglyceride with minimal change in membrane phospholipid composition. Unlabeled exogenous oleate was omitted from the reaction (this did not alter the selectivity of labeling). The incubation time was extended to 60 min. Concentration dependence experiments like that shown in Fig. 1A were performed with each lysophospholipid that was examined, utilizing both [3H]oleate (at 1 μM) and [3H]arachidonate (at 0.05 μM) for acylation. Arachidonate labeled all tested lysophospholipids more selectively than oleate (not shown). The following lysophospholipid concentrations yielded maximal specific labeling with arachidonate and were utilized in all later experiments: lyso-PC, 1-alkyl-2-lyso-PC, and lyso-PS, 100 μM; lyso-PI, 50 μM; lyso-PE, 250 μM; lyso-PE-plasmalogen, 500 μM; and lyso-PG, 250 μM. The variations in optimal lysophospholipid concentrations and fatty acid efficiencies are likely to be due to the different physicochemical properties of the lysophospholipids, as well as the lysophospholipid/fatty acid preferences of the acyl-CoA transferases involved. The labeling selectivity achieved with [3H]arachidonate was highest for PC (93% of the total label in phospholipids was found in PC), followed by 90% for PI, 87% for alkyl-PC, 85% for PE, 81% for PE-plasmalogen, 71% for PG, and 59% for PS (Table I). ~50% of the [3H]arachidonate was incorporated into the membranes and, of that, 98% incorporated into phospholipids. Neutral lipids and residual arachidonate that was not washed out during the termination of the labeling reaction accounted for the rest of the membrane-bound radioactivity. Utilizing radioactively labeled lyso-PC and lyso-PE we determined that ~10% of the lysophospholipid added (7.5 μM of lyso-PC or 20 μM of lyso-PE) remained in the membranes after the labeling procedure (data not shown). The additional lyso-PC increased the intrinsic lyso-PC level by about 15%. We have further examined the effect of lyso-PE-plasmalogen on PLD activity. No effects of the lysophospholipid were observed at concentrations up to 100 μM; higher concentrations inhibited PLD activity, with an IC50 of 1 mM (not shown). It might therefore be concluded that, although part of added lysophospholipids remains in the membranes after the labeling procedure, the resulting changes in the lysophospholipid content of the membranes are minor ones and do not affect the activity of neutral SPM-PLD toward endogenous substrates. Consequently, the labeling conditions established here provided us with synaptic membranes, labeled in vitro in selected phosphoglycerides, which could be assayed separately as potential substrates in a subsequent PLD assay.

PLD Assay with Selectively Labeled Endogenous PC—PLD activity was determined by measuring the production of PA and PPr, products of the PLD-catalyzed hydrolysis and transphatidylidylation reactions, respectively. In the presence of Na-oleate as an activator, SPM-PLD could utilize endogenous PC from synaptic membranes labeled with [3H]arachidonate. Production of both [3H]PA (in the presence of 100 mM 1-propanol) and [3H]PPr (in the absence of alcohol) was stimu-
Synaptic membranes were labeled in selected phospholipids with 0.05 μM \(^{3}H\)arachidonate (1 μCi/30 μg of membrane protein) and the respective lysophospholipids as detailed under “Experimental Procedures.” Lysophospholipid concentrations employed were: lyso-PC, lyso-alkyl-PC, and lyso-PS, 100 μM; lyso-PI, 50 μM; lyso-PE and lyso-PG, 250 μM; and lyso-PE-plasmalogen, 500 μM. Results are expressed as mean ± S.D. The numbers in parentheses represent the number of determinations. The total percentages slightly exceed 100% due to the unequal number of determinations.

Table I

| Phospholipid | PC/alkyl-PC | PS | PI | PE/PE-plasmalogen | PG | PA |
|--------------|-------------|----|----|-------------------|----|----|
| % cpm of total phospholipids | % cpm of total phospholipids | % cpm of total phospholipids | % cpm of total phospholipids | % cpm of total phospholipids | % cpm of total phospholipids | % cpm of total phospholipids |
| Lysophospholipid acceptor | Lysophospholipid acceptor | Lysophospholipid acceptor | Lysophospholipid acceptor | Lysophospholipid acceptor | Lysophospholipid acceptor | Lysophospholipid acceptor |
| PC/alkyl-PC | 93.1 ± 1.3 (7) | 87.0 ± 4.0 (5) | 14.4 ± 2.8 (4) | 5.1 ± 2.2 (4) | 3.5 ± 0.7 (4) | 5.9 ± 1.6 (4) |
| PS | 0.8 ± 0.8 (3) | 0.6 ± 0.5 (2) | 59.2 ± 3.6 (4) | 4.9 ± 3.7 (2) | 2.3 ± 0.0 (1) | 1.6 ± 0.0 (1) |
| PI | 4.8 ± 1.2 (7) | 7.1 ± 1.2 (5) | 12.1 ± 2.1 (4) | 30.2 ± 6.2 (5) | 8.8 ± 1.7 (4) | 8.4 ± 1.3 (4) |
| PE/PE-plasmalogen | 1.1 ± 0.6 (7) | 3.4 ± 0.7 (6) | 12.8 ± 1.8 (4) | 2.2 ± 1.1 (5) | 85.1 ± 2.3 (4) | 81.3 ± 2.7 (4) |
| PG | 0.3 ± 0.2 (7) | 0.4 ± 0.1 (5) | 1.0 ± 0.2 (4) | 0.4 ± 0.3 (5) | 1.1 ± 0.5 (4) | 1.9 ± 0.8 (4) |
| PA | 0.8 ± 0.1 (3) | 3.2 ± 2.3 (3) | 2.6 ± 0.0 (1) | 0.5 ± 0.2 (2) | 3.7 ± 0.0 (1) | 8.2 ± 0.0 (1) |

Fig. 4. Stimulation of PLD activity by sodium oleate. Synaptic membranes were labeled in PC with 0.05 μM \(^{3}H\)arachidonate (1 μCi/30 μg membrane protein) and 100 μM lyso-PC as described under “Experimental Procedures.” For assaying PLD activity, the labeled membranes (0.25 mg/ml) were then incubated for 10 min at 37 °C, at increasing concentrations of Na-oleate as indicated, either with or without 100 mM 1-propanol, in the presence of 1 mM MgCl₂. Production of \(^{3}H\)PPr (solid circles) and \(^{3}H\)PA (open circles) was determined as detailed under “Experimental Procedures.”

Fig. 5. Specificity of PLD for choline phospholipids. Batches of synaptic membranes were labeled in different phospholipids with 0.05 μM \(^{3}H\)arachidonate (1 μCi/30 μg membrane protein) and the respective lysophospholipids as described under “Experimental Procedures.” Lysophospholipid concentrations employed were, lyso-PC, 1-alkyl-2-lyso-PC, and lyso-PS, 100 μM; lyso-PI, 50 μM; lyso-PE, 250 μM; and lyso-PE-plasmalogen, 500 μM. For assaying PLD activity, the labeled membranes (0.25 mg/ml) were incubated either with or without 0.3 mM Na-oleate, in the presence of 100 mM 1-propanol and 1 mM MgCl₂, for 10 min at 37 °C. Oleate-stimulated production of \(^{3}H\)PPr was determined. A, production of \(^{3}H\)PPr during the PLD assay is expressed as % cpm of the major labeled phospholipid in the respective determination. B, production of \(^{3}H\)PPr during the PLD assay is expressed as % cpm of labeled PC in the respective determination. The data are means of 6 (PC and alkyl-PC), 3 (PS and PI) or 2 (PE and PE-plasmalogen) independent experiments. The error bars represent the S.E.

plasmalogen (Fig. 5A). The highest rate of conversion of non-choline phospholipids was observed in PS-labeled membranes which were also less specifically labeled, and where PC accounted for 14% of the label in phospholipids (Table I). This suggested that most of the PPr formed in the PS-labeled membranes was derived from PC. Assuming the PC conversion rate to be 0.34%, it was calculated that about 80% of PPr formed in PS-labeled membranes is indeed PC-derived. To further evaluate the contribution of PC to PPr formation, the latter was expressed as a percentage of the labeled PC present in each preparation. In PS-labeled membranes, 0.24% of the labeled PC were converted to PPr within 10 min; 0.33% of the PC were converted in PI-labeled membranes and 0.34% in PE-labeled membranes (Fig. 5B). Hence, the low PLD activity observed in SPM labeled in phospholipids other than PC or alkyl-PC most likely originates from residual labeled PC present in such preparations. (The low PLD activity (<100
production of $[^3H]$PPr was determined and is expressed here as cpm of the labeled PC in the respective determination. The data are variance between some experiments.) The conversion of PG was not measurable, since a labeled degradation product of PG comigrated with PPr on the TLC plate; this degradation product was not formed by PLD action, since it was also present in zero time controls. Altogether, these results show that SPM-PLD is highly specific for PC and alkyl-PC.

Preference for Fatty Acids in C-2 Position of PC—Batches of synaptic membranes were labeled in PC with $1 \mu$Ci/30 $\mu$g of membrane protein of either $[^3H]$arachidonate, $[^3H]$palmitate, $[^3H]$myristate, or $[^3H]$oleate, in the presence of optimal lysophosphatidylcholine concentrations, without adding unlabeled fatty acid. Optimal lysophosphatidylcholine concentrations for selective PC labeling were 50 $\mu$M for labeling with oleate and 100 $\mu$M for labeling with other fatty acids. PLD utilized the different C-2-fatty acylated PC isomers in the following order: 2-oleoyl-PC (0.67% of labeled 2-oleoyl-PC were converted to PPr in 10 min) > 2-myristoyl-PC (0.60%) > 2-palmitoyl-PC (0.46%) > 2-arachidonoyl-PC (0.34%) (Fig. 6). Although the differences were not statistically significant, the results seem to suggest that PLD prefers certain fatty acids in the C-2 position of the choline-containing substrate molecules. It should also be noted that the egg-yolk lysophosphatidylcholine utilized in these experiments as an acceptor is itself somewhat heterogenous, a fact that possibly masked differences that are due to the variation in the C-2 fatty acyl chain.

DISCUSSION

Problems Associated with Determination of Phospholipase Substrate Specificity—Determination of the substrate specificity of lipolytic enzymes is not a trivial biochemical problem. Lipids aggregate in an aqueous environment in various forms, each according to its physicochemical properties and other prevailing conditions (20). Obviously, the lipid aggregate form would influence its susceptibility to enzymic hydrolysis by affecting parameters such as the effective surface concentration. This difficulty is compounded in the case of lipases which are integral membrane proteins, as fusion of substrate-containing and enzyme-containing vesicles may be rate limiting for catalysis. Such fusion is likely to be greatly affected by the nature of the lipid substrate, again influencing apparent rates of hydrolysis. One approach to this problem involves the use of a detergent-phospholipid mixed micellar system, which had been introduced by Dennis et al. (21). The advantage of this approach are (i) in introducing the phospholipid substrate in a defined physical environment (the detergent micelle) and, (ii) in enabling quantitative kinetic analysis of enzyme-substrate interaction based on the phospholipid surface concentration. This approach had been utilized to analyze phospholipases such as phospholipase A$_2$ (21) and phosphatidic acid phosphohydrolase (23), as well as enzymes that require lipid cofactors, e.g. protein kinase C (22). However, one disadvantage of mixed micellar systems is that the phospholipid is not present in its physiological environment, i.e. the membrane phospholipid bilayer. In addition, the presence of a detergent may conceivably affect substrate-enzyme interaction and, hence, apparent substrate specificity. Finally, phospholipases which are integral membrane proteins must be solubilized prior to their analysis in a mixed micellar system; their removal from the bilayer, which involves exchange of the "annular" lipids with detergent molecules, is liable to affect their substrate specificity.

We described here a novel alternative approach to that problem which is complementary to the use of mixed micelles. An in vitro procedure for selective labeling of endogenous phospholipids in synaptic membranes was developed, thus providing a labeled substrate for neutral synapostomal PLD within its native membranes. The feasibility of this approach stems from the presence of two enzymes, acyl-CoA synthetase and acyl-CoA:lyso-phospholipid acyltransferase, in synapticosomal membranes (24). These enzymes reacylate endogenous lysophospholipids normally produced by phospholipase A$_2$ (29). Selectivity of phospholipid labeling was accomplished in the present study by incorporating excess of a chosen lysophospholipid into membranes and its dominance over intrinsic lysophospholipids (mostly lyso-PC) in the competition for fatty acid acylation by $[^3H]$arachidonate. (It is noteworthy that our approach would have yielded still higher selectivity of labeling had we used radiolabeled lysophospholipids and acylated them with unlabeled fatty acids; however, labeled lysophospholipids other than lyso-alkyl-PC and lyso-PC are not commercially available to date.) The major advantage of this approach over previously described ones is in providing a nearly physiological milieu for phospholipase-phospholipid interaction. The lysophospholipid reacylating enzymes are also present in microsomes from most tissues (29), which could therefore be labeled by a procedure similar to that which was used here for synaptic membranes. In addition to avoiding the problems associated with use of sonicated vesicles and mixed micellar substrates, the present approach may potentially be used to study enzyme activation by physiological mechanisms, e.g. receptors, G proteins, and protein kinases.

Substrate Specificity of Neutral Rat Brain SPM-PLD—The present approach was employed for studying the substrate specificity of neutral rat brain SPM-PLD, thus demonstrating its utility. We showed that neutral synapostomal PLD is specific for the choline-phosphoglycerides diacyl-PC and alkyl-acyl-PC. The small PLD activity observed in PS-labeled membranes is most likely derived from residual labeled PC in these membranes. This could be deduced by expressing the PLD activity as percent of the labeled PC in those preparations. Thus, a nearly absolute specificity of this enzyme for choline-phosphoglycerides was demonstrated. In addition, suggestive evidence is provided for some specificity of SPM-PLD for the C-2 fatty acyl moiety of its PC substrate. These properties clearly distinguish the neutral rat brain PLD from certain other mammalian PLDs which utilize other phospholipids as substrate (see below). A similar preference for PC over PE was reported for the transphosphatidylation activity.

![Fig. 6. Influence of fatty acid in position C-2 of PC on PLD activity.](image-url)
of the acid-active rat brain PLD by Chalifour et al. (30). Qian and Drewes (31) have studied PC hydrolysis by a neutral GTPγS-activated PLD from canine brain; the substrate specificity of this enzyme has not been examined, and its relationship to the neutral rat brain PLD discussed here is unknown. Numerous studies carried out in [3H]choline-labeled intact cells have indicated the existence of a PC-hydrolyzing PLD (for review, see Refs. 32 and 33); the neutral rat brain SPM-PLD activity assayed in vitro (Ref. 19 and the present study) is likely to represent one or more of these cellular enzymes.

Labeling intact cells with other phospholipid head groups has suggested the existence of PLDs which hydrolyze PE (9) and PI (34). This conclusion is borne out in vitro studies, employing exogenous sonicated phospholipid substrates, which demonstrate the existence of PLDs that prefer non-choline-containing phospholipids. A PLD that hydrolyzes PE rather than PC was described in membranes isolated from bovine neutrophil extracts (13, 14) and human plasma (15). An N-acetyl-PE-specific PLD was found in dog brain and rat heart (11, 12). Recently, a cytosolic PLD utilizing PE, PC, and PI in that order of preference was demonstrated in various bovine tissues (10). Another soluble PLD, which specifically releases PI-glycan-anchored cell-surface proteins from the membrane, was purified from human plasma and bovine serum, cloned, and sequenced (16-18). As sonicated phospholipid vesicles were utilized in the above-mentioned studies, the substrate specificity of these PLDs, when it was examined, cannot be regarded as conclusive. The present approach of selective labeling of membrane phospholipids in vitro can be employed to elucidate the substrate specificity of these phospholipases and help establish their physiological significance.

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