Modulation of Phosphatidylinositol-specific Phospholipase C Activity by Phospholipid Interactions, Diglycerides, and Calcium Ions*

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We have investigated the effects of different lipids on the activity of a phosphatidylinositol (PI)-specific phospholipase C isolated from sheep seminal vesicular glands. Dispersions of PI in the absence of detergent are hydrolyzed at 1-3 pmol/min/mg of protein, a rate only 3-10% of that obtained when optimal concentrations of sodium deoxycholate are present. When hydrolysis of PI in microsomes from mouse L-cells is measured, only 0.01 pmol of PI is hydrolyzed/min/mg of protein. Lipid dispersions prepared from extracts of microsomes are also poor substrates. We added varying amounts of other phospholipids to PI dispersions to explore the effect on PI hydrolysis. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) had only modest effects on PI hydrolysis, but phosphatidylcholine (PC) was markedly inhibitory. Diglycerides stimulated PI hydrolysis in dispersions by about 10-fold. Using small unilamellar vesicles containing PI and PE (1:0.4), we found 50 pmol of PI hydrolyzed/min/mg of protein. Incorporation of PC in increasing amounts into these vesicles inhibited PI hydrolysis by 78% at 1 PC/PI, and at 4 PC/PI, no hydrolysis occurred. PC incorporation into vesicles separate from those containing PI was not inhibitory. Incorporation of PS into vesicles containing PI and PC overcame the inhibition of PI hydrolysis in a calcium ion- and PS-dependent manner. PI hydrolysis at 3 mM Ca²⁺ using vesicles containing PI/PC/PS/PE in proportions of 1:4:2:0.4 was 2.5 pmol/min/mg of protein, compared to undetectable hydrolysis in similar vesicles without PS. Addition of diglyceride further increased the rate of hydrolysis by 2.5-fold. We conclude that PC inhibits PI hydrolysis by interacting with the substrate. The inhibition is overcome by negatively charged lipids and high concentrations of calcium ions. Diglyceride, a product of PI hydrolysis, further stimulates PI breakdown. Since negatively charged lipids are concentrated on the inner leaflet of most cell membranes, we postulate that calcium fluxes resulting from stimulation of cell surface receptors may initiate PI breakdown which is further accelerated as diglyceride accumulates.

The physiological significance of agonist-stimulated PI hydrolysis is not fully elucidated. Recent experiments in platelets (1) and other tissues (2, 3) indicate that diacylglyceride, which is produced from PI by the action of a PI-specific phospholipase C following stimulation, is further degraded to release arachidonic acid for conversion to prostaglandins, thromboxanes, leukotrienes, and other arachidonate metabolites that are collectively designated as eicosanoids (4). In addition, diglyceride itself has been shown to stimulate a CAMP-independent protein kinase present in membranes (5).

The availability of free arachidonate controls the rate of synthesis of arachidonate metabolites. PL-specific phospholipase C is thought to regulate arachidonate release from PI, partly because agents that elevate platelet and arachidonate release (6) and because the enzyme requires calcium ions for activity. Calcium fluxes are important in the activation of platelets and other cells. The release of free calcium ions from platelet intracellular storage granules following thrombin stimulation (7) may allow the PI-specific phospholipase C to hydrolyze PI in the membrane and thus trigger arachidonate release.

PI-specific phospholipase C activity has been described in crude extracts from many mammalian tissues (8). We have recently identified two immunologically distinct PI-specific phospholipase C enzymes in sheep seminal vesicles and have purified one of them to homogeneity. While the enzyme readily metabolizes pure dispersions of PI, the PI in cellular membranes is a poor substrate. These findings contrast with studies of bacterial PL-specific phospholipase C enzymes, which do not require calcium ions and readily hydrolyze PI from cell membranes. Irvine et al. (9) have studied the ability of the mammalian enzyme in crude homogenate to hydrolyze PI in various lipid dispersions and have concluded that PC inhibits the enzyme and that acidic phospholipids and negatively charged amphiphiles overcome the inhibition. In the current report, we have investigated the effects of various lipids on homogeneous PI-specific phospholipase C using SUV of defined lipid composition. We find that PC inhibits enzyme activity by "masking" substrate PI. The inhibition is overcome by increasing the concentration of PS in the vesicles in a manner which is dependent on calcium ions. Diglycerides further enhance enzyme activity, suggesting that this product of PI-specific phospholipase C may have a physiological role in enhancing the rate of PI breakdown.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylcholine (egg), phosphatidylethanolamine (egg), phosphatidylinositol (grade III, soybean, ammonium salt), sodium deoxycholate, triolein, 1-monolein, cholesterol, bovine serum albumin (recrystallized and lyophilized), and EGTA were from Sigma. Phosphatidylserine (bovine brain, Sigma) was purified by silicic acid column chromatography (10). The purity of phospholipids was monitored by thin layer chromatography with activated Silica Gel 60 plates (MCB Manufacturing Co., Cincinnati, OH) developed in chloroform/methanol/acetic acid/H₂O (80:30:10:3:2) and visualized with iodine vapor. The fatty acid compositions of the phospholipids used...
TABLE I
Fatty acid compositions of phospholipids used in this study

| Phospholipid            | Fatty acid (mol %) |
|-------------------------|--------------------|
|                         | 16:0 | 18:0 |
| Phosphatidylinositol (soybean) | 35.2 | 9.4  |
| Phosphatidylcholine (egg)    | 34.5 | 12.5 |
| Phosphatidylethanolamine (egg) | 19.1 | 31.8 |
| Phosphatidylserine (bovine brain) | 1.2  | 55.0 |

TABLE II
Rate of PI hydrolysis under different assay conditions

| Assay conditions       | µmol PI hydrolyzed/min/mg protein |
|------------------------|----------------------------------|
| 1. No deoxycholate, brief sonication | 1-3 |
| 2. +1 mg/ml deoxycholate, brief sonication | 25 |
| 3. +100 µM 1,2-diolein, brief sonication | 17 |
| 4. +50 µM arachidonic acid | 16 |
| 5. +250 µM arachidonic acid | 30 |
| 6. PI in SUV made from LMC-cell membrane phospholipids | 52 |

RESULTS

We initially investigated the rate of PI hydrolysis using our homogeneous, semisal vesicle PI-specific phospholipase C with sonicated dispersions of PI with or without other phospholipids. When PI is dispersed by brief sonication in the absence of detergent at pH 7.0, 1-3 µmol of PI are hydrolyzed/min/mg of protein as shown in Table II. The rate of hydrolysis was stimulated 10-fold by the addition of the amionic detergent sodium deoxycholate, which presumably forms mixed micelles with the substrate (Table II and Ref. 13). When PI is dispersed in the presence of arachidonate or other fatty acids (9), hydrolysis is similar to that obtained using deoxycholate (Table II). Addition of varying proportions of PE or PS had minimal effects on PI hydrolysis (data not shown). PC markedly inhibited PI hydrolysis as illustrated below. An unexpected result was that a diglyceride (1,2-diolein) enhanced the rate of PI hydrolysis, as shown in Table II. This observation is of potential physiological significance since diglyceride is a product of the PI-specific phospholipase C.

Both 1,2- and 1,3-diglyceride stimulated PI hydrolysis as shown in Fig. 1. The inhibition of PI hydrolysis observed at high 1,2-diolein concentrations was associated with the formation of a precipitate in reaction mixtures. Mixtures of 1,2-diarylodon also stimulated hydrolysis but did not inhibit (or precipitate) at higher concentrations. Other neutral lipids including 1,2-distearin, triolein, and 1-monoolein only slightly stimulated the rate of PI hydrolysis. The ability of diglyceride...
approximately 0.02 pmol of PI hydrolyzed/min/mg of protein shown). When diolein was added to intact microsomes, there was a stimulation of hydrolysis. The slow PI hydrolysis was caused by lipid interactions rather than by masking of PI by proteins because dispersions of extracted microsomal lipids give results similar to intact microsomes, as shown in Fig. 2A. In this case, high concentrations of calcium ions were also stimulatory, with an initial rate of hydrolysis of approximately 0.01 μmol of PI hydrolyzed/min/mg of protein at 50 mM calcium ions. The effect of Ca2+ is not merely due to increased ionic strength because increasing NaCl concentrations up to 0.75 M had little effect on PI hydrolysis (data not shown). When diolein was added to intact microsomes, there was a 2-fold enhancement in the rate of PI hydrolysis (Fig. 3A) even though the access of diglyceride to microsomal phospholipids under these conditions is probably minimal. When 30 μM diolein was added to microsomal lipids, there was a 3-fold stimulation of PI hydrolysis (Fig. 3B) which was not increased by higher diolein concentrations.

PI Hydrolysis in Small Unilamellar Vesicles—The highest rates of PI hydrolysis were observed using SUV (Table II). PI with PE in a ratio of 1:4 is rapidly hydrolyzed (13-30 μmol/min/mg at 60 μM PI) until 60-70% of the total PI is hydrolyzed. Although 60 μM PI is less than saturating, this concentration was used in all subsequent experiments in order to conserve material. When the SUV concentration was raised to 250 μM PI, approximately 2-fold greater rates of hydrolysis were observed. Vesicles prepared using only PI were equally rapidly hydrolyzed but were unstable. Therefore, PE was included in all vesicle preparations. Inclusion of PC in PI/PE vesicles inhibited PI hydrolysis by 75% in SUV containing 1 PC/PI; PI hydrolysis was completely inhibited in SUV containing 4 PC/PI, as shown in Fig. 4. When PC-containing vesicles were added to PI/PE vesicles, there was almost no effect on PI hydrolysis, indicating that PC and PI must be in the same

**TABLE III**
The amount of 1,2-diolein required for activation of PI hydrolysis depends on PI concentration

| [PI] | Maximum activity [DO]required for 90% of maximal activity [PI]/[DO] |
|------|---------------------------------------------------------------|
| μM   | units/mg | μM          |
| 10   | 0.4      | 1.5         | 6.7    |
| 50   | 7.0      | 8.1         | 6.3    |
| 200  | 19.3     | 31.3        | 7.1    |

to enhance PI hydrolysis is due to an effect on the substrate rather than the enzyme since the optimal activation of hydrolysis varies with substrate concentration at constant amounts of enzyme, as shown in Table III. Approximately 1 diglyceride molecule/7 PI molecules gives half-maximal activation over a wide range of substrate concentrations. Diglyceride is more potent than deoxycholate under these conditions since approximately 3 deoxycholate molecules/PI give similar stimulation of hydrolysis.

Hydrolysis of PI in Microsomal Membranes and Lipids—PI was hydrolyzed poorly when microsomes derived from L8 cells were used as substrate, as shown in Fig. 2A. In this case, hydrolysis was stimulated by calcium ions at much higher concentrations than in dispersions of PI where calcium concentrations above 1.0 mM were inhibitory. The initial rate of PI hydrolysis at 50 mM calcium ions was approximately 0.01 μmol of PI hydrolyzed/min/mg of protein. The slow PI hydrolysis was caused by lipid interactions rather than by masking of PI by proteins because dispersions of extracted microsomal lipids give results similar to intact microsomes, as shown in Fig. 2B. In this case, high concentrations of calcium ions were also stimulatory, with an initial rate of hydrolysis of approximately 0.02 μmol of PI hydrolyzed/min/mg of protein at 50 mM calcium ions. The effect of Ca2+ is not merely due to increased ionic strength because increasing NaCl concentrations up to 0.75 M had little effect on PI hydrolysis (data not shown). When diolein was added to intact microsomes, there was a 2-fold enhancement in the rate of PI hydrolysis (Fig. 3A) even though the access of diglyceride to microsomal phospholipids under these conditions is probably minimal. When 30 μM diolein was added to microsomal lipids, there was a 3-fold stimulation of PI hydrolysis (Fig. 3B) which was not increased by higher diolein concentrations.

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**FIG. 2.** Time course of PI cleavage in intact L8-cell microsomes (A) or in extracted microsomal lipids (B) and effect of added calcium. The enzyme concentration was 4.6 μg/ml, and the total phospholipid concentration was 0.15 mM. We determined that PI accounts for 5.3 ± 1.2% of the total phospholipid in these microsomal preparations by high performance liquid chromatography as described under "Experimental Procedures." Therefore, the PI concentration is approximately 8 μM (compare to PI/PE vesicles at this concentration, 1.5 units/mg at 1 mM Ca2+). The buffer contained 50 mM Hepes, pH 7.0, 100 mM NaCl, and 0.5 mg/ml of bovine serum albumin. A free Ca2+ concentration of approximately 4 μM was attained using a CaCl2/EGTA buffer ([CaCl2]/[EGTA] = 0.9) as previously described (13).

**FIG. 3.** Effect of 1,2-diolein on the time course of PI cleavage in intact microsomes (A) or in extracted microsomal lipids (B). Assay conditions are the same as described in the legend to Fig. 2. In A, the 1,2-diolein (D.O.) in ether solution was placed in the bottom of assay tubes, and the solvent was evaporated under a stream of N2. The other components of the assay mixture were then added and the mixture was sonicated for 15 s × 50 watts. In B, the microsomal lipids and 1,2-diolein (D.O.) were mixed first in organic solvent and the assays were performed as described above.
vesicle in order for PC to be inhibitory. It appears that PC inhibits PI hydrolysis by masking substrate PI rather than by affecting the enzyme. Calcium ion concentrations above 1 mM did not stimulate PI hydrolysis in PC/PI/PE vesicles. In contrast, rates of PI hydrolysis using SUV made from LM-cell microsomal lipids were 0.03, 0.05, and 0.06 pmol of PI/min/mg of protein at 1, 10, and 50 mM CaCl₂, respectively.

PC/PE vesicles do not inhibit hydrolysis of [³H]PI in PC/PE vesicles, as shown in Fig. 5. In fact, PC/PI/PE vesicles also failed to inhibit, indicating that the enzyme has no apparent affinity for the unlabeled PI in the PC-containing vesicles, as compared to unlabeled PI/PE vesicles which were inhibitory (Fig. 5). PC/PS/PE-containing vesicles were only slightly inhibitory, indicating that the enzyme does not have a high affinity for the acidic phospholipid PS in PC-containing vesicles. However, when PS was incorporated into vesicles containing PC/PI/PE, there was a marked increase in PI hydrolysis from undetectable to 2 μmol of PI hydrolyzed/min/mg of protein at 0.5 PS/PI, as shown in Fig. 6. The rate of PI hydrolysis was further stimulated in vesicles containing additional PS, and the reaction was dependent on high calcium ion concentrations at higher proportions of PS. Above 3 mM calcium ions, the vesicles aggregated and precipitated. Similar results were seen using SUV at 250 μM PI (data not shown). The ability of PS to overcome inhibition by PC is not due to a direct effect of PS on either enzyme or PI since PS in vesicles without PC (i.e., PI/PE/PS) did not stimulate PI hydrolysis, but was actually inhibitory (data not shown). 1,2-Diolein did not stimulate PI hydrolysis unless the vesicles contained PC, PI, and PE in a ratio of 4:1:0.4. PS and diglycerides (DG) were present in a molar ratio with PI as follows: [1] no PS or DG; [2] 1 DG; [3] 2 PS; [4] 3 PS and 1 DG. A, 2 PS and 2 DG. The PI concentration in all assays was 60 μM. The PS to PI ratio was varied: [1] 0; [2] 0.5; [3] 1; [4] 2; [5] 4. Values represent data from two experiments giving similar results. Enzyme concentration was 0.46 μg/ml. Assays were performed for 2 min at 37°C.
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PI hydrolysis in small unilamellar vesicles containing phospholipids in proportions which mimic the cytoplasmic face of the membrane bilayer.  

**Fig. 8.** PI hydrolysis in small unilamellar vesicles containing phospholipids in proportions which mimic the cytoplasmic face of the membrane bilayer. O, PC/PS/PI/PE = 4:2:1:3; C, PC/PS/PI/PE/cholesterol = 4:2:1:3:5. PI concentration was 60 μM, and enzyme concentration was 0.46 pg/ml. Assays were performed for 2 min at 37 °C.

PI breakdown occurs rapidly when platelets are activated by thrombin. The PI-specific phospholipase C appears to be equally active when assayed in crude extracts from thrombin-stimulated versus unstimulated platelets. Studies of the distribution of phospholipids of the platelet membrane indicate that PI is contained almost exclusively on the inner leaflet (17, 18). In unstimulated cells, the cytoplasmic PI-specific phospholipase C is prevented from hydrolyzing PI. In the current work, we have investigated the effects of various lipids on PI hydrolysis. The most informative experiments are those using SUV, where the lipids are available in a fairly uniform state. We find that PI is rapidly hydrolyzed in such vesicles unless they contain PC. When the PC content approaches that seen in platelets or other cells, PI hydrolysis is completely inhibited. PC inhibits by preventing the enzyme from interacting with PI (17, 18). PI breakdown occurs rapidly when platelets are activated by thrombin. The PI-specific phospholipase C appears to be equally active when assayed in crude extracts from thrombin-stimulated versus unstimulated platelets. Studies of the distribution of phospholipids of the platelet membrane indicate that PI is contained almost exclusively on the inner leaflet (17, 18). In unstimulated cells, the cytoplasmic PI-specific phospholipase C is prevented from hydrolyzing PI. In the current work, we have investigated the effects of various lipids on PI hydrolysis. The most informative experiments are those using SUV, where the lipids are available in a fairly uniform state. We find that PI is rapidly hydrolyzed in such vesicles unless they contain PC. When the PC content approaches that seen in platelets or other cells, PI hydrolysis is completely inhibited. PC inhibits by preventing the enzyme from interacting with PI (17, 18). PI breakdown occurs rapidly when platelets are activated by thrombin. The PI-specific phospholipase C appears to be equally active when assayed in crude extracts from thrombin-stimulated versus unstimulated platelets. Studies of the distribution of phospholipids of the platelet membrane indicate that PI is contained almost exclusively on the inner leaflet (17, 18). In unstimulated cells, the cytoplasmic PI-specific phospholipase C is prevented from hydrolyzing PI. In the current work, we have investigated the effects of various lipids on PI hydrolysis. The most informative experiments are those using SUV, where the lipids are available in a fairly uniform state. We find that PI is rapidly hydrolyzed in such vesicles unless they contain PC. When the PC content approaches that seen in platelets or other cells, PI hydrolysis is completely inhibited. PC inhibits by preventing the enzyme from interacting with PI (17, 18). PI breakdown occurs rapidly when platelets are activated by thrombin. The PI-specific phospholipase C appears to be equally active when assayed in crude extracts from thrombin-stimulated versus unstimulated platelets. Studies of the distribution of phospholipids of the platelet membrane indicate that PI is contained almost exclusively on the inner leaflet (17, 18). In unstimulated cells, the cytoplasmic PI-specific phospholipase C is prevented from hydrolyzing PI. In the current work, we have investigated the effects of various lipids on PI hydrolysis. The most informative experiments are those using SUV, where the lipids are available in a fairly uniform state. We find that PI is rapidly hydrolyzed in such vesicles unless they contain PC. When the PC content approaches that seen in platelets or other cells, PI hydrolysis is completely inhibited. PC inhibits by preventing the enzyme from interacting with PI (17, 18). PI breakdown occurs rapidly when platelets are activated by thrombin. The PI-specific phospholipase C appears to be equally active when assayed in crude extracts from thrombin-stimulated versus unstimulated platelets. Studies of the distribution of phospholipids of the platelet membrane indicate that PI is contained almost exclusively on the inner leaflet (17, 18). In unstimulated cells, the cytoplasmic PI-specific phospholipase C is prevented from hydrolyzing PI. In the current work, we have investigated the effects of various lipids on PI hydrolysis. The most informative experiments are those using SUV, where the lipids are available in a fairly uniform state. We find that PI is rapidly hydrolyzed in such vesicles unless they contain PC. When the PC content approaches that seen in platelets or other cells, PI hydrolysis is completely inhibited. PC inhibits by preventing the enzyme from interacting with PI (17, 18).

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