Partial Purification and Characterization of a (Glycosyl) Inositol Phospholipid-specific Phospholipase C from Peanut*

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We have isolated a glycosyl inositol phospholipid (GIP) anchor-hydrolyzing activity from peanut seeds by a series of column chromatographic steps. The activity has a pH optimum below 6.0, requires calcium, and is inhibited by sulfhydryl reagents. It cleaves the GIP anchors of solubilized acetylcholinesterase from bovine erythrocytes and variant surface glycoprotein from Trypanosoma brucei. On the other hand, it does not act on membrane-bound GIP-anchored substrate or on inositol-acylated GIP anchor of human erythrocyte acetylcholinesterase. The only product released from $^3$H]myristate-labeled variant surface glycoprotein following treatment with the activity from peanut was $^3$H-labeled diacylglycerol. Together, these findings identify the activity from peanut seeds as a GIP anchor-hydrolyzing phospholipase C. The enzyme has been found to hydrolyze not only protein GIP anchors but also phosphatidylinositol, whereas it shows no activity against other phospholipids. The water-soluble products of phosphatidylinositol hydrolysis by peanut phospholipase C were characterized as a mixture of inositol 1,2-cyclic phosphate and inositol phosphate.

The covalent attachment of proteins to glycosyl inositol phospholipids (GIP) is recognized as an important means of anchoring proteins to membranes (for recent reviews, see Refs. 1-3). GIP-anchored proteins are functionally diverse and include ectoenzymes, complement regulatory factors, antigenic cell-adhesion molecules, prions, tumor markers, and protozoan cell surface antigens. One proposed role for the anchor-hydrolyzing phospholipases is needed. Interestingly, a particular subgroup of these enzymes, the bacterial PI-PLCs have recently been characterized in terms of their ability to hydrolyze PI and protein GIP anchors (24-26).

GIP anchor-hydrolyzing phospholipases include a GIP-specific phospholipase C (GIP-PLC) purified from trypanosomes (4-6) and GIP-PLCs from rat liver (7) and mouse brain (8). In addition, GIP anchor-specific phospholipases D (GIP-PLD) were isolated from human and bovine serum (9-12), as well as from bovine brain (13). Although these phospholipases efficiently cleave GIP anchors in vitro, the physiological role of GIP hydrolysis by anchor-specific phospholipases is still unclear. Although GIP-PLD is abundantly present in mammalian serum, it does not seem to be active therein as it is inhibited almost completely at physiological serum bicarbonate levels (14). Furthermore, it has been shown that serum GIP-PLD hydrolyzes GIP structures only in the presence of detergent and is not active on membrane-bound GIP-anchored proteins (15). Similarly, GIP-PLC from trypanosomes seems to be active on GIP anchors only in the presence of membrane-disrupting agents or when the phospholipase is present in the same membrane as the GIP substrate (4, 16). Only recently, Scallon et al. (17) were able to demonstrate a possible role for GIP-PLD in vivo. They showed that COS cells co-transfected with GIP-PLD and alkaline phosphatase released the majority of the alkaline phosphatase into the medium whereas in the absence of GIP-PLD the alkaline phosphatase was incorporated into the membrane (17). Although these results suggest that intracellular compartments may provide a more favorable environment for the action of GIP-PLD, further evidence for a biological role of GIP anchor-hydrolyzing phospholipases is needed.

Phosphatidylinositol-specific phospholipases C (PI-PLC) have long been known and, in light of their central roles in signal transduction events, have been studied extensively over the past decades (reviewed by Refs. 18 and 19). PI-PLCs hydrolyze inositol phospholipids, thereby generating the second messengers, diacylglycerol and inositol phosphates (20, 21).

Interestingly, a particular subgroup of these enzymes, the bacterial PI-PLCs, was found to hydrolyze not only phosphatidylinositol (PI) but also protein GIP anchors (22, 23). In fact, it was this unique property of bacterial PI-PLCs that initially led to the discovery of GIP-anchored proteins. Several of these bacterial PI-PLCs have recently been characterized in terms of their ability to hydrolyze PI and protein GIP anchors (24-26).

While most of the studies involving PI-PLCs from eukaryotic cells have been done on animal cells, PI-PLCs have also been described in plants (27-30). However, these studies were carried out on crude preparations, and no detailed information on the enzymatic properties of plant PI-PLCs is currently available. In this report we describe the identification, partial purification, and characterization of a PI-hydrolyzing phospholipase C from peanut seeds. Interestingly, the phospholipase was found to also hydrolyze protein GIP anchors. To our knowledge, this is the first detailed description of a plant
phospholipase, or of a nonbacterial PI-PLC, capable of hydrolyzing GIP anchors of proteins.

**MATERIALS AND METHODS**

Unless otherwise stated, all reagents used were of analytical grade and either from Fluka (Buchs, Switzerland), Sigma, or Merck (Darmstadt, Germany). DEAE-cellulose DE53 was from Whatman Biosystems ( Maidstone, England). Triton X-100, reduced, was from Aldrich (Steinheim, Germany). PI-PLC from *Bacillus thuringiensis* was from ICN Biochemicals. Phospholipases D from peanut (Type II and Type III), cabbage (Type I), and *Streptomyces chrysomallus* (Type VI) were from Sigma. Phospholipase D from cowpea (No. 79488) and PI-PLC from *Bacillus cereus* were from Fluka (Buchs, Switzerland). Alkaline phosphatase was from Boehringer Mannheim. Edrophonium chloride (Tension) was a gift from Hoffmann-La Roche (Basel, Switzerland). Fresh peanut seeds imported from Thailand and China were bought at a local food market.

**GIP-anchored Proteins**

Bovine erythrocyte ghost membranes were prepared by hypotonic lysis of washed erythrocytes and stored at −20 °C (31). Bovine and human erythrocyte acetylcholinesterase (AChE) was purified by affinity chromatography as described before (32). The final suspension contained purified AChE in 10 mM Tris, pH 7.4, 144 mM NaCl, containing 0.05–0.1% Triton X-100. [H]Myrystate-labeled variant surface glycoprotein (VSG) from *Trypanosoma brucei*, prepared as described by Hereld et al. (33), was kindly provided by Dr. A. K. Menon (Rockefeller University).

**Assays**

**GIP Anchor Hydrolysis**—GIP anchor hydrolysis activity was assayed using purified membrane form AChE from bovine erythrocytes as substrate. Bovine erythrocyte AChE has a covalently attached GIP as substrate. Bovine erythrocyte ghost membranes were prepared by hypotonic lysis of washed erythrocytes and stored at −20 °C (31). Bovine and human erythrocyte acetylcholinesterase (AChE) was purified by affinity chromatography as described before (32). The final suspension contained purified AChE in 10 mM Tris, pH 7.4, 144 mM NaCl, containing 0.05–0.1% Triton X-100. [H]Myrystate-labeled variant surface glycoprotein (VSG) from *Trypanosoma brucei*, prepared as described by Hereld et al. (33), was kindly provided by Dr. A. K. Menon (Rockefeller University).

**Hydrolysis of Phosphatidylserine and Inositol Phospholipids**—Hydrolysis of phosphatidylserine, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate was determined by incubating a mixture of these phospholipids and PI (0.5–10 mM final phospholipid concentration) with peanut phospholipase in 100 μl of 50 mM MES, pH 6.5, 0.5 mM CaCl₂, containing 0.2–2% deoxycholate or 0.2–0.5% Triton X-100, for 16 h at 37 °C. After incubation, lipids were extracted as above and separated by TLC in CHCl₃:MeOH:acetone:acetic acid:H₂O (40:15:15:12:8, v/v). The iodine-stained lipid spots were scraped, and lipid phosphorus was measured as above. The phosphoinositide mixture was prepared by incubation (Sigma, No. P-0023) consisted of 46.2 ± 1.5% phosphatidylinositol 4-monophosphate, 34.4% phosphatidylinositol, 6.9 ± 0.5% PI, and 12.4 ± 0.4% phosphatidylserine (means ± S. D. from four individual determinations). Recovery of phospholipids from samples incubated in the presence of peanut phospholipase relative to control incubations in the absence of the enzyme was calculated based on the recovery of phosphatidylserine (used as internal standard) and found to be approximately 90%.

**Phosphatidic Acid Hydrolysis**—The presence of phosphatidic acid and phosphohydrolase activity in the samples was tested by incubating phosphatidic acid (0.2–4 mM) with peanut phospholipase for 4 h under standard incubation conditions for AChE and PI hydrolysis.

**Hydrolysis of Phosphatidylserine and Inositol Phospholipids**—Hydrolysis of phosphatidylserine, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate was determined by incubating a mixture of these phospholipids and PI (0.5–10 mM final phospholipid concentration) with peanut phospholipase in 100 μl of 50 mM MES, pH 6.5, 0.5 mM CaCl₂, containing 0.2–2% deoxycholate or 0.2–0.5% Triton X-100, for 16 h at 37 °C. After incubation, lipids were extracted as above and separated by TLC in CHCl₃:MeOH:acetone:acetic acid:H₂O (40:15:15:12:8, v/v), and phosphorus was measured in the aqueous and organic phases. The sum of phosphorus in the two phases was always constant and reflected the amount of phosphatidic acid added (>95% recovery). The lipid products in the organic phase were analyzed by TLC as described above.

**Identification of the Reaction Products after Phospholipase Treatments**—Incubation with PI-PLC (40–140 nmol in each of six separate Eppendorf tubes) was incubated in 50 μl of 20 mM Tris, pH 7.4, containing 0.5 mM CaCl₂ and 0.4% Triton X-100, in the presence of peanut phospholipase for 4 h at 37 °C. The reaction was stopped by two-phase extraction with 250 μl of CHCl₃:MeOH:HCl (66:33:1, v/v), and the phases were separated by centrifugation. The amount of PI hydrolysis was determined by phosphorus determination of inorganic phosphorus in the organic phases. The final phosphatidylinositol aqueous phases were treated as follows. In sample A, total phosphorus was determined according to the standard procedure (inclusion of perchloric acid with subsequent heating at 180 °C). In sample B, phosphatidic acid was determined by phosphorus analysis in perchloric acid without the heating step. Samples C and D were acidified with 2 μl of 6 M HCl and incubated for 15 min at 95 °C. After cooling to room temperature, the pH was adjusted to 8.2 by the addition of 120 μl of 0.2 M NaHCO₃, and the samples were incubated in the absence or in the presence of (2 IU) of alkaline phosphatase for 18 h at 37 °C. Phosphorus determinations were done as described for sample B. Samples E and F were adjusted to pH 8.2 by addition of 17 μl of 0.2 M NaHCO₃ and incubated in the presence of alkaline phosphatase as above. Determination of PI-PLC from *B. cereus* or *B. thuringiensis* instead of the protein enzyme, as well as incubations with inositol 1,2-cyclic monophosphate or inositol 1-monophosphate standards.

**Purification of GIP Anchor-hydrolyzing Phospholipase**

**Solubilization of Crude Enzyme**—Batches of 5–10 g of dry peanut seeds were ground with a mortar and pestle to a fine powder. To 10 g of the ground powder, the seeds were minced and then homogenized with 50 ml of buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.25 mM β-mercaptoethanol, 0.25 mM succrose) for 1 min in a Vibris-type blender at low speed. The resulting homogenate was stirred at room temperature for 2 h and subsequently centrifuged at 50,000 × g for 15 min at 4 °C in a refrigerated centrifuge. The clear supernatant was discarded, and the debris and the floating fat were carefully removed and centrifuged overnight at 105,000 × g as above. The clear infranatant was again
removed and used for (NH4)2SO4 precipitation. 

(NH4)2SO4 Precipitation—The soluble extract was brought to 20% (w/v) by the slow addition of ground (NH4)2SO4, and the mixture was centrifuged at 35,000 × g for 20 min at 4 °C. The resulting supernatant was adjusted to 35% (NH4)2SO4 and centrifuged as above. The two precipitates were resuspended in small volumes (1–2 ml) of 50 mM MES, pH 6.5. The 35% (NH4)2SO4 precipitate was found to contain the majority of GIP anchor-hydrolyzing activity and was used for further purification.

Subsequent purification of GIP anchor-hydrolyzing activity from peanut seeds and from commercially available phospholipase D preparations from peanut (Sigma, Type II) was done by sequential column chromatography. The enzymes from the two sources behaved identically under these conditions. The following detailed description pertains to a representative extract from peanut seeds.

DEAE-cellulose Chromatography—The crude enzyme solution from the 35% (NH4)2SO4 precipitate was applied to a 10-ml DEAE-cellulose column equilibrated with 20 mM Tris-HCl, pH 7.4. The column was washed with 50 ml of 20 mM Tris-HCl, pH 7.4, and eluted stepwise with 50 ml of 20 mM Tris-HCl, pH 7.4, containing 200 mM NaCl, followed by 70 ml of 20 mM Tris-HCl, pH 7.4, containing 400 mM NaCl. Fractions of 1 ml were collected and assayed for GIP anchor-hydrolyzing activity, PC hydrolysis, and protein.

Oxyt-Sephrose Chromatography—Fractions containing GIP anchor-hydrolyzing activity were pooled (8.5 ml), brought to 400 mM NaCl, and applied to a 5-ml oxyt-Sephrose column equilibrated with 20 mM Tris-HCl, pH 7.4, containing 400 mM NaCl. The column was washed with 50 ml of 20 mM Tris-HCl, pH 7.4, and eluted stepwise with 50 ml of 20 mM Tris-HCl, pH 7.4, containing 25 ml of 3 mM Tris-HCl, pH 7.4. GIP anchor-hydrolyzing activity was eluted with 3 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100, reduced. Fractions of 1 ml were collected and assayed for GIP anchor-degrading activity, PC hydrolysis, and protein.

Concanavalin A-Sepharose Chromatography—Fractions containing GIP anchor-hydrolyzing activity were pooled (10.2 ml), brought to 400 mM NaCl, and applied to a 2-ml concanavalin A-Sepharose column equilibrated with 20 mM Tris-HCl, pH 7.4, containing 400 mM NaCl. The column was washed with 50 ml of the same buffer followed by 40 ml of 20 mM Tris-HCl, pH 7.4, containing 25 ml of 3 mM Tris-HCl, pH 7.4. GIP anchor-hydrolyzing activity was eluted with 3 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100, reduced. Fractions of 1 ml were collected and assayed for GIP anchor-degrading activity, PC hydrolysis, and protein. Fractions containing GIP anchor-hydrolyzing activity were pooled (5.2 ml) and dialyzed overnight at 4 °C against 50 mM MES, pH 6.5.

All aliquots removed during the purification procedure were dialyzed overnight at 4 °C against 50 mM MES, pH 6.5, and stored at 4 °C.

Cleavage Specificity

[3H]Myristate-labeled VSG—Aliquots containing GIP anchor-converting activity were incubated with small amounts of [3H]myristate-labeled VSG in 200 μl of 50 mM MES, pH 6.5, 0.5 mM CaCl2, containing 5% SDS (12, 45) and 20% glycerol (VSG preparation), for 1–4 h at 37 °C. Released hydrophobic products were sequentially extracted with 2 X 200 μl of toluene, followed by two extractions with 200 μl of water-saturated butanol (39). The organic extracts as well as the remaining aqueous phases were counted for radioactivity in a scintillation counter. Alternately, the organic extracts were dried under nitrogen, resuspended in a small volume of the same solvent, and applied on TLC plates. The plates were developed in benzene:ether:NH4OH (100:80:0.1, v/v) or CHCl3:MeOH:H2O (40:15:15:12:8, v/v). Lanes were scraped in small increments and, after incubation in 0.5 ml of MeOH:HCl (50:3, v/v) for 1 h at room temperature, counted for radioactivity in a scintillation counter. [3H]Dimyristoylphosphatidic acid and [3H]myristoylglycerol were located by co-migration with the corresponding iodine-stained lipid standards on the same TLC plate. In some experiments, the phosphatidic acid phospholipase D inhibitors, pronanol and chlorpromazine, were added to the incubation mixture.

Human Erythrocyte AChE—Human erythrocyte AChE contains a fatty acid esterified to the inositol of the GIP moiety (40) which prevents hydrolysis of the anchor by bacterial PI-PLC. On the other hand, inositol acylation does not affect anchor hydrolysis by GIP-PLD (31, 41). Thus, the ability of the peanut phospholipase to hydrolyze the GIP anchor of human erythrocyte AChE was used as a criteria to assess its cleavage specificity. Purified human erythrocyte AChE was treated with peanut phospholipase for 20 h at 37 °C in 50 mM MES, pH 6.5, containing 0.5 mM CaCl2. Subsequently, inositol-linked fatty acids were removed by incubation of the samples in 0.8 m hydroxylamine, 0.1 m triethylamine, pH 11.0, containing 1.0 mM edrophonium chloride (added as reversible inhibitor of AChE to protect against inactivation), and 2 mM EDTA, for 20 h at 25 °C. After dialysis for 4 h at 4 °C against 10 mM Tris, pH 7.4, containing 144 mM NaCl, 2 mM EDTA, 2 m hydrolysis was determined by phase partitioning in Triton X-114 (see above). Control samples included human erythrocyte AChE incubated with peanut phospholipase alone, with hydroxylamine alone, with hydroxylamine followed by peanut phospholipase, as well as an identical series of samples using GIP-PLD from serum or PI-PLC from B. thuringiensis instead of the peanut enzyme.

Hydrolysis of Membrane-bound AChE

The ability of peanut phospholipase to hydrolyze membrane-bound GIP-anchored AChE was tested by incubating bovine erythrocyte ghosts (250 μg of membrane protein) with peanut phospholipase in 50 mM MES, pH 6.5, containing 0.5 mM CaCl2 and 0–0.5% Triton X-100, for 16 h at 37 °C. Subsequently, the extent of AChE hydrolysis was determined by phase partitioning in Triton X-114 as described above. Control incubations were done in the presence of PI-PLC form B. thuringiensis or GIP-PLD from bovine serum, as well as in the absence of phospholipases.

Miscellaneous

AChE activity was determined according to Ellman et al. (42). Protein determination using bichinchoninic acid (Pierce) was done as described elsewhere (43). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to Laemmli (44) with 5–15% acrylamide gradient gels. SDS-PAGE and subsequent transfer of proteins to polyvinylidene difluoride membranes was done as described elsewhere (35). The cross-reacting determinant (CRD) of AChE was detected using rabbit anti-CRD antibody (obtained from Dr. M. A. J. Ferguson, University of Dundee, Scotland) as first, and swine anti-rabbit IgG peroxidase-conjugated antibody as second antibody. Phosphorus analysis was done according to Rouser et al. (36). In some experiments, the heating step (30 min at 180 °C, in perchloric acid) to hydrolyze phosphate esters, was omitted. This modification allows (in our case) the distinction between free phosphate and inositol-bound phosphate in the aqueous phase of the two-phase lipid extraction. The heating step used for color development (5 min at 100 °C, in aqueous environment) does not hydrolyze inositol 1-phosphate or inositol 1,2-cyclic phosphate. The detection limit of the phosphate assay was at 2 nmol of phosphate as determined by standard curves using phosphate buffer.

RESULTS AND DISCUSSION

GIP Anchor-degrading Activities in Plant Extracts

We noted that treatment of GIP-anchored AChE from bovine erythrocytes with commercially available preparations of phospholipase D from peanut resulted in conversion of AChE from the membrane form to the soluble form, as assayed by phase partitioning in Triton X-114 (results not shown). These observations are in line with recent reports describing the use of commercially available preparations of plant phospholipases D in the characterization of GIP-anchored proteins (45–47). In these reports, it was assumed that the GIP anchors became hydrolyzed by D-type phospholipases; however, the cleavage specificities were not determined (45–47). As an extension of our own and the above-mentioned observations, we tested (commercially available) plant phospholipase D preparations for the presence of GIP anchor-hydrolyzing activities. We found that various extracts from peanut and cabbages, as well as from S. chromofuscus contained GIP anchor-degrading activities. The relative efficiencies of the phospholipase D extracts to hydrolyze GIP anchors decreased in the order of peanut, Type II (Sigma) >> S. chromofuscus (Sigma) >> peanut (Fluka) ≈ cabbage, Type I (Sigma) >> peanut, Type III (Sigma). In order to study the

P. Büttikofer, unpublished data.
putative GIP anchor-hydrolyzing activities, we decided to purify the activities from commercially available peanut extracts as well as from fresh peanut seeds.

**Purification of GIP Anchor-hydrolyzing Phospholipase from Peanut Seeds**

Using GIP-anchored bovine erythrocyte AChE as substrate, we purified a GIP anchor-degrading activity from peanut seeds by ammonium sulfate precipitation followed by a series of column chromatographic steps. The solubilized crude enzyme was found to precipitate almost quantitatively at 35% (NH₄)₂SO₄ together with 40–50% of the total protein (Table I). The resuspended precipitate was subsequently applied on a DEAE-cellulose anion exchange column, and the bound protein was eluted with an increasing NaCl step gradient (Fig. 1A). GIP anchor-degrading activity was recovered in the flow through. The majority of PC-hydrolyzing phospholipase D activity bound to the column and eluted at NaCl concentrations of 200–400 mM, which is in line with a previous report on the purification of PC-hydrolyzing phospholipase D from peanut (48). The fractions containing GIP anchor-degrading activity were pooled and subsequently chromatographed on an octyl-Sepharose column. While the majority of protein, including the remaining PC-hydrolyzing activity, was found in the flow-through, GIP anchor-hydrolyzing activity efficiently bound to the column and eluted only after the addition of detergent to the low ionic strength buffer (Fig. 1B). Fractions containing GIP anchor-hydrolyzing activity were found to also hydrolyze PI; however, they were devoid of PC-hydrolyzing activity. GIP anchor-degrading fractions were again pooled and applied to a concanavalin A-Sepharose column from where the bound activity eluted after the addition of methyl α-D-mannopyranoside (Fig. 1C). The overall recovery of GIP anchor-hydrolyzing activity after ammonium sulfate precipitation and column chromatography was around 30% with a 250-fold purification (Table I). Partial purification by column chromatography of the GIP anchor-hydrolyzing activity from commercially available peanut phospholipase D extracts (Type II, Sigma) showed no differences as compared to the enzyme isolated from fresh peanuts (results not shown).

The resulting specific activity of the enzyme from commercial peanut extracts was somewhat higher than that of the enzyme isolated from peanut seeds (Table I). Separation of the partially purified enzymes by SDS-PAGE (20 μg of protein) followed by visualization of protein with Coomassie Brilliant Blue and silver staining revealed candidate bands at 18, 35, and 42 kDa (results not shown).

All subsequent studies were done using the partially purified enzymes after concanavalin A-Sepharose. We noted no differences in any of the described assays between the enzyme isolated from fresh peanut seeds and that isolated from commercially available extracts.

**Cleavage and Substrate Specificity**

**GIP Anchor Hydrolysis**—In order to determine the cleavage specificity, aliquots of fractions containing GIP anchor-degrading activity were incubated with [3H]myristate-labeled VSG, and the released products were characterized by sequential extraction with organic solvents or by TLC using two different solvent systems. Treatment of [3H]myristate-labeled VSG with GIP anchor-hydrolyzing activity released a product that was extractable to more than 90% with toluene, and on two different TLC systems co-migrated with diacylglycerol standard (Fig. 2). Less than 10% of the radioactivity was extracted by butanol (following toluene extraction), and no radiolabeled products co-migrated with phosphatidic acid standard on TLC (results not shown). In order to exclude the possibility that diacylglycerol was generated through the combined action of a D-type phospholipase and a phosphatidic acid phosphohydrolase, [3H]myristate-labeled VSG was treated with peanut phospholipase in the presence of the phosphatidic acid phosphohydrolase inhibitors, propranolol and chlorpromazine (each at 10 mM final concentration; results not shown). Addition of these compounds to the incubation mixtures inhibited GIP anchor-hydrolyzing activity by 20 and 80%, respectively; however, [3H]diacylglycerol was again the only hydrolysis product detected. Furthermore, treatment of [3H]phosphatidic acid, obtained by serum GIP-PLD treatment of radiolabeled VSG, with peanut phospholipase did not result in dephosphorylation of phosphatidic acid (>80% of radiolabel was recovered in phosphatidic acid after a 4-h incubation with peanut phospholipase). These data suggest that the GIP anchor-hydrolyzing activity from peanut extract is a C-type phospholipase.

Additional evidence for the cleavage specificity of the anchor-degrading phospholipase was obtained using purified AChE from human erythrocytes as substrate. Human erythrocyte AChE contains a fatty acid linked to the inositol of the GIP anchor (39). This modification prevents anchor hydrolysis by bacterial PI-PLCs; however, it does not affect anchor degradation by serum GIP-PLD (31, 41). Sensitivity to PI-PLC, on the other hand, can be obtained by removal of the inositol-linked fatty acid. Furthermore, it was shown that the single fatty acid linked to the inositol is sufficient to retain the detergent interaction of human erythrocyte AChE after GIP-PLD cleavage (41). Therefore, in order to be able to determine the extent of anchor hydrolysis by phase partitioning, GIP-PLD-treated AChE is incubated with hydroxyamine to

### Table I

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<th>Source</th>
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<th>Activity*</th>
<th>Recovery*</th>
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* Corrected for amounts removed.
Fig. 1. Purification of GIP anchor-hydrolyzing activity from peanut. A, the 35% (NH₄)₂SO₄-precipitable fraction of peanut extracts was applied on DEAE-cellulose, washed extensively with low ionic strength buffer, and eluted with 200 mM NaCl (fractions 63-116), followed by 400 mM NaCl (fractions 117-174). Fractions containing GIP anchor-degrading activity (7-15) were pooled and subsequently chromatographed on an octyl-Sepharose column (B), from where the bound activity eluted after addition of Triton X-100 to the buffer. Fractions containing GIP anchor-degrading activity (142-151) were again pooled and applied on a concanavalin A-Sepharose column (C). Bound activity eluted after the addition of methyl α-D-mannopyranoside, and the fractions containing GIP anchor-hydrolyzing activity (46-50) were pooled and dialyzed. For details, see "Materials and Methods." ○, protein; ●, GIP anchor-hydrolyzing activity.

which removes inositol-linked fatty acid and renders AChE water-soluble (31, 41).

Treatment of human erythrocyte AChE with anchor-degrading activity from peanut, followed by incubation with hydroxylamine, showed no hydrolysis of the GIP anchor (Table II). In contrast, if human erythrocyte AChE was first treated with hydroxylamine followed by exposure to peanut phospholipase, more than 90% of AChE was recovered in the aqueous phase. Essentially identical results were obtained with B. thuringiensis PI-PLC (Table II). On the other hand, treatment of human erythrocyte AChE with GIP-PLD from bovine serum resulted in hydrolysis of the GIP anchor without prior removal of the inositol-linked fatty acid (Table II). We noted that, in contrast to serum GIP-PLD and bacterial PI-PLC, the peanut phospholipase was inhibited only slowly by exposure to hydroxylamine (results not shown). Therefore, following peanut phospholipase treatment of human erythrocyte AChE, it is important to completely inhibit phospholipase activity by addition of EDTA (see below) to the hydroxylamine buffer, as otherwise, hydrolysis (up to 50%) of
AChE GIP anchor can occur which may lead to misinterpretations regarding the cleavage specificity of the phospholipase. 

**Phospholipid Hydrolysis**—The substrate specificity of the peanut phospholipase was studied using various phospholipid suspensions. PI was readily hydrolyzed by the peanut enzyme in the presence of deoxycholate or Triton X-100 (see below). On the other hand, no hydrolysis of PC was observed in the presence of these two detergents, or of SDS (i.e. <2% of PI hydrolyzing activity). In addition, while PI in mixed suspensions containing PI and PC (2 mM of each phospholipid) was completely hydrolyzed, no degradation of PC was observed (i.e. <2% of PI-hydrolyzing activity). When phospholipid mixtures (0.5–10 mM phospholipid) consisting of phosphatidylserine, PI, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate were incubated with peanut phospholipase, the ratio of phosphatidylserine to phosphatidylinositol 4,5-bisphosphate were incubated with peanut phospholipase, the ratios of phosphatidylserine to phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate were not recognized as substrates by the phospholipase (i.e. <2% activity against these classes as compared to PI).

The cleavage specificity of the peanut phospholipase was further assessed by characterization of the PI hydrolysis products in the organic and aqueous phases after two-phase extraction. The lipid products in the organic phase after complete hydrolysis of PI by peanut phospholipase were analyzed by TLC using two different solvent systems and showed the exclusive presence of diacylglycerol (results not shown). In addition, we found that less than 3% of phosphorus in the aqueous phase was present as free phosphate, ruling out the possibility that the diacylglycerol recovered in the organic phase was generated through the combined action of a phospholipase D and a phosphatidic acid phosphohydrolase. Furthermore, after treatment of phosphatidic acid with peanut phospholipase, less than 5% of total phosphorus was recovered in the aqueous phase after two-phase extraction, confirming the absence of a phosphatidic acid phosphohydrolase activity. These results indicate that PI was hydrolyzed by a C-type phospholipase generating diacylglycerol and inositol mono-phosphate.

**Analysis of the Water-soluble Reaction Products after Phospholipase Treatment of PI**—PI hydrolysis by bacterial PI-PLCs results in the almost exclusive formation of inositol 1,2-cyclic phosphate (49), whereas mammalian PI-PLCs produce a mixture of inositol 1,2-cyclic phosphate and inositol 1-phosphate (50). It has been shown that alkaline phosphatase (a phosphomonoesterase) is able to release free phosphate from inositol 1-phosphate, whereas inositol 1,2-cyclic phosphate is not hydrolyzed (51). Thus, in order to determine the type of inositol phosphate generated after cleavage of PI by peanut phospholipase, we characterized the water-soluble products of PI hydrolysis by treatment with alkaline phosphatase and determination of free phosphate (Table III). Our results on peanut phospholipase-treated PI demonstrate that approximately half of the inositol-bound phosphate was released by alkaline phosphatase as free phosphate. In control incubations using PI hydrolyzed by bacterial PI-PLCs, essentially no free phosphate was detected after alkaline phosphatase treatment of the water-soluble products. Similarly, no phosphate could be released by alkaline phosphatase from the inositol 1,2-cyclic phosphate standard. On the other hand, more than 85% of phosphate was released by alkaline phosphatase from the inositol 1-phosphate standard (Table III).

<table>
<thead>
<tr>
<th>Substrate/Treatment</th>
<th>Phosphorus (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI + peanut phospholipase</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>PI + PI-PLC</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Inositol 1-phosphate</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Inositol 1,2-cyclic phosphate</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

The numbers represent the means of two independent experiments; each series of treatments was repeated at least twice using different amounts of substrates (40–140 nmol); the results were similar to the ones shown.

Identification of the reaction products after phospholipase treatment of PI

PI (approximately 140 nmol) was incubated with peanut phospholipase or bacterial PI-PLC, and the reaction products were characterized as described under "Materials and Methods." The various treatments included determination of phosphates (P0 in perchloric acid with (180°C) or without heating, acid treatment (H'), and incubation in the presence of alkaline phosphatase (AP).

**TABLE III**

**Enzymatic Properties**

**pH Dependence**—The peanut phospholipase C was active over a broad pH range, with maximal AChE GIP anchor-degrading activity between pH 5.5 and 6.0 (Fig. 3A).

**Calcium Dependence**—GIP anchor-hydrolyzing phospholipase C was activated by calcium. Half-maximal activity was obtained around 2 μM CaCl₂; whereas maximum activity was observed at around 1 mM calcium (Fig. 3B). Both EDTA and EGTA completely inhibited GIP anchor hydrolysis. A similar calcium dependence of the phospholipase, i.e. activation at millimolar concentrations of calcium and complete inhibition by EDTA, was also observed with PI as substrate (results not shown).

Inhibitors—With AChE as substrate, phospholipase C activity was inhibited by millimolar concentrations of EDTA or EGTA (>95% inhibition), ZnCl₂, or p-chloromercuriphenyl-sulfonic acid (50% inhibition at 1 mM and 10 mM, respectively), as well as by the phosphatase inhibitors, propranolol (50% inhibition at 10 mM), NaF (>90% inhibition at 50 mM), and sodium o-vanadate (80% inhibition at 10 mM). MgCl₂ was unable to substitute for CaCl₂, o-Phenanthroline (6 mM) did not alter AChE anchor-hydrolyzing activity. Similar effects on phospholipase C activity, i.e. inhibition by millimolar concentrations of EDTA or ZnCl₂, as well as ineffectiveness
Glycosyl Inositol Phospholipid-specific Phospholipase C

peanut phospholipase C (0), GIP-PLD from bovine serum

by peanut phospholipase C was affected by the presence of Triton X-100 in the assay (Fig. 4). As compared to incubations carried out in the presence of 0.02% Triton X-100, the activity was lower by 2- to 3-fold in detergent concentrations below the critical micellar concentration (~0.015%). Furthermore, GIP anchor hydrolysis was increasingly inhibited by Triton X-100 concentrations ranging from 0.05% (50% inhibition) to 0.5% (>80% inhibition). A similar detergent-dependent inhibition of GIP anchor hydrolysis was observed for GIP-PLD from bovine serum and PI-PLC from B. thuringiensis (Fig. 4). These effects can be explained by the physical state of the substrate, bovine erythrocyte AChE, in detergent-containing solutions. Below the critical micellar concentration of Triton X-100, AChE occurs as aggregates (52). Apparently, the phospholipases, in particular peanut phospholipase C and serum GIP-PLD, are unable to use aggregated AChE as substrate. However, AChE becomes an optimal substrate for the phospholipases at around the critical micellar concentration, GIP anchor hydrolysis is inhibited which might be explained by a direct inhibitory effect of Triton X-100 on the phospholipases themselves. Interestingly, PI-PLC from B. thuringiensis did not show the narrow range of optimal activity just above the critical micellar detergent concentration; in fact, PI-PLC was most active slightly below this concentration. The reason for this behavior is not known; however, it does not seem to be an artifact since we have previously observed a similar detergent dependence of PI-PLC from B. cereus.4

With PI as substrate, increasing amounts of detergent resulted in increased PI hydrolysis; e.g. at a given concentration of PI of 2 mM, a 3-fold or 4-fold increase in PI hydrolysis was observed going from 0.5% to 1.5% deoxycholate or from 0.1% to 1.0% Triton X-100, respectively.

Kinetics of AChE and PI Hydrolysis—In order to compare the kinetics of hydrolysis of the two substrates, experiments were carried out under identical conditions, i.e. at constant concentrations of Triton X-100 and CaCl2 (final concentrations of 0.2% and 0.5 mM, respectively) at pH 7.0. We observed a linear increase in the rate of AChE hydrolysis in the range between 80 nM and 2 µM AChE; higher concentrations were not used due to the limited amounts of substrate available. The rate of PI hydrolysis also increased linearly between 0.2 mM and 2 mM PI; increasing the concentration of PI above 4 mM resulted in inhibition of hydrolysis which may be due to the decreased ratios of detergent to PI. At selected substrate concentrations of 1.1 µM AChE and 1.0 mM PI, the rates of substrate hydrolysis were 120 fmol/min and 133 pmol/min, respectively.

Treatment of Bovine Ghosts with Peanut Phospholipase—In order to test if the phospholipase C from peanut also hydrolyzes membrane-attached GIP anchors, we determined GIP anchor hydrolysis of membrane-bound bovine erythrocyte ghost AChE. Treatment of ghost membranes with bacterial PI-PLC quantitatively hydrolyzed the GIP anchor of membrane-bound GIP-PLD (Table IV). This finding is consistent with the ability of B. thuringiensis PI-PLC to release GIP-anchored proteins from membranes (22). In contrast, peanut phospholipase C was largely ineffective in hydrolyzing membrane-bound AChE GIP anchor (Table IV). A similar result was obtained with GIP-PLD from bovine serum which has previously been shown to be ineffective on membrane-bound substrates (15). On the other hand, following solubilization of ghost membranes with Triton X-100, both peanut phospholipase C and GIP-PLD hydrolyzed the GIP anchor of AChE (Table IV).

The formation of the two types of inositol phosphates may be phosphate and inositol 1-phosphate. Thus, it is possible that pholipase C from peanut, we compare it with other known various plants (celery, cauliflower, onions, and daffodils) re-proteins. The peanut enzyme is unable to hydrolyze mem-

pholipase C and trypanosome GIP-PLC readily hydrolyze under appropriate conditions the trypanosome enzyme hydro-
albeit at a much lower rate (4-6). In light of our findings that in terms of the water-soluble products formed. PI hydrolysis classification of trypanosome GIP-PLC might have to be trypanosome phospholipase is commonly known as GIP-spe-
clearly shares similarities with GIP anchor-specific phospho-
proteins from cell surfaces (24; reviewed by Refs. 1 and 2).

nut phospholipase C generates a mixture of inositol 1,2-cyclic phosphate and inositol 1-phosphate. It is a characteristic of plant PI-PLCs. It is worth mentioning that a mechanism for the simultaneous formation of inositol 1,2-cyclic phosphate and inositol 1-phosphate by totally inde-pendent pathways has recently been considered for mamma-

lian PI-PLCs (53). Alternatively, it is possible that hydrolysis of PI by peanut phospholipase C proceeds sequentially via an inositol 1,2-cyclic phosphate intermediate, as it has been demonstrated for bacterial PI-PLCs (49, 53).

Hydrolysis of GIP-anchored proteins by bacterial PI-PLCs and by trypanosome GIP-PLC results in the formation of an epitope on the GIP anchor, the CRD, that is recognized on immunoblots by specific anti-CRD antibodies. Although it has been shown that different epitopes on the anchor are necessary for antibody binding, the dominant determinant seems to involve the cyclic phosphate formed by PI-PLC treatment of the GIP anchor (64). We also used anti-CRD antibodies in our study to probe the formation of the CRD; however, we were repeatedly unable to detect any reactivity of the antibody toward the GIP anchor of peanut phospholi-

pase C-treated AChE (results not shown). In control samples on the same immunoblots, the antibody reacted with the GIP anchor of AChE that was either completely (>80%) or only partly (15% and 27%) hydrolyzed by bacterial PI-PLC. Thus, although our results using PI as substrate indicate the formation of inositol 1,2-cyclic phosphate by peanut phospholipase C, the characteristic determinant on the GIP anchor of peanut phospholipase C-treated AChE seems to be absent. At present, we have no explanation for this apparent discrepancy.

Despite the many similarities of the peanut phospholipase C with other GIP anchor-degrading phospholipases, it seems to be unique with regard to its calcium dependence. Whereas bacterial PI-PLCs (22, 23) as well as T. brucei GIP-

PLC (4-6) show no specific ion requirements, peanut phospholipase C needs calcium for its activity and is inhibited by EDTA. On the other hand, inhibition by sulfhydryl reagents seems to be common to all anchor-hydrolyzing phospholipases C. Interestingly, calcium dependence as well as inhibition by thiol reagents of the peanut phospholipase C is characteris-
tics shared by other recently described inositol phospholipid-
specific phospholipases C from plants (27-30). It is, however, not known if these phospholipases also hydrolyze GIP an-

chor.

**Conclusions**

We identified and partially purified an enzyme from peanut seeds with an activity of a (glycosyl) inositol phospholipid-specific phospholipase C. It is the first description of a GIP anchor-hydrolyzing phospholipase in plants. It will be interesting to see if the ability of the phospholipase C to cleave GIP anchors is a unique feature of the peanut enzyme or if this characteristic is shared by other inositol phospholipid-specific plant phospholipases C.

Until now, there have been no reports on GIP-anchored proteins in plants, and, thus, it may be questioned that GIP anchor hydrolysis by plant phospholipases has a physiological role. However, only very recently, evidence for the existence of GIP-anchored proteins in lower plants has been obtained. In light of these findings, our identification of a (glycosyl) phosphatidylinositol-specific phospholipase C in peanut is of particular interest as this may initiate a search for GIP-anchored proteins, the putative natural substrates of the phospholipases, in plants.

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rification as well as for providing purified bovine serum GIP-PLD, and R. Gentinetta for purification of erythrocyte AChEs. We also thank Dr. A. M. Mezon for the gift of [14C]myristate-labeled VSG and for critical comments on the manuscript, and Dr. M. A. J. Ferguson for providing anti-CRD antibodies.

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