Purification of a phosphatidylinositol-glycan-specific phospholipase C from liver plasma membranes: A possible target of insulin action

(diacylglycerol/glycosylated phosphatidylinositol membrane protein anchors)

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ABSTRACT Insulin stimulates the hydrolysis of a phosphatidylinositol-glycan, resulting in the generation of two related inositol phosphate-glycan enzyme modulators and diacylglycerol. A phosphatidylinositol-glycan-specific phospholipase C that catalyzed this reaction was found in the plasma-membrane fraction of rat liver. The enzymatic activity was measured by the release of diacylglycerol from the glycosylated-phosphatidylinositol-membrane-anchored variant surface glycoproteins of African trypanosomes. The enzyme also catalyzed the production of an inositol phosphate-glycan from an insulin-sensitive phosphatidylinositol-glycan precursor. The enzyme was solubilized with neutral nonionic detergent and purified to near homogeneity by anion-exchange chromatography on DEAE-cellulose, followed by hydrophobic chromatography on butyl-agarose. The resulting enzyme preparation was purified ≈20,000-fold from whole liver and exhibited one major silver-stained band of M_r 52,000 on NaDodSO$_4$/PAGE. Gel permeation chromatography of the purified activity revealed a Stokes radius of 35 Å and an apparent molecular weight of 62,000, suggesting that the enzyme was tightly associated with lipid or detergent but existed as a monomer in its active form. The enzyme was specific for glycosylated phosphatidylinositol; no hydrolysis of phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate, or phosphatidylcholine was observed. The enzyme was calcium independent and thiol activated. These data suggest a role for the phosphatidylinositol-glycan-specific phospholipase C as an effector for some of the metabolic actions of insulin.

We have reported (1–3) that insulin stimulates the phosphodiesteratic hydrolysis of a phosphatidylinositol (PtdIns)-glycan. Two structurally related, water-soluble products of this reaction were identified as inositol phosphate (InsP)-glycans, which acutely modulated the activities of certain intracellular, insulin-sensitive enzymes (1, 2, 4). The rapid generation of diacylglycerol (acyl$_2$Gro) was also observed in response to insulin over the same time course. The insulin-sensitive acyl$_2$Gro appeared to differ in fatty acid composition from the α-adrenergic-sensitive species and probably arose from the hydrolysis of the PtdIns-glycan precursor, rather than from a pool of hormonally sensitive inositol phospholipids (3). Both acyl$_2$Gro and the water-soluble InsP-glycans were also generated in response to exogenously added PtdIns-specific phospholipase C (PLC) purified from *Staphylococcus aureus* suggesting that some of the acute actions of insulin may involve the activation of a selective cellular phospholipase C (1).

The PtdIns-glycan precursor shares limited structural homology with the glycolipid anchor of the membrane form of variant surface glycoproteins (mfVSG) from *Trypanosoma brucei* (1–3, 5). Similar glycolipid moieties apparently anchor certain eukaryotic proteins to plasma membranes, including alkaline phosphatase (6, 7), acetylcholine esterase (8), 5′-nucleotidase (8), Thy-1 antigen (9), decay-accelerating factor (10), neural cell-adhesion molecule (11), T-cell activating protein (12), and heparan sulfate (13). The functional significance of this unusual covalent linkage is unknown (14–16). Both the PtdIns-glycan and the glycolipid anchor for mfVSG contain nonacetylated glucosamine covalently linked to PtdIns and can be hydrolyzed to water-soluble forms by the *S. aureus* PtdIns-specific PLC as well as a PLC purified from *T. brucei* that is specific for glycosylated PtdIns, although neither PLC recognizes polyphosphorylated inositol phospholipids (1–3, 17–19).

While insulin is known to cause the increased labeling of several phospholipids (20–25), it has not been found to stimulate the hydrolysis of the inositol phospholipids (25, 26) and does not share the activity of those hormones that induce calcium mobilization through the generation of inositol trisphosphate (25, 27). Thus the insulin-dependent production of acyl$_2$Gro and the InsP-glycans is likely due to the stimulation of a PLC that selectively hydrolyzes the PtdIns-glycan precursor. We report here the purification to near homogeneity of such an enzyme from rat liver plasma membranes that appears highly specific for molecules containing glycosylated PtdIns. This PtdIns-glycan-specific PLC catalyzes the phosphodiesteratic hydrolysis of the insulin-sensitive PtdIns-glycan precursor (1–3) and mfVSG from *T. brucei* (28, 29) to produce soluble InsP-glycans and acyl$_2$Gro, but does not appear to mediate the analogous reactions for other phospholipids, including the inositol phospholipids. The location of the enzyme in the plasma membrane and its substrate specificity are consistent with a role as an effector in mediating some of the actions of insulin.

EXPERIMENTAL PROCEDURES

Materials. All reagents were from Sigma, with the exceptions of Nonidet P-40 (NP-40) (Calbiochem), DE52 DEAE-cellulose (Whatman), Silica G and Silica 60 plates (Fisher), Extracti-Gel D (Pierce), fetal calf serum (GIBCO), Dulbecco's modified Eagle's medium, bovine achilles tendon collagen (Worthington), and silver-staining kit and protein assay (Bio-Rad). [9,10-^{3}H]Myristic acid, [^{3}H]inositol, [^{3}H]phosphatidylinositol 4,5-bisphosphate, [9,10-^{3}H]phosphatidylcholine, Econofluor, and Aquasol were from New England Nuclear. The BC$_3$H1 myocyte cell line was the kind gift of Mary Standaert (University of South Florida, Tampa, FL).

Preparation of mfVSG, C-terminal Glycopeptide, and Dimyristoylated PtdIns. Trypanosomes (10^{10} cells) of the Molteno Trypanozoon antigen type (MITat) 1.4 purified from

infected rat blood (30) were biosynthetically labeled by incubation with [9,10-^{3}H]myristate (29). mfVSG was extracted from the labeled cells with 0.1% trifluoroacetic acid and purified by HPLC using a 5-μm reversed-phase C_{18} preparative Ultrapore column (Altex, Berkeley, CA) as described (17, 31). Purified mfVSG was digested with Pronase for 36 hr to obtain the C-terminal glycopeptide (32). Dimyristoylated PtdIns was prepared from mfVSG by nitrous acid deamination (5). The identities of the two mfVSG derivatives were confirmed by TLC (5, 32).

Purification of PtdIns-Glycan Precursor. Confluent cultures of BC_3H1 myocytes were deprived of serum and labeled with [^{3}H]inositol (0.5 μCi/ml of medium; 1 Ci = 37 GBq) for 48 hr in serum-free medium. The labeled cells were extracted with chloroform/methanol/HCl (1 M), 200:100:1 (vol/vol), and the PtdIns-glycan precursor was prepared by TLC, followed by HPLC as described (3).

Protein Assay and NaDodSO$_4$/PAGE. The protein concentration of PtdIns-glycan PLC was determined by the method of Lowry *et al.* (33) after precipitation with trichloroacetic acid using prepackaged reagents from Sigma. mfVSG protein was assayed by the method of Bradford (34) with the Bio-Rad assay. NaDodSO$_4$/PAGE was performed using 10% polyacrylamide gels in Laemmli buffers (35). Proteins were visualized either by staining with Coomassie blue or with silver.

Purification of Rat Liver Plasma Membranes. Plasma membranes were prepared from fresh livers obtained from five to seven adult male Sprague–Dawley rats (350–500 g) (Charles River Breeding Laboratories) by slight modification of the technique described by Neville (36). Briefly, livers were minced and homogenized in 1 mM sodium bicarbonate buffer, pH 8.0, that contained 10 mM EGTA, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin at 10 μg/ml, leupeptin at 10 μg/ml, pepstatin A at 10 μg/ml, and chymostatin at 10 μg/ml. All other buffers used during membrane purification contained 0.1 mM phenylmethylsulfonyl fluoride. The liver homogenate was centrifuged for 10 min at 400 × g, and the pellets were resuspended in 60% (wt/vol) sucrose to achieve a final sucrose concentration of 44% (wt/vol). Ten milliliters of 40% (wt/vol) sucrose was layered over 25 ml of the homogenate, and the preparation was centrifuged for 2 hr in a Sorvall SW27 rotor at 26,000 rpm. The purified membranes were washed twice with Hanks' buffered saline solution and finally with 25 mM Tris·HCl, pH 8.0, at 4°C.

Detergent Solubilization of PtdIns-Glycan PLC. Freshly prepared rat liver plasma membranes were suspended in 20 ml of 1% NP-40, 25 mM Tris·HCl buffer (pH 8.0) containing aprotinin at 20 μg/ml, chymostatin at 10 μg/ml, and leupeptin at 10 μg/ml. The suspension was homogenized in a loose-fitting teflon–glass homogenizer for 10 min at 4°C and centrifuged for 1 hr at 100,000 × g. The resulting supernatant contained >80% of PtdIns-glycan PLC activity.

Assay of PtdIns-Glycan PLC Activity. Enzyme activity was assayed at 37°C in a final volume of 1 ml with [^{3}H]myristate-labeled C-terminal glycopeptide in 25 mM Tris·HCl (pH 7.4)

with 0.1% NP-40. The reaction was terminated after 1 hr by extraction with 1 ml of toluene. Organic and aqueous phases were separated by brief centrifugation at 100 × g, and the organic phase was taken to dryness *in vacuo*, redissolved in 40 μl of chloroform/methanol, 2:1 (vol/vol), containing 5 μg of dimyristoylglycerol, and spotted on a Silica G TLC plate that had been preactivated by baking at 125°C for 1–2 hr. The plate was developed twice in petroleum ether/diethyl ether/acetic acid, 70:30:2 (vol/vol), and then stained with iodine vapor to localize acyl$_2$Gro. The acyl$_2$Gro region was scraped and counted. Alternatively, 0.5 ml of the organic phase was taken directly for liquid scintillation counting.

RESULTS

Purification of PtdIns-Glycan PLC. The purification of PtdIns-glycan PLC from rat liver plasma membranes is summarized in Table 1. Purified liver plasma membranes were found to be highly enriched in PtdIns-glycan-specific PLC activity, assayed by measuring the hydrolysis of the C-terminal glycopeptide of mfVSG. Activity was not detected in the cytosolic or nuclear fractions. Neutral nonionic detergents such as *n*-octyl glucopyranoside and NP-40 were most effective at solubilizing enzyme activity. The zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and the anionic detergent sodium deoxycholate were partially successful while cationic detergents either completely inhibited or failed to solubilize the activity (data not shown). Twenty milliliters of the solubilized enzyme was applied to a 20-ml DEAE-cellulose anion-exchange column. Activity was eluted with buffer that contained 50 mM NaCl (Fig. 1). A broad peak of fractions that extracted radiolabel into the organic phase was observed. However, when analyzed by TLC only a few of the fractions exhibited significant acyl$_2$Gro-releasing activity.

The acyl$_2$Gro-producing fractions were pooled and passed through a column of Extracti-Gel D of equal volume to remove detergent. PtdIns-glycan PLC was then adsorbed onto a 10-ml column of butyl-agarose (37). The enzyme was specifically eluted with 0.1% NP-40 in 25 mM Tris·HCl buffer (pH 7.5) at 0°C. Interestingly, total activity increased after this step, suggesting the removal of some inhibitory factor by butyl-agarose (Fig. 2, Table 1). Analysis of the lipid products after digestion of the C-terminal glycopeptide of mfVSG with fractions of the partially purified enzyme (prior to butyl-agarose chromatography) showed free fatty acid production in addition to acyl$_2$Gro (data not shown). Contamination of this preparation with phospholipase A or diglyceride lipase activities could result in anomalously low PLC activity. PtdIns-glycan PLC was purified 800-fold from purified plasma membranes. Seventy grams of rat liver yielded ≈120 μg of enzyme. Taking into account yield of enzyme, this reflects a purification of ≈20,000-fold over whole liver.

Samples from each stage of the purification were subjected to NaDodSO$_4$/PAGE in 10% gels. Silver staining of the enzyme after chromatography on butyl-agarose (Fig. 3, lane

Table 1. Purification of PtdIns-glycan PLC

Step	Total protein, mg	Total activity, pmol·min^{-1}	Specific activity, pmol·min^{-1}·mg^{-1}	Purification, fold	Yield, %
Detergent-solubilized membranes	125	4.25	0.034	1	100
Supernatant after centrifugation	36	3.42	0.10	2.8	80
DE52	2.1	1.48	0.50	14.7	35
Butyl-agarose	0.12	3.18	26.5	780	76

Samples from each stage of the purification were incubated in 1 ml with 25 nM [^{3}H]mfVSG for 2 hr at 37°C and then extracted twice with 1 ml of chloroform/methanol, 200:1 (vol/vol). The organic phases were taken to dryness and analyzed by TLC. Each assay was performed in duplicate and was consistent to within 10%. Specific activity is expressed as pmol of mfVSG consumed per min per mg protein but does not reflect activities at saturating concentration of substrate.

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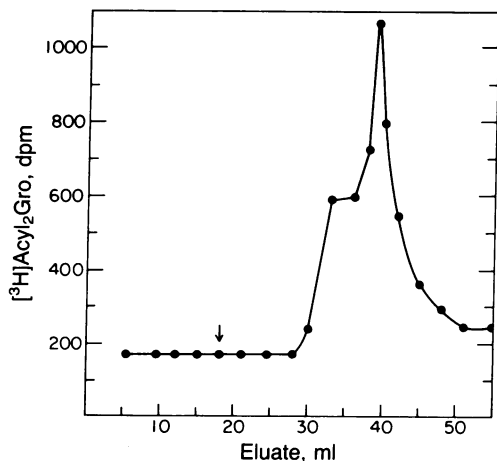*Proc. Natl. Acad. Sci. USA 84 (1987)* 2665

FIG. 1. DEAE-cellulose (DE52) chromatography of PtdIns-glycan PLC. The supernatant fraction of the solubilized rat liver plasma membranes was applied to a 20-ml DE52 column (2.5 cm × 5 cm) equilibrated in 1% NP-40/25 mM Tris·HCl, pH 8.0, at 0°C. PtdIns-glycan PLC was eluted with 50 mM NaCl in 0.5% NP-40/25 mM Tris·HCl, pH 8.0, at 0°C at a flow rate of 20 ml/hr. All buffers contained leupeptin at 10 μg/ml, aprotinin at 10 μg/ml, and chymostatin at 10 μg/ml. Enzyme activity was localized by assay with ^{3}H-labeled C-terminal glycopeptide of mfVSG for 1 hr at 37°C, followed by toluene extraction, and analysis by TLC. The arrow refers to where elution with 50 mM NaCl was begun. dpm refers to counts of acyl$_2$Gro released.

4) revealed one major band with an apparent molecular weight of 52,000.

The molecular size of PtdIns-glycan PLC was also evaluated on a 50-ml AcA 44 column, eluted with 0.5% NP-40 (Fig. 4). Activity was eluted with ≈28 ml, indicating an apparent molecular weight of 62,000 with a Stokes radius of ≈35 Å. Comparison of the electrophoretic mobility and gel permeation profile of the enzyme indicates some association of the major silver-stained band with detergent or lipid, or the possibility that activity resides in one of the minor, higher molecular weight bands, but suggests that the catalytic activity of the enzyme is essentially monomeric in its active form.

PtdIns-Glycan PLC Catalyzes the Hydrolysis of Glycosyl-

FIG. 3. NaDodSO$_4$/PAGE of PtdIns-glycan PLC. Samples from each stage of the purification were electrophoresed on 10% polyacrylamide gels. Lanes: 1, 25 μg of solubilized plasma membranes; 2, 25 μg of supernatant after centrifugation of solubilized plasma membranes; 3, 25 μg of PtdIns-glycan PLC after DE52 chromatography; 4, 25 μg of PtdIns-glycan PLC after butyl-agarose chromatography. The enzyme in lane 4 was incubated on ice with 10% (wt/vol) trichloroacetic acid for 1 hr. Following centrifugation in an Eppendorf microfuge for 0.5 hr, the pellet was washed twice with cold ether and dissolved by boiling in sample buffer. Protein was visualized by silver staining with the Bio-Rad kit. Molecular weight markers were from Sigma.

ated-PtdIns Substrates. The activity of the purified phospholipase was assayed using [^{3}H]myristate-labeled mfVSG, C-terminal glycopeptide, and the BC$_3$H1-derived [^{3}H]inositol-labeled PtdIns-glycan as substrate. The [^{3}H]-labeled myristoylated substrates were incubated with the enzyme, and the lipid reaction products were extracted with toluene or chloroform/methanol, 200:1 (vol/vol), and analyzed on a neutral lipid system (Fig. 5). Purified preparations of PtdIns-glycan PLC catalyzed the cleavage of the phosphodiester bond and released acyl$_2$Gro from both substrates.

The enzymatic hydrolysis of the [^{3}H]inositol-labeled PtdIns-glycan derived from BC$_3$H1 myocytes was evaluated by chromatography of the aqueous products (Fig. 6). The purified PtdIns-glycan PLC was reconstituted by sonication of the HPLC purified PtdIns-glycan (3) in lipid vesicles containing 1:1 molar mixtures of phosphatidylcholine/phosphatidylethanolamine. Following a 2-hr incubation, the re-
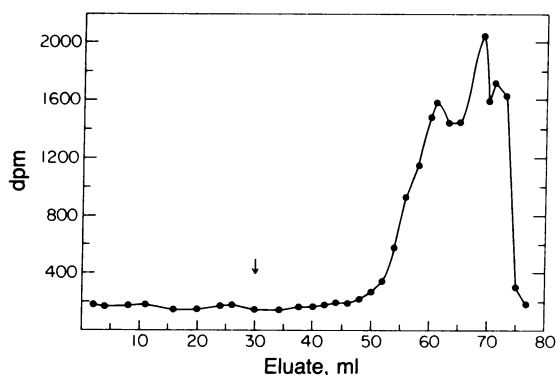

FIG. 2. Butyl-agarose chromatography of PtdIns-glycan PLC. PtdIns-glycan PLC from DE52 was passed through a column of Extracti-Gel D of equivalent volume to remove detergent and absorbed onto a 10-ml butyl-agarose column (1.5 cm × 6 cm) equilibrated in 25 mM Tris (pH 8.0) at 0°C. The column was washed with 20 ml of the same buffer, and then PtdIns-glycan PLC activity was eluted with 0.1% NP-40/25 mM Tris·HCl, pH 8.0, at 0°C at a flow rate of 20 ml/hr (↓). Enzyme activity was identified by assay for 1 hr with C-terminal glycopeptide of mfVSG. dpm refers to counts present in the organic phase following toluene extraction of the incubation mixture.
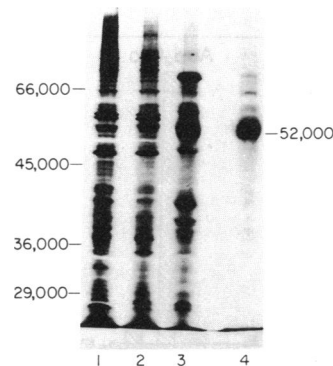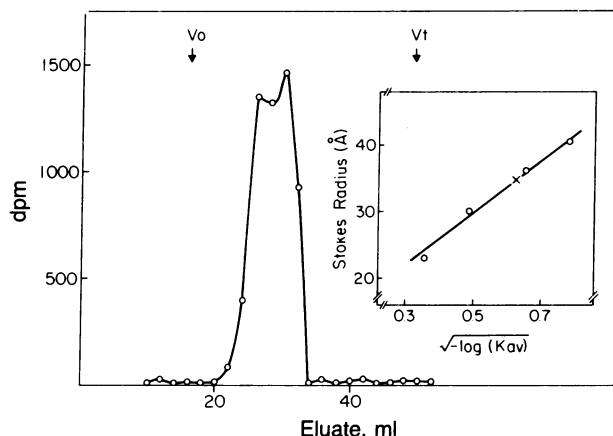

FIG. 4. AcA 44 molecular sizing of PtdIns-glycan PLC. A 50-ml AcA 44 sizing column was equilibrated in 0.5% Nonidet P-40, 20% (vol/vol) glycerol, 25 mM Tris·HCl (pH 8.0) at 0°C. Activity was localized by assay with C-terminal glycopeptide of mfVSG. The flow rate of the column was 3 ml/hr. (*Inset*) The column was standardized with myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase. ×, PtdIns-glycan PLC. Vo, void volume. Vt, total volume.
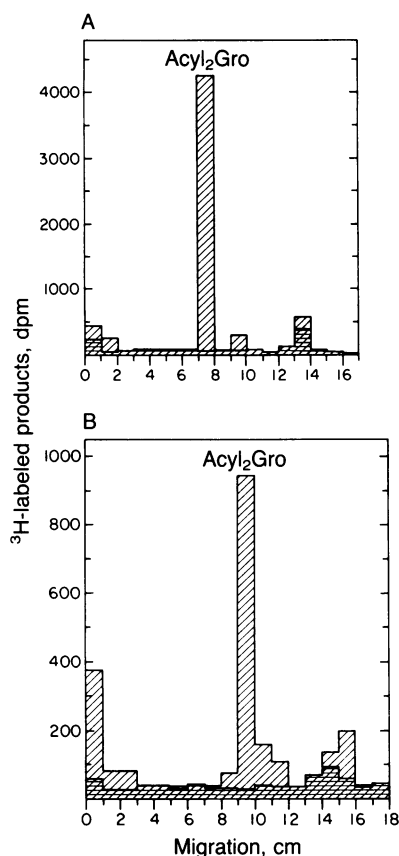
FIG. 5. Product analysis of PtdIns-glycan PLC catalyzed hydrolysis of [³H]myristate-labeled substrates. Butyl-agarose purified PtdIns-glycan PLC was incubated with mfVSG (*A*) or C-terminal glycopeptide (*B*) for 2 hr. The incubation mixtures were twice extracted with chloroform/methanol and analyzed by TLC. The position of acyl₂Gro on the chromatogram was localized both by iodine staining of dimyristoylglycerol standard and by [³H]acyl₂Gro produced by trypanosomal phospholipase C.

action mixture was extracted and phase-separated as described in Fig. 6, and the aqueous products were directly chromatographed on an HPLC anion-exchange column (1). The enzyme caused the production of InsP-glycan, eluting at 15 min. Further chemical and chromatographic evaluation of this product confirmed its identity as the insulin-sensitive enzyme modulator (data not shown).

Although PtdIns-glycan PLC was active against mfVSG and the BC₃H1-derived PtdIns-glycan, the enzyme exhibited no activity in hydrolysis of PtdIns, phosphatidylinositol-4,5-bisphosphate, or phosphatidylcholine under a variety of reaction conditions, including variations in calcium concentration, thiol reagents, other phospholipids, and additional cold substrate. These findings suggest the clear specificity of the enzyme for glycosylated inositol phospholipids.

Characterization of PtdIns-Glycan PLC Activity. The effects of cations and thiol reagents on the activity of the purified enzyme are summarized in Table 2. The presence of calcium was not required for enzyme activity, and the enzyme was stimulated by treatment with the chelator EGTA. Like the PtdIns-glycan PLC purified from *T. brucei* (17), the analogous activity from rat liver plasma membranes was activated by exposure to sulfhydryl reagents such as 2-mercaptoethanol and inhibited by mercurial compounds. The activity is stable for ≈1 week at 4°C or 1 month at −20°C. Enzyme activity is stabilized by glycerol and ethylene glycol but is destroyed with repeated freeze-thawing.
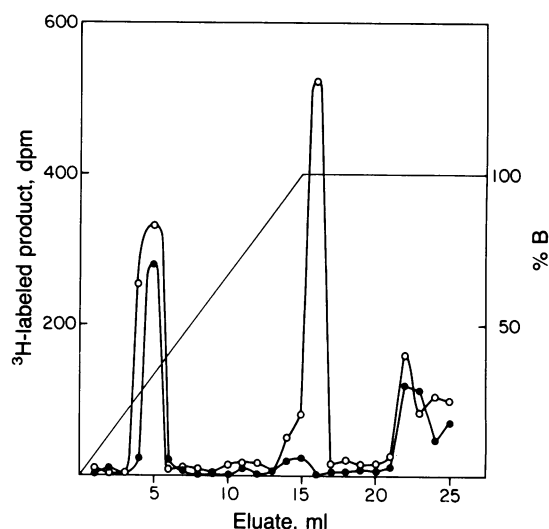

FIG. 6. Product analysis of PtdIns-glycan PLC-catalyzed hydrolysis of [³H]inositol-labeled PtdIns-glycan precursor. The purified enzyme was incubated with [³H]inositol-labeled PtdIns-glycan purified from BC₃H1 cells as described (3). After a 2-hr incubation in a final volume of 0.25 ml, 1 ml of chloroform/methanol/HCl (1 M), 200:100:1 (vol/vol), was added, followed by 0.5 ml of 10 mM formic acid. Solutions were centrifuged at 500 × g for 5 min, and the upper aqueous phase was directly injected onto a SAX HPLC column and eluted as described (1). Resulting fractions were analyzed by liquid scintillation counting. ●, Control; ○, enzyme.

DISCUSSION

We have described (1–3) the insulin-sensitive hydrolysis of a PtdIns-glycan. This reaction results in the generation of InsP-glycans that regulate certain intracellular enzymes (1–2, 4) and of acyl₂Gro, the endogenous activator of protein kinase C (38). The PtdIns-glycan was isolated following metabolic labeling of cells with [³H]inositol, [³H]glucosamine, and [³H]myristate and served as a substrate for a phosphodiesteratic hydrolysis stimulated by insulin in intact cells or by exogenously added PtdIns-specific PLC in a reconstituted system (1–3). Both the insulin- and PtdIns-PLC-dependent hydrolysis of this glycolipid resulted in the generation of identical products, suggesting that this action of insulin was mediated by a PLC. Moreover, the substrate specificity of this putative enzyme activity was suggested by the apparent inability of insulin to cause the hydrolysis of the inositol phospholipids or the subsequent mobilization of intracellular calcium (25, 27), a consequence of the hormonally sensitive PtdIns-specific PLC. The prediction of a PtdIns-glycan-specific PLC was verified by the identification of such an enzyme activity in rat liver plasma membranes. This enzyme is clearly distinct from the PtdIns-specific PLC activities by virtue of its calcium independence and specificity for glycosylated PtdIns as substrate. Identification of

Table 2. Effects of cations and sulfhydryl reagents on PtdIns-glycan PLC

Addition	Activity, dpm/2 hr	% control
None	3970	100
100 μM EGTA	5900	148
100 μM EGTA/100 μM CaCl₂	6250	157
50 mM 2-mercaptoethanol	7564	190
100 μM HgCl₂	677	17

Purified PtdIns-glycan PLC was incubated with 25 nM mfVSG in the presence of the indicated reagents for 2 hr at 37°C. Enzyme activity was assayed as described for Table 1. All assays were performed in duplicate. Variance was less than 10%.

the catalytic products of this PLC as the water-soluble and -insoluble insulin-dependent enzymatic products found in intact cells infers the insulin sensitivity of this enzyme, and suggests its role as an effector of insulin action.

The PtdIns-glycan PLC purified from rat liver plasma membrane shares some properties with the analogous activity purified from *T. brucei*. Both enzymes are membrane associated, calcium independent, thiol stimulated, and inhibited by mercurials (17). Each activity catalyzes the hydrolysis of glycan-hexosaminyl-PtdIns-containing molecules, while PtdIns and its phosphorylated derivatives are poor substrates. Although the mammalian liver-derived enzyme is larger than that purified from trypanosomes, the enzymes may share some homology, particularly at the active site.

In addition to its possible role in signal transduction during insulin-dependent metabolism, the PtdIns-glycan PLC may also be involved in the regulation of those proteins anchored to the plasma membrane by PtdIns. A report (39) has described the reduction of cellular alkaline phosphatase by insulin in the rat osteoblast-like osteogenic sarcoma line ROS 17/2.8. Alkaline phosphatase is known to be covalently linked to PtdIns (6, 7), so that the insulin-dependent reduction of alkaline phosphatase may result from the hormone-sensitive PtdIns-glycan PLC activity. In addition, insulin was reported to cause the acute release of heparan sulfate from cultured hepatocytes (13). This protein is also anchored to the cell surface via a covalent linkage with glycosylated PtdIns (13). Thus, in addition to a putative role in mediating some of the metabolic actions of insulin by catalyzing the generation of Ins$P$-glycans and acyl$_2$Gro, the plasma membrane-associated PtdIns-glycan PLC may also play some role in regulating the dissociation of certain proteins from the plasma membrane. The further molecular characterization of PtdIns-glycan PLC and the elucidation of the chemical structures of its substrates may provide further insights into the molecular mechanisms of insulin action.

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