Insulin Activates Glycerol-3-phosphate Acyltransferase (de Novo Phosphatidic Acid Synthesis) through a Phospholipid-Derived Mediator. Apparent Involvement of Giα and Activation of a Phospholipase C†

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ABSTRACT: We studied the mechanism whereby insulin activates de novo phosphatidic acid synthesis in BC3H-1 myocytes. Insulin rapidly activated glycerol-3-phosphate acyltransferase (G3PAT) in intact and cell-free preparations of myocytes in a dose-related manner. The apparent K_m of the enzyme was decreased by treatment with insulin, whereas the V_{max} was unaffected. No activation was found by ACTH, insulin-like growth factor-I, angiotensin II, or phenylephrine, but epidermal growth factor, which, like insulin, is known to activate de novo phosphatidic acid synthesis in intact myocytes, also stimulated G3PAT activity. In homogenates or membrane fractions, the effect of insulin on G3PAT was fully mimicked by nonspecific or phosphatidylinositol (PI)-specific phospholipase C (PLC). An antiserum raised against PI–glycan–PLC completely blocked the effect of insulin on G3PAT. Although the above findings suggested involvement of a PLC in insulin-induced activation of G3PAT, neither diacylglycerol nor protein kinase C activation appeared to be involved. On the other hand, insulin stimulated the release of a cytosolic factor, which activated membrane-associated G3PAT. This cytosolic factor had a molecular weight of less than 5K as determined by Sephadex G-25 chromatography. NaF, a phosphatase inhibitor, blocked the activation of G3PAT by insulin, suggesting involvement of a phosphatase. Insulin-induced activation of G3PAT was also blocked by pretreatment of intact myocytes with pertussis toxin and by prior addition, to homogenates, of an antiserum that recognizes the C-terminal decapeptide of Giα. Our results suggest that insulin activates a pertussis toxin sensitive, Giα-protein-requiring PI–glycan–PLC in BC3H-1 myocytes, resulting in the release of a cytosolic, low molecular weight factor, which decreases the K_m of G3PAT, probably by a phosphatase-mediated mechanism. This activation of G3PAT may account for insulin-induced increases in de novo synthesis of phosphatidic acid, which, in turn, may amplify diacylglycerol–protein kinase C signaling and provide a mechanism to replenish phospholipids that are hydrolyzed during insulin action.

Insulin increases diacylglycerol (DAG) in BC3H-1 myocytes (Farese et al., 1984, 1985, 1987) through (a) hydrolysis of preexisting phospholipids (Saltiel et al., 1987; Nair et al., 1988) and (b) de novo synthesis of phosphatidic acid (PA) (Farese et al., 1984). Glycerol-3-phosphate acyltransferase (G3PAT) is responsible for converting glycerol 3-phosphate and fatty acyl-CoA to PA, and activation of this enzyme may be responsible for the insulin effect on de novo PA synthesis. Previous studies in rat skeletal muscle have suggested that insulin releases a soluble factor, which activates G3PAT (Stevens & Husbands, 1988), and this factor appears to be similar to other putative insulin "mediators", which are postulated to be responsible for certain effects of the hormone on a variety of enzymes [for review, see Larner (1988)]. Moreover, it has been postulated that soluble "mediators" may be released by activation of a specific phospholipase C (PLC), which hydrolyzes a phosphatidylinositol (PI)–glycan (Saltiel et al., 1986, 1988; Saltiel & Cuatrecasas, 1988; Mato et al., 1987; Mato, 1989). In the present study, we examined the possibility that insulin stimulates de novo PA synthesis in BC3H-1 myocytes through activation of G3PAT by a lipid-derived mediator. We also examined the possibility that a G-protein may be involved in this activation.

Experimental Procedures

Materials. Crystalline zinc porcine insulin was purchased from Elanco. [2-^{3}-H]Glycerol 3-phosphate (10.6 Ci/mmol) was obtained from Du Pont/NEN. Dioctanoylglycerol (DiC_8) was purchased from Molecular Probes. PI-specific PLC (*Staphylococcus aureus*) and staurosporine were purchased from Calbiochem. Nonspecific phospholipase C (PLC) (Clostridium perfringens; type IX) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma. Pertussis toxin was purchased from List Biological Laboratories Inc. Anti-PI–glycan-specific PLC antiserum was prepared by Judy A. Fox by immunizing rabbits with highly purified PI–glycan-specific PLC [see Fox et al. (1987)] and Freund's adjuvant. Anti-Giα, Goα, and Gsα antisera were prepared by Graeme Milligan [see Ali et al. (1989) and Milligan et al. (1989)]. To minimize the effect of factors other than γ-globulin, all antisera, as well as nonimmune serum, were incubated at 56 °C for 30 min and centrifuged at 600*g* for 15 min to obtain the γ-globulin-containing supernatants, which were used experimentally. [Similar results were obtained with untreated sera (data not shown).]

Cell Culture and Preparation of Cell Extracts. Myocytes were cultured for 10–14 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with controlled process serum replacement (Sigma) as described (Farese et al., 1984).

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On the day of the experiment, the medium was removed and replaced with Dulbecco's phosphate-buffered saline containing 0.1 mM $CaCl_2$, glucose (1 mg/mL), and albumin (1 mg/mL) (DPBSGA). Cells were incubated for 1 h at 37 °C and treated with different agents as indicated. After incubation, cells were chilled, washed three times with DPBSGA, gently scraped, and centrifuged. The cell pellet was washed with homogenization buffer containing 10 mM Tris/HCL (pH 7.4), 0.25 M sucrose, and 0.7 mM dithiothreitol (DTT). Cells were homogenized in the same buffer at a concentration of 4 mg of protein/mL with 10 strokes of a motor-driven Teflon pestle in a Potter-Elvejhem tube. Routinely, homogenates were first centrifuged at 600*g* for 5 min to remove unbroken cells and nuclei. The pellets were discarded and the supernatants were used directly ("homogenates") or centrifuged at 105000*g* for 30 min to obtain cytosol (supernatants) and "postnuclear" membrane fractions.

Determination of G3PAT Activity. G3PAT activity was measured by a slight modification of the method of Lawson et al. (1981). The incubation mixture contained 250 mM KCl, 50 mM Tris/HCl (pH, 7.4), 0.7 mM DTT, 0.2 mM (except in kinetic studies) [^{3}H]glycerol 3-phosphate (1 μCi), 136 μM palmitoyl-CoA (a saturating concentration), 2 mg/mL albumin, and G3PAT enzyme in homogenate or subcellular fraction, in a final volume of 0.5 mL. Enzyme and all other additions were separately equilibrated for 3 min at 37 °C. The reaction was started by adding the enzyme to the incubation mixture. After 1 min, incubation was stopped with 2 mL of water-saturated butanol, followed by 1.5 mL of butanol-saturated water. The butanol phase was separated and washed twice, and an aliquot was counted for radioactivity. The rate of product formation was linear for at least 2 min. The labeled products formed were primarily PA (70–75%) and lyso-PA (20–25%), as reported previously (Stevens & Husbands, 1987). In the experiments reported below, insulin increased PA labeling to a greater extent than lyso-PA.

Determination of Proteins. Proteins were measured according to the method of Bradford (1976).

Results

Subcellular Localization and Kinetic Parameters of G3PAT. As in other tissues [see Bell and Coleman (1980)], G3PAT activity was localized to the postnuclear membrane fraction of BC3H-1 myocytes. No activity was dected in the cytosol, although this fraction stimulated membrane G3PAT activity (see below). G3PAT has been suggested to be comprised of two isozymes, one in the outer mitochondrial membrane and the other in the endoplasmic reticulum, both having their active sites facing the cytosol (Coleman & Bell, 1983). The microsomal isozyme is inhibited by *N*-ethylmaleimide (NEM), whereas the mitochondrial isozyme is insensitive to NEM (Bell & Coleman, 1980; Coleman & Bell, 1983). Accordingly, we found that G3PAT activity of a postmitochondrial, or "microsomal fraction" (i.e., the supernatant obtained after centrifugation of homogenates at 10000*g* for 10 min), was completely inhibited by treatment with 2 mM NEM (incubation for 15 min at 4 °C, followed by 2 mM DTT to neutralize NEM), whereas the "mitochondrial fraction" (i.e., the 10000*g*-pellet) contained both NEM-sensitive and NEM-insensitive G3PAT activity (data not shown). Since insulin-induced changes in G3PAT activity (see below) were fully inhibited by NEM, since there were considerable losses of microsomes to the "mitochondrial" fraction, and since apparent mitochondrial (NEM-insensitive) G3PAT activity accounted for only 15% of total G3PAT activity, all subsequent experiments were conducted with preparations that included all

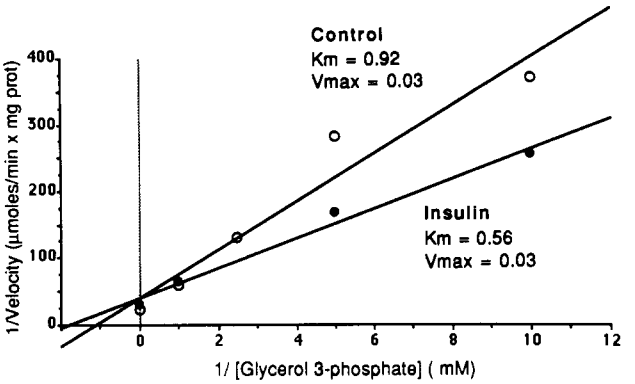

FIGURE 1: Kinetic parameters of G3PAT activation in control and insulin-treated BC3H-1 myocytes. Cells were treated with 100 nM insulin or vehicle for 5 min. Homogenates were then prepared and G3PAT activity was measured, as described in methods, but using different concentrations of glycerol 3-phosphate.

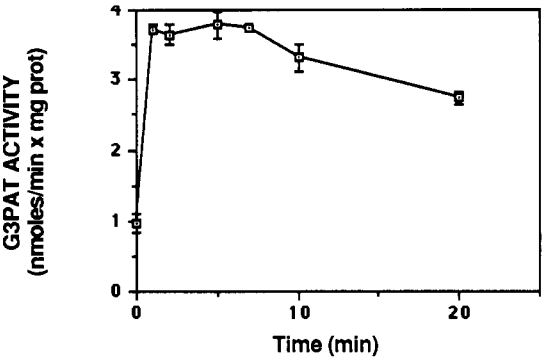

FIGURE 2: Time course of insulin-induced activation of G3PAT. Intact BC3H-1 myocytes were treated with 100 nM insulin for the times indicated in the figure. Then homogenates were prepared and G3PAT activity was measured. Values shown are means of duplicates of a representative experiment repeated five times with similar results.

postnuclear membranes, with or without cytosol as described.

The apparent K_m and V_{max} for conversion of glycerol 3-phosphate into lipids were first examined in postnuclear homogenates derived from control and insulin-treated (100 nM × 5 min) intact myocytes. Insulin treatment provoked a decrease in the apparent K_m of the enzyme, without significant alteration of the V_{max} (Figure 1). The apparent K_m of control cells was 0.81 ± 0.09 mM (n = 4) versus 0.51 ± 0.13 mM (n = 4) for insulin-treated cells, and the percent decrease in the insulin-treated cells was 41 ± 10% (mean ± SE, n = 4), which was statistically significant ($p < 0.025$; paired t test). Similar effects of insulin on the K_m of the G3PAT were observed in kinetic experiments in which microsomal G3PAT activity was assessed by subtraction of NEM-insensitive activity from total G3PAT activity (data not shown). We measured enzyme activity in all the following experiments by using 0.2 mM glycerol 3-phosphate, which proved to be a very sensitive concentration for examining agonist-induced alterations in enzyme activity.

Effects of Insulin and Other Agonists on G3PAT Activation in Intact Myocytes. Within 1 min of addition of 100 nM insulin to intact myocytes, G3PAT activity of subsequently prepared homogenates was increased 2–3-fold, and this effect was maintained for 20 min, after which it tended to decrease (Figure 2). Dose-related effects of insulin on G3PAT activity of intact myocytes are shown in Figure 3. These time- and dose-related effects of insulin on G3PAT activation are similar to previously reported effects of insulin on incorporation of [^{3}H]glycerol into PA, DAG, and other lipids (Farese et al., 1987, 1988).

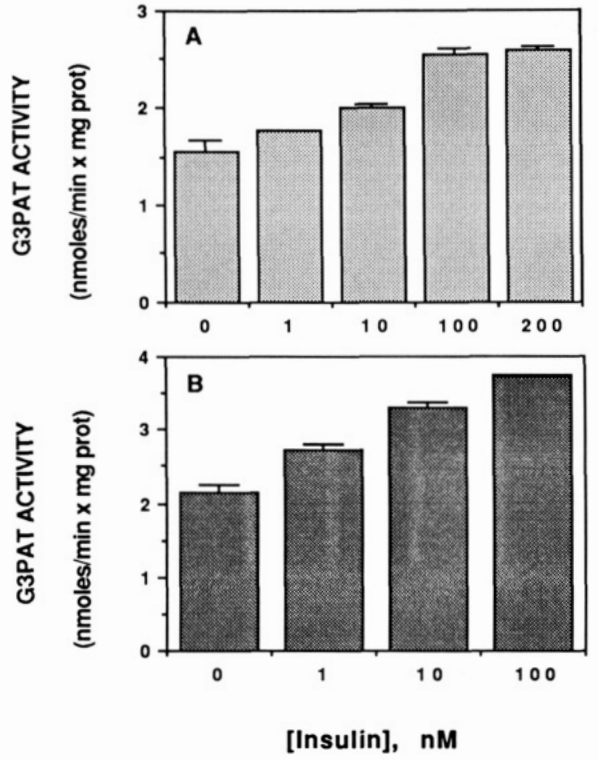

FIGURE 3: Dose-dependent activation of G3PAT by insulin. (A) Homogenates of BC3H-1 myocytes were treated in vitro with different concentrations of insulin for 1 min and G3PAT activity was then measured. (B) Intact myocytes were treated with insulin for 5 min. Homogenates were then prepared for assay of G3PAT activity. Values are from a representative experiment repeated three times with similar results.

Table I: Effects of Insulin and Other Agents on Glycerol-3-phosphate Acyltransferase Activity in BC3H-1Myocytes[a]

treatment	G3PAT act. [nmol of G3P converted min^{-1} (mg of protein)$^{-1}$]	rel act. (%)	n
none (control)	1.20 ± 0.10	100	7
insulin (100 nM)	2.30 ± 0.01	193 ± 9[c]	7
ACTH (0.1 μM)	1.28 ± 0.12	109 ± 2	2
phenylephrine (1 μM)	1.31 ± 0.12	110 ± 3	2
angiotensin II (0.1 μM)	1.07 ± 0.07	102 ± 11	2
EGF (100 ng/mL)	2.15 ± 0.07	184 ± 5[b]	4
IGF-I (50 ng/mL)	1.15 ± 0.10	95 ± 6	2
Me_2SO (0.7% v/v)	1.07 ± 0.09	95 ± 3	5
TPA (500 nM) in Me_2SO	1.10 ± 0.10	100 ± 2	2
staurosporine (16 μM)	1.03 ± 0.01	93 ± 7	2
staurosporine + insulin	2.12 ± 0.01	178 ± 2	2

[a] Intact BC3H-1 myocytes were incubated for 5 min (or 20 min in the case of treatment with Me_2SO or TPA) with the indicated treatments. (Where indicated, staurosporine was added 5 min before treatment with either insulin or vehicle.) Homogenates were prepared and G3PAT activity was assayed as described in methods. Means ± SE of duplicate determinations from a representative experiment are shown at the left. Means ± SE of G3PAT activity relative to controls (set at 100%) observed in n separate experiments are shown at the right [b] $p < 0.005$. [c] $p < 0.001$ (paired t test versus control).

The effect of insulin on G3PAT was relatively specific, as there was no significant effect of IGF-I, ACTH, angiotensin II, or phenylephrine (Table I). However, epidermal growth factor (EGF) also stimulated G3PAT activity, and this agrees with our finding of EGF-induced stimulation of de novo PA synthesis (i.e., [^{3}H]glycerol incorporation) in intact BC3H-1 myocytes (Farese et al., 1989).

Effects of Insulin and Phospholipase C on G3PAT Activity in Cell-Free Preparations of BC3H-I Myocytes. Direct addition of insulin to postnuclear homogenates also stimulated G3PAT activity (Table II). Moreover, this effect of insulin was fully mimicked by the addition of either nonspecific PLC or PI-specific PLC (Table II). As in the intact cells, the stimulation of G3PAT by insulin in these homogenates was dose dependent (Figure 3).

Table II: Effects of Insulin and Phospholipase C (PLC) on G3PAT Activity of Homogenates and Membrane Fractions from BC3H-1 Myocytes[a]

treatment	G3PAT act. [nmol of G3P converted min^{-1} (mg of protein)$^{-1}$]	rel act. (%)	n
Homogenates			
none (control)	1.04 ± 0.07	100	8
insulin (100 nM)	1.89 ± 0.19	188 ± 4[e]	8
nonspecific PLC (0.5 units/mL)	2.12 ± 0.02	189 ± 11[d]	4
PI-specific PLC (1 μg/mL)	2.20 ± 0.09	183 ± 9[e]	8
Membrane Fractions			
none (control)	1.45 ± 0.13	100	6
insulin (100 nM)	2.34 ± 0.03	178 ± 6[e]	6
nonspecific PLC (0.5 units/mL)	2.37 ± 0.01	170 ± 6[c]	3
PI-specific PLC (1 μg/mL)	2.52 ± 0.08	168 ± 8[b]	3
NaF (100 mM)	1.60 ± 0.08	110 ± 9	4
NaF + insulin	1.52 ± 0.12	113 ± 6	4

[a] Homogenate and membrane fractions were prepared from BC3H-1 myocytes and equilibrated for 3 min at 37 °C. Indicated treatments were then added, and, after 1 min, G3PAT activity was measured. In the case of NaF, membranes were pretreated for 3 min with this compound before adding either insulin or vehicle. Means ± SE of duplicate determinations from a representative experiment are shown at the left. Means ± SE of G3PAT activity relative to controls (set at 100%) observed in n separate experiments are shown at the right. [b] $p < 0.025$. [c] $p < 0.01$. [d] $p < 0.005$. [e] $p < 0.001$ (paired t test versus control).

Release of a "soluble mediator" after treatment of membranes with insulin or PI–PLC has been reported previously (Larner et al., 1979; Seals & Czech, 1981; Saltiel, 1987). We therefore conducted similar experiments with BC3H-1 myocyte membranes and found that insulin, nonspecific PLC, and PI-specific PLC activated G3PAT in these postnuclear membrane fractions (Table II). (As described below, insulin and PI-PLC also stimulated the release of a soluble G3PAT stimulatory factor from membranes.)

Effects of Antibodies to PI–Glycan-Specific Phospholipase C on Insulin-Induced Activation of G3PAT. Short pretreatment of either postnuclear homogenates or membrane fractions with an antiserum raised to PI–glycan-specific PLC (final dilution 1:100) completely blocked the subsequent activation of G3PAT by insulin, without altering basal G3PAT activity (Table III). A 10-fold greater dilution of the antiserum provoked a lesser effect (data not shown). On the other hand, serum obtained from unimmunized rabbits was without effect (Table III).

Evidence That Insulin or Phospholipase C Stimulates the Release of a Cytosolic Factor That Activates G3PAT. To further test the possibility that a soluble mediator, released or generated during insulin or PLC treatment, may activate G3PAT, we examined the effects of cytosols from intact control or insulin-treated (for 5 min) myocytes on the G3PAT activity of control or insulin-treated membranes. G3PAT activity of control membranes was stimulated by 43 ± 9% (mean ± SE; $n = 3$; $p < 0.05$; paired t test) by the addition of control cytosol, but the stimulation was greater with insulin-treated cytosol, viz., 89 ± 7% ($n = 3$; $p < 0.01$ versus no cytosol and $p < 0.025$ versus control cytosol). G3PAT activity of the membrane fraction alone was also increased by insulin treatment (e.g., 0.63 versus 1.02 nmol of G3P converted min^{-1} (mg of protein)$^{-1}$; control versus insulin in a representative

Table III: Effects of Antisera to (A) PI–Glycan-Specific Phospholipase C and (B) Various G-Proteins on Insulin-Induced Activation of G3PAT in Cell-Free Preparations of BC3H-1 Myocytes[a]

treatment	G3PAT act. [nmol of G3P converted min^{-1} (mg of protein)$^{-1}$]	rel act. (%)	n
A			
control	1.17 ± 0.06	100	
insulin	2.65 ± 0.05	190 ± 15[c]	6
control + nonimmune serum	1.33 ± 0.14	101 ± 7	4
insulin + nonimmune serum	2.81 ± 0.02	199 ± 18[c]	6
control + PI–glycan–PLC antiserum	1.12 ± 0.10	95 ± 3	4
insulin + PI–glycan–PLC antiserum	1.28 ± 0.12	93 ± 4	6
B			
control	1.73 ± 0.08	100	5
insulin	3.40 ± 0.13	168 ± 9[c]	5
insulin + nonimmune serum	3.26 ± 0.08	171 ± 8[d]	5
insulin + anti-Giα antiserum	1.84 ± 0.05	108 ± 2	4
insulin + anti-Goα antiserum	2.90 ± 0.05	158 ± 6[c]	4
insulin + anti-Gsα antiserum	3.35 ± 0.15	169 ± 13[b]	3

[a] Homogenate or membrane fractions of BC3H-1 myocytes were equilibrated at 37 °C for 2 min and then incubated for 3 min in the presence of immune or nonimmune serum (final dilution 1:100). Insulin (100 nM) or vehicle were then added, and, after 1 min, G3PAT activity was measured as described in the methods. Means ± SE of duplicate determinations from a representative experiment are shown at the left. Means ± SE of activity relative to controls (set at 100%) observed in n separate experiments are shown at the right. [b] $p < 0.05$. [c] $p < 0.005$. [d] $p < 0.001$ (paired t test versus control).

experiment), and these insulin-treated membranes were further activated by addition of cytosol, particularly that derived from insulin-treated cells (52 ± 11% and 70 ± 9% increases in G3PAT activity with addition of control and insulin-treated cytosols, respectively). Thus, after insulin treatment, there were increases in the activity of both membrane-bound G3PAT and a cytosolic stimulatory factor. To further characterize the latter, cytosols derived from control and insulin-treated myocytes were chromatographed on a Sephadex G25 column, and column fractions were examined for their effect on G3PAT activity of control membranes. As shown in Figure 4, a G3PAT activator was eluted after the column void volume (i.e., in the inclusion volume), suggesting that this activator has a molecular weight of <5000, comparable to that of activators reported on previously (Larner et al., 1979; Kiechle et al., 1981; Seals & Czech, 1981; Saltiel & Cuatrecasas, 1986; Stevens & Husbands, 1987): moreover, insulin-treated cytosol contained more G3PAT activator than control cytosol. Similar results were also obtained following direct treatment of control postnuclear membranes with insulin: a soluble G3PAT stimulatory factor was released and recovered in the inclusion volume fractions from Sephadex G25 columns (Table IV).

Failure of Diacylglycerol–Protein Kinase C Signaling To Stimulate G3PAT. We also evaluated the possibility that DAG (directly or through activation of protein kinase C) might mediate the insulin effect on G3PAT, since DAG is also produced during PLC action. Addition of DiC_8 (100 μg/mL) or TPA (20 or 100 nM), DAG analogues, and known activators of protein kinase C in BC3H-1 myocytes (Cooper et al., 1987; Standaert et al., 1988) to homogenates (data not shown) or intact myocytes (Table I) had no effect on G3PAT

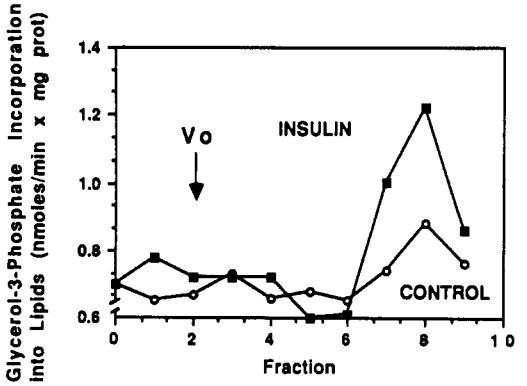

FIGURE 4: Gel filtration chromatography of cytosols from control and insulin-treated myocytes. Cytosols (200 μL) were chromatographed through a Sephadex G-25 column (total bed volume: 5 mL) equilibrated with buffer containing 10 mM Tris/HCl, (pH 7.4), 0.25 M sucrose, and 0.7 mM DTT. Fractions (0.5 mL) were collected and aliquots (70 μL) were tested for their ability to stimulate G3PAT activity of control membranes (containing 70 μg of protein). Absorbance (at 280 nm) of the fractions was measured to identify the void volume (V_0). Shown here are mean values of duplicate determinations of a representative experiment, repeated three times with similar results.

Table IV: Release of Soluble Mediator from Membranes of BC3H-1 Myocytes Treated with Insulin or PI-Specific PLC[a]

addition to assay	G3PAT act. [nmol of G3P converted min^{-1} (mg of protein)$^{-1}$]
none	0.39 ± 0.01
control column fraction	0.42 ± 0.01
insulin column fraction	0.76 ± 0.03
PI-sp PLC column fraction	0.71 ± 0.02

[a] Equal amounts (400 μg of protein) of control membranes were incubated at 37 °C with 100 nM insulin, PI-specific PLC (1 μg/mL) or vehicle (control) for 2 min. Reaction mixtures were then centrifuged at 105000g for 30 min to obtain supernatant fractions, which were chromatographed on a Sephadex G-25 column. The inclusion volume fractions (pooled fractions 7–9 in Figure 4) were collected and their effect (80 μL) on control membrane (50 μg protein) G3PAT activity was tested. The data shown are means (±SE) of duplicates from a representative experiment repeated twice with similar results.

activity. In addition, when myocytes were pretreated with 16 μM staurosporine, a fully effective inhibitor of protein kinase C and phorbol ester-dependent effects (e.g., glucose transport) in these cells (unpublished observations), the insulin effect on G3PAT activity was not altered (Table I). These findings suggested that neither DAG nor protein kinase C mediates the insulin effect on G3PAT.

Effects of NaF on Insulin-Induced Activation of G3PAT. As the effects of "insulin mediators" on certain enzymes may involve phosphatase activation (Saltiel, 1987; Kiechle et al., 1981), we questioned whether insulin activates G3PAT through a similar mechanism. We therefore used NaF, a phosphatase inhibitor (Saltiel, 1987; Kiechle et al., 1981), and found that NaF did not affect control G3PAT activity but fully inhibited insulin-induced stimulation of the enzyme (Table II).

Effects of Pertussis Toxin on Insulin-Induced Activation of G3PAT. Since it has been proposed that insulin signaling in BC3H-1 myocytes may involve G-protein mediated activation of PLC (Luttrel et al., 1988), we studied the effect of pertussis toxin on insulin-induced activation of G3PAT. When myocytes were pretreated with pertussis toxin for 18 h, no effect of insulin on G3PAT activity could be detected during a subsequent incubation of intact cells (Table V). [As will be reported elsewhere, pertussis toxin also inhibited insulin effects on [^{3}H]glycerol incorporation (but not basal incorpo-

Table V: Effect of Pertussis Toxin on the Activation of G3PAT by Insulin or Phospholipase C[a]

treatment	G3PAT act. [nmol of G3P converted min^{-1} (mg of protein)$^{-1}$]	rel. act. (%)	n
none (control)	1.36 ± 0.02	100	6
insulin	2.66 ± 0.04	195 ± 15	6
pertussis toxin	1.31 ± 0.10	90 ± 2	4
pertussis toxin + insulin	1.55 ± 0.05	99 ± 9	6
phospholipase C	2.53 ± 0.07	188 ± 2	4
pertussis toxin + phospholipase C	2.38 ± 0.08	166 ± 9	2

[a] BC3H-1 myocytes were pretreated with pertussis toxin (100 ng/mL) for 18 h and then treated with 100 nM insulin, 1 μg/mL PI-specific phospholipase C, or vehicle (control) for 20 min. Cells were scraped, centrifuged, and washed. Homogenates were prepared and G3PAT activity was assayed. Means ± SE of duplicate determinations from a representative experiment are shown at the left. Means ± SE of G3PAT activity relative to controls (set at 100%) observed in n separate experiments are shown at the right.

ration) into lipids in BC3H-1 myocytes.] On the other hand, the ability of PI-specific PLC to stimulate G3PAT activity (Table V) was not compromised by pertussis toxin. These results suggested that pertussis toxin inhibited a factor (possibly a Gi-protein) whose function precedes, rather than follows, PLC activation.

Effects of Antiserum to G-Proteins on Insulin-Induced Activation of G3PAT. To further evaluate the possibility that Gi-proteins are involved in G3PAT activation by insulin, we tested the effect of an antiserum that recognizes a C-terminal decapeptide, which is common in the α subunits of Gi1α and Gi2α (Ali et al., 1989; Milligan et al., 1989). [This antiserum has been used to uncouple the interaction of δ-opioid receptor with Gi (McKenzie et al., 1988), and we have documented the presence of Giα in BC3H-1 myocytes by demonstration of 35–40-kDa protein by immunoblotting with anti-Giα antiserum.] This antiserum completely blocked insulin-induced activation of G3PAT in cell-free preparations (Table III). Complete inhibition was obtained with dilutions of the Gi antiserum from 1:100 to 1:10000, and lesser inhibition was observed at greater dilutions (Figure 5). On the other hand, nonimmune serum and antisera that recognize the C-terminal decapeptides of Gsα (Milligan et al., 1989) and Goα (Mulaney & Milligan, 1989) were without effect on control or insulin-stimulated G3PAT activity, even when used at final dilutions of 1:100 (Table III) or 1:10 (results not shown).

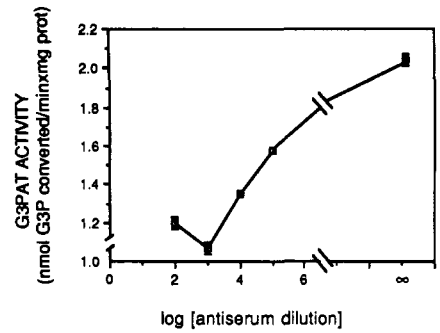

FIGURE 5: Dose-related inhibition of insulin-stimulated G3PAT activity by anti-Giα antiserum. Homogenates of BC3H-1 myocytes were treated with the indicated dilutions of Giα antiserum for 3 min before adding insulin. G3PAT activity was then measured (see legend to Table III). Means plus or minus variations of duplicate determinations are shown.

Discussion

The present findings suggest that previously reported effects of insulin on de novo synthesis of PA (Farese et al., 1984, 1985, 1987, 1988) may be largely due to activation of G3PAT. Insulin-induced increases in the activity of G3PAT have been observed in adipose (Soorana & Saggerson, 1976) and hepatic tissues (Bates et al., 1977), but these effects were relatively small and poorly defined. In the present study, the activation of microsomal G3PAT in BC3H-I myocytes was quite sizeable and involved a decrease in the K_m (rather than a change in the V_{max}) of the enzyme. Interestingly, activation of G3PAT by insulin was observed in intact cells, homogenates, and membrane preparations. The activation in homogenates and membrane preparations was mimicked by treatment with either nonspecific PLC or PI-specific PLC, and the insulin effect on G3PAT in these cell-free preparations was blocked by anti-PI-glycan-PLC antiserum. Moreover, the insulin effect appeared to be due, at least partly, to a cytosolic, membrane-derived, relatively low molecular weight factor (<5K), which activated the enzyme. Therefore, on the whole, our findings are in keeping with the hypothesis (Saltiel et al., 1987; Larner, 1988; Saltiel & Cuatrecasas, 1988; Mato, 1989) that insulin activates a PLC that hydrolyzes a PI-glycan or other phospholipid(s), resulting in the release of a cytosolic factor(s), which in turn activates other enzyme systems. Along the latter lines, in preliminary experiments, we have found that insulin stimulated the hydrolysis of a [^{3}H]glucosamine-containing lipid(s) in postnuclear membrane preparations of BC3H-1 myocytes (unpublished observations).

As with previously studied insulin-stimulated mediator(s), inhibitory effects of NaF suggested that G3PAT may be activated through a mechanism involving a phosphatase (Saltiel, 1987; Kiechle et al., 1981). The latter is in keeping with previous observations in adipose tissue, suggesting that G3PAT is activated by phosphatases and inhibited by factors that promote phosphorylation (Nimmo & Houston, 1978). On the other hand, it is also possible that NaF inhibits factors other than a phosphatase(s), to explain the present inhibition of insulin effects on G3PAT.

The apparent K_m of the enzyme (with respect to glycerol 3-phosphate) was found to be approximately 0.8 mM, and this is reasonably close to the concentration of glycerol 3-phosphate previously estimated to be present in BC3H-I myocytes, viz., 0.4 mM (Farese et al., 1988). It may therefore be surmised that a decrease in the K_m of G3PAT should lead to enhanced de novo PA synthesis in intact myocytes. It should further be noted that it was important to conduct assays with glycerol 3-phosphate concentrations close to or below K_m, rather than at or above V_{max}, as this was instrumental in allowing us to observe relatively large, agonist-induced changes in G3PAT activity.

The failure of DAG generation and protein kinase C activation to influence G3PAT activity is noteworthy. Activation of phosphatidylcholine (PC) hydrolysis [see Nair et al. (1988) and Farese et al. (1988)] through DAG–PKC signaling during insulin activation has been suggested from observed increases in PC hydrolysis during TPA treatment of BC3H-1 myocytes (Nair et al., 1988), and it was logical to question whether the activation of G3PAT occurred through a similar mechanism. Clearly, this is not the case for G3PAT activation by insulin.

The observation of a direct stimulatory effect of insulin on G3PAT in postnuclear homogenates and membrane preparations allowed us to use antisera directed against either PI-glycan-specific PLC or Giα-protein to evaluate their importance during insulin action in cell-free systems. Indeed, both antisera fully blocked the stimulatory effects of insulin on G3PAT activity, and this is in keeping with the possibility that insulin effects on G3PAT in BC3H-I myocytes are mediated

through a pertussis toxin sensitive Giα-protein and activation of PI-glycan-specific PLC. In contrast, we have found that insulin-induced activation of a phospholipase that hydrolyzes PC is not pertussis toxin sensitive (unpublished observations).

The finding of activation of G3PAT by EGF was of further interest, since both insulin and EGF have been found to provoke sustained increases in [^{3}H]glycerol incorporation into PA, DAG, and other lipids in intact BC3H-1 myocytes (Farese et al., 1987, 1988, 1989). IGF-I, on the other hand, provokes only a small and very transient increase in [^{3}H]glycerol incorporation into lipids in intact myocytes (Farese et al., 1989), and, accordingly, we were unable to observe a significant change in G3PAT enzyme activity in the present study. The failure to observe an effect of IGF-I on G3PAT provides clear evidence that the insulin effect on G3PAT (and presumably Giα and PI-glycan-PLC) is not mediated through the IGF-I receptor.

In summary, the present findings suggest that insulin activates microsomal G3PAT, and therefore de novo PA synthesis, in BC3H-1 myocytes, through a soluble mediator, which is released from membrane phospholipids (possibly a PI-glycan) by activation of a Giα-coupled PLC. Since newly synthesized PA is largely converted directly to DAG, G3PAT activation may provide a mechanism to amplify DAG production and protein kinase C activation during PI-glycan (or other glycolipid) hydrolysis. In addition, G3PAT activation and de novo synthesis of PA, DAG, and PC (Farese et al., 1987, 1988), along with hydrolysis of PC (Nair et al., 1988; Farese et al., 1988), may provide integrated mechanisms to replenish PI and the PI-glycan during stimulated hydrolysis of the latter lipid. Further studies are needed to test these possibilities.

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Registry No. G3PAT, 9029-96-3; PLC, 9001-86-9; insulin, 9004-10-8; epidermal growth factor, 62229-50-9; phosphatase, 9013-05-2; glycerol 3-phosphate, 57-03-4; PI-specific PLC, 63551-76-8.

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