# Insulin Stimulates Hydrolysis of Plasmanylinositol-Glycan and Phosphatidylinositol-Glycan in Rat Adipocytes

## Insulin-Induced Generation of Inositol Glycan, Alkylacylglycerol, and Diacylglycerol

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**Insulin binding to its receptor has been known to induce hydrolysis of phosphatidylinositol-glycan and release inositol-glycan and diacylglycerol, two putative second messengers of insulin actions. We metabolically labeled and purified PIG in rat cultured adipocytes. The treatment of [$^3$H]glycerol-labeled PIG with phosphatidylinositol-specific phospholipase C released [$^3$H]glycerol-labeled DAG and [$^3$H]glycerol-labeled 1-alkyl,2-acyl-glycerol, suggesting that PIG has not only PIG but also plasmanylinositol-glycan moiety. Insulin induced hydrolysis of PIG/PMIG and generation of IG, DAG, and AAG in a dose-dependent manner. This report shows the first demonstration of insulin-sensitive PMIG in rat adipocytes. These results provide evidence that insulin-induced generation of IG, DAG, and AAG might be early events in the insulin-signaling mechanism in rat adipocytes, and insulin-releasable AAG seems to mediate some actions of insulin. *Diabetes* 42:988–94, 1993**

Insulin is the key anabolic hormone regulating the metabolism of carbohydrate, lipids, and proteins. Despite extensive investigation, the mechanism of insulin action is not completely understood. It has been established that the tyrosine kinase activity associated with the insulin receptor is essential for the signal transduction mechanism. However, only limited information is available on the molecular events subsequent to insulin receptor occupancy and directly responsible for the biological effects of the hormone (1). It has been shown previously that in different cell systems, insulin promotes the hydrolysis of a novel phosphoglycolipid, PIG, eliciting the release of molecules of DAG and IG (2–4). IG and DAG display several insulinlike effects (5–7). It has been reported that IG can affect in vitro the activity of some cellular enzymes known to be regulated in vivo by insulin (8–12). In addition, the addition of IG to intact cells results in an insulinlike effect on glucose oxidation (8), lipogenesis (8), antilipolysis (13), amino acid transport (14), phospholipid methyltransferase (15), pyruvate dehydrogenase (8), glycogen phosphorylase (16), pyruvate kinase (16), protein phosphorylation/dephosphorylation (17), and growth promotion (18) in intact cells. It has been observed that the DAG moiety of the insulin-sensitive PIG contains mostly saturated fatty acid (19). Insulin-releasable IG and DAG are speculated to conduct pleiotropic intracellular insulin actions.

Several laboratories have now demonstrated the presence of insulin-sensitive PIG in various cell lines, including BC3H-1 myocytes (2,3,20), CHO cells (21,22), splenic T-cells (23), rat hepatocytes (24), and rat adipocytes (25). Mato et al. (26) reported the existence of insulin-sensitive PMIG in H35 hepatoma cells. PMIG has been identified in mature erythrocyte-associated acetylcholinesterase, decay-accelerating factor and CD59 (27–30). Macaulay and Larkins (25) demonstrated the presence of insulin-sensitive PIG in primary cultured rat adipocytes and impaired insulin-stimulated hydrolysis of PIG in STZ-induced diabetic rats. However, the insulin-dependent generation of IG in rat adipocytes was not known.

In this study, we report on the first demonstration of the presence of insulin-sensitive PMIG in cultured rat adipocytes. We show the insulin-dependent turnover of PIG/PMIG, IG, DAG, and AAG in rat adipocytes. The putative

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PIG, phosphatidylinositol-glycan; IG, inositol glycan; DAG, diacylglycerol; PI, phosphatidylinositol; AAG, 1-alkyl,2-acyl-glycerol; PMIG, plasmanylinositol-glycan; CHO, Chinese hamster ovary; STZ, streptozocin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; KRPB, Krebs-Ringer phosphate buffer; BSA, bovine serum albumin; $PIP_2$, phosphatidyl-inositol 4,5 biphosphate; IL-3, interleukin 3.

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role of insulin-releasable AAG as one of the second messengers of insulin action is discussed.

## RESEARCH DESIGN AND METHODS

[6-$^3$H]glucosamine hydrochloride, [6-$^3$H]galactose, [2-$^3$H]glycerol, [9,10-$^3$H]myristic acid, and [U-$^{14}$C]glucose were purchased from DuPont-NEN (Boston, MA). DMEM and FBS were from Gibco Oriental (Tokyo, Japan). Dowex 1-X8 resin (formate form) was from Bio-Rad (Richmond, CA). Silica gel and cellulose TLC plates were from Merck Japan (Tokyo, Japan). Partisil SAX-HPLC column (10 mm, 4.6×25 cm) was from Whatman. *Bacillus cereus* PI-specific phospholipase C was purchased from Funakoshi (Tokyo, Japan). Statistical analysis was performed using the Student's *t* test. Statistical significance was determined at the 0.01 level.

**Primary culture of adipocytes.** Male Sprague-Dawley rats (120–150 g) were obtained from Funabashi and were allowed free access to food and water. Isolated adipocytes were prepared in KRPB, pH 7.4, containing 10 mM HEPES, 3% BSA, and 5 mM glucose. The number of cells was determined with a hemocytometer. Isolated adipocytes were cultured by the method of Garvey (31) with minor modifications. The cells were cultured at 37°C in DMEM supplemented with 5% FBS, 3% BSA, and 10 mM HEPES (pH 7.4) for 8 h.

**Glucose oxidation and lipogenesis.** Primary cultured or fresh prepared adipocytes ($10^5$ cells/ml) were incubated with insulin in KRPB, pH 7.4, 10 mM HEPES, 3% BSA in the presence of 5 mM [U-$^{14}$C] glucose (0.5 μCi/tube) for 30 min at 37°C with gentle shaking in a total volume of 0.4 ml (20). Glucose oxidation was monitored by evaluation of $^{14}CO_2$ from [U-$^{14}$C]glucose. Lipogenesis was measured by the incorporation of [U-$^{14}$U]glucose into lipids.

**Purification of PIG/PMIG, AAG, and DAG.** Freshly prepared adipocytes ($10^5$ cells) were cultured in DMEM supplemented with 5% FBS, 3% BSA, and 10 mM HEPES in the presence of [$^3$H]glucosamine (2 μCi/ml), [$^3$H]galactose (2 μCi/ml), [$^3$H]glycerol (10 μCi/ml) or [$^3$H]myristic acid (5 μCi/ml) for 8 h at 37°C. The cells were extensively washed with KRPB (pH 7.4) containing 10 mM HEPES and 3% BSA, and then incubated with 1 nM insulin at 37°C in a total volume of 1 ml. The reaction was terminated by the removal of medium and addition of 2 ml of chilled methanol. The cells were extracted with chloroform:methanol:0.01 N HCl (2:1:1 by vol) and centrifuged (2000 g for 10 min) to separate lipid and aqueous layers (20). Both lipid and aqueous extracts were dried in a vacuum spin. PIG/PMIG was purified on activated silica TLC plates using solvent 1 (chloroform:acetone:methanol:glacial acetic acid/water, 10:4:2:2:1 by vol) and solvent 2 (chloroform:methanol:$NH_4OH$:water, 45:35:3.5:10 by vol) as described previously (20). [$^3$H]glucosamine-labeled PIG, which was purified from BC3H-1 myocytes, was chromatographed on parallel silica gel TLC to identify PIG from adipocytes. DAG and AAG were purified on the activated silica gel HPTLC plates in solvent 3 (petroleum ether:diethylether:acetic acid, 70:30:2 by vol) as described previously (20).

**Selective degradation of PIG.** [$^3$H]glucosamine-, [$^3$H]glycerol-, or [$^3$H]myristic acid-labeled PIG was resuspended in 50 mM HEPES buffer, pH 7.4, 0.1% BSA, 0.05% cholic acid by sonication and incubated with *Bacillus cereus* PI-specific phospholipase C at 37°C for 1 h (20). The incubation was terminated by the addition of chloroform:methanol:10 mM KCl (2:1:1 by vol). DAG and AAG were extracted into the organic phase and purified by silica gel TLC using solvent 3.

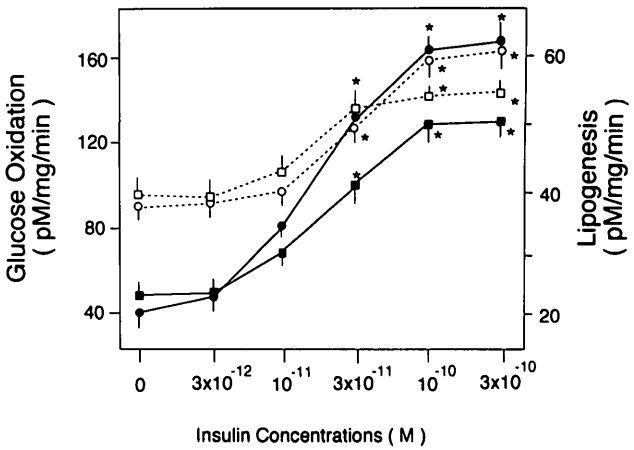


**FIG. 1. Effects of insulin on glucose oxidation and lipogenesis before and after culturing of adipocytes. Primary cultured (○, □) and fresh adipocytes (●, ■) were prepared as described in METHODS. The cells were incubated with the indicated concentrations of insulin in KRPB, pH 7.4, 10 mM HEPES, and 3% BSA in the presence of 5 mM [U-$^{14}$C]glucose (0.5 μCi/tube) at 37°C for 30 min. Glucose oxidation (○, ●) and lipogenesis (□, ■) were assayed as described in METHODS. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The activities reported were compared with treatment in buffer alone. *$P < 0.01$.**

**Purification of IG.** IG was purified by C18 Sep Pak columns, Dowex 1-X8, cellulose TLC and SAX-HPLC, as described previously (20). Briefly, the aqueous extracts were passed through C18 Sep Pak columns and then applied onto Dowex 1-X8 columns (0.5 × 2 cm, formate form). IG was eluted with 2 vol of 0.1 N HCl and purified by cellulose TLC developed in isopropranol:pyridine:acetic acid:water (8:8:1:4 by vol). IG (migrated at Rf = 0.34) was extracted from the plates with water, lyophilized, redissolved in 25 mM pyridine-HCl buffer, pH 6.5, and then applied onto an analytical SAX-HPLC column (4.6 × 30 cm) equilibrated with 25 mM pyridine-HCl, pH 6.5. The column was subjected to a 10-min isocratic elution in 25 mM pyridine-HCl buffer, pH 6.5, followed by a linear 30-min gradient of 25 mM-0.5 M pyridine-HCl buffer, pH 6.5, at 1 ml/min. The IG was eluted in a major peak at 35 min.

## RESULTS

**Insulin responsibility of primary cultured adipocytes.** Primary culture of adipocytes was conducted as described in METHODS. No significant loss of cell numbers was seen in 8-h cultured adipocytes compared with freshly prepared adipocytes (data not shown). Insulin responsiveness of the cultured adipocytes was compared with that of fresh adipocytes. As shown in Fig. 1, treatment of cultured adipocytes with 3 × $10^{-10}$ M insulin caused a 1.9-fold stimulation of glucose oxidation and a 1.4-fold increase of lipogenesis, compared with a 4.1-

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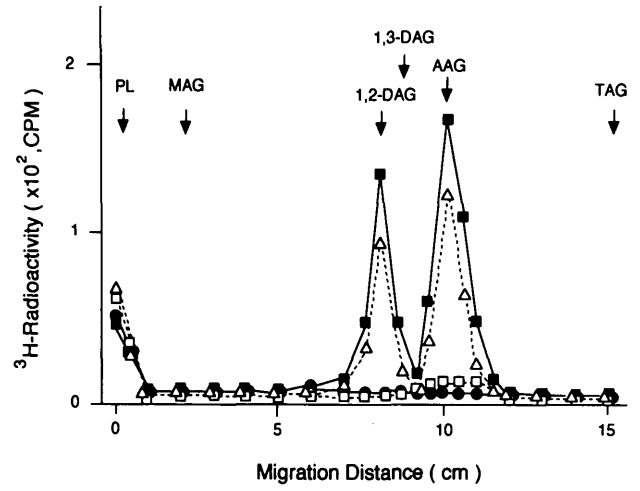


**FIG. 2. Silica gel HPTLC of 1,2-DAG and AAG released from PIG by PI-specific phospholipase C. The [$^3$H]glucosamine- (●), [$^3$H]galactose- (□), [$^3$H]glycerol-(■), or [$^3$H]myristic acid-labeled (△) PIG was incubated with PI-specific phospholipase C. After a two-phase separation, the lower layer was collected and subjected to silica gel HPTLC run in solvent 3. The 0.5-cm regions were scraped and counted. Data are mean values obtained from three 35-mm plates in a representative experiment with identical results performed in three repeated experiments. (*MAG*), 1-monoacylglycerol; (*TAG*), triacylglycerol.**

fold stimulation of glucose oxidation and a 2.2-fold increase of lipogenesis in fresh adipocytes. Insulin sensitivity on glucose oxidation and lipogenesis was not significantly different from those of fresh adipocytes. The decreased stimulation of glucose oxidation and lipogenesis was a consequence of increased basal activities of glucose oxidation and lipogenesis in cultured adipocytes. These data demonstrated that the cultured adipocytes maintained insulin responsiveness on glucose oxidation and lipogenesis after an 8-h culture.

**Identification of PMIG.** PIG in adipocytes was labeled with [$^3$H]*myo*-inositol, [$^3$H]glucosamine, [$^3$H]galactose, [$^3$H]glycerol, [$^3$H]myristic acid, and [$^3$H]palmitic acid (data not shown). However, [$^3$H]galactosamine, [$^3$H]mannose, and [$^3$H]ethanolamine were not incorporated into PIG (data not shown). When the lipid phase of [$^3$H]glycerol-labeled PIG after phospholipase C treatment was analyzed on silica gel HPTLC using solvent 3, 33% of the original radioactivity was detected at the position of the 1,2-DAG standard, whereas 41% of that was shown at the position of AAG, as shown in Fig. 2. Silica gel HPTLC of [$^3$H]myristic acid-labeled PIG after PI-phospholipase C treatment demonstrated that 32% of the original radioactivity was detected at the position of the 1,2-DAG standard, whereas 33% of that was shown at the position of AAG (Fig. 2). Further evidence that PIG contains a PMIG structure was obtained by testing the alkali-resistance of the PIG. After 15 min at room temperature in 2 N NaOH in methanol, 56% of the original radioactivity in the PIG labeled with [$^3$H]glycerol remained associated in the organic phase (data not shown). These data demonstrate that PIG contained PMIG and PIG moieties.

**Insulin-stimulated PMIG/PIG turnover and generation of AAG, DAG, and IG in primary cultured adipocytes.** The effects of insulin on PIG/PMIG metabolism were

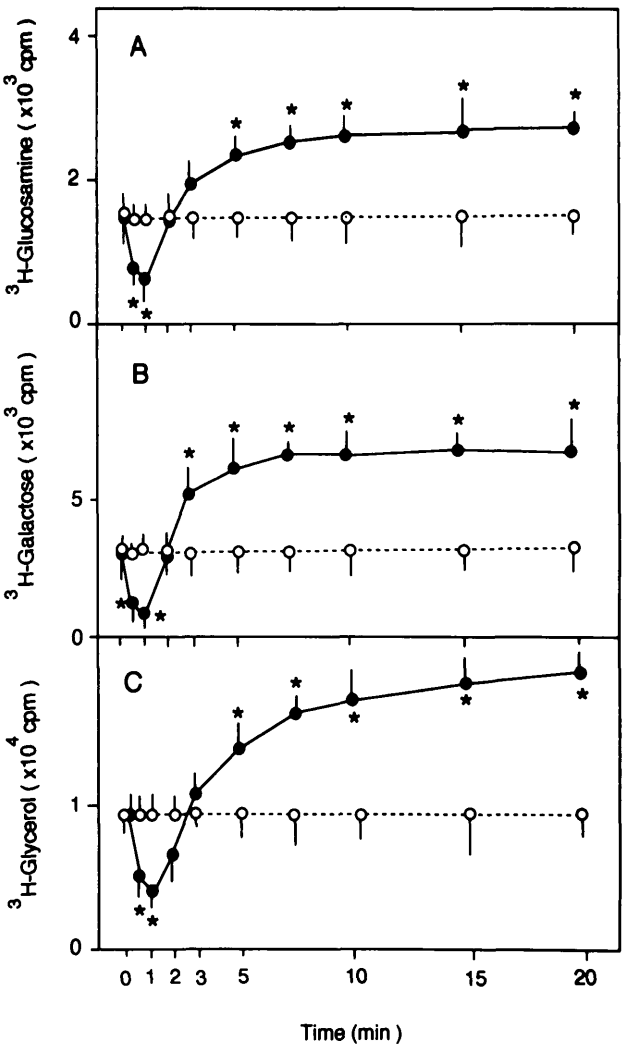


**FIG. 3. Time course of insulin-stimulated turnover of PIG/PMIG. Adipocytes were labeled with [$^3$H]glucosamine (*A*), [$^3$H]galactose (*B*), or [$^3$H]glycerol (*C*) for 8 h and then treated with (●) or without (○) 1 nM insulin for the indicated time intervals. The cells were extracted with chloroform/methanol/HCl and purified on silica gel TLC using solvent 1 and 2. The area where PIG/PMIG migrated was scraped off and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.**

studied in cultured adipocytes separately prelabeled with [$^3$H]glucosamine, [$^3$H]galactose, [$^3$H]glycerol, or [$^3$H]myristic acid. The addition of insulin to the cells produced a significant decline in the [$^3$H]galactose radioactivity in PIG/PMIG within 30 s. This decline was maximal at 2 min and was followed by a substantial increase of radioactivity after 5 min (Fig. 3). This gradual increase in counts after 5 min seems to reflect resynthesis of PIG/PMIG. Similar patterns of PIG/PMIG turnover were seen in [$^3$H]glucosamine- or [$^3$H]glycerol-labeled cells, as shown in Fig. 3. Similar patterns of PIG/PMIG turnover were shown in [$^3$H]myristic acid-labeled adipocytes (data not shown). The time course of [$^3$H]glucosamine- or [$^3$H]galactose-labeled IG production was evaluated in response to insulin. As shown in Fig. 4, insulin evoked a rapid increase in [$^3$H]glucosamine-

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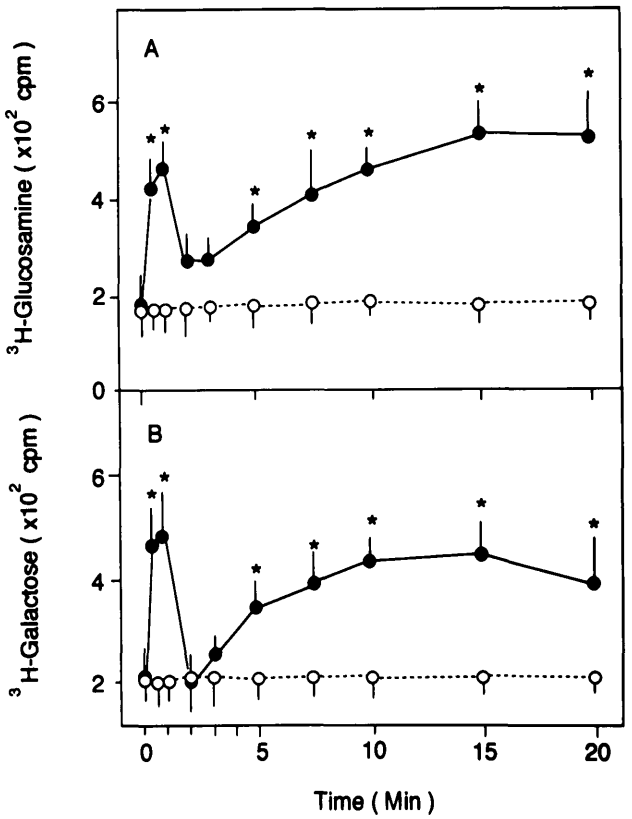


FIG. 4. Time course of insulin-stimulated generation of IG. Adipocytes were labeled with [$^3$H]glucosamine (*A*) or [$^3$H]galactose (*B*) for 8 h and then treated with (●) or without (○) 1 nM insulin for the indicated time intervals. IGs were purified as described in METHODS. The fractions where IG eluted from SAX-HPLC column were collected and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.

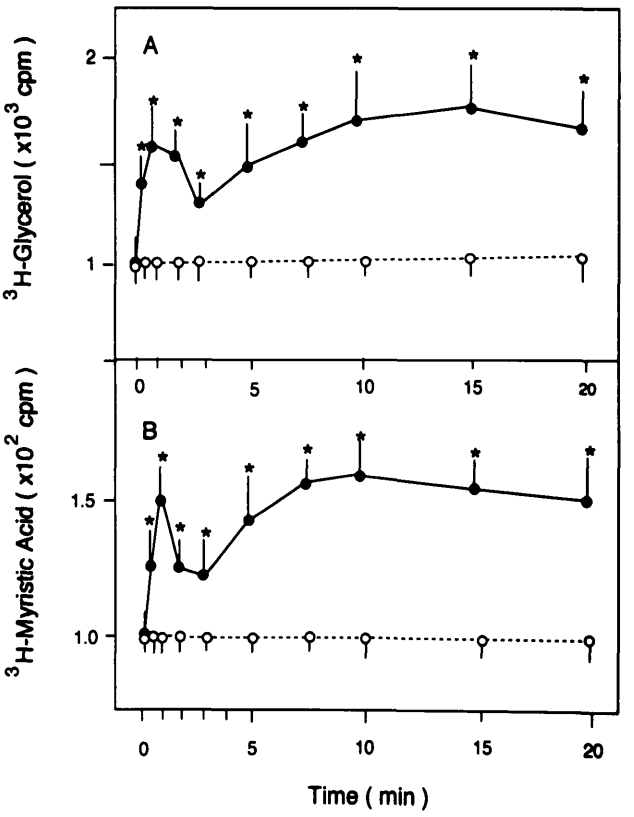


FIG. 5. Time course of insulin-stimulated generation of DAG. Rat adipocytes were labeled with [$^3$H]glycerol (*A*) or [$^3$H]myristic acid (*B*) for 8 h and then treated with (●) or without (○) 1 nM insulin for the indicated time intervals. DAG was extracted with chloroform/methanol/HCl and purified on silica gel HPTLC using solvent 3. The area where 1,2-DAG migrated was scraped off and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.

labeled IG within 1 min, followed by a decline by 2 min, and a significant increase thereafter. The time courses of insulin-stimulated IG production labeled with [$^3$H]galactose were identical to those labeled with [$^3$H]glucosamine, as shown in Fig. 4. The time course of generation of DAG also was evaluated in adipocytes labeled with [$^3$H]glycerol or [$^3$H]myristic acid in response to insulin treatment. Insulin induced a rapid increase in [$^3$H]glycerol-labeled DAG within 30 s, which was maximal at 1 min and was followed by a decline by 3 min and a slow increase after 5 min, as shown in Fig. 5. In the case of [$^3$H]myristic acid labeling, insulin treatment caused a similar biphasic pattern of DAG generation (Fig. 5). The time course of generation of AAG in response to insulin treatment also was evaluated in cultured adipocytes labeled with [$^3$H]glycerol. Insulin induced a rapid increase in [$^3$H]glycerol-labeled AAG within 30 s, which was maximal at 1 min. This declined by 3 min, then slowly increased after 5 min, as shown in Fig. 6. Insulin treatment was shown to cause a biphasic pattern of AAG generation, similar to DAG turnover.

We studied the dose-response relationship between insulin and [$^3$H]glucosamine-labeled or [$^3$H]galactose-labeled IG generation at 1 min after insulin exposure. As shown in Fig. 7, treatment with insulin caused concentration-dependent generation of [$^3$H]glucosamine-labeled and [$^3$H]galactose-labeled IG. Maximal effective concentrations of insulin were 1 nM, and half-maximal concentrations were 30 pM, respectively. We studied the dose-response relationship between insulin and [$^3$H]glycerol-labeled DAG generation at 1 min after insulin exposure. As shown in Fig. 8, treatment with increasing concentrations of insulin caused a concentration-dependent release of DAG. A maximal effective concentration of insulin was 1 nM, and half-maximal concentration was 30 pM. Insulin stimulated the generation of AAG in a dose-dependent manner (Fig. 8). A maximal concentration of insulin was 1 nM and half-maximal concentration was 30 pM.

We investigated the insulinlike bioactivity of purified IG from insulin-treated fresh adipocytes on glucose oxidation and lipogenesis in rat adipocytes. Extracellular addition of IG to adipocytes stimulated glucose oxidation and lipogenesis significantly in a dose-dependent manner (data not shown). The IG did not have a significant effect on glucose transport (data not shown). Nitrous acid treatment of IG abolished its stimulatory activities on glucose oxidation and lipogenesis. This insulinlike bioactivity of IG also was sensitive to the treatment with nitrous acid (data not shown).

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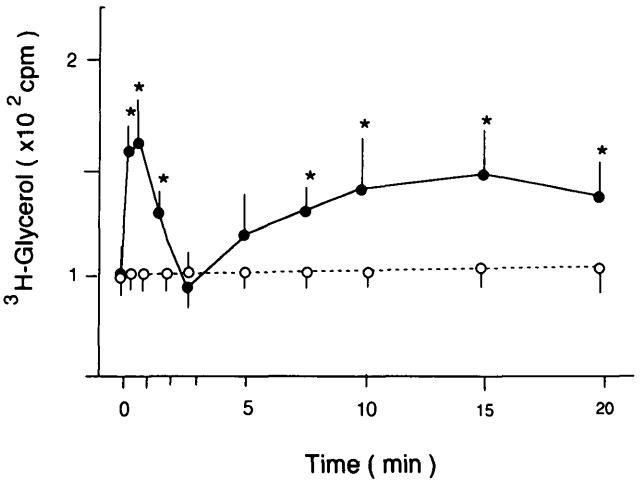


**FIG. 6. Time course of insulin-stimulated generation of AAG. Rat adipocytes were labeled with [$^3$H]glycerol for 8 h and then treated with (●) or without (○) 1 nM insulin for the indicated time intervals. AAG was extracted with chloroform/methanol/HCl and purified on silica gel HPTLC using solvent 3. The area where AAG migrated was scraped off and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.**

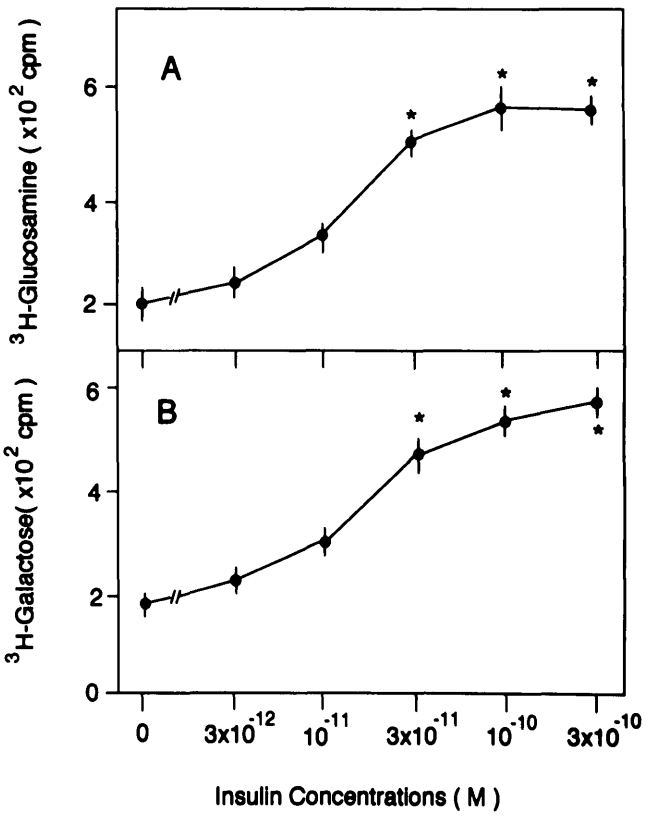


**FIG. 7. Dose-related effects of insulin on the generation of IG. Rat adipocytes were labeled with [$^3$H]glucosamine (*A*) or [$^3$H]galactose (*B*) for 8 h and then treated with the indicated concentrations of insulin for 1 min. IG was purified and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.**

## DISCUSSION

Recent evidence suggests that insulin promotes the phosphodiesteratic hydrolysis of PIG or protein-anchoring PIG in plasma membranes, resulting in the release of DAG, IGs, and the anchored proteins (5,7,10). An additional putative proteolytic step also was shown to be necessary to release IG and proteins from protein-anchoring PIG (10,32). Released DAG and IG are speculated to serve as intracellular second messengers of insulin actions. Insulin-stimulated release of alkaline phosphatase (32,33), heparan sulfate proteoglycan (34), and lipoprotein lipase (35) into the medium might be linked to some extracellular insulin actions. Previous studies by two laboratories demonstrated that existence of at least two species of IGs based on their bioactivities, chromatographic characteristics, and chemical compositions (4,34). One type of IG acts on mitochondrial pyruvate dehydrogenase phosphatase, contains D-*chiro*-inositol, non-*N*-acetylated galactosamine, mannose, and phosphate (Ins/GalN/Man modulator) (4). The second type of IG inhibits cAMP-dependent protein kinase, contains *myo*- and *chiro*-inositol, non-*N*-acetylated glucosamine, galactose, and phosphate (Ins/GlcN/Gal modulator) (36). These insulin-sensitive PIG/PMIG have been labeled with saturated fatty acids in BC3H-1 myocytes (2,3,20), rat hepatocytes (24), rat adipocytes (25), CHO cells (21,22) and splenic T-cells (23). PIG in adipocytes was labeled with [$^3$H]*myo*-inositol, [$^3$H]glucosamine, [$^3$H]galactose, [$^3$H]glycerol, [$^3$H]myristic acid, and [$^3$H]palmitic acid. However, [$^3$H]galactosamine, [$^3$H]mannose, and [$^3$H]ethanolamine were not incorporated into PIG. The treatment of PIG with PI-specific phospholipase C followed by silica gel HPTLC analysis of released DAG moiety shows that the major part of PIG has PMIG structure and the minor part has PIG structure. PMIG has been identified in mature erythrocyte-associated acetylcholinesterase, decay-accelerating factor, placental cell alkaline phosphatase, and CD 59 proteins (28–30,37,38). Previous research has uncovered PMIG-anchoring proteins are demonstrable on a wide spectrum of eukaryotic cell types, including molds, parasites, and diverse cell types of all mammalian species studied to date (39). Mato et al. (26) reported the presence of insulin-sensitive PMIG in H35 hepatoma cells, which were labeled with [$^3$H]palmitic acid and hydrolyzed by insulin within 1 min after insulin addition. Despite potential differences within the lipid moiety of PMIG/PIG, both were predominantly labeled with [$^3$H]*myo*-inositol, [$^3$H]glucosamine, [$^3$H]galactose, [$^3$H]glycerol, and [$^3$H]myristic acid, were not separated by silica TLC run in solvent 1 and 2, and functioned as a substrate for PI-specific phospholipase C, suggesting that both are structurally related. Both were also insulin-sensitive in primary cultured rat adipocytes.

The time courses of the PIG/PMIG turnover and the generation of IG, AAG, and DAG were superimposable, suggesting that radiolabeled IG, AAG, and a part of DAG released by insulin were generated from the hydrolysis of PIG/PMIG. The generation of IG, AAG, and DAG was an insulin concentration–dependent process. These results suggest that hydrolysis of PIG/PMIG and the subsequent generation of IG, AAG, and DAG were insulin-specific

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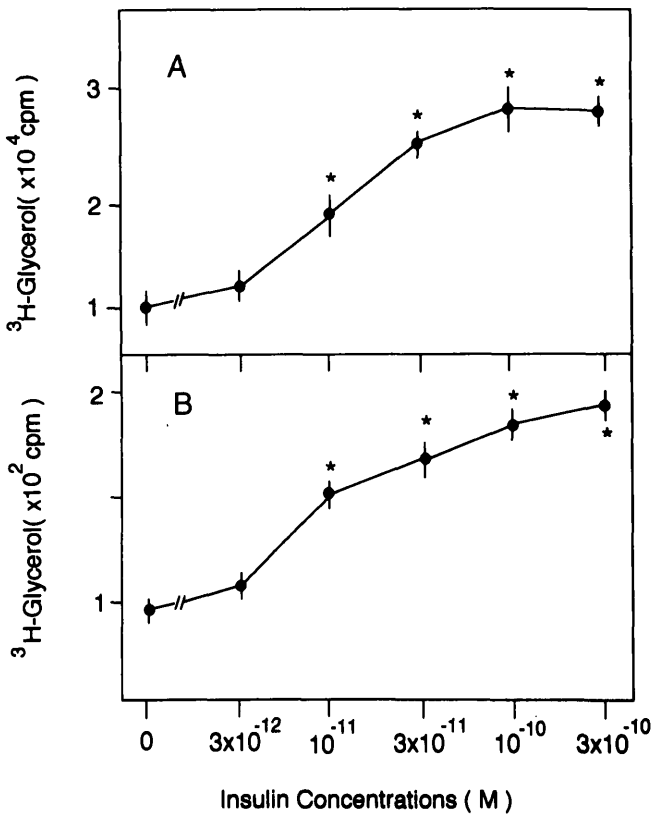


**FIG. 8. Dose-related effects of insulin on the generation of 1,2-DAG (*A*) and AAG (*B*). Rat adipocytes were labeled with [$^3$H]glycerol for 8 h and then treated with the indicated concentrations of insulin for 1 min. DAG and AAG were purified and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in three other experiments. The radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.**

events in adipocytes. Purified IG stimulated glucose oxidation and lipogenesis in isolated adipocytes.

Insulin-stimulated hydrolysis of PIG and generation of the Ins/GlcN/Gal modulators have been shown in BC3H-1 myocytes (2,3,20), H35 hepatoma cells (26), rat hepatocytes (24), rat adipocytes (25), CHO cells (21,22), and H4 hepatoma cells (S.S., unpublished observations). In addition, the Ins/GlcN/Gal modulator has been reported to affect the activity of several cellular enzymes such as low $K_m$ phosphodiesterase (3), adenylate cyclase (40), acetyl-CoA carboxylase (40), cAMP-dependent protein kinase (12), phospholipid methyltransferase (15), and pyruvate dehydrogenase phosphatase (40). The Ins/GlcN/Gal modulator stimulates glucose oxidation and lipogenesis (8) and inhibits lipolysis (13), as insulin does when added to intact cells. Alemany et al. (17) have shown that the Ins/GlcN/Gal modulator faithfully mimicked the insulin-directed effects on phosphorylation/dephosphorylation of key target enzymes such as ATP citrate lyase, hormone sensitive lipase, and glycogen phosphorylase. An IG fragment from the structurally characterized *Trypanosoma brucei* variant surface glycoprotein glycosyl-PI anchor has been demonstrated to conduct insulin-mimetic antilipolytic activity (41). IG inhibited microsomal glucose-6-phosphatase and cytosolic fructose-1,6-bisphosphatase and glucose production from pyruvate in hepatocytes. In addition, it has been reported that anti-IG antibodies raised against the oligosaccharide anchor of membrane proteins selectively blocked insulin-induced activation of pyruvate dehydrogenase in BC3H-1 myocytes (42). These lines of evidence suggest that insulin-induced hydrolysis of PIG/PMIG and subsequent release of IGs may be responsible for some of insulin's actions.

The involvement of DAG and protein kinase C in insulin action has been suggested (5). In isolated rat adipocytes, insulin increased intracellular DAG levels without affecting of $PIP_2$ (43). A part of insulin-increased DAG is generated from PIG/PMIG hydrolysis in response to insulin, whereas other portions are speculated to be generated from insulin-stimulated de novo synthesis and insulin-induced hydrolysis of phosphatidylcholine and phosphatidyl-ethanolamine (5). It has been controversial whether insulin does activate protein kinase C activity or not (5,44). Previous data provide evidence that insulin increases DAG, induces translocation of protein kinase C from cytosol to plasma membranes, and activates membrane-associated protein kinase C in adipocytes, and this activation is involved in some of insulin's intracellular actions, such as glucose transport (45–48).

This study shows that insulin induced generation of AAG from PMIG in adipocytes. Exogenous addition of AAG elicited varied biological responses in appropriate target systems, including hypotension (49), leukemic cell differentiation (50), and stimulated amino acid transport (51). Several investigators demonstrated that AAG activated protein kinase C (52–54). Robinson (55,56) reported that IL-3 and phorbol ester induced AAG generation in an IL-3–dependent cell line. We speculate that AAG released from PMIG by insulin might be a putative second messenger of insulin action. Physiological significance of AAG should be investigated. The molecular mechanism of insulin-induced generation of IG, AAG, and DAG is poorly understood. Further investigations into the molecular process of insulin-stimulated generation of IG, AAG, and DAG may clarify many molecular mechanisms of insulin action.

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