Original Articles

Mutated Insulin Receptor Val[996] Reduces Insulin-Dependent Generation of Inositol Glycan and Diacylglycerol

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We evaluated whether insulin-receptor tyrosine kinase activity is required for activation of PDH, insulin-induced hydrolysis of PIG and generation of IG and 1,2-DAG. For the analysis, we used stable-transfected CHO cell lines expressing wild-type human insulin receptor (CHO-wt cells) or the mutant receptor (Val[996]) that lacks tyrosine kinase activity (CHO-mut cells) (1,2). Insulin stimulated PDH activity in three CHO cell lines in a dose-dependent manner. Half-maximal concentrations of insulin to activate PDH was 7 × 10^{-11} M in the CHO-wt cells, 10^{-9} M in the parental cells, and 8 × 10^{-9} M in the CHO-mut cells. Insulin stimulated hydrolysis of PIG and generation of IG and DAG in three CHO cell lines in a dose-dependent manner. Half-maximal concentrations of insulin to induce generation of IG was 8 × 10^{-11} M in the CHO-wt cells, 10^{-9} M in the parental CHO cells, and 10^{-8} M in the CHO-mut cells. ED_{50} for the stimulation of DAG generation was 7 × 10^{-11} M in the CHO-wt cells, 10^{-9} M in the parental cells, and 10^{-8} M in the CHO-mut cells. It is concluded that insulin-dependent PDH activation, PIG hydrolysis, and IG and DAG generation are mediated by the wild-type but not by the mutated insulin receptor of Val[996]. This study suggests that tyrosine kinase activity of the insulin receptor might be a prerequisite for insulin-stimulated generation of IG and DAG.
***Diabetes* 41:1373–79, 1992**

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Received for publication 5 December 1991 and accepted in revised form 2 April 1992.

PDH, pyruvate dehydrogenase; PIG, phosphatidylinositol glycan; IG, inositol glycan; DAG, 1-diacylglycerol; CHO, Chinese hamster ovary; CHO-wt, CHO cells expressing wild-type human insulin receptor gene; CHO-mut, Chinese hamster ovary cells expressing mutant receptor of Val[996]; IG, immunoglobulin; S6, species antigen-6; PI, phosphatidylinositol; FBS, fetal bovine serum; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; WGA, wheat germ agglutinin.

Several lines of recent evidence have shown that insulin promotes the phosphodiesteratic hydrolysis of PIG or protein-anchoring PIG in plasma membranes, resulting in the release of IG, DAG, and the anchored proteins (3–7). An additional putative proteolytic step also was shown to be necessary to release IG and proteins from protein-anchoring PIG (3,5). Released IG and DAG are speculated to serve as intracellular second messengers of insulin actions to conduct some of insulin's actions (3,4).

The insulin receptor is an insulin-dependent protein tyrosine kinase. Several lines of evidence suggest that its tyrosine kinase activity is necessary for many actions of insulin, including the stimulation of S6 kinase, 2-deoxy-D-glucose uptake, glycogen synthesis, thymidine incorporation into DNA, and receptor down-regulation (8–11). We have provided evidence that insulin-stimulated generation of an insulin mediator (PDH activator) is enhanced markedly in rat liver plasma membranes in the presence of ATP and Mn^{2+}, known to be required for the activation of insulin receptor kinase (12). A recent report noted that insulin-dependent hydrolysis of PIG is mediated by the wild-type but not by the kinase-defect insulin receptor of Ala[1018] (13). However, no information is available about the requirement for tyrosine kinase activity in the generation of IG and DAG.

In this report, we have identified insulin-sensitive PIG and IG in the transfected CHO cell lines, expressing the CHO-wt cells or CHO-mut cells (1,2) by metabolically labeling. We evaluate the effect of insulin on the hydrolysis of PIG and the generation of IG and DAG to investigate the requirement for insulin-receptor tyrosine kinase activity for the generation of these two putative second messengers of insulin.

RESEARCH DESIGN AND METHODS

Bacillus cereus PI-specific phospholipase C was the gift of Dr. Guillermo Romero (University of Virginia, Charlottesville, VA). [1-^{14}C]pyruvic acid, *myo*-[2-^{3}H]inositol, [6-^{3}H]glucosamine hydrochloride, [6-^{3}H]galactose, and [2-^{3}H]glycerol were purchased from Du Pont-NEN (Boston, MA). Ham's F12 medium, FBS, and G418 were from Gibco Oriental (Tokyo, Japan). Dowex 1 × 8 resin (formate form) was from Bio-Rad (Richmond, CA). Silica gel and cellulose TLC plates were from Merck. Partisil SAX HPLC column (10 μm, 4.6 × 250 mm) was from Whatman (Clifton, NJ). Statistical analysis was performed with the Student's *t* test. Statistical significance was determined at the 0.01 level.

Cell culture and metabolic labeling of IG, DAG, and PIG. The transfected CHO-wt and CHO-mut, and the parental CHO cells were provided by Dr. M. Kasuga (Kobe University, Kobe, Japan). The maintenance of the three CHO cell lines was done as described by Yamamoto-Honda et al. [2]. The CHO-wt and CHO-mut cells in 35-mm dishes were cultured and grown in Ham's F-12 medium, 10% FBS, and 600 μg/ml G418 to confluence. The cells were serum-starved in F-12 medium, 0.1% BSA and 10 mM HEPES for 24 h in the presence of [^{3}H]*myo*-inositol (10 μCi/ml, 24 h), [^{3}H]glucosamine (2 μCi/ml, 24 h), [^{3}H]galactose (2 μCi/ml, 24 h), or [^{3}H]glycerol (10 μCi/ml, 2 h). The dishes were washed extensively with F-12 medium, 0.1% BSA, 10 mM HEPES, and then incubated with or without 10 nM insulin at 37°C. The reaction was terminated by removing medium and adding chilled methanol (14). The contents of the plates were scraped, transferred to glass tubes, extracted with chloroform/methanol/0.01N HCl (2:1:1, by vol.) and centrifuged (2000 g for 10 min) to separate lipid and aqueous layers. Both lipid and aqueous extracts were dried in a vacuum spin.

Purification of IG, DAG, and PIG. The lipid extracts were resolved in chloroform/methanol/1N HCl (100:100:1, by vol.) and analyzed by silica TLC with three solvent systems (14). For the separation of PIG, the lipid extracts were chromatographed on activated silica TLC plates impregnated with 0.1% potassium oxalate, with solvent 1 (chloroform/acetone/methanol/glacial acetic acid/water, 10:4:2:2:1, by vol.). The PIG-containing fractions were collected and extracted with methanol for 30 min at 37°C. The PIG fractions were rechromatographed in solvent 2 (chloroform/methanol/NH_4OH/water, 45:35:3.5:10, by vol.). The PIG-containing fractions were collected and extracted with methanol (14). For the separation of DAG, the lipid extracts were chromatographed on the activated silica gel TLC plates in solvent 3 (petroleum ether/diethylether/acetic acid, 70:30:2, by vol.) (14).

The aqueous extracts were resuspended in water by sonication, passed through C18 Sep Pak columns to remove any residual lipids, and then applied onto Dowex 1 × 8 columns (0.5 × 2 cm, formate form) (14). The columns were washed extensively with 10 vol of water and 10 vol of 10 mM formic acid. IG was eluted with 2 vol of 0.1N HCl and lyophilized. The lyophilizate was resuspended in water and applied onto cellulose TLC plates. The plates were developed in solvent 4 (*n*-propanol/water, 7:3, by vol.). One-centimeter areas around origins were scraped, extracted with water, lyophilized, resuspended with water, and applied onto cellulose TLC plates. The plates were chromatographed in solvent 5 (isopropanol/pyridine/acetic acid/water, 8:8:1:4, by vol.). One-centimeter regions were scraped, extracted with water, and counted. The extracted fractions from the IG-migrating area (R_F = 0.34) were lyophilized, dissolved in 25 mM pyridine-HCl buffer, pH 6.5, and then applied into an analytical SAX HPLC column 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 minor peak of A at 25 min and in a major peak of B (denoted as IG) at 35 min, as described previously (14).

Insulin-receptor tyrosine kinase assay. Partially purified insulin receptors were isolated from transfected cells with WGA-agarose, as described previously (15). Tyrosine kinase activity was determined by a modification of the method of Grunberger et al. (16). Equal amounts of the lectin-purified receptors, as assessed by insulin binding, were incubated with various concentrations of insulin for 10 min at 24°C, followed by incubation at 24°C for 10 min with the exogenous substrate poly-Glu:Tyr (4:1; final 2.5 mg/ml) in 50 mM HEPES buffer, pH 7.4, 0.1% Triton X-100, containing 50 μM ATP (cold plus 12.5 μCi of [γ-^{32}P]ATP) and 2 mM $MnCl_2$. Reaction was stopped by spotting two aliquots onto 2-cm squares of filter paper, followed by extensive washing in 10% trichloroacetic acid, 10 mM pyrophosphate. Radioactivity was determined by Cerenkov counting.

PDH assay. PDH activity was monitored by measuring the conversion of [^{14}C]pyruvate to [^{14}C]O_2, as described previously (12). The parental CHO, CHO-wt and CHO-mut cells were serum-starved for 24 h and then incubated with insulin (0–10^{-6} M) for 10 min. The incubation was terminated by adding cold quench buffer (50 mM KH_2PO_4, pH 8.0, 50 mM KF, 10 mM dichloroacetic acid, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol). The cells were washed with cold quench buffer and scraped. The cell extracts were homogenized with Polytrone and centrifuged at 3000 *g* for 1 min. Four hundred microliters of the supernatant was added to 100 μl of the assay buffer to give final concentrations of 0.1 mM CoA, 0.1 mM cocarboxylase, 0.5 mM β-NAD, 0.1 mM dithiothreitol, and 0.25 mM [1-^{14}C]pyruvic acid (3.3 μCi/ml). After incubation at 37°C for 5 min, the reaction was terminated by the addition of 200 μl of 8 M H_2SO_4. Released $^{14}CO_2$ was trapped to 250 μl of phenetylamine for 1 h at 24°C and counted by liquid scintillation spectrometry.

RESULTS

Insulin-stimulated tyrosine kinase activity of the transfected human insulin receptor. Insulin binding to the parental CHO, CHO-wt, and CHO-mut cells was assessed by Scatchard analysis of binding curves obtained at 4°C. Scatchard analysis indicates that the

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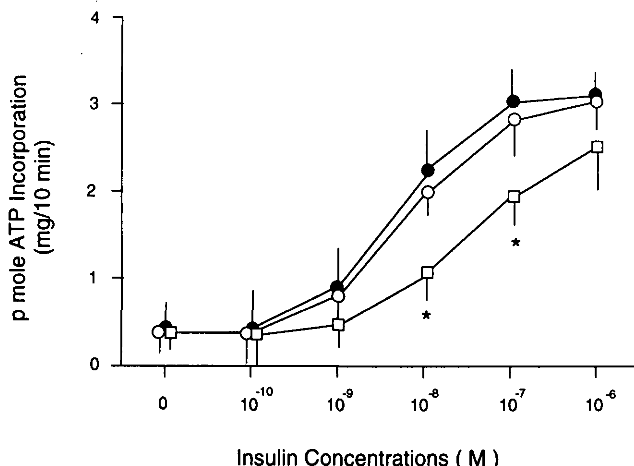

FIG. 1. Insulin-stimulated tyrosine kinase activity in insulin receptors from transfected CHO cell lines. Insulin receptors were partially purified from parental CHO, CHO-wt, and CHO-mut cells with WGA-agarose. Equal amounts of lectin-purified receptors were incubated with various concentrations of insulin and assayed for tyrosine kinase activities. Data are means ± SD of quinticate determinations. Identical results were obtained in 3 other experiments. Data in CHO-wt and CHO-mut cells were compared with those in parental CHO cells. *$P < 0.01$.

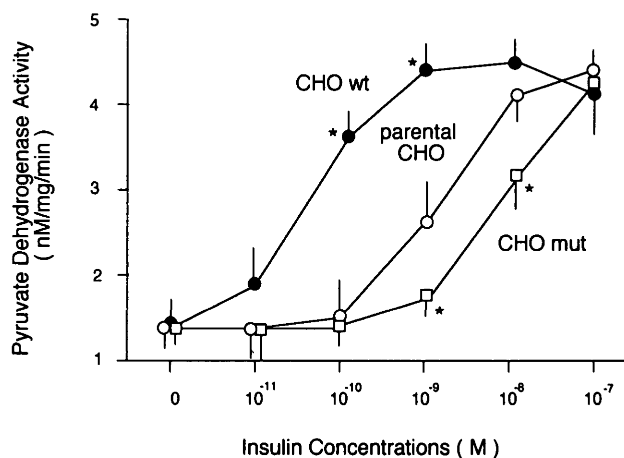

FIG. 2. Effect of insulin on PDH activity in parental CHO, CHO-wt, and CHO-mut cells. Parental CHO, CHO-wt, and CHO-mut cells were serum-starved for 24 h and then incubated with indicated concentrations of insulin for 10 min. Incubation was terminated by addition of cold quench buffer, and cells were scraped off and homogenized. PDH activity was assayed as described in METHODS. Data are means ± SD of 3 experiments, each performed in triplicate. Data were compared with those of parental CHO cells. *$P < 0.01$.

parental CHO, CHO-wt, and CHO-mut cells have ~3000, 39,000, and 40,000 insulin receptor/cells, respectively (data not shown). No significant difference was noted in the affinity between the CHO-wt and CHO-mut cells. Insulin receptors were partially purified from the parental CHO, CHO-wt, and CHO-mut cells. Insulin-stimulated tyrosine kinase activity was assessed with poly-Glu:Tyr (4:1) as an exogenous substrate in vitro. As shown in Fig. 1, basal and maximal ^{32}P incorporation into poly-Glu:Tyr (4:1) was the same for the receptors from the CHO-wt and the parental CHO cells, as was the ED_{50} value of 3 nM. In contrast, the dose-response curve for tyrosine kinase activity of the receptors from the CHO-mut cells was shifted to right with the ED_{50} of 30 nM compared with those of the receptors from the parental CHO and CHO-wt cells. Because the CHO-mut cells were speculated to have ~3000 native Chinese hamster receptors and 27,000 mutant human receptors of Val^{996}, the native Chinese hamster receptors were speculated to conduct most tyrosine kinase activity, suggesting that the mutant receptor of Val^{996} has little or no intrinsic tyrosine kinase activity. Yamamoto-Honda et al. reported that receptor of Val^{996} in the CHO-mut cells was demonstrated to abolished insulin-induced autophosphorylation of the β-subunit (2).

Effect of insulin on PDH activity in the parental CHO, CHO-wt, and CHO-mut cells. We studied the effects of insulin on PDH activity in the parental CHO, CHO-wt, and CHO-mut cells. Insulin stimulated PDH activity in the parental CHO cells (Fig. 2). The maximally stimulated activity was ~2.6-fold greater than basal, and the half-maximal response was elicited by 10^{-9} M insulin. Insulin stimulation of PDH was enhanced in the CHO-wt cells. The maximally stimulated activity was ~threefold greater than basal, and the half-maximal response was elicited by 7×10^{-11} M insulin in the CHO-wt cells. PDH stimulation in the CHO-mut cells was less sensitive to insulin than in the parental CHO cells, and the half-maximal response was elicited by 8×10^{-9} M insulin.

We studied the effects of insulin on lipogenesis and 2-deoxy-D-glucose uptake in the parental CHO, CHO-wt, and CHO-mut cells. The maximally stimulated activity was ~3.4-fold greater than basal, and the half-maximal response was elicited by 10^{-9} M insulin in the parental CHO cells (data not shown). Insulin stimulation of lipogenesis was enhanced in the CHO-wt cells. The maximally stimulated lipogenesis was ~fourfold greater than basal, and the half-maximal response was elicited by 10^{-10} M insulin in the CHO-wt cells. Stimulation of lipogenesis in the CHO-mut cells was less sensitive to insulin than in the parental CHO cells, and the half-maximal response was elicited by 10^{-8} M insulin. Half-maximal concentrations of insulin to stimulate 2-deoxy-D-glucose were 6×10^{-11} M in the CHO-wt cells, 1.5×10^{-9} M in the CHO cells and 2×10^{-9} M in the CHO-mut cells (data not shown).

Insulin-stimulated PIG turnover and generation of IG and DAG in the parental CHO, CHO-wt, and CHO-mut cells. The effect of insulin on PIG metabolism was studied in the parental CHO, CHO-wt, and CHO-mut cells labeled with [^{3}H]glucosamine. In the parental CHO cells, the addition of 10 nM insulin to the cells produced a significant decline of the radioactivity in PIG at 0.5–1 min that seems to reflect hydrolysis of PIG. This decline was followed by a substantial increase in radioactivity after 5 min (Fig. 3). This gradual increase in counts after 5 min seems to reflect resynthesis of PIG. In [^{3}H]*myo*-inositol-, [^{3}H]galactose-, or [^{3}H]glycerol-labeled parental cells, the addition of insulin to the cells decreased the radioactivities of PIG at 1 min and then increased the radioactivities significantly after 5 min (data not shown). Insulin stimulated a decline of [^{3}H]glucosamine radioactivity in PIG at 0.5–1 min and an increase of radioactivity in PIG after 5

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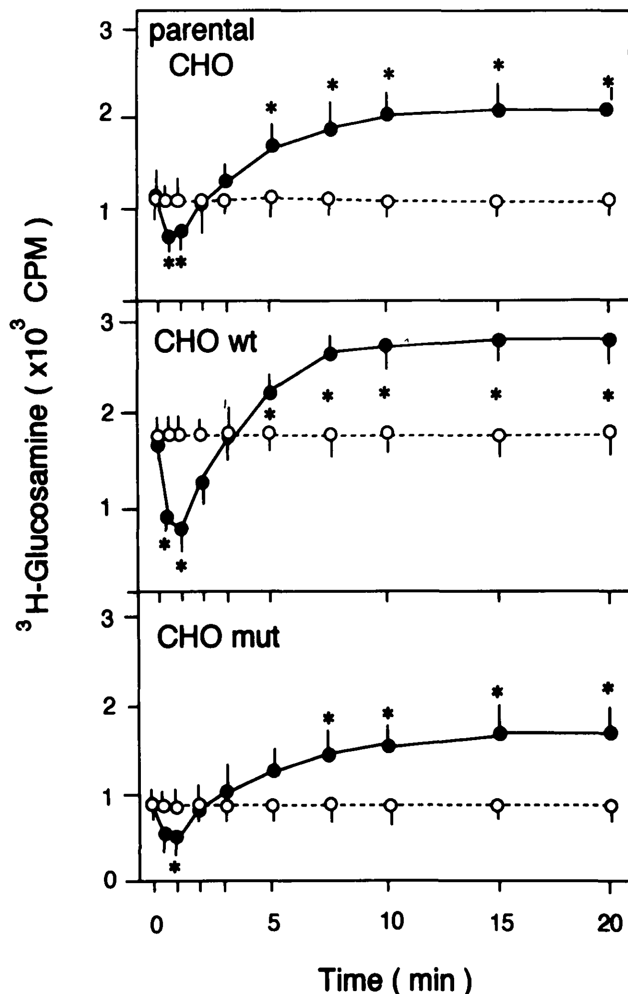

FIG. 3. Time course of insulin-induced hydrolysis of phosphatidylinositol glycan in parental CHO, CHO-wt, and CHO-mut cells. The 3 CHO cell lines were preincubated with separately [^{3}H]glucosamine for 24 h. Cells were then incubated with (●) or without 10 nM insulin (○) for indicated time intervals. Peak A lipids were purified by silica gel TLC with solvent system 1 and 2. Area where peak A lipids migrated was scraped and counted. Data are means ± SD of quintiicate determinations. Identical results were obtained in 3 other experiments. Radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.

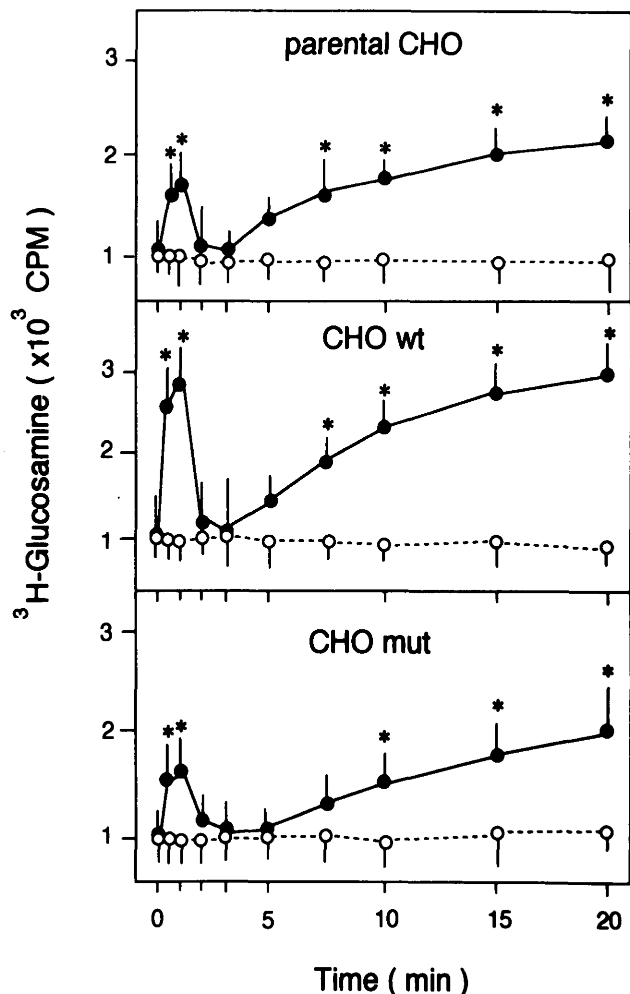

FIG. 4. Time course of insulin-induced generation of inositol glycan in parental CHO, CHO-wt, and CHO-mut cells. The 3 CHO cell lines were incubated with [^{3}H]glucosamine for 24 h. Cells were treated with (●) or without 10 nM insulin (○) for indicated time intervals and extracted with chloroform/methanol/HCl. IG fractions were purified and counted. Data are means ± SD of 5 determinations. Identical results were obtained in 3 other experiments. Radioactivities reported were compared with treatment in buffer alone. *$P < 0.01$.

min (Fig. 3). The insulin-induced turnover of [^{3}H]*myo*-inositol-, [^{3}H]galactose-, or [^{3}H]glycerol-labeled PIG had a similar pattern (data not shown). Insulin induced a similar pattern of [^{3}H]glucosamine-labeled PIG turnover in the CHO-mut cells (Fig. 3).

The time course of IG production in response to insulin was evaluated in the parental CHO, CHO-wt, and CHO-mut cells. In the [^{3}H]glucosamine-labeled parental cells (Fig. 4), insulin evoked a rapid increase in [^{3}H]glucosamine-labeled IG within 30 sec, which was maximal at 1 min. Insulin induced a decline at 2 min and a significant increase after 5 min. The time courses of insulin-stimulated IG production labeled with [^{3}H]*myo*-inositol or [^{3}H]galactose were identical to those labeled with [^{3}H]glucosamine in the parental cells (data not shown). Similar biphasic insulin-stimulated generation of [^{3}H]glucosamine-labeled IG was demonstrated in the CHO-wt cells (Fig. 3). Insulin also induced biphasic generation of [^{3}H]*myo*-inositol- or [^{3}H]galactose-labeled IG in the CHO-wt cells (data not shown). [^{3}H]glucosamine-labeled IG generation also was shown in the CHO-mut cells (Fig. 4).

The time course of generation of DAG in response to insulin also was evaluated in the three CHO cell lines. In the [^{3}H]glycerol-labeled parental cells, insulin induced an increase in labeled DAG at 1 min. It was followed by a decline and a slow increase after 10 min, as shown in Fig. 5. In the [^{3}H]myristic acid–labeled parental cells, insulin treatment caused a similar biphasic [^{3}H]myristic acid-labeled DAG generation in the parental cells (data not shown). The generation of [^{3}H]glycerol-labeled DAG was stimulated at 1 and 5–20 min by insulin in the CHO-wt cells (Fig. 4). [^{3}H]glycerol-labeled DAG generation also was stimulated at 1 and 10–15 min after insulin treatment in the CHO-mut cells (Fig. 5).

We studied the dose-response relationship between insulin and IG generation at 1 min after insulin exposure

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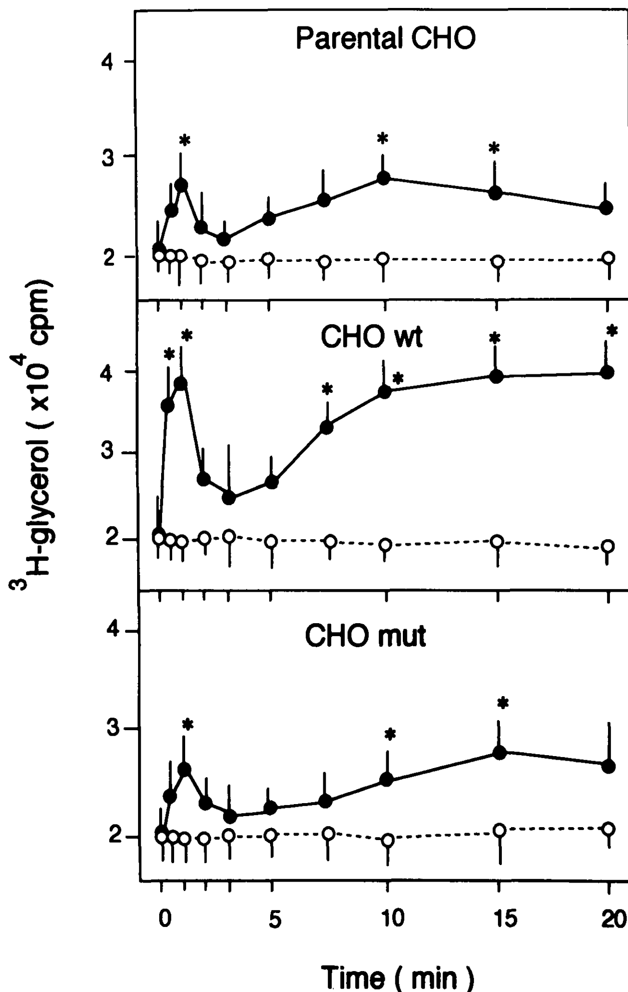

FIG. 5. Time course of insulin-induced generation of diacylglycerol in parental CHO, CHO-wt, and CHO-mut cells. The 3 CHO cell lines were incubated with [^{3}H]glycerol for 2 h. Cells were incubated with (●) or without 10 nM insulin (○) for indicated time intervals. Cells were extracted with chloroform/methanol/HCl, and lipid phases were purified on silica gel TLC run in solvent 3. Area where DAG migrated was scraped off and counted. Data are means ± SD of 5 determinations. Identical results were obtained in 3 other experiments. Data were compared with treatment in buffer only. *$P < 0.01$.

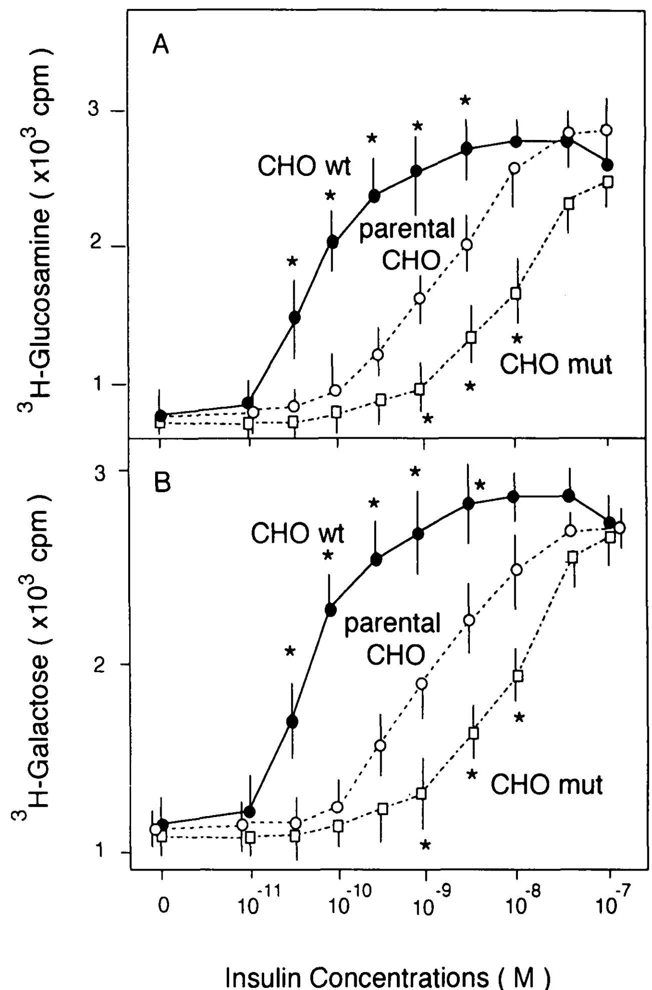

FIG. 6. Dose-related effects of insulin on generation of inositol glycan in parental CHO, CHO-wt, and CHO-mut cells. The 3 CHO cell lines in 35-mm dishes were labeled with [^{3}H]glucosamine (*A*) or [^{3}H]galactose (*B*) for 24 h. Cells were incubated with indicated concentrations of insulin for 1 min and then extracted with chloroform/methanol/HCl. IG fractions were purified and counted. Data are means ± SD of quintuplicate determinations. Identical results were obtained in 3 other experiments. Data in CHO-wt and CHO-mut cells were compared with those in parental cells. *$P < 0.01$.

to the three CHO cells labeled with [^{3}H]glucosamine. In the parental cells, insulin causes stimulation of IG generation with an ED_{50} of 10^{-9} M (Fig. 6*A*). In the CHO-wt cells, the ED_{50} for stimulation of IG generation is 8×10^{-11} M. Introduction of the wild-type human insulin receptors leads to increased insulin sensitivity. The CHO-mut cells, on the other hand, show insulin-induced stimulation of IG generation only at high insulin concentrations, with an ED_{50} of 10^{-8} M. In the cases of [^{3}H] galactose labeling, half-maximal concentrations of insulin needed to induce IG generation were 8×10^{-11} M in the CHO-wt cells, 10^{-9} M in the parental cells, and 10^{-8} M in the CHO-mut cells (Fig. 6*B*). The dose-response curve actually is shifted to the right in the CHO-mut cells compared with the parental CHO cells.

We studied the dose-response relationship between insulin and DAG generation at 1 min after insulin exposure to the three CHO cells labeled with [^{3}H]glycerol. As shown in Fig. 7, treatment with increasing concentrations of insulin caused a concentration-dependent generation of DAG. The maximal effective concentration of insulin was 10^{-7} M and half-maximal was 10^{-9} M in the parental CHO cells. The dose-response curve of DAG generation was left shifted in the CHO-wt cells, because the ED_{50} was 7×10^{-11} M. The insulin-induced DAG generation in the CHO-mut cells was reduced compared with the parental CHO cells, and the ED_{50} was 10^{-8} M.

DISCUSSION

Several lines of recent evidence from site-specific mutagenesis studies of insulin-receptor genes suggest that its tyrosine kinase activity may be necessary for conducting some of insulin's actions, including the stimulation of S6 kinase, 2-deoxy-D-glucose uptake, glycogen synthesis, thymidine incorporation into DNA, and receptor down-regulation (8–10). However, the mutated kinase-defect

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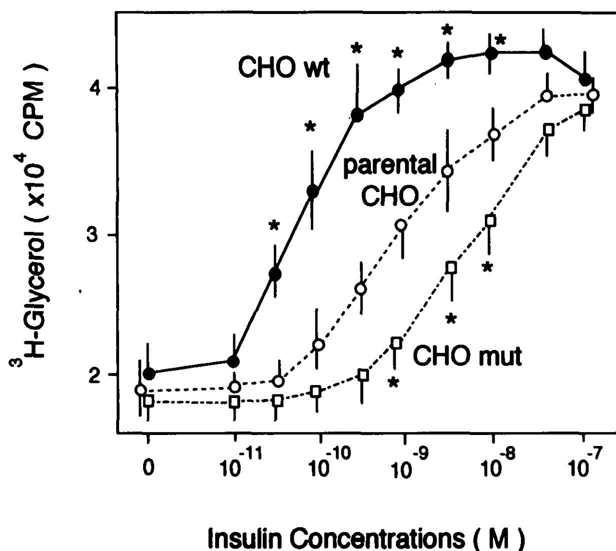

FIG. 7. Dose-related effects of insulin on generation of diacylglycerol in parental CHO, CHO-wt, and CHO-mut cells. The 3 CHO cell lines in 35-mm dishes were labeled with [^{3}H]glycerol for 2 h. Cells were incubated with indicated concentrations of insulin for 1 min and then extracted with chloroform/methanol/HCl. Radioactivity of DAG was counted. Data are means ± SD of quinticate determinations. Identical results were obtained in 3 other experiments. Data in CHO-wt and CHO-mut cells were compared with those in parental cells. *$P < 0.01$.

receptors of Phe^{1146} stimulated glycogen synthesis equally in the CHO cells compared with the wild-type receptor (17). There was enhanced insulin stimulation of thymidine incorporation into DNA in the CHO cells transfected by the mutated receptor in which tyrosines at 1162 and 1163 in the autophosphorylation site of the β-subunit were replaced with phenylalanines (18). Gottschalk reported that cells expressing the mutant receptor of Ala^{1030} failed to mediate insulin-signaling to glucose transport, but glycogen synthesis, however, completely mediated insulin stimulation of PDH (19). These lines of recent evidence suggest that tyrosine kinase activity of the insulin receptor is not a prerequisite for signaling all of insulin's actions (17–22). This study demonstrated the Val^{996} receptor had normal insulin-binding affinity but little or no tyrosine kinase activity toward an artificial substrate, poly-Glu:Tyr (4:1). Yamamoto-Honda et al. reported that insulin-stimulated autophosphorylation was reduced severely with the mutant of Val^{996} [2]. These results proved that the Val^{996} receptor is completely inactive with respective to tyrosine kinase activity. In this study, the effect of insulin on PDH and lipogenesis was studied with the wild-type and the Val^{996} insulin-receptor transfectants. The overexpression of the wild-type human insulin receptor results in increased insulin sensitivity toward PDH and lipogenesis. In contrast, the overexpression of Val^{996} receptor in the CHO cells results in reduced insulin sensitivity to PDH and lipogenesis compared with the parental CHO cells, suggesting that the Val^{996} receptor fails to conduct insulin signaling to PDH and lipogenesis and abolishes insulin's effect on PDH and lipogenesis, which were mediated through the native insulin receptor. In contrast, the Val^{996} receptor failed to mediated insulin's stimulation of 2-deoxy-D-glucose uptake and suppressed the native receptor-mediating stimulation of 2-deoxy-D-glucose uptake (data not shown). This study shows that the CHO-wt cells demonstrated increased insulin sensitivity for PIG hydrolysis and generation of IG and DAG compared with the parental CHO cells. The Val^{996} receptor does not mediate insulin signaling to PIG hydrolysis and generation of IG and DAG, and it suppresses insulin's effect on the turnover of PIG, IG, and DAG, which is mediated through the native insulin receptor. Villalba et al. (13) demonstrated that overexpression of wild-type human insulin receptor–enhanced insulin-dependent PIG hydrolysis in the CHO cells, and the kinase-inactive receptor of Ala^{1018} did not mediate insulin's effects on PIG hydrolysis (11). These lines of evidence have provided proof that insulin-induced hydrolysis of PIG and the generation of IG and DAG are insulin receptor–mediated processes.

The Ala^{1018} receptor in which lysine 1018 in the ATP-binding site of the tyrosine kinase domain was exchanged for alanine was known to lack completely tyrosine kinase activity and to fail to conduct some insulin actions, such as 2-deoxy-D-glucose uptake and glycogen synthesis (19). The Ala^{1018} receptor in the transfected RAT 1 A/K1018 and CHO-mut cells retains full ability to stimulate PDH activity (19). In contrast, the Val^{996} receptor, in which the third glycine in the conserved Gly-X-Gly-X-X-Gly motif is the putative binding site for ATP, was not able to conduct insulin's PDH stimulatory activity. Gottschalk speculated that insulin's action on PDH might be mediated not through the insulin-receptor tyrosine kinase–signaling pathway, but through a pertussis toxin–sensitive G protein–signaling pathway (19). However, Villalba et al. (13) reported that the Ala^{1018} receptor in the same CHO-mut cells failed to mediate insulin signal to PIG hydrolysis. Romero et al. (23) reported that the extracellular addition of anti–inositol glycan antibody to BC3H-1 myocytes reduced insulin-stimulated PDH activation, suggesting that the major portion of PDH activation by insulin is mediated by insulin-releasable inositol glycan. This study shows that the Val^{996} receptor inhibits insulin signaling toward PIG hydrolysis and IG and DAG generation. The purified IG from the CHO-wt cells activated PDH in a cell-free system (data not shown). These data suggested the possibility that IG might contribute to insulin-induced PDH activation as a second messenger.

Several previous studies have suggested that the interaction of insulin with its receptor in the presence of ATP leads to a conformation change in the insulin-receptor intracellular domain (18,22,24). One ATP binding-defective receptors, Ala^{1018}, has full PDH stimulatory capacity (19), but another mutant, Val^{996}, failed to stimulate PDH activity. These data raise the possibility that insulin-associated conformation changes may be an essential or minimal step in the ability of the insulin receptor to transmembrane-signal. ATP-binding–induced conformational changes of the receptor might be an amplifying step in the ability of the receptor to transmembrane-signal. The precise mechanism by which the Ala^{1018} receptor is able to mediate insulin's effect on PDH should be investigated.

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Overexpression of the kinase-inactive receptors in rodent cells that have their own normal endogenous receptors shifts the dose-response curves for insulin stimulation of biological functions, such as glucose transport, glycogen synthesis, S6 kinase activation, c-*fos* induction, and thymidine uptake to the right compared with the parental CHO cells (9–11). Overexpression of the Val^{996} receptor results in a slight reduction of insulin sensitivity and maximal response of PDH activation, lipogenesis, PIG hydrolysis, and generation of IG and DAG, suggesting that the Val^{996} receptor conducts a possible negative interaction with the native insulin receptors. Yamamoto-Honda et al. reported that the Val^{996} receptor conducted the same insulin stimulation of glycogen synthesis and DNA synthesis as the parental CHO cells (2). The reasons for the difference is not clear. The discrepancy may be explained by the difference in incubation time used; generation of IG and DAG occurred in a few minutes, whereas synthesis of glycogen and DNA were estimated to occur in a few hours. Another possibility is that the Val^{996} receptor truly inhibits the function of the native insulin receptors mediating PDH activation and lipogenesis stimulation, but does not affect stimulation of glycogen synthesis and thymidine incorporation. It has been speculated that a great deal of spare kinase activity for dispersed insulin bioactions exists (17–22). Insulin-induced stimulation of PDH and lipogenesis might need more kinase activation of insulin receptors, whereas insulin's effects on glycogen synthesis and thymidine incorporation may require less kinase activation. The precise mechanism by which the kinase-inactive receptor inhibits some of the native insulin receptor-mediating actions, but not all insulin actions, should be elucidated.

The molecular mechanism of insulin-induced generation of IG and DAG, especially the coupling of the insulin-receptor kinase to PI-specific phospholipase C, should be elucidated. The three CHO cell lines, in which extent of signaling from the insulin-receptor tyrosine kinase differ, might be good models for investigation of molecular mechanism of the insulin-receptor signaling process.

ACKNOWLEDGMENTS

This investigation was supported in part by a Grant for Diabetes Research from the Ministry of Health and Welfare (Japan).

We would like to thank Dr. Joseph Larner and Dr. Charles F.W. Schwartz for their critical reading of the manuscripts. We are grateful to Dr. Masato Kasuga and Dr. Guillermo Romero for supplying the CHO cell lines and *Bacillus cereus* PI-specific phospholipase C.

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