THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 49, Issue of December 3, pp. 34795–34802, 1999

Printed in U.S.A.

β_3-Adrenergic Stimulation Differentially Inhibits Insulin Signaling and Decreases Insulin-induced Glucose Uptake in Brown Adipocytes*

(Received for publication, February 16, 1999, and in revised form, August 9, 1999)

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Activity of the sympathetic nervous system is an important factor involved in the pathogenesis of insulin resistance and associated metabolic and vascular abnormalities. In this study, we investigate the molecular basis of cross-talk between β_3-adrenergic and insulin signaling systems in mouse brown adipocytes immortalized by SV40 T infection. Insulin-induced tyrosine phosphorylation of the insulin receptor, insulin receptor substrate 1 (IRS-1), and IRS-2 was reduced by prestimulation of β_3-adrenergic receptors (CL316243). Similarly, insulin-induced IRS-1-associated and phosphotyrosine-associated phosphatidylinositol 3-kinase (PI 3-kinase) activity, but not IRS-2-associated PI 3-kinase activity, was reduced by β_3-adrenergic prestimulation. Furthermore, insulin-stimulated activation of Akt, but not mitogen-activated protein kinase, was diminished. Insulin-induced glucose uptake was completely inhibited by β_3-adrenergic prestimulation. These effects appear to be protein kinase A-dependent. Furthermore inhibition of protein kinase C restored the β_3-receptor-mediated reductions in insulin-induced IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity. Together, these findings indicate cross-talk between adrenergic and insulin signaling pathways. This interaction is protein kinase A-dependent and, at least in part, protein kinase C-dependent, and could play an important role in the pathogenesis of insulin resistance associated with sympathetic overactivity and regulation of brown fat metabolism.

The sympathetic nervous system has long been recognized to play an important role in the pathogenesis of insulin resistance and associated metabolic and vascular abnormalities, such as type 2 diabetes, obesity, dyslipidemia, and hypertension (for a recent review see Ref. 1). At a molecular level, insulin has been shown to phosphorylate tyrosyl residues in the C terminus of the β_2-adrenergic receptor (2), whereas β-adrenergic stimulation can inhibit the activation of the insulin receptor in some tissues (3–7). However, the potential molecular mechanisms downstream of these interactions and their effects remain poorly elucidated.

To investigate the cross-talk between the insulin and adrenergic signaling systems, we have utilized brown adipocytes. These provide an attractive cell model for several reasons. Brown adipose tissue (BAT)[1] is highly regulated by the sympathetic nervous system and expresses different subtypes of adrenergic receptors (8–10), including the β_3-adrenergic receptor, a potential target for anti-obesity and anti-diabetic drug therapy (11, 12). BAT is important in controlling energy balance in rodents by its capacity to uncouple mitochondrial respiration, a process mediated by the expression of the uncoupling protein-1 (UCP-1) (for recent review see Ref. 13 and references therein). And finally, BAT is an insulin-sensitive tissue and contains the main elements of the insulin signaling system (14–17). Thus, in the cells binding of insulin to its receptor leads to activation of the receptor kinase and tyrosine phosphorylation of several insulin receptor substrates (IRS) including IRS-1 and IRS-2. These, in turn, interact with Src homology 2 (SH2) domain-containing proteins such as phosphatidylinositol 3-kinase (PI 3-kinase), Grb2, SHP2, and others. Activation of PI 3-kinase leads to activation of the main downstream effector Akt and stimulation of glucose uptake, glycogen synthesis, and protein synthesis. Association of IRS proteins with Grb2 leads to recruitment of SOS and RAS and results in activation of the MAP kinase pathway, a major regulatory pathway for gene expression (for recent reviews see Refs. 18–21).

In this study, we present a cell model of immortalized brown adipocytes and demonstrate molecular evidence for divergent β_3-adrenoreceptor-mediated alterations at multiple levels of the insulin signaling system, including glucose uptake as a final biological end point.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS) was purchased from Sigma, bovine serum albumin (BSA) from Arnel Products Co., Inc. (New York), adenosine deaminase and collagenase from Roche Molecular Biochemicals, phosphoinositol from Avanti Polar Lipids (Alabaster, AL), thin layer chromatography plates from VWR (Bridgeport, NJ), nitrocellulose from Schleicher & Schuell, membranes for Northern blotting from Mi-

* This work was supported in part by National Institutes of Health Grants DK 33201 and Joslin's Diabetes and Endocrinology Research Center Grant DK 36836. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Supported by Grant Kl-1131/1 from the Deutsche Forschungsgemeinschaft.

‖ Supported by a grant from the Studienstiftung des deutschen Volkes.

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[1] The abbreviations used are: BAT, brown adipose tissue; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; UCP-1, uncoupling protein-1; FBS, fetal bovine serum; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

cron Separations, Inc. (Westborough, MA), and electrophoresis supplies from Bio-Rad.

The β_3-receptor agonist CL316243 was kindly provided by Dr. Kurt Steiner (Wyeth Ayerst Research, Princeton, NJ). Antibodies used for immunoprecipitation and immunoblotting included the following: anti-UCP-1 (Alpha Diagnostic International, San Antonio, TX), anti-IRS-1 (raised in rabbit against C terminus), anti-IRS-2 (raised in rabbit against PH domain and C terminus), antiphosphotyrosine 4G10 (kindly provided by Dr. Morris White, Joslin Diabetes Center, Boston, MA), anti-insulin receptor (kindly provided by Dr. Bentley Cheatham, Joslin Diabetes Center, Boston, MA), polyclonal anti-PI 3-kinase p85 (Upstate Biotechnology, Inc., Lake Placid, NY), antiphosphospecific MAP kinase, antiphosphospecific Akt (New England Biolabs, Beverly, MA), and anti-Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein A-Sepharose was from Amersham Pharmacia Biotech, and the PKC assay system was from Promega (Madison, WI). Penicillin/streptomycin and the PKA assay system were from Life Technologies, Inc.; forskolin, the PKC inhibitors GF109203X and Ro-31–8245 were from Calbiochem; 2-deoxy-[^{3}H]glucose and [γ-^{32}P]ATP were from NEN Life Science Products, and ^{125}I-protein A was supplied by ICN Biochemicals, Inc. (Costa Mesa, CA). All other supplies were from Sigma.

Cell Isolation and Culture—Interscapular brown adipose tissue was isolated from newborn FVB mice (Taconic), minced, and subjected to collagenase digestion (2 mg of collagenase in 2 ml of isolation buffer containing 0.123 M NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 100 mM Hepes, and 4% BSA) for 30 min. The digested tissue was filtered through a 100-μm nylon screen. Collected cells were centrifuged (200 × *g*) for 5 min. The pellet consisting of precursor cells was washed once in isolation buffer and centrifuged again. Cells were resuspended in 2 ml of culture medium (Dulbecco's modified Eagle medium (DMEM) containing 25 mM glucose, 20% FBS, 20 mM Hepes, 100 units/ml penicillin/streptomycin), seeded on two 35-mm plates, and grown in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every day. After reaching 80% confluence, cells were passed to 10-cm plates and infected with the puromycin resistance retroviral vector pBabe encoding SV40 T antigen (kindly provided by Dr. J. DeCaprio, Dana Farber Cancer Institute, Boston) for 24 h. Following infection, the brown adipose precursor cells were maintained in culture medium for 72 h and then subjected to selection with puromycin (1 μg/ml) for at least 3 weeks.

For differentiation, selected preadipocytes were grown to confluence in culture medium supplemented with 20 nM insulin and 1 nM T3 (differentiation medium). Confluent cells were incubated for 24 h in differentiation medium further supplemented with 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethasone, and 0.125 mM indomethacin (induction medium). Subsequently, the cells were maintained in differentiation medium for 4–5 days until exhibiting a fully differentiated phenotype with massive accumulation of multilocular fat droplets. Experiments were carried out after starving the cells in serum-free medium for 16–18 h. Unless indicated otherwise, cells were pretreated for 30 min with adenosine deaminase (2 units/ml), followed by the β_3-adrenergic agent for 30 min, and then stimulated with insulin for 5 min. All experiments were performed within 20 passages following immortalization.

Immunoprecipitation and Western Blot Analysis—At the end of the stimulation period, cells were washed twice with ice-cold PBS and lysed in extraction buffer (50 mM Hepes, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 10% glycerol, 1% Igepal CA-630, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4). Cell lysates were clarified by centrifugation at 12,000 × *g* for 10 min at 4 °C. The fat cake was removed with cotton-tipped applicators, and the supernatants were transferred to fresh tubes. Protein amount was determined by the Bradford method (22) using BSA as standard, and the dye reagent concentrate was from Bio-Rad. Equal amounts of protein (100 μg and 1 mg, respectively) were either directly solubilized in Laemmli sample buffer (LSB) or immunoprecipitated for at least 2 h at 4 °C with the indicated antibodies. Immune complexes were collected by adding 50 μl of a 50% slurry of protein A-Sepharose in phosphate-buffered saline (PBS) for 1 h at 4 °C. After three washes in extraction buffer immunoprecipitates were solubilized in LSB. Lysates or immunoprecipitates were boiled for 2 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked in TBS (10 mM Tris, 0.15 mM NaCl, 0.05% Tween, pH 7.2) containing 3% BSA for 30 min, incubated with the respective antibodies for 2 h, washed 3 times for 5 min each in TBS, and incubated with ^{125}I-protein A for 45 min. After three washes for 5 min each, the immunoblots were exposed on a PhosphorImager screen, and signals were quantified using a Molecular Dynamics densitometer.

PI 3-Kinase Assays—Cells were incubated with or without the indicated hormones in non-serum-containing DMEM with 25 mM glucose for the indicated periods, and lysates were obtained as described above. Supernatants containing 1 mg of protein were immunoprecipitated for 2 h at 4 °C with the appropriate antibodies, and the immune complexes were collected by adding 50 μl of a 50% slurry of protein A-Sepharose in PBS for 1 h at 4 °C. The immune complexes were washed twice with PBS containing 1% Igepal-CA 630, twice with 0.5 M LiCl, 0.1 M Tris, pH 7.5, and twice in reaction buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA). Sepharose beads were resuspended in a mixture containing 50 μl of reaction buffer, 10 μl of 100 mM $MgCl_2$, and 10 μl of phosphatidylinositol (2 μg/μl, previously dried under a gentle argon stream and resuspended in 10 μl of 10 mM Tris, pH 7.5, 1 mM EGTA by sonication at 4 °C). Reactions were initiated by addition of 5 μl of a solution containing 880 μM ATP, 20 mM $MgCl_2$, and 10 μCi of [γ^{32}P]ATP (3.000 Ci/mmol) per tube. The reactions were stopped after 10 min by adding 20 μl of 8 N HCl and 160 μl of $CHCl_3$/methanol (1:1). After a brief centrifugation in a desktop centrifuge, 50 μl of the lower organic phase of each sample were spotted on a silica gel thin layer chromatography plate. The plate was developed in $CHCl_3$/methanol/H_2O/NH_4OH (120:94:23:2.4), dried, exposed to a PhosphorImager screen, and quantitated with a Molecular Dynamics densitometer.

Protein Kinase Assays—Protein kinase B/Akt assays were performed essentially as described (23). Briefly, immunoprecipitates with anti-Akt antibody were washed and resuspended in 40 μl of reaction buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 40 μM ATP, 3 μCi of [γ-^{32}P]ATP, 5 μg Crosstide). After 20 min at 30 °C the reaction was stopped, and aliquots were spotted on squares of P-81 paper, washed, and counted by Cerenkov. Protein kinase A and C assays were done according to the manufacturer's instructions.

Northern Blot Analysis—Northern blot analysis was performed according to standard techniques using denaturing formaldehyde-containing agarose gels (24). After stimulation with the indicated reagents for 4 h, total cellular RNA was isolated by using RNA Stat-60 (Tel-Test "B", Inc., Friendswood, TX). 20 μg of RNA was subjected to electrophoresis in 1.2% agarose gels. Ethidium bromide staining of the gels confirmed equal loading and integrity of the RNA. RNA was transferred to nylon membranes (Micron Separations, Inc., Westborough, MA), and blots were hybridized with 25 ng of a UCP-1-specific cDNA probe previously labeled by using a random prime labeling kit (Promega, Co., Madison, WI) for 2 h at 65 °C. Blots were washed once in 2× SSC, 0.1% SDS at room temperature for 20 min and once in 0.1× SSC, 0.1% SDS at 65 °C for 20 min. Membranes were allowed to air-dry and exposed on a PhosphorImager Screen followed by quantitation with a Molecular Dynamics densitometer.

Glucose Uptake Assays—Cells were assayed for glucose uptake essentially as described (25). Differentiated monolayers of brown adipocytes were pretreated with 2 units/ml adenosine deaminase for 30 min. Unless indicated otherwise, subsequent pretreatment with the β_3-selective agonist CL316243 was for 30 min, before insulin was added for another 30 min. At the end of the stimulation period, cells were exposed to 50 μl of 2-deoxy-[^{3}H]glucose (0.5 μCi/ml final concentration) for 3 min. The incorporated radioactivity was determined by liquid scintillation counting of triplicate points.

Statistical Analysis—Results are indicated as mean ± S.E. for all data. Unpaired Student's *t* tests were used for analysis of differences between various cell treatments. p values <0.05 are considered significant and <0.01 highly significant.

RESULTS

Immortalized Preadipocytes Can Be Differentiated into Mature Brown Adipocytes—Brown fat precursor cells were isolated from interscapular brown fat of newborn mice and immortalized by SV40 T antigen infection as described under "Experimental Procedures." Preadipocytes showed a spindle-shaped morphology similar to fibroblasts (Fig. 1*A, top panel, left*). Northern blot analysis revealed no detectable UCP-1 mRNA in brown fat precursor cells (data not shown), and no fat accumulation could be seen using the fat-specific Oil Red O staining (Fig. 1*A, bottom panel, left*). Following differentiation with insulin, T3, indomethacin, isobutylmethylxanthine, and dexamethasone, confluent cells became smaller, rounded up, accumulated fat, and the cytoplasm/nucleus-ratio increased dramatically. Once fully differentiated, multilocular fat drop-

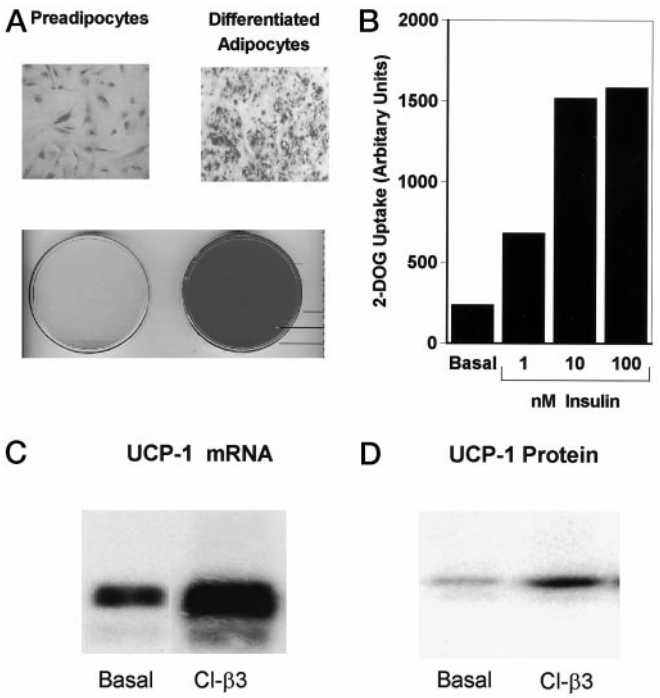

FIG. 1. **Brown adipocyte cell model.** Brown adipose tissue precursor cells were isolated from newborn mice as described under "Experimental Procedures." *A,* after selection with puromycin (1 μg/ml) for 3 weeks, SV40 T-immortalized cells were maintained in DMEM containing 25 mM glucose and 20% FBS. When the cells reached confluence, differentiation was induced with dexamethasone, indomethacin, and isobutylmethylxanthine. 5 days later massive accumulation of multilocular fat droplets in differentiated adipocytes is demonstrated microscopically ($\times$ 40 magnification, hematoxylin-stained) and by Oil Red O staining. *B,* cells were differentiated in 12-well plates, starved for 48 h in DMEM containing 25 mM glucose, and subsequently assayed for glucose uptake in triplicate at the indicated insulin concentrations. *C,* differentiated brown adipocytes were stimulated for four hours with the β_3-receptor-specific agonist CL316243 (100 μM). UCP-1 mRNA of non-stimulated and stimulated cells was detected by Northern blotting using a γ-^{32}P-labeled UCP-1-specific cDNA. *D,* differentiated brown adipocytes were treated with 10 μM CL316243 for 18 h. UCP-1 protein in cell lysates was visualized by immunoblotting with a UCP-1-specific antibody and ^{125}I-labeled protein A.

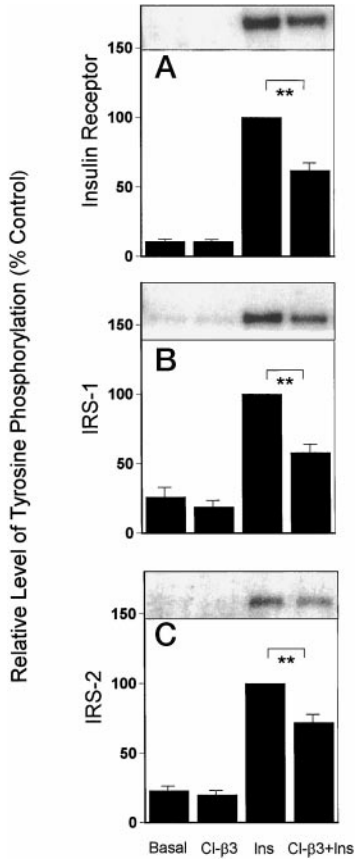

FIG. 2. **Insulin-induced tyrosine phosphorylation of the insulin receptor, IRS-1 and IRS-2, is reduced by β_3-adrenergic prestimulation.** Brown adipocytes were starved in serum-free medium (DMEM containing 25 mM glucose) for 18 h, pretreated with adenosine deaminase (2 units/ml) for 30 min, and stimulated with insulin (*Ins,* 100 nM) for 5 min, either with or without prior treatment for 30 min with the β_3-receptor-specific agonist CL316243 (*Cl-β3*, 100 nM). Protein lysates were subjected to immunoprecipitation with antibodies against the insulin receptor β-subunit, IRS-1, and IRS-2, respectively, followed by SDS-PAGE and Western blotting using a phosphotyrosine-specific antibody. A representative experiment and the statistical analysis of at least six independent experiments with the S.E. are shown. ** denotes $p < 0.01$ comparing insulin stimulation alone with insulin treatment after β_3-agonist pretreatment.

lets could be detected both microscopically (Fig. 1*A, top panel, right*) and by Oil Red O staining (Fig. 1*A, bottom panel, right*). UCP-1 mRNA was expressed in the basal state and could be further increased by 3–4-fold upon β_3-receptor-specific (CL316243) stimulation (Fig. 1*C*). This was paralleled by changes in UCP-1 at the protein level (Fig. 1*D*). An important functional characteristic of adipocytes, including brown adipocytes, is insulin-dependent glucose uptake. Insulin-induced glucose uptake in our cell lines was dose-responsive with a submaximal, approximately 6-fold increase at an insulin concentration of 10 nM (Fig. 1*B*). For cross-talk experiments, we used a maximally stimulatory concentration of insulin of 100 nM, unless indicated otherwise.

Insulin-induced Tyrosine Phosphorylation of the Insulin Receptor, IRS-1, and IRS-2 Is Reduced by β_3-Adrenergic Pretreatment—To investigate the molecular basis of an interaction between adrenergic and insulin signaling systems, we first assessed changes at proximal steps of the insulin signaling cascade. Insulin alone produced an approximately 10-fold increase in tyrosine autophosphorylation of the β-subunit of its receptor in differentiated brown adipocytes. This was diminished by 40% in β_3-receptor agonist (CL316243)-pretreated brown adipocytes as compared with insulin treatment alone (Fig. 2*A*), a statistically highly significant reduction. Insulin-induced IRS-1 tyrosine phosphorylation was reduced in parallel by 45% when cells were pretreated with the CL316243 as compared with insulin treatment alone (Fig. 2*B*). By contrast, the decrease in insulin-induced tyrosine phosphorylation of IRS-2 was reproducibly less and averaged only 28% under the same treatment conditions (Fig. 2*C*). These alterations in insulin-mediated IRS-1 and IRS-2 tyrosine phosphorylation were highly statistically significant. Treatment with the β_3-adrenoreceptor agonist alone had no significant effect on the basal tyrosine phosphorylation neither of the insulin receptor nor of IRS-1 or IRS-2.

β_3-Adrenergic Prestimulation Reduces Insulin-induced p85 Binding to IRS-1 but Not IRS-2—A pivotal regulator for most of the metabolic actions of insulin is activation of the lipid kinase PI 3-kinase. This occurs by binding of the p85 regulatory subunit of PI 3-kinase to tyrosine-phosphorylated insulin receptor substrates such as IRS-1 and IRS-2 with subsequent activation of the p110 catalytic subunit of the enzyme. By immunoprecipitating cell lysates with IRS-specific antibodies and immunoblotting with an antibody detecting the p85-subunit, we found that insulin treatment alone induced a 4-fold increase in p85 binding to IRS-1 as compared with the basal state. Activation of the β_3-adrenergic receptor prior to insulin stimulation reduced the insulin-induced increase in p85 bind-

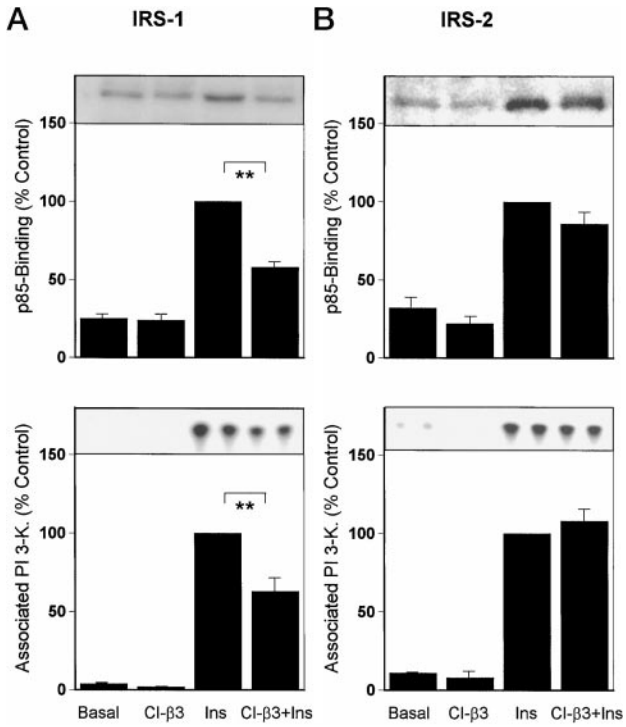

FIG. 3. **Insulin-induced p85 binding to IRS-1 and IRS-1-associated PI 3-kinase activity are reduced by β_3-adrenergic stimulation but not IRS-2 p85 binding and IRS-2-associated PI 3-kinase activity.** After starving cells for 18 h in serum-free medium (DMEM containing 25 mM glucose), adipocytes were pretreated with adenosine deaminase (2 units/ml) for 30 min and then stimulated for 5 min with insulin (*Ins,* 100 nM), with or without prior β_3-adrenergic stimulation (*Cl-β3,* 100 nM) for 30 min. Protein lysates were subjected to immunoprecipitation with antibodies against IRS-1 (*A*) or IRS-2 (*B*), respectively, followed by SDS-PAGE and Western blotting using an anti-PI 3-kinase p85 antibody. PI 3-kinase activities in immunoprecipitates were measured in duplicate as described under "Experimental Procedures." A representative experiment and the statistical analysis with the S.E. of at least three independent experiments are shown. ** denotes $p < 0.01$ comparing insulin stimulation alone with insulin treatment after β_3-agonist pretreatment.

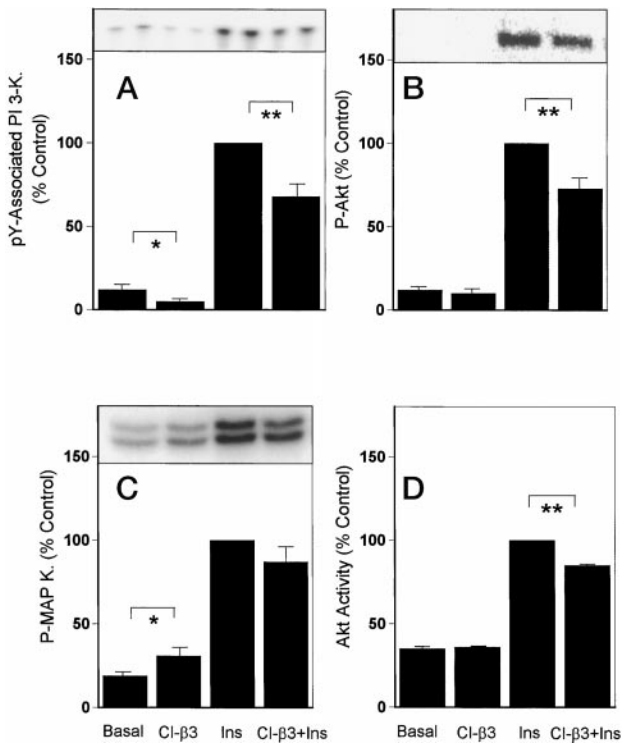

FIG. 4. **Insulin-stimulated phosphotyrosine-associated PI 3-kinase activity as well as Akt phosphorylation and activation but not phosphorylation of MAP kinase are reduced by β_3-adrenoreceptor agonist treatment.** Brown adipocytes, serum-starved for 18 h and pretreated with adenosine deaminase (2 units/ml) for 30 min, were treated for 5 min with insulin (*Ins,* 100 nM) following β_3-adrenergic prestimulation (*Cl-β3,* 100 nM) for 30 min where indicated. *A,* PI 3-kinase activities in immunoprecipitates were measured in duplicates as described under "Experimental Procedures." *B* and *C,* Western blot analysis using phospho-specific antibodies detecting the active forms of Akt and the p42/p44 isoforms of MAP kinase. *D,* Akt kinase activity was measured as described under "Experimental Procedures." The statistical analysis with S.E. for at least six independent experiments is depicted in the *bar graphs.* *, $p < 0.05$; **, $p < 0.01$; *pY*, phosphotyrosine.

ing to IRS-1 by 45%. This alteration was highly statistically significant (Fig. 3*A, top panel*). The association of IRS-2 with p85 was also increased 3-fold upon insulin treatment alone. However, in contrast to the changes seen with IRS-1, pretreatment with the β_3-adrenoreceptor agonist resulted in only a 15% decrease in p85 binding to IRS-2, and this change did not reach the level of statistical significance (Fig. 3*B, top panel*). Thus, β_3-adrenergic stimulation reduced insulin receptor phosphorylation, IRS-1 phosphorylation, and p85 binding to IRS-1 about 40–45% but had a much smaller effect on IRS-2 phosphorylation and no significant effect on p85 docking to IRS-2.

Insulin-induced IRS-1- but Not IRS-2-associated PI 3-Kinase Activity Is Decreased after β_3-Agonist Pretreatment—To determine the effects of these changes in p85 subunit binding, we performed *in vitro* PI 3-kinase assays on IRS-1- and IRS-2-associated PI 3-kinase, as described under "Experimental Procedures." IRS-1-associated PI 3-kinase activity was strongly increased upon insulin treatment. β_3-Agonist pretreatment prior to insulin stimulation led to a 40% decrease in activity as compared with insulin treatment alone (Fig. 3*A, bottom panel*). This change was statistically highly significant and paralleled the reduction in p85 binding. IRS-2-associated PI 3-kinase activity was also strongly induced by insulin. However, pretreatment with the β_3-receptor agonist did not result in a significant change of insulin-induced PI 3-kinase activity consistent with the lack of effect on p85 binding to this insulin receptor substrate (Fig. 3*B, bottom panel*).

β_3-Receptor Agonist Pretreatment Diminishes Insulin-stimulated Phosphotyrosine-associated PI 3-Kinase Activity and Activation of Akt but Not MAP Kinase—As a parameter for the total insulin-induced PI 3-kinase activity, we performed PI 3-kinase activity assays in phosphotyrosine immunoprecipitates. Again, insulin treatment alone induced a strong increase in PI 3-kinase activity. β_3-Adrenergic prestimulation reduced this increase by 30% ($p < 0.01$) (Fig. 4*A*). β_3-Adrenergic stimulation alone also significantly reduced the basal activity level, an effect less appreciated with the individual substrates (Fig. 4*A*).

PI 3-kinase mediates the activation of Akt, and this has been closely linked to glucose transport. By immunoblotting with a phospho-specific antibody to activated Akt, we found a 10-fold increase in phosphorylation of this enzyme over basal levels by insulin stimulation alone. Following β_3-adrenergic prestimulation, the insulin-mediated Akt phosphorylation was reduced by 25% ($p < 0.01$) (Fig. 4*B*). This decrease was accompanied by a 15% reduction in Akt kinase activity ($p < 0.01$) (Fig. 4*D*). Phosphorylation of MAP kinase, another key regulator in insulin and growth factor signaling pathways, was also increased 5-fold by insulin stimulation alone, as determined by immunoblots using a phospho-specific antibody against the activated isoforms p42 and p44. Stimulation with the β_3-adrenoreceptor agonist CL316243 alone also led to a small but significant increase in MAP kinase phosphorylation over the basal state.

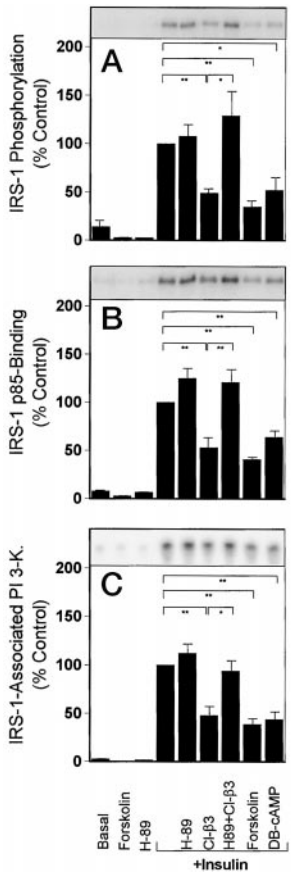

FIG. 5. **The β_3-adrenergic inhibitory effect on insulin-induced IRS-1 tyrosine phosphorylation, IRS-1 p85 binding, and IRS-1-associated PI 3-kinase activity is PKA-dependent.** After 18 h starvation, cells were pretreated with adenosine deaminase (2 units/ml) for 30 min and then incubated with the compounds indicated. Pretreatment with H-89 (10 μM) was for 1 h, with forskolin (50 μM), dibutyryl cAMP (*DB-cAMP,* 1.5 mM) and CL316243 (*Cl-β3,* 100 nM) for 30 min. Where indicated adipocytes were treated with insulin (100 nM) for 5 min at the end of this incubation period. Protein lysates were subjected to immunoprecipitation with an anti-IRS-1-specific antibody and blotted using an anti-phosphotyrosine antibody (*A*) and an anti-PI 3-kinase p85 antibody (*B*), respectively. PI 3-kinase assays in IRS-1 immunoprecipitates were performed (*C*) as described under "Experimental Procedures." A representative experiment is shown together with the statistical bar graph analysis with the S.E. of at least three independent experiments. *, $p < 0.05$; ** $p < 0.01$.

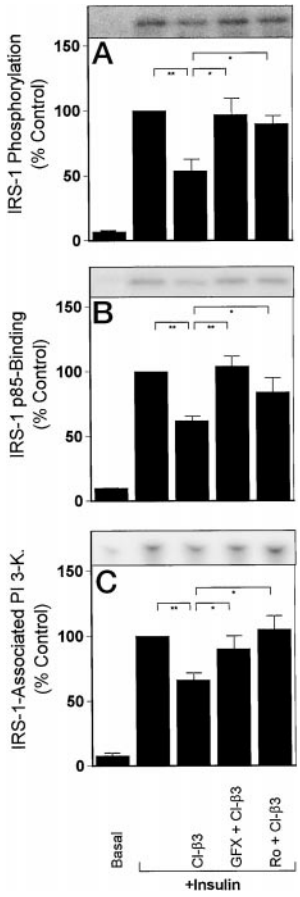

FIG. 6. **Inhibition of PKC restores the β_3-adrenergic inhibitory effect on insulin-induced IRS-1 tyrosine phosphorylation, IRS-1 p85 binding, and IRS-1-associated PI 3-kinase activity.** After 18 h starvation, cells were pretreated with adenosine deaminase (2 units/ml) for 30 min and then incubated with the compounds indicated (*GFX,* general PKC inhibitor GF109203X, 5 μM; *Ro,* general PKC inhibitor Ro-31-8425, 10 μM; *Cl-β3,* CL316243, 100 nM). Pretreatment with GF109203X and Ro-31-8425 was for 30 min, followed by the β_3-adrenoreceptor agonist for 30 min and insulin (100 nM) for 5 min where indicated. Protein lysates were subjected to immunoprecipitation with an anti-IRS-1-specific antibody and blotted using an anti-phosphotyrosine antibody (*A*) and an anti-PI 3-kinase p85 antibody (*B*), respectively. PI 3-kinase assays in IRS-1 immunoprecipitates were performed (*C*) as described under "Experimental Procedures." A representative experiment is shown together with the statistical bar graph analysis with the S.E. of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

However, β_3-adrenergic prestimulation did not further augment the insulin-mediated increase but tended to rather decrease it, although this change was not significant ($p = 0.15$) (Fig. 4*C*).

β_3-Adrenoreceptor-mediated Alterations of Insulin Signaling Components Are PKA-dependent—Gα subunits of β-adrenergic receptors activate membrane-bound adenylyl cyclase thereby increasing intracellular cAMP-levels which, in turn, activate protein kinase A (PKA). Indeed, pretreatment of brown adipocytes with dibutyryl cAMP and the adenylyl cyclase-activating compound forskolin could mimic the molecular changes observed with β_3-adrenergic pretreatment (Fig. 5). Insulin-induced IRS-1 phosphorylation (Fig. 5*A*), p85 binding to IRS-1 (Fig. 5*B*), and IRS-1-associated PI 3-kinase activity (Fig. 5*C*) as well as insulin receptor, IRS-2, and Akt phosphorylation (data not shown) were decreased significantly after pretreating brown adipocytes with these compounds. Additionally, pretreatment of these cells with the specific PKA inhibitor H-89 could reverse the IRS-1-associated effects observed after CL316243 treatment (Fig. 5, *A–C*), as well as the changes in insulin receptor, IRS-2, and Akt phosphorylation (data not shown).

Inhibition of PKC Restores the β_3-Adrenoreceptor-mediated Decrease in Insulin-induced IRS-1 Tyrosine Phosphorylation, IRS-1 p85 Binding, and IRS-1-associated PI 3-Kinase Activity but Leaves IRS-2 Alterations Unaffected—Gα subunits of G protein-coupled receptors have been shown to activate PKC isoforms. To determine whether PKC might play a role in the signaling pathways responsible for the alterations observed, the influence of general PKC inhibition on the effects of β-adrenergic agents was studied using the general PKC inhibitors GF109203X and Ro-31-8425. The decrease in insulin-induced tyrosine phosphorylation of IRS-1 caused by β_3-adrenergic prestimulation was restored by inhibition of PKC (Fig. 6*A*). Furthermore, the β_3-adrenoreceptor-mediated reductions in insulin-induced IRS-1 binding to the p85 subunit of PI 3-kinase and IRS-1-associated PI 3-kinase activity also were rescued by PKC inhibition (Fig. 6, *B* and *C*). PKC inhibition alone or in combination with insulin did not have significant effects on the basal or the insulin-stimulated levels of phosphorylation or p85 binding and associated PI 3-kinase activity (data not shown). The rescuing effect of PKC inhibition was specific for IRS-1. Inhi-

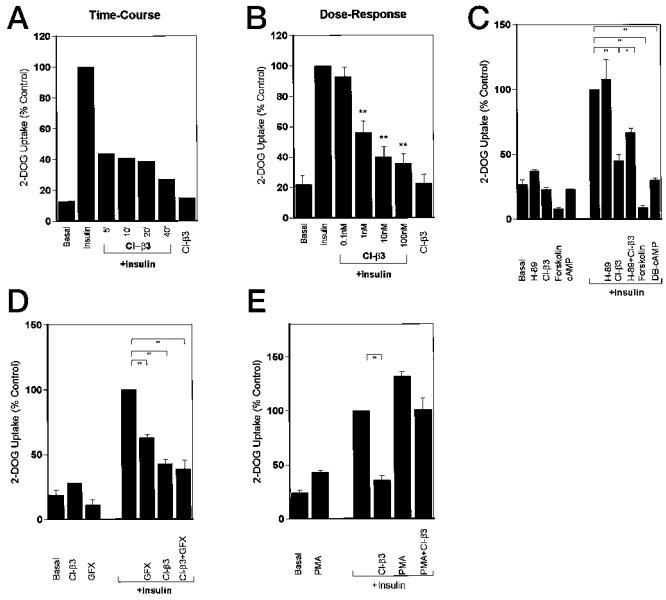

FIG. 7. **Insulin-induced glucose uptake is inhibited by β_3-adrenergic stimulation.** Brown adipocytes, serum-starved for 48 h and pretreated with adenosine deaminase (2 units/ml) for 30 min, were stimulated with insulin (100 nM) for 30 min. The β_3-adrenoreceptor agonist CL316243 (*Cl-β3*) was added at the indicated times prior to the insulin treatment at a concentration of 100 nM (*A*) or at the indicated concentrations 30 min prior the insulin treatment (*B*), respectively. *C,* pretreatment with H-89 (10 μM) was for 1 h, with forskolin (50 μM), dibutyryl cAMP (*DB-cAMP*, 1.5 mM), and CL316243 (*Cl-β3,* 100 nM) for 30 min before insulin (100 nM) was added to the medium for 30 min, as indicated. *D,* pretreatment with GF109203X was for 30 min, followed by the β_3-adrenoreceptor agonist for 30 min and insulin (100 nM) for 30 min where indicated. *E,* brown adipocytes were either non-treated or incubated overnight with PMA. *Cl-β3* (CL316243, 100 nM) was added for 30 min prior to insulin treatment (100 nM, 30 min), as indicated. 2-Deoxyglucose (*2-DOG*) uptake is presented as percentage of the insulin-induced uptake (100%). The *graphs* in *B–E* present the average and the S.E. of at least three independent experiments. *, a statistically significant difference ($p < 0.05$); **, a highly significant difference ($p < 0.01$) as compared with insulin treatment alone (*B–E*) or CL316243 + insulin (*C*).

bition of PKC did not result in significant changes in insulin-induced tyrosine phosphorylation of the receptor β-subunit, IRS-2 tyrosine phosphorylation, p85 binding, or IRS-2-associated PI 3-kinase activity after CL316243 treatment (data not shown).

Insulin-induced Glucose Uptake Is Reduced by β_3-Adrenergic Pretreatment—One of the major effects of insulin signaling is stimulation of glucose uptake. Insulin alone induced an approximately 6-fold increase in glucose uptake in these differentiated culture brown adipocytes. Pretreating cells with the β_3-adrenoreceptor agonist CL316243 for only 5 min prior to insulin treatment reduced the insulin-stimulated glucose uptake by 55%. The inhibition became almost complete after 40 min of treatment with CL316243 (Fig. 7*A*). The inhibitory effect of β_3-adrenergic prestimulation on insulin-induced glucose uptake was dose-dependent. Pretreatment of cells with CL316243 at a concentration of 1 nM reduced the insulin-mediated glucose uptake by 50%, and insulin-induced glucose uptake was almost completely abolished at a concentration of 100 nM (Fig. 7*B*). Treatment with 100 nM CL316243 alone did not have a significant effect on the basal level of glucose uptake. The inhibition of insulin-induced glucose uptake by β_3-adrenergic prestimulation could be mimicked with dibutyryl cAMP and forskolin treatment (Fig. 7*C*). Additionally, the decrease in insulin-induced glucose uptake after CL316243 pretreatment could be partially rescued by inhibition of PKA with H-89 (Fig. 7*C*). In contrast, the PKC inhibitor GF109203X itself had a negative effect on insulin-induced glucose uptake and did not rescue glucose uptake after β_3-agonist prestimulation (Fig. 7*D*). However, when brown adipocytes were treated overnight with 1 μg/ml PMA to down-regulate classical (α, β, and γ) and novel (δ, ϵ, ϕ, and η) PKC isoforms, insulin-induced 2-deoxyglucose uptake was only reduced by 24% after β_3-agonist pretreatment in PMA-treated cells compared with 64% in non-PMA-treated adipocytes (Fig. 7*E*).

DISCUSSION

By using a brown adipocyte cell model, we have studied the molecular mechanisms of interaction between sympatho-adrenergic and insulin signaling pathways and action. We have utilized SV40 T antigen-immortalized brown adipocytes, since these cells exhibit a highly differentiated phenotype and can be established from different animal models of insulin resistance from a single newborn mouse.

Brown fat is a characteristic target tissue for β_3-adrenergic agents that regulate adaptive thermogenesis in these cells by virtue of expression of the mitochondrial protein UCP-1. These cells also exhibit the classical function of insulin in fat, *i.e.* stimulation of glucose transport. Our cell lines demonstrate both insulin-induced glucose uptake and expression of UCP-1 at mRNA and protein level in the basal state as well as in response to β_3-adrenergic stimulation. As compared with other well studied white adipose cell lines, *e.g.* 3T3-L1 cells, insulin-stimulated glucose uptake in differentiated brown adipocytes is similarly robust (6-fold stimulation) and sensitive (submaximal response at 10 nM insulin). Furthermore, these cells exhibit the typical morphology of mature brown adipocytes with massive accumulation of multilocular fat.

In this study, we find that β_3-adrenergic prestimulation results in a decrease in insulin-stimulated receptor tyrosine phosphorylation. This most likely reflects decreased receptor kinase activity and is consistent with a number of previous studies in rat and human adipocytes showing a decrease in receptor kinase activity by nonspecific β-adrenergic stimulation with isoproterenol (3–7). The decrease in receptor tyrosine phosphorylation was not accompanied by a shift in mobility of the receptor β-subunit on SDS gels (data not shown), suggesting that it is not due to increased serine/threonine phosphorylation of the receptor. This finding is in accordance with studies by Issad *et al.* (3) who were unable to detect increased serine/threonine phosphorylation of the insulin receptor by phosphopeptide mapping following adrenergic stimulation.

In parallel with the decrease in receptor tyrosine phosphorylation, insulin-induced tyrosine phosphorylation of IRS-1, and to a lesser extent IRS-2, was also reduced by β_3-adrenergic pretreatment. This is due to a decrease in receptor tyrosine kinase activity as indicated by the diminished receptor tyrosine phosphorylation. However, this differentially affects IRS-1 and IRS-2. In this context, it should be noted that the C-terminal regions of IRS-1 and IRS-2 are rather poorly conserved (35% identity) (21), and the presence of different receptor binding domains such as the "kinase regulatory loop binding domain" which is present in IRS-2, but not IRS-1 (26), might well explain the difference observed. Different compartmentalization and trafficking of IRS-1 and IRS-2 have been observed in fat cells and might also play a role (27). Furthermore, as IRS-2 is more rapidly phosphorylated than IRS-1 (data not shown), we cannot exclude the possibility that CL316243-induced changes in p85 binding to IRS-2 and IRS-2-associated PI 3-kinase activity can be observed at different time points of insulin stimulation.

At the level of PI 3-kinase activity, insulin-induced phosphotyrosine-associated PI 3-kinase activity was decreased by β_3-adrenergic prestimulation, both in the basal state and after

insulin stimulation. This suggests insulin receptor-independent and receptor-dependent cross-talking mechanisms. In agreement with this work, Ohsaka *et al*. (28) have demonstrated that insulin-stimulated PI 3-kinase activity is suppressed by β_3-adrenergic stimulation in rat adipocytes via a direct cAMP-dependent mechanism. Furthermore, this decrease in PI 3-kinase activity is associated with a reduction in insulin-induced activation of Akt. It is likely to be caused, at least in part, by the decreased PI 3-kinase activity observed, but Akt has also been demonstrated to be regulated by PI 3-kinase-independent stimuli including stress (29, 30). Thus, again a number of separate signaling pathways is likely to interact at this level.

MAP kinase in brown adipocytes is activated by both insulin and β_3-adrenergic stimulation (present study and Ref. 31). The potential mechanism for this G protein-coupled receptor-mediated MAP kinase activation is under intensive investigation. One possible pathway involves G$\beta\gamma$ subunits (reviewed in Ref. 32). Also, evidence has been presented for $G_s\alpha$ initiating MAP kinase activation (33, 34). Interestingly, β_3-adrenergic stimulation prior to insulin treatment did not result in a further increase of MAP kinase activation but rather tended to decrease it. In the context of our data, a plausible explanation is that the inhibition of insulin-induced signaling pathways upstream of MAP kinase might prevent a sufficient insulin-stimulated increase.

Surprisingly, despite only modest decreases in PI 3-kinase and even smaller decreases in Akt activation, the inhibition of insulin-induced glucose uptake by β_3-adrenergic prestimulation was almost complete. From these observations two nonmutually exclusive conclusions could be drawn. On the one hand, it is possible that a small decrease in Akt activation as detected on immunoblots and by *in vitro* kinase assays might be sufficient to cause a considerable inhibition of glucose uptake. Alternatively, inhibition of insulin-induced glucose uptake after β_3-agonist treatment might be mediated by Akt-independent pathways. Recent studies using a dominant negative Akt mutant lend support to the latter hypothesis (35).

In our cells, β_3-adrenergic stimulation alone did not result in a significant increase in glucose uptake. This is in contrast to previous reports that demonstrated an approximately 2-fold increase in glucose uptake in response to (nor)adrenergic stimulation using cultured rat brown adipocytes (36, 37). We do not know the cause for this discrepancy. It is conceivable that differences in the experimental systems such as cell starvation periods, stimulation times, the presence or absence of adenosine in the culture media, and the usage of different adrenergic compounds at different concentrations are responsible.

Finally, our data suggest both PKA-dependent and PKC-dependent signaling pathways mediating the β_3-adrenergic effects on the insulin signaling system. Treatment of brown adipocytes with dibutyryl cAMP and forskolin resulted in significant changes similar to CL316243 treatment, and inhibition of PKA by H-89 reversed all changes detected after β_3-agonist pretreatment. Furthermore, PKC inhibition rescued the β_3-receptor-mediated decreases in insulin-induced IRS-1 tyrosine phosphorylation, p85 binding, and IRS-1-associated PI 3-kinase activity. We performed PKA activity assays to exclude the possibility that this rescuing effect of the PKC inhibitors could be explained by an additional PKA inhibitory effect of GF109203X and Ro-31-8425. Whereas the PKA inhibitor H-89 decreased PKA kinase activity by 75%, no change of this protein kinase was detectable after PKC inhibitor treatment (data not shown). Whereas adrenergic activation of PKA has long been known to alter insulin receptor tyrosine kinase activity (38) in brown adipocytes, at present, the signaling pathway from β-adrenergic receptors to activation of PKC in general and its consequences are poorly understood. PKC has been shown to be activated by the β_2-adrenergic receptor (39). In Swiss 3T3 fibroblasts, PKCα and other unidentified isoforms are involved in the β-adrenergic receptor coupling to adenylate cyclase (40). However, as we did not see changes in total or isoform-specific PKC activity after exposure of adipocytes to CL316243 (data not shown), the detailed mechanisms of activation and the identity of PKC isoforms involved remain unknown. On the other hand, our observation that inhibition of PKC decreased the insulin-stimulated glucose uptake also suggests the involvement of PKC isoforms as positive regulators. This is consistent with recent studies demonstrating a role for the atypical PKC isoforms ζ and λ in mediating this insulin effect (41–43).

In summary, we have characterized the cross-talk between adrenergic and insulin signaling pathways in a brown adipocyte cell model at different levels. We find that β_3-adrenergic stimulation inhibits insulin signaling and insulin-induced glucose uptake. The mechanisms presented here might constitute a molecular basis important in the pathogenesis of insulin resistance in states of adrenergic overactivity and in brown adipocyte metabolism.

Acknowledgments—We are grateful to Dr. Kurt Steiner (Wyeth-Ayerst Research, CN 8000, Princeton, NJ) for the kind gift of the β_3-adrenoreceptor agonist CL316243. We gratefully acknowledge Dr. James DeCaprio (Dana Farber Cancer Institute, Boston) for providing us with the retroviral pBabe vector coding for SV40T. We are indebted to Pere Puigserver for helpful advice and comments at many experimental stages of this work and to Terri-Lyn Azar and Catherine Remillard for excellent secretarial assistance.

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