Rottlerin Inhibits Insulin-Stimulated Glucose Transport in 3T3-L1 Adipocytes by Uncoupling Mitochondrial Oxidative Phosphorylation

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There is increasing evidence that protein kinase C (PKC) isoforms modulate insulin-signaling pathways in both positive and negative ways. Recent reports have indicated that the novel PKCδ mediates some of insulin’s actions in muscle and liver cells. Many studies use the specific inhibitor rottlerin to demonstrate the involvement of PKCδ. In this study, we investigated whether PKCδ might play a role in 3T3-L1 adipocytes. We found that PKCδ is highly expressed in mouse adipose tissue and increased on 3T3-L1 adipocyte differentiation, and insulin-stimulated glucose transport is blocked by rottlerin. The phosphorylation state and activity of PKCδ are not altered by insulin, but the protein translocates to membranes following insulin treatment. In contrast to the results with rottlerin, inhibition of PKCδ activity or expression has no effect on glucose transport in adipocytes, unlike muscle cells. Lastly, we found that rottlerin lowers adenosine triphosphate levels in 3T3-L1 cells by acting as a mitochondrial uncoupler, and this is responsible for the observed inhibition of glucose transport. (Endocrinology 143: 3884–3896, 2002)

THE PROTEIN KINASE C (PKC) family mediates downstream signaling from many growth factors and G protein-coupled receptors. This family of serine/threonine kinases comprises at least 10 isoforms that can be divided into three general categories: conventional PKCs (α, β, and γ) are dependent on calcium and diacylglycerol (DAG); the novel PKCs (δ, ε, η, and θ) do not depend on either calcium or DAG but are activated by inositol-phospholipids or phosphatidic acid (1–4). The regulatory region of the conventional and novel PKCs contain C1 and C2 domains that bind DAG and calcium, respectively. There is also a related class of DAG-dependent kinases, including PKCμ/ PKD and PKCτ, which have somewhat larger regulatory regions that contain dual C1 domains (5–7). All of the DAG-dependent PKC isoforms can be artificially activated by phorbol esters.

Whether activation of PKC is involved in insulin’s metabolic effects has long been a matter of controversy. It was not until the identification, cloning, and characterization of the different PKC isoforms described above that definitive studies could be performed. There is now good evidence that PKC isoforms do indeed impinge upon insulin-signaling pathways in both positive and negative ways (8). Overexpression of the conventional PKCs has been shown to directly inhibit the kinase activity of the insulin receptor in vitro through serine phosphorylation (9). Hyperglycemia activates the PKCβ isoform selectively, thus leading to insulin resistance (10). On the other hand, ectopic expression of βII enhances insulin-stimulated glucose transport in the NIH3T3 cell, and insulin has been shown to modulate the alternative splicing of the PKCβ isoform, favoring the βII splice variant, in L6 muscle cells (11, 12). Preventing the change in splicing blocks insulin-stimulated glucose transport and artificially causing the βII splice by transfection of the splicing factor SRp40 mimic the effect of insulin (13). Thus, PKCβ has both negative and positive effects on insulin-signaling depending on the specific splice variant expressed.

More recently, the atypical PKCs λ and ζ have been shown to be activated by the phosphoinositide-dependent kinase 1 leading to glucose transporter-4 (GLUT4) translocation and stimulation of glucose transport in 3T3-L1, rat adipocytes, and L6 muscle cells (14–18). Overexpression of wild-type PKCζ/λ stimulates both basal and insulin-stimulated glucose transport, whereas a dominant-negative PKCζ/λ blocks transport. More importantly, adenoviral overexpression of PKCζ in the tibialis anterior muscle in rats elevated basal glucose transport in the perfused hind limb (19). Interestingly, PKCζ and λ have identical effects on glucose transport, the λ isoform having a predominant role in 3T3-L1 adipocytes, in which ζ is not expressed, and the ζ isoform having a major role in primary adipocytes and muscle (15–18). The interpretation of these studies is often complicated by the fact that many of the reagents used to inhibit ζ or λ are not isoform specific but will inhibit both. Once again, overexpression of an atypical PKC can also have negative effects because PKCζ phosphorylates insulin receptor substrate (IRS)-1, impairing its ability to activate phosphatidylinositol 3-kinase (PI3K) in response to insulin in NIH3T3 and Fao cells (20, 21). These

Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-dependent kinase; DAG, diacylglycerol; DNP, dinitrophenol; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; GLUT4, glucose transporter 4; HDM, high-density microsome; IR, insulin receptor; IRS-1, insulin receptor substrate 1; LDM, low-density microsome; P38K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl-fluoride.
isoforms are also activated by phosphatidic acid, generated by non-Pi3K-dependent mechanisms, and may be the point of convergence in signaling to GLUT4 translocation (22, 23).

A number of previous papers have proposed a role for novel PKCs in insulin action in muscle and liver, but there are no reports implicating PKCδ in adipocytes (24, 25). Over-expression of PKCs δ and θ inhibits the kinase activity of the insulin receptor, and elevated free fatty acids specifically activate the PKCθ isoform in muscle, causing insulin resistance (26–29). On the positive side, expression of PKCδ or ε enhances insulin-stimulated glucose transport in NIH3T3 cells (11). Consistent with this, insulin increases PKCδ activity in L6 muscle cells, HepG2 hepatoma cells, primary rat skeletal muscle cells, and mouse skin keratinocytes. Blockade of PKCδ signaling via expression of a dominant-negative protein or transfection of antisense oligonucleotides prevents the stimulation of glucose transport in rat skeletal muscle cells, proliferation of skin keratinocytes, and stimulation of pyruvate dehydrogenase in HepG2 and L6 cells (24, 25, 30).

Many studies of PKCδ signaling have used rottlerin as a specific inhibitor, in conjunction with other methods, as evidence for the involvement of PKCδ in a variety of biological processes (31).

In this report, we investigated whether insulin signals via PKCδ in the 3T3-L1 adipocyte cell model as has been shown for liver and muscle. We show that insulin causes a relocation of PKCδ to different membrane fractions but does not change its phosphorylation state or activity. Inhibition of PKCδ activity or expression has no effect on insulin-stimulated glucose transport. However, rottlerin inhibits glucose transport in a PKCδ-independent manner by acting as a mitochondrial uncoupler.

Materials and Methods

Materials and cell culture
Porcine insulin was kindly provided by Eli Lilly & Co. (Indianapolis, IN). The PKC inhibitors were obtained from Calbiochem (San Diego, CA). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was from Sigma (St. Louis, MO). Protein A/G-agarose, src, and fyn antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ERK1/2 antibodies (M12320), antibodies to individual PKC isoforms, and antiphosphotyrosine antibodies (pY20) were from BD-Transduction Laboratories, Inc. (Lexington, KY). Phospho-ERK antibodies (anti-AC-TIVE MAPK) were from Promega Corp. (Madison, WI). Phospho-Akt (Ser473), phospho-PKCδ (Thr505) antibodies were from Cell Signaling Inc. (Beverly, MA). PKCα antibodies for immunoprecipitation were from Zymed Laboratories, Inc. (South San Francisco, CA) and Santa Cruz Biotechnology, Inc.. Enhanced chemiluminescence reagents were from Amersham (Piscataway, NJ). Myristoylated cell-permeable inhibitory peptides to PKCα (SIYRRGARRWRKL), PKCβ (AVRMDRQTVAVG-VIKAV), and PKCγ (AALVRQAHAVAFFFK) were synthesized by Bioresource Technologies, Inc. (Camarillo, CA) or United Biochemical Research Inc. (Seattle, WA). [3H]-2-Deoxyglucose (50 μCi/ml mmol) was from Perkin-Elmer/NEN Life Science Products (Boston, MA). Unless noted, all other reagents were supplied by Sigma or Fisher Scientific Co. (Springfield, NJ). Mice were housed and handled under a protocol that had been approved by the University of California San Diego Animal Subjects Committee in accordance with Institutional Animal Care and Use Committee guidelines.

Cell culture

The 3T3-L1 cells were maintained in DMEM with 25 mm glucose with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum in a 10% CO₂ environment. Cells were then differentiated 2 d after confluence by the addition of the media containing 500 μm isobutylmethylxanthine (IBMX), 10 μm dexamethasone, and 4 μg/ml insulin. After 2 d, cells were grown in media containing only insulin for another 3 d. Differentiated adipocytes were replated into appropriate dishes. Subsequently, medium was changed to DMEM regular glucose with 10% fetal calf serum and replaced every 2 d until the cells were well differentiated (d 10) with many lipid droplets. For the antisense experiments, cells in 12-well plates were transfected with the PKCδ antisense oligonucleotide (GAAGGAGATGCCTCGGAA) or the corresponding sense oligonucleotide (TTCCAGGCGATCTCTCCT) using Fugene 6 (Roche, Indianapolis, IN) following the manufacturer’s instructions. Phosphorothioate oligonucleotides were synthesized by GENSET Corp. (La Jolla, CA). Cells were transfected with oligos every 2 d until use.

Glucose transport

Cells in 12-well plates were rinsed three times with PBS at 23 C and incubated with 0.5 ml Krebs-Ringer phosphate-HEPES buffer containing 0.5% fatty acid-free BSA for 2 h. Cells were stimulated with insulin (50 ng/ml) at 37 C for 30 min. In some experiments, cells were pretreated with various pharmacological inhibitors or cell-permeable peptides for 30 min. The transport reaction was initiated by adding 50 μl Krebs-Ringer phosphate-HEPES buffer containing 1.1 mm 2-deoxyglucose (50 μM final) with [3H]-2-deoxyglucose (0.2 μCi/well). After a 10-min incubation, cells were washed three times with ice-cold PBS containing 100 mm phloretin, solubilized in 1% SDS, 0.1% NaOH, neutralized, and counted. Nonspecific uptake was measured in the presence of 25 μM cytochalasin B.

PKCδ activity

PKCδ activity was measured with the Signatect assay system (Promega Corp.) following a protocol similar to Braiman et al. (24). Briefly, 3T3-L1 adipocytes were resuspended in cold lysis buffer [25 mm Tris (pH 7.4), 0.05% Triton X-100, 0.5 mm EDTA, 0.5 mm EGTA, 0.5 mm phenylmethylsulfonylfluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml apro tinin] and lysed with a cold Dounce homogenizer. The clarified lysate was immunoprecipitated with anti-PKCδ antibodies (Zymed Laboratories, Inc. or Santa Cruz Biotechnology, Inc.). Immune complexes were washed five times with ice-cold lysis buffer with 0.2 M NaCl and two times with kinase buffer [20 mm Tris (pH 7.5), 5 mm MgCl₂, 0.25 mm EGTA, 0.4 mm CaCl₂, 0.1 mg/ml BSA, 0.32 mg/ml phosphatidylysine]. Some reactions also contained 32 mg/ml diacylglycerol. The kinase reaction was performed in the same buffer containing 0.5 μCi[^32P]-ATP, 100 μM ATP, and 100 μM biotinylated Neurogranin (28–43) peptide (AAKIAKSSFRGHMARKK) or a biotinylated EF-1α peptide (AVRDM-RQTVAVGVIKAV) as a substrate for 5 min at 30 C and was terminated by the addition of half the volume of 7.5 M guanidine hydrochloride. Samples were spotted onto SAM capture membranes, washed extensively, and counted.

Immunoblotting

The 3T3-L1 cells were serum starved for 16 h in 12-well plates and then stimulated with insulin for 5 or 10 min at 37 C. The cells were washed with ice-cold PBS and lysed in radiommunoprecipitation assay buffer containing 20 mm Tris-HCl (pH 7.5), 1 mm EDTA, 140 mm NaCl, 1% Nonidet P-40, 1 mm sodium orthovanadate, 1 mm PMSF, and 10 μg/ml aprotinin. Samples were centrifuged at 2500 rpm for 10 min, fat aspirated, and pellet discarded. Equal amounts of protein from the control and insulin-treated lysates were solubilized in 2× SDS-sample buffer. The proteins were denatured by boiling for 5 min, separated by electrophoresis on 7.5% SDS-PAGE, and transferred to polyvinylidenefluoride membranes. The filter was blocked with 3% BSA in Tris-buffered saline-0.2% Tween-20 for 30 min and incubated with the antiphosphotyrosine, anti-phospho-Akt (Ser473), anti-phospho-ERK (Thr202/Tyr204), anti-phospho PKCδ (Thr505), or anti-PKCδ antibodies. For immunoblotting of individual PKC isoforms, visualized by autoradiography, extracts from mouse white adipose tissue, 3T3-L1 fibroblasts, or 3T3-L1 adipocytes were made directly with 2× SDS-sample buffer containing 2 mm sodium orthovanadate and 200 mm sodium fluoride. Samples were boiled and fractionated by SDS-PAGE, transferred to polyvinylidenedi-
fluoride, and immunoblotted in a multichannel blotter (Hoefer, San Francisco, CA) using antibodies at a dilution of 1:1000. The filters were washed with Tris-buffered saline-0.2% Tween-20 for 30 min and incubated with horseradish-peroxidase conjugated secondary antibodies, and tyrosine-phosphorylated proteins were visualized by enhanced chemiluminescence.

Subcellular fractionation

Cells were stimulated with 100 ng/ml insulin for 20 min and then washed three times with ice-cold PBS. Cells were scraped into ice-cold HES buffer [255 mM sucrose, 20 mM HEPES (pH 7.4), and 1 mM EDTA] supplemented with 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mg/ml benzamidine, 0.5 mM PMSF). Cells were then homogenized using a Teflon/glass homogenizer. Subcellular fractionation was carried out as described previously (32). Internal and plasma membrane fractions were kept at −70 C before protein quantification and immunoblotting with PKCδ antibodies.

Immunostaining

The 3T3-L1 adipocytes were plated on 10-mm acid-washed glass coverslips and stimulated with insulin for 30 min. Cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Following two washes in PBS, the cells were permeabilized and blocked in PBS containing 5% BSA and 0.5% Nonidet P-40 for 10 min. Coverslips were incubated with the anti-PKCδ antibody (1:400 dilution) for 60 min at room temperature, washed once in PBS, and then incubated with fluorescein isothiocyanate-conjugated antirabbit IgG antibody (1:100 dilution) in PBS with 5% BSA and 0.5% Nonidet P-40 for 30 min at room temperature. Finally, the coverslips were extensively washed with PBS, rinsed with water, and mounted in PBS containing 15% gelvatol (polyvinyl alcohol), 33% glycerol, and 0.1% sodium azide.

Measurement of intracellular ATP

The 3T3-L1 cells in 12-well plates were treated with increasing concentrations of rottlerin or FCCP for 30 min. Cells were washed twice with cold PBS and lysed in 0.1 M NaOH/0.5 mM EDTA. Extracts were incubated at 60 C for 20 min and frozen at −20 C. Extracts were diluted 1:200 into 0.2 M NaOH/0.5 mM EDTA, and ATP was measured on 20 μl of the dilution by adding 100 μl luciferase/luciferin (Sigma) and measuring light output for 15 sec in a Turner Designs luminometer. Serial dilutions of ATP from 30 μM to 30 nm were used to generate a standard curve.

Results

PKC isoform expression in fat cells

Initially we examined expression of PKC isoforms in mouse epididymal fat pads, and 3T3-L1 adipocytes before and after differentiation. Whole-cell extracts were immunoblotted for the conventional PKC isoforms α, β, and γ; the novel PKC isoforms δ, ε, and θ; and the atypical PKC isoforms ι, λ, and ζ. We also blotted for the less related PKCμ isoform. Epididymal fat pads express the novel isoforms δ and ε, the atypical isoforms ι and λ, and a lower level of the conventional isoforms α and β (Fig. 1A). The antibodies to ι and λ cross-react, so we cannot distinguish between these two isoforms. The 3T3-L1 adipocytes express high levels of the novel isoforms δ and ε, and the atypical isoforms ι and λ, both before and after differentiation. The conventional PKC isoforms α, β, and γ are expressed highly in the preadipocyte cells, but expression drops with differentiation (Fig. 1A). PKCμ is expressed at similar levels in all three extracts, but PKCθ and ζ are not detectable in any of the three. The lack of expression of PKCζ confirms previous findings (18). These results confirmed that the novel PKC isoform δ is expressed at a high level in fat and is one of the major isoforms expressed in 3T3-L1 adipocytes. We then sought to determine whether PKCδ is involved in insulin-stimulated glucose transport as has been shown for primary neonatal rat muscle cells and L6 muscle cells (Fig. 1B). Increasing concentrations of the inhibitor rottlerin caused a dose-dependent decrease in insulin-stimulated glucose transport (half-maximal effective dose, 2–3 μM) but had no effect on basal transport (data not shown). This finding suggested that PKCδ might be involved in insulin-stimulated transport in adipocytes as has been shown for muscle cells.

Insulin does not activate PKCδ

If insulin signals to glucose transport via PKCδ, then it is reasonable that insulin might activate the kinase. PKCδ activation was monitored using an antibody against phospho-Thr505. This phosphorylation site lies on the activation loop of PKCδ and is phosphorylated by phosphoinositide-dependent kinase 1 (33). Whole-cell extracts from 3T3-L1 adipocytes stimulated with insulin were immunoblotted with the
antibody to pThr505 PKCδ (Fig. 2A). This site was phosphorylated in the basal, unstimulated cells and phosphorylation did not increase with insulin stimulation for 2–30 min. The blots were stripped and rebotted for phospho-ERK to verify that the cells had been adequately starved of serum and that insulin caused an increase in ERK phosphorylation. Thus, phosphorylation of the activation loop residue is constitutive and not stimulated by insulin. We confirmed the immunoblotting data by measuring PKCδ kinase activity. PKCδ was immunoprecipitated from 3T3-L1 cells that had been stimulated with insulin for increasing times. The immunoprecipitates were subjected to an in vitro kinase assay using a biotinylated peptide substrate. Two different antibodies and two substrate peptides were used in independent experiments. No increase in total PKCδ kinase activity was observed with insulin treatment in agreement with the immunoblotting data (Fig. 2B). This is in contrast to reports on muscle and liver cells in which insulin increases PKCδ phosphorylation and activity.

**Insulin causes a redistribution of PKCδ**

The novel PKC isoforms are calcium independent but are activated by DAG generated by phospholipase C. The DAG also serves to localize the kinase to membrane compartments. We have previously shown that insulin causes an increase in DAG levels in 3T3-L1 adipocytes (32). Although insulin does not activate PKCδ, the intracellular localization of the kinase may be altered by insulin treatment. Consequently, we monitored the cellular localization of PKCδ in different membrane fractions of insulin-stimulated 3T3-L1 adipocytes. Insulin causes a biphasic increase in DAG in these cells, an initial transient peak at 30 sec, followed by a slower more sustained rise at 10–30 min. Starved cells were stimulated for increasing times with insulin and membrane fractions prepared by sucrose gradient centrifugation. Initially we prepared plasma membrane and mitochondrial/nuclear fractions because PKCδ has been shown to translocate to these membranes in other cells. Insulin caused an increase in PKCδ localization in both plasma membrane and mitochondrial/nuclear fractions by immunoblotting with either the pThr505 antibody or an antibody against native PKCδ (Fig. 3A).

Interestingly, the redistribution of PKCδ coincides with the sustained increase in DAG at 15–30 min, rather than the acute peak of DAG at 30 sec. We subsequently investigated whether PKCδ might relocate to other microsomal fractions. Cells were stimulated with insulin for increasing times and then fractionated into plasma membrane, mitochondrial/nuclear, and low (LDM)- and high-density microsomal (HDM) fractions by sucrose gradient centrifugation. A longer time course of insulin treatment was used because the translocation occurred at 15–30 min in the initial experiments. Insulin caused an increase in pThr505-PKCδ in both the mitochondrial/nuclear and plasma membrane fractions as before (Fig. 3A). Insulin also caused an increase in pThr505-PKCδ in the LDM fraction and a concomitant decrease in PKCδ in the HDM fraction (Fig. 3B). The redistribution was confirmed by immunofluorescence. Cells were stimulated with insulin, fixed, and stained with an antibody to PKCδ. In the unstimulated state, PKCδ staining is homogenous throughout the cell. Stimulation with insulin causes a redistribution of PKCδ staining to membrane structures (Fig. 3C). Thus, insulin alters the intracellular localization but not activity of PKCδ in 3T3-L1 adipocytes. This would be consistent with insulin signaling via PKCδ and agrees with the relocation of PKCδ observed in muscle and liver cells.

**PKCδ is associated with PKCλ in 3T3-L1 adipocytes**

PKCδ has been shown to associate with a number of signaling proteins including the insulin receptor in muscle cells and PI3K in TF-1 erythroleukemia cells (26, 34). PKCδ is tyrosine phosphorylated in response to insulin, phorbol 12-myristate 13-acetate (PMA), platelet-derived growth factor, epithelial growth factor, ligation of the IgE receptor and various apoptotic stimuli, and serine phosphorylated by PKCζ (24, 35–39). Therefore, we investigated whether PKCδ could be found in association with components of these signaling pathways in 3T3-L1 adipocytes. Cell lysates from unstimulated and insulin-stimulated adipocytes were immunoprecipitated with antibodies to PKCδ. The pellets and supernatants were then immunoblotted for phosphotyrosine (Fig. 4A). Insulin causes an increase in tyrosine phosphorylation of the insulin receptor (IR) and IRS-1 in the supernatants, but no tyrosine-phosphorylated proteins were found in the pellets. Reblotting for PKCδ demonstrated that the antibodies precipitated more than 70% of the protein. This result demonstrated that PKCδ does not associate with the IR in 3T3-L1 cells, unlike muscle cells, and furthermore showed that PKCδ is not tyrosine phosphorylated either in the basal or insulin-stimulated state. We subsequently in-
investigated whether PKCδ is associated with PKCα, PKCµ, src, or fyn. Three of these kinases, PKCα, src, and fyn, have been shown to phosphorylate PKCδ so they might be expected to interact. Cell lysates from unstimulated or insulin-stimulated cells were immunoprecipitated with antibodies for PKCα, PKCµ, src, or fyn, and then the pellets and supernatants were immunoblotted for PKCδ. Only antibodies to PKCα were able to precipitate PKCδ (Fig. 4B). The finding of a complex between PKCδ and PKCα is intriguing because PKCα has been shown to be critical for insulin signaling to GLUT4 translocation and stimulation of glucose transport in these cells.
Inhibition of PKCδ activity does not inhibit glucose transport

The alteration in cellular localization of PKCδ is consistent with a role in insulin signaling. The earlier results with rotterlin suggested a requirement for PKCδ activity in insulin-stimulated glucose transport. To confirm this, we performed a direct comparison of the effect of rotterlin on glucose transport with other well-characterized PKC inhibitors. The 3T3-L1 adipocytes were pretreated with increasing doses of rotterlin, calphostin C (a DAG antagonist), bisindolylmaleimide I (an ATP-binding site inhibitor selective for the α, β, γ, δ, and ε isoforms of PKC), Go6976 (a PKCa, β, and μ inhibitor), or Ro 31–8220 (an inhibitor of conventional and novel PKCs) for 30 min, stimulated with insulin for 30 min, and then 2-deoxyglucose transport measured. As before, rotterlin caused a dose-dependent decrease in transport. Calphostin C, BIM, or Go6976 did not inhibit transport at concentrations that would inhibit PKC. Only Ro 31–8220 inhibited transport at 10–20 μM, a concentration that inhibits the atypical PKC isoforms ζ, λ, and ε (Fig. 5A). Therefore, these results are inconsistent with insulin signaling via PKCδ to transport but are consistent with the published data on PKCα.

All the chemical inhibitors used have nonspecific effects, so we used cell-permeable peptide inhibitors to inhibit individual PKC isoforms. Cells were pretreated with each cell-permeable peptide (100 μM) for 30 min before stimulation with insulin. The PKCζ pseudosubstrate inhibitor, which also inhibits the other atypical PKCs, blocked insulin-stimulated glucose transport, as has been published previously, but equivalent peptides for PKCδ and PKCμ were without effect (Fig. 5B). We verified that the PKCδ peptide was able to inhibit glucose transport in L6 muscle cells. Differentiated L6 myotubes were preincubated with rotterlin (10 μM) or the PKCδ inhibitory peptide (100 μM) for 30 min before stimulation with insulin for 30 min. Insulin causes an approximate 2-fold increase in 2-deoxyglucose transport in these cells. Both rotterlin and the PKCδ peptide reduced insulin-stimulated transport to basal levels (Fig. 5C), neither agent altered basal transport rates (data not shown). This result again argues against a role for PKCδ in insulin-stimulated glucose transport in 3T3-L1 adipocytes unlike L6 muscle cells.

The novel and conventional PKC isoforms are all DAG dependent. Consequently, these isoforms can be downregulated by chronic treatment with phorbol-esters. Cells were treated overnight with 100 nM PMA and whole-cell extracts immunoblotted for PKC isoform expression as before. The conventional isoforms α, β, and γ and the novel isoforms δ, ε, ζ, η were all decreased in 3T3-L1 adipocytes, whereas the novel isoforms δ, ε, ζ, η were only decreased in L6 muscle cells.
PKC isoform δ were not detected showing complete down-regulation (Fig. 6A). Expression of the novel PKC isoform ε was reduced greater than 90%, but the atypical PKC isoforms τ and λ and PKCμ were not altered. Basal and insulin-stimulated glucose transport were measured on the PMA down-regulated cells. PMA treatment raised basal transport 2.5-fold but had no effect on insulin-stimulated transport (Fig. 6B). Therefore, insulin is still able to stimulate glucose transport in the absence of PKCδ. Interestingly, pretreatment of down-regulated cells with rottlerin still blocked insulin-stimulated transport, strongly suggesting that the effect of rottlerin is not due to inhibition of PKCδ. Transport is still sensitive to wortmannin, so the mechanism of insulin stimulation is likely unchanged by the PMA down-regulation.

Inhibition of PKCδ expression does not inhibit glucose transport

The pharmacological methods used above to inhibit PKCδ activity could be criticized for nonspecific effects. To obtain evidence for a role for PKCδ in insulin-stimulated glucose transport using an independent approach, we reduced PKCδ expression using antisense oligonucleotides. We initially determined the stability of the PKCδ protein by inhibiting protein synthesis with cycloheximide. The half-life of PKCδ in 3T3-L1 adipocytes is 24 h. Complete elimination of the protein can be seen by 96 h (Fig. 7A). Consequently, we transfected cells with two doses of antisense oligonucleotides to PKCδ for 4 and 5 d. The oligonucleotide sequence was published previously (40). Whole-cell extracts were immunoblotted for PKCδ. Transfection of cells with 5 μg antisense oligonucleotide for 5 d causes an 80% reduction in PKCδ (Fig. 7B). These conditions were then used for transport studies. Adipocytes were transfected with antisense or sense oligonucleotides to PKCδ for 5 d. Basal and insulin-stimulated transport were measured. The antisense oligonucleotide to PKCδ had no effect on basal or insulin-stimulated glucose transport (Fig. 7C). Therefore, neither inhibition of PKCδ activity or reduction of PKCδ expression reduces glucose transport.

Rottlerin is a mitochondrial uncoupler in 3T3-L1 adipocytes

The results from the previous sections indicate that PKCδ is not involved in glucose transport, but rottlerin, a supposedly specific PKCδ inhibitor, reduces insulin-stimulated transport. Obviously, rottlerin must have another target within the cell. Rottlerin does not inhibit glucose transport directly when added to a transport assay but requires pretreatment (data not shown). It was recently shown that rottlerin functions as a mitochondrial uncoupler to increase oxygen consumption and reduce ATP levels in rat parotid acinar cells (41). To test whether rottlerin uncouples 3T3-L1 adipocytes, cells were treated with increasing doses of rottlerin (1, 3, and 10 μm) or the chemical uncoupler FCCP (1, 3, and 10 μm) for 30 min and intracellular ATP levels were measured on cell extracts using luciferase/luciferin. Both rottlerin and FCCP cause a dose-dependent decrease in cellular ATP levels. At the maximal concentration (10 μm), both agents reduce ATP levels by 80% (Fig. 8A). In parallel, we determined the effects of increasing doses of rottlerin and FCCP on glucose transport. Both agents cause a similar dose-dependent decrease in insulin-stimulated glucose transport (Fig. 8B). This is the first demonstration that FCCP inhibits glucose transport in adipocytes and suggests that the ability of rottlerin to inhibit transport is related to its ability to act as a mitochondrial uncoupler. Dinitrophenol (DNP) was used as an antiobesity drug for many years because it increases energy expenditure by uncoupling oxidative phosphorylation. We tested whether reduction of ATP levels using DNP would also inhibit glucose transport. Cells were treated with 0.5 mM DNP for 30 min before stimulation with insulin for 30 min and measurement of 2-deoxyglucose transport. In parallel, cellular ATP levels were determined using luciferase/luciferin. Treatment of 3T3-L1 cells with DNP caused an 80% reduction in ATP and a more than 90% reduction in glucose transport (Fig. 8C), confirming the correlation between ATP levels and transport that was seen with rottlerin and FCCP.

Rottlerin reduces insulin-stimulated activation of Akt and ERK

Given the ability of rottlerin to reduce ATP levels, one conceivable mechanism for the inhibition of transport is due to reduced kinase activity. To investigate this possibility, cells were treated with increasing doses of rottlerin for 30 min and then stimulated with insulin. Whole-cell extracts were immunoblotted with antibodies to phosphotyrosine. Tyrosine phosphorylation of the insulin receptor and IRS-1 are
preserved, even at the highest concentration of rottlerin that reduces ATP levels by 80% (Fig. 9A). The effects on Akt activation are more pronounced however. Phosphorylation of Akt on Ser473 is reduced in a dose-dependent manner with increasing concentrations of rottlerin (Fig. 9B). Rottlerin has shown to be a short activation of ERK in PC12 cells (40). Therefore, cells were treated with 10 μm rottlerin for 30 min and stimulated with insulin for increasing times. Whole-cell extracts were immunoblotted with antibodies to the dually phosphorylated form of ERK (Thr204/Tyr204). Insulin can still activate ERK at early time points (5 and 10 min) in the presence of rottlerin, but the kinase is rapidly dephosphorylated by 30 min (Fig. 9C). Thus, the prolonged activation of ERK by insulin in 3T3-L1 adipocytes is prevented by rottlerin.

These studies suggested that rottlerin may inhibit insulin signaling because phosphorylation of a number of key proteins is reduced. To determine whether this defect is responsible for the reduced glucose transport, we performed a time course of rottlerin treatment in 3T3-L1 adipocytes. Cells were treated with insulin (50 ng/ml) for 30 min before measuring 2-deoxyglucose transport. Rottlerin (10 μm) was added at different times relative to the insulin: 30 and 15 min before insulin; at the same time as insulin; or 5, 10, and 15 min after insulin. Addition of rottlerin before or at the same time as insulin blocked glucose transport (Fig. 9D). More importantly, addition of rottlerin 5 min after insulin was still able to block glucose transport, but the inhibitory effect was progressively lost at later times. This is significant because activation of signaling via the insulin receptor occurs within the first 5 min after insulin stimulation. The reduced glucose transport is therefore not simply a reflection of impaired signaling.

Rottlerin causes irreversible uncoupling of mitochondria

Reductions in ATP levels with rottlerin and FCCP are observed rapidly, within 5 min of addition. To determine whether rottlerin is acting as a chemical uncoupler like FCCP or DNP, we performed a wash-out experiment. Cells were treated with 10 μm rottlerin or FCCP for 30 min, and then the cells were washed and the medium was changed to medium lacking the uncouplers. Cells were allowed to recover for 0, 30, 60, or 90 min, and then ATP levels were measured using luciferase/luciferin. The reduced ATP levels because of treatment with FCCP returned to normal after a 30-min wash-out (Fig. 10A). Unexpectedly, ATP levels following rottlerin treatment did not recover, even after a 90-min wash-out. In parallel cells, we measured insulin-stimulated glucose transport. Following the wash-out of rottlerin and FCCP for different times, cells were stimulated with insulin for 30 min and 2-deoxyglucose transport measured. The impaired glucose transport in cells treated with rottlerin did not recover, even after a 90-min wash-out consistent with the continued reduction in ATP levels (Fig. 10B). The results with FCCP were more striking. Glucose transport was normalized in cells that were stimulated with insulin immediately after removal of the FCCP. Allowing cells to recover for 30 min or longer before insulin stimulation led to a superinduction of transport. Therefore, rottlerin is not acting as a chemical uncoupler but must irreversibly inhibit a component of the electron transport chain.

Discussion

Because of the evidence for PKCδ involvement in insulin action in other cell types, we decided to investigate whether this PKC isoform might play a role in 3T3-L1 adipocytes. Initial experiments demonstrated that PKCδ is highly expressed in 3T3-L1 adipocytes and mouse fat, and rottlerin inhibited glucose transport in a dose-dependent manner. However, we were not able to document an increase in either phosphorylation of PKCδ or activity with insulin. The insulin receptor has been shown to associate with PKCδ in muscle and NIH3T3 cells, but we did not observe any association in 3T3-L1 cells, which may explain the lack of phosphorylation. We did observe, however, the translocation of PKCδ to the plasma membrane, LDM, and nuclear/mitochondrial fractions by sucrose gradient centrifugation and immunofluorescence. This would be consistent with insulin causing a relocation of PKCδ without changing its inherent activity.

**Fig. 7.** Antisense elimination of PKCδ does not inhibit glucose transport. A, 3T3-L1 adipocytes were treated with cycloheximide (100 μg/ml) and harvested at varying times thereafter. Equal amounts of protein were immunoblotted for PKCδ. B, 3T3-L1 adipocytes were transfected with two doses of antisense oligonucleotide to PKCδ every 2 d. Cells were harvested after 4 and 5 d and equal amounts of protein immunoblotted for PKCδ. C, 3T3-L1 adipocytes were transfected with 5 μg antisense or sense oligonucleotide to PKCδ every 2 d for 5 d. Cells were stimulated with insulin (50 ng/ml) and 2-deoxyglucose transport measured. Results are mean and SEM of two experiments in triplicate.
PKCδ activity was inhibited using a variety of pharmacological agents or cell-permeable inhibitor peptides. We were able to document the importance of PKCA, as has been published, but inhibition of PKCδ had no effect on glucose transport. Similarly, elimination of PKCδ using antisense oligonucleotides or down-regulation with chronic PMA also had no effect. Therefore, we were left with the conundrum that rottlerin inhibited glucose transport in a PKCδ-independent manner.

The ability of rottlerin to inhibit PKCδ has been questioned recently. Davies et al. (42) tested a series of kinase inhibitors, including rottlerin, against a panel of recombinant kinases. Rottlerin was unable to inhibit PKCδ in this assay but was able to inhibit MAPK-activated kinase 2 (IC₅₀ 5.4 μM) and p38-regulated/activated kinase (IC₅₀ 1.9 μM). It has also been reported to inhibit calmodulin-dependent kinase III (31). Similarly, in another study rottlerin was unable to inhibit immunoprecipitated PKCδ activity in the presence of DAG and phosphatidylserine and unexpectedly stimulated kinase activity in their absence (41). In this latter study, they showed that rottlerin reduces cellular ATP levels by uncoupling mitochondrial oxidative phosphorylation. The drop in ATP levels was sufficient to prevent the tyrosine phosphorylation of PKCδ in response to carbachol or PMA. This effect was seen only in primary parotid acinar cells but not in the PC12 and RPG1 cell lines. It was proposed that primary cells rely on oxidative phosphorylation for ATP production, whereas cultured cell lines rely on both glycolysis and oxidative phosphorylation. Hence, rottlerin did not function as an uncoupler in either PC12 or RPG1 cells, even though rottlerin increased oxygen consumption in these cells.

We show here that rottlerin is able to uncouple 3T3-L1 adipocytes similar to the chemical uncouplers FCCP and DNP. This would suggest that ATP production in these adipocytes is primarily driven by oxidative phosphorylation rather than glycolysis. This is consistent with a switch in energy metabolism during differentiation. We also show that the drop in ATP levels is sufficient to inhibit insulin signaling because Akt and ERK phosphorylation are impaired. Both FCCP, DNP, and rottlerin decreased insulin-stimulated glucose transport in parallel with the decrease in ATP. Basal transport was not altered by these agents, so the ability of transporters already resident in the plasma membrane to transport glucose is not dependent on ATP. There are two possible explanations for the decreased transport, either an insulin-signaling defect because of the impaired kinase phosphorylation or a failure of GLUT4-containing vesicles to translocate to the plasma membrane. The trafficking of GLUT4 vesicles is mediated by ATP-dependent microtubule motors, so the lowered ATP level may directly inhibit vesicle motion. The impairment in signaling is not the sole explanation for the reduced transport because addition of rottlerin 5 min after insulin was still able to inhibit transport. Activation of PI3K signaling is maximal within 5 min of insulin stimulation, so the inhibition by rottlerin is unrelated to signaling in this experiment. We also found that the uncoupling effect of rottlerin is irreversible. It is unlikely, therefore, that rottlerin is a chemical uncoupler, but it may directly inhibit a component of the electron transport chain. This notion is supported by the observation that the mitochondrial toxin rotenone, which is a competitive inhibitor of complex I (nicotinamide-adenine dinucleotide-coenzyme Q) of the electron transport chain, also inhibits...
glucose transport in a manner similar to the chemical uncouplers (data not shown).

A number of papers have investigated the effects of lowering ATP levels on glucose transport. Glucosamine causes insulin resistance and reduces cellular ATP levels. Hresko et al. (43) found that treatment of 3T3-L1 adipocytes with glucosamine for 2.5 h in glucose-free medium blocked insulin-stimulated glucose transport and caused an 80% reduction in ATP levels. Severe depletion of ATP with azide mimicked the effect of glucosamine and impaired insulin signaling and GLUT4 translocation. The authors reported a tight correlation between cellular ATP levels and the magnitude of the insulin-induced increase in glucose transport. Kang et al. (44) reported that treatment of 3T3-L1 adipocytes with glucosamine for 5 h in medium containing glucosed lowered ATP levels by a more modest 15% but still impaired insulin-stimulated glucose transport by 50%. In the second article, glucosamine treatment was performed in the presence of glucose, which is preferentially transported over glucosamine, resulting in a milder reduction in ATP. Treatment of 3T3-L1 cells with 0.05 mM dinitrophenol or 3 mM azide to obtain a similar reduction in ATP was insufficient to inhibit insulin-stimulated glucose transport but, unlike glucosamine, increased basal glucose transport 4-fold. Higher doses of DNP or azide to reduce ATP levels by 40% were able to inhibit insulin signaling, but the effects on transport were identical with the lower doses leading the authors to conclude that a mild reduction in ATP levels per se does not cause insulin resistance. The results presented here are consistent with the report of Hresko et al. The acute reduction in ATP levels with rottlerin and FCCP is very dramatic (>80%). This reduction is sufficient to impair insulin signaling and reduce glucose transport without activating basal transport.

The induction of basal transport by a prolonged mild reduction of ATP reported by Kang et al. (44) is similar to effects seen in muscle. Treatment of muscle strips or L6 muscle cells with mitochondrial uncouplers leads to an increase in glucose transport (45–48). One possible explanation for this increase is the activation of PKC signaling because of the release of calcium from the mitochondria upon depletion of ATP (46). Elevation of intracellular calcium to levels insufficient to cause contraction increases glucose transport (49). Alternatively, reduction of ATP levels and concomitant increase in the ADP/ATP ratio leads to the activation of the AMP-dependent kinase (AMPK) (50). This kinase can directly activate glucose transport by causing translocation of GLUT4 vesicles (51). The kinase can be activated artificially by the compound 5-aminoimidazole-4-carboxamide ribonu-

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**FIG. 9.** Rottlerin impairs insulin activation of Akt and ERK. The 3T3-L1 cells were treated with increasing doses of rottlerin for 30 min and stimulated with insulin for 5 min. Whole-cell extracts were immunoblotted for phosphotyrosine. B, Cells were treated with increasing doses of rottlerin and stimulated with insulin. Whole-cell extracts were immunoblotted for phospho-Akt (Ser473). C, Cells were treated with rottlerin (10 μM) for 30 min and stimulated with insulin for the indicated times. Whole-cell extracts were immunoblotted for phospho-ERK (Thr202/Tyr204). D, Cells were stimulated with insulin (50 ng/ml) for 30 min before measurement of 2-deoxyglucose transport. Rottlerin (10 μM) was added to the cultures 30 and 15 min before insulin stimulation (−30 and −15); at the same time as insulin stimulation (0); or 5, 10, or 15 min after insulin stimulation (+5, +10, and +15). Results are the mean and SEM of three determinations. Asterisks indicate statistical significance vs. insulin alone (P < 0.05).
cleoside (AICAR), which is phosphorylated inside the cell to 5-aminoimidazole-4-carboxamide ribonucleotide, an AMP mimic. Treatment of muscle cells or strips with AICAR stimulates glucose transport to the same extent as insulin or contractions (52–54). Indeed, contractions activate AMPK/H₂, and the effects of AICAR and contractions are not additive, which has lead to the suggestion that AMPK mediates exercise-induced transport (55, 56). Treatment of 3T3-L1 adipocytes with AICAR stimulates basal transport only 2-fold and impairs insulin-stimulated transport by 50% (57). The increase in basal transport is similar to the 4-fold induction due to chronic 5-h treatment with low doses of DNP or azide as reported by Kang et al. (44). So the stimulatory effect observed with mild ATP depletion may be linked to activation of AMPK. We did not observe an increase in basal transport with acute reductions in ATP, but it may take longer to manifest an effect on AMPK.

In summary, we have shown that PKCδ is highly expressed in 3T3-L1 adipocytes. Its phosphorylation state and activity are not altered by insulin, but the protein translocates to membranes following insulin treatment. Inhibition of PKCδ activity or expression has no effect on glucose transport in adipocytes, unlike muscle cells. Lastly, the effects of rottlerin to inhibit glucose transport are most likely related to its ability to act as a mitochondrial uncoupler and reduce ATP levels.

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