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Protein Kinase Cδ But Not PKCα Is Involved in Insulin-Induced Glucose Metabolism in Hepatocytes

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ABSTRACT

The liver is a major insulin-responsive tissue responsible for glucose regulation. One important mechanism in this phenomenon is insulin-induced glycogen synthesis. Studies in our laboratory have shown that protein kinase Cs delta (PKCδ) and alpha (α) have important roles in insulin-induced glucose transport in skeletal muscle, and that their expression and activity are regulated by insulin. Their importance in glucose regulation in liver cells is unclear. In this study we investigated the possibility that these isoforms are involved in the mediation of insulin-induced glycogen synthesis in hepatocytes. Studies were done on rat hepatocytes in primary culture and on the AML-12 (alpha mouse liver) cell line. Insulin increased activity and tyrosine phosphorylation of PKCδ within 5 min. In contrast, activity and tyrosine phosphorylation of PKCα were not increased by insulin. PKCδ was constitutively associated with IR, and this was increased by insulin stimulation. Suppression of PKCδ expression by transfection with RNAi, or overexpression of kinase dead (dominant negative) PKCδ reduced both the insulin-induced activation of PKB/Akt and the phosphorylation of glycogen synthase kinase 3 (GSK3) and reduced significantly insulin-induced glucose uptake. In addition, treatment of primary rat hepatocytes with rottlerin abrogated insulin-induced increase in glycogen synthesis. Neither overexpression nor inhibition of PKCα appeared to alter activation of PKB, phosphorylation of GSK3 or glucose uptake in response to insulin. We conclude that PKCδ, but not PKCα, plays an essential role in insulin-induced glucose uptake and glycogenesis in hepatocytes. J. Cell. Biochem. 113: 2064–2076, 2012.

KEY WORDS: PKC ISOFORMS; PKCδ; PKCα; INSULIN SIGNALING; GLYCOGENESIS; HEPATOCYTES

The binding of insulin to its receptor initiates a cascade of events leading to its many biological effects. The first step in this cascade is activation of the insulin receptor intrinsic tyrosine kinase, which phosphorylates endogenous substrate proteins, primarily members of the insulin receptor substrate (IRS) family [White, 1997]. Tyrosine phosphorylated motifs in these substrates serve as docking sites for the recruitment and activation of a number of signaling proteins, including certain members of the protein kinase C (PKC) family of serine threonine kinases. PKC comprises a family of serine–threonine kinases that play an important regulatory role in a variety of biological phenomena [Azzi et al., 1992; Liu, 1996]. The family is composed of a number of individual isoforms that are categorized according to their mechanisms of activation. It is generally believed that the enzymes, when quiescent are located in the cytoplasm and that upon activation they translocate to the plasma membrane or to membranes of cytoplasmic organelles to become fully activated in the presence of specific cofactors [Hug and Sarre, 1993; Gschwendt, 1999; Stempka et al., 1999].

A number of PKC isoforms have been implicated in insulin signal transduction [Sampson and Cooper, 2006]. We reported that insulin stimulates glucose uptake and induces tyrosine phosphorylation, translocation, and activation of PKCs α, βII, δ, and ζ in skeletal muscle [Braiman et al., 1999b; Rosenzweig et al., 2002; Cipok et al., 2006]. Activation of PKCs βII and ζ occurs via a PI3-kinase dependent pathway, whereas PKCs δ and α are activated within 1–5 min independent of PI3 Kinase. We recently showed that this activation involves participation of Src tyrosine kinase [Rosenzweig et al., 2004; Cipok et al., 2006]. Studies from our laboratory have further shown that PKCδ is induced by insulin to physically associate with the IR and to regulate subsequent events in IR signaling such as IR tyrosine phosphorylation and internalization, and, ultimately, glucose uptake [Braiman et al., 1999a, 2001b]. In

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contrast, PKCα does not associate with IR, but is constitutively associated with IRS-1 and appears to exert a basic constitutive inhibition of IR signaling; insulin stimulation induces disassociation of PKCα from IRS-1, thereby removing this inhibition [Rosenzweig et al., 2004; Cipok et al., 2006]. Thus, both PKC isoforms play a role in insulin signaling in skeletal muscle.

The role of PKCs α and δ on insulin signaling in liver cells is as yet unclear. Some studies have been indirect and have relied on use of PKC activators to mimic or otherwise modulate insulin-induced effects without identification of the specific PKC isoforms involved [Donchenko et al., 1994; Probst et al., 2003]. Although PKCζ was shown to be activated by insulin in primary rat hepatocytes, its inhibition did not alter insulin-induced activation of glycogen synthase 3 (GS3) [Lavoie et al., 1999]. Other isoforms were not studied. A role for PKCδ has been both implicated [Caruso et al., 2001; Chen et al., 2006] and denied [Probst et al., 2003] in certain insulin-induced effects in hepatic cells. It was reported recently that PKCδ may play a negative role in hepatic insulin signaling [Bezy et al., 2011].

In the present study, we have attempted to identify more precisely the roles of PKCs α and δ in IR signaling in hepatocytes because of the clear role of these proteins in skeletal muscle. Studies were performed on hepatocytes from adult rat liver in primary culture and on the AML-12 mouse liver cell line. Our results demonstrate that insulin stimulates PKCδ and that this isoform plays an essential role in insulin-induced glucose uptake and glycogen synthesis in hepatocytes, whereas PKCα appears to have no discernible role in insulin effects in these cells.

MATERIALS AND METHODS

All animal experimentation was conducted in accord with accepted standards of humane animal care as required by the Ethics Committee of the Israel National Committee on Use of Experimental Animals. Principles of animal care (NIH publication no. 85–23, revised 1985) were followed.

MATERIALS

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Antibodies to various proteins were obtained from the following sources: anti-PKCα (sc-208; C-terminus-C20); anti-PKCδ (sc-213; C-terminus-C17); anti-pPKCδ tyr332 (sc18365); anti-pPKCδ tyr187 (sc18363); anti-pPKCδ tyr311 (sc18364R); and anti-insulin receptor (IRβ, sc711; C-terminus-C19), antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphotyrosine from Upstate Biotechnology (Boston, MA), anti-skeletal muscle actin from Sigma Chemicals (St. Louis, MO); anti-pPKB and anti-pGSK3 antibodies from Cell Signaling (Danvers, MA); enhanced chemical luminescence (ECL) was performed with antibodies purchased from BioRad (Hercules, CA) and reagents from Sigma Chemicals. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were obtained from BioRad. TPA, leupeptin, aprotinin, PMSF, DTT, orthovanadate, and pepstatin were purchased from Sigma Chemicals. Insulin (HumulinR—recombinant human insulin) was purchased from Lilly France SA (Fergersheim, France).

CELL CULTURE

AML-12 mouse liver cells were obtained from ATCC. AML-12 cells were grown in modified Eagle's medium supplemented with 10% fetal calf serum for 4 days post-confluence, with media changed daily. Twenty-four hours prior to study, cells were transferred to low glucose (4.5 mM), serum-free DMEM containing 1% bovine serum albumin. On the day of the study, cells were transferred to phosphate-buffered saline (pH 7.4) containing 2 mM glucose for 10 min prior to addition of insulin (100 nM).

Primary hepatocyte cultures were prepared as described [Rosa, 2004; Rosa and Baraba, 2005; Rosa and Skala, 2005]. In all experiments, male adult Wistar rats (250–325 g) were used. Rats were housed individually in wire cages in a temperature-controlled room (21 ± 1°C), on 12 h light–dark cycle, with free access to food and water. Rats were anaesthetized with Phenobarbital (10 mg/100 g body weight). Hepatocytes were isolated by a modified collagenase-perfusion technique; calcium-free Swim's S-77 medium containing collagenase (0.5 g/l) was used for liver perfusion through a portal cannula. After approximately 10 min the liver was transferred to a plastic beaker containing 20 ml of the enzyme perfusion medium. The Glisson capsule was cut with microdissecting scissors and the cells were dispersed by gently drawing them up and down in a wide-bore Pasteur pipette. The suspension was filtered first through nylon gauze of 253 μm mesh and then through a smaller nylon mesh (64 μm) into a plastic centrifuge tube. The cells passing through were mainly hepatocytes. Usually more than 90% of cells excluded trypan blue as the measure of viability. After washing twice with the same collagenase-free medium, the cells were suspended to a final concentration of 10^6 cells per ml M199 serum-free medium. Three ml of cell suspension was placed in 60-mm Petri dishes previously coated with collagen. Culture dishes were kept at 37°C in an atmosphere of 5% CO_2 and 95% air. The culture medium was replaced with fresh medium 4 h later to remove unattached cells and hepatocytes were incubated for the next 24 h in M199 serum-free medium. On the day of study, cells were transferred to phosphate-buffered saline (pH 7.4) containing 2 mM glucose for 10 min prior to addition of insulin (100 nM).

PREPARATION OF CELL LYSATES FOR IMMUNOPRECIPITATION

Culture dishes (90 mm; Nunc) containing the liver cells were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached in RIPA buffer (50 mM Tris–HCl pH 7.4; 150 mM NaCl; 1 mM EDTA; 10 mM NaF; 1% Triton X-100; 0.1% SDS; 1% Na deoxycholate) containing a cocktail of antiproteases (20 μg/ml leupeptin; 10 μg/ml aprotinin; 0.1 mM PMSF; 1 mM DTT) and antiphosphatases (200 μM ortho-vanadate; 2 μg/ml pepstatin). After scraping, the preparation was centrifuged at 20,000*g* for 20 min at 4°C. The supernatant from this was used for immunoprecipitation.

IMMUNOPRECIPITATION

To 0.5 ml of cell lysate, 25 μl of A/G Sepharose was added and the suspension was rotated continuously for 30 min at 4°C. The preparation was then centrifuged at 20,000*g* at 4°C for 10 min, and the supernatant was mixed with 30 μl of A/G Sepharose along with specific antibodies to the individual PKC isoforms (dilution 1:100). Samples were rotated overnight at 4°C. The suspension was

then centrifuged at 2,000*g* for 1 min at 4°C, and the pellet was washed twice with TBST with centrifugation at 2,000*g* for 1 min at 4°C. To this, 25 μl of sample buffer (0.5 M Tris–HCl pH 6.8; 10% SDS; 10% glycerol; 4% 2-beta-mercaptoethanol; 0.05% bromophenol blue) was added. The suspension was again centrifuged at 500*g* (4°C for 10 min), boiled for 5 min, and then subjected to SDS–PAGE.

WESTERN BLOT ANALYSIS

Western blots were performed as described [Horovitz-Fried et al., 2006a; Jacob et al., 2010]. Equal protein loading of Western blots was confirmed by immunoblotting for β-actin.

PKC ISOFORM VIRAL INFECTION

When the cells were approximately 80% confluent, the culture medium was aspirated and cultures were infected with the viral medium containing PKCδ, or control vector recombinant adenoviruses for 1 h as described [Braiman et al., 1999a, 2001b]. The cultures were then washed with DMEM and transferred to growth medium. Twenty-four hours prior to study, cells were transferred to low glucose (4.5 mM), serum-free DMEM containing 1% bovine serum albumin.

RNAi TRANSFECTION

Duplex RNAi primer sequences (20 μM) for PKCδ and control (Stealth RNAi, Invitrogen, Carlsbad, CA) were transfected into AML-12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The RNA sequences (5′–3′) transfected were as follows:

RNAi control—CCC UCC GCG UAC CUC UUC ACU UAU U
RNAi PKCδ—CCC UUC CUG CGC AUC UCC UUC AAU U

PKC ACTIVITY ASSAY

Activity of specific PKC isoenzymes was determined as described [Braiman et al., 1999b, 2001b; Rosenzweig et al., 2002]. Briefly, PKCα and PKCδ immunoprecipitates were obtained from freshly prepared whole-cell lysates of untreated or insulin-treated AML-12 cultures. These lysates were prepared in RIPA buffer without NaF. Activity was measured with the SignaTECT Protein Kinase C Assay System (Promega, Madison, WI: The SignaTECT® Protein Kinase C (PKC) Assay System uses the biotinylated peptide Neurogranin) (AAKIQAS*FRGHMARKK), a specific substrate commercially available for PKC activity. The biotinylated, ^{32}P-labeled substrate is recovered from the reaction mix using the SAM^{2}® Biotin Capture Membrane, which is a novel streptavidin matrix. A reaction mixture was prepared that contained PKC biotinylated pseudosubstrate, [γ-^{32}P]ATP (10 μCi/ml), diacylglycerol, and phospholipids; equal amounts (80–100 μg protein/μl) of immunoprecipitates were aliquoted on SignaTECT membranes. Following incubation for 10 min at 30°C, the reaction was terminated with termination buffer, scintillation fluid was added, and an aliquot was counted on a β-counter for determination of amount of inorganic phosphate formed.

GLUCOSE UPTAKE

Glucose transport was measured in triplicate samples in 6-well plates with the use of [^{3}H]2-deoxy-D-glucose (1 mCi/ml, American Radiolabeled Chemicals, St. Louis, USA), as earlier described [Aga-Mizrachi et al., 2008]. After insulin treatment of cells in growth medium (100 nM for 30–40 min), cells were washed three times with warm (37°C) PBS, the final wash being replaced immediately with 0.75 ml PBS containing 0.5 μCi/ml [^{3}H]2-deoxy-D-glucose and glucose at a concentration of 0.1 mM. Cells were then incubated for 10 min at 37°C, washed three times with cold (4–6°C) PBS and then lysed by addition of 1 ml 0.1% SDS and incubated for 30 min in 37°C. The contents of each well were transferred to counting vials and 3.5 ml scintillation fluid was added to each vial and vortexed. Samples were counted in the ^{3}H window of a Tricarb scintillation counter. Values were normalized to the protein content of each well.

GLYCOGEN SYNTHESIS

The rate of ^{14}C-glucose incorporation into glycogen was studied as a measure of glycogen synthesis, as described [Rosa, 2004; Rosa and Skala, 2005]. After 24 h culture, the medium was removed and the cells were incubated in the same medium containing ^{14}C-glucose without (control) or with insulin. Two hours later, the incubation medium was removed and after three washes with cold saline, hepatocytes were frozen immediately in liquid nitrogen. The cells were digested in 0.2 N NaOH and an aliquot was taken for the determination of glycogen and protein [Lowry et al., 1951] as well as determination of radioactivity in a liquid scintillation counter.

STATISTICAL ANALYSIS

Values are presented as mean ± SEM. Statistical differences between the treatments and controls were tested by unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA). A difference of $P < 0.05$ or less in the mean values was considered statistically significant.

RESULTS

We initially examined the levels of various PKC isoforms in AML-12 hepatocytes and in cells from adult rat liver in primary culture. We found that PKC isoforms α, βII, δ, ε, and ζ were readily detected in both cell types, in general agreement with other studies [see Probst et al., 2003; Patel et al., 2004]. It has long been known that insulin stimulates these isoforms in preparations of skeletal muscle and fat cells [Sampson and Cooper, 2006], but effects in liver cells are inconsistent depending on the cell line studied. We have also reported that in skeletal muscle insulin rapidly upregulates PKCδ and PKCα expression [Horovitz-Fried et al., 2006ab; Brand et al., 2010]. Therefore, in additional preliminary experiments, we examined effects of insulin on protein levels of the various isoforms in hepatocytes and found that only PKCδ was upregulated (Fig. 1). PKCα appeared to be slightly down regulated, less so in AML-12 cells than in primary hepatocytes, while changes in the levels of the remaining isoforms were not detected. Densitometry measurements of protein levels for PKCs α and δ were made on Western blots and the results are shown in Figure 1B,C. A further comparison between

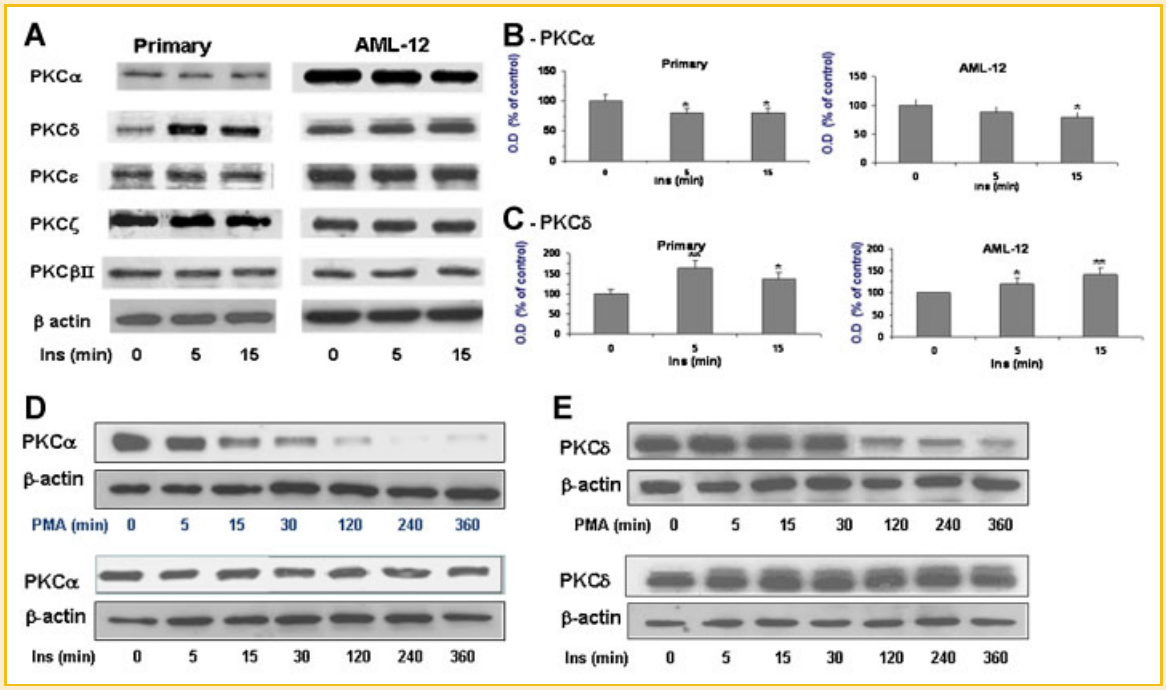

Fig. 1. Effects of insulin on protein levels of PKC isoforms in primary cultures of rat hepatocytes (left) and AML-12 mouse liver cells. Hepatic cells in primary culture and AML-12 mouse liver cells were grown in culture and transferred to low glucose serum-free medium for 18 h, following which insulin (100 nM) was added for the times indicated. Following insulin stimulation, cell lysates were subject to SDS-PAGE and immunoblotting with specific anti-PKC antibodies. A: Representative Western blots of effects of insulin on individual PKC isoforms. B,C: Graphs represent densitometry measurements of Western blots showing effects of insulin on protein levels of PKCα (B) and PKCδ (C). Each bar is the mean ± s.e. of measurements made in three experiments ($^{*}P < 0.05$; $^{**}P < 0.01$ compared to time 0). D,E: Effects of PMA (upper blots) and insulin (lower blots) on PKCα (D) and PKC δ (E) protein levels in AML-12 over a 6-h time period. Each blot is representative of results obtained in three individual experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

effects of insulin on PKCα and PKCδ in AML-12 cells is shown in Figure 1D,E. In this set of experiments PKC protein levels following treatment for up to 6 h with either insulin or the phorbol ester PMA, which down regulates classical (such as α) and novel (such as δ) PKC isoforms. As shown in the upper blots of Figure 1D,E, PMA downregulated both PKCα and PKCδ. In contrast, insulin caused no detectable change in PKCα protein levels but strikingly increased PKCδ levels.

Because of the effects of insulin on levels of PKCα and δ, and because these isoforms were shown to regulate IR signaling in skeletal muscle through interactions with IR and IRS-1 [Braiman et al., 2001b; Rosenzweig et al., 2002; Cipok et al., 2006], we examined the possibility that they may be involved in insulin signaling in hepatocytes.

INSULIN ACTIVATES PKCδ BUT NOT PKCα IN HEPATOCYTES

Previous studies on various models of skeletal muscle in our laboratory have shown that PKCδ and PKCα are stimulated by insulin within 5 min. We, therefore, decided first to examine the effect of insulin on PKCδ and PKCα activities (Fig. 2). PKCδ or PKCα was immunoprecipitated from control and insulin-stimulated hepatocytes, and PKC activity was measured as described in the Materials and Methods Section. As can be seen, insulin induced an increase in PKCδ activity in both AML-12 cells and hepatocytes in primary culture (Fig. 2A,B). In contrast, PKCα activity in both cell types was not increased and appeared to be decreased by insulin stimulation (Fig. 2C,D).

PKC isoforms, when quiescent, are distributed in the cytoplasm and when activated are translocated to the plasma membrane or to membranes of cytoplasmic organelles. In order to verify the difference between PKCα and PKCδ in liver cells by another criterion, we examined and compared the distribution of PKCs δ and α in AML-12 cells following their stimulation by phorbol esters, known activators of classical and novel PKC isoforms, and insulin. Examples of the results obtained are shown in Figure 2D (PKCδ) and Figure 2E (PKCα). As shown, PMA stimulation induced translocation of both PKCδ and PKCα to the plasma membrane (Fig. 2D,E, upper photographs). Insulin, in contrast, induced translocation of PKCδ to cytosolic locations (Fig. 2D, lower photographs; arrows) but had no discernible effect on the distribution of PKCα in hepatocytes (Fig. 2E, lower photographs).

We and others [Braiman et al., 1999b; Rosenzweig et al., 2002; Steinberg, 2004; Cipok et al., 2006] have shown that changes in tyrosine phosphorylation state are associated with activation of various PKC isoforms, including and especially PKCs α and δ. Accordingly, we examined effects of insulin on tyrosine phosphorylation of PKCs α and δ in hepatocytes in primary culture and in AML-12 cells. Following stimulation with insulin, cells were lysed and subjected to immunoprecipitation with specific anti-PKCδ and anti-PKCa antibodies. Following SDS-PAGE, immunoblotting was performed with anti-phosphotyrosine antibodies. As shown in

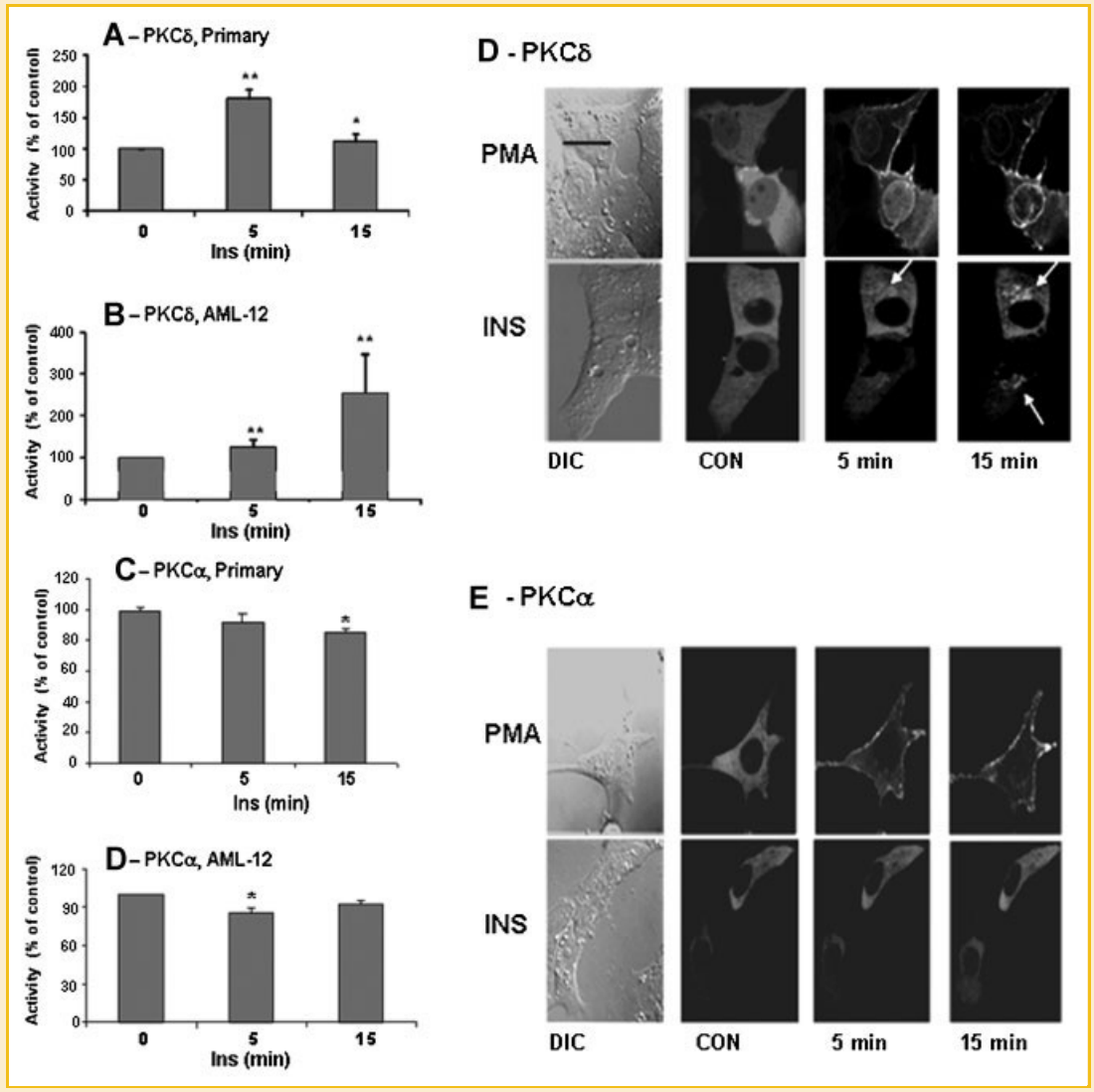

Fig. 2. Insulin increases activity of PKCδ but not PKCα in hepatocytes. Hepatic cells in primary culture and AML-12 mouse liver cells were grown in culture and transferred to low glucose serum-free medium for 18 h as described in the Materials and Methods Section. A–C: Activity of PKCα and δ following treatment with insulin for the times indicated. PKCδ and PKCα were immunoprecipitated from whole cell lysates of hepatocytes in primary culture (A) or AML-12 mouse liver cells (B,C) with specific anti-PKC antibodies. Activity of the immunoprecipitates was measured as described in the Materials and Methods Section. Each bar represents the mean ± s.e. from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$ compared to time 0). D,E: Photomicrographs of GFP-labeled PKC δ (D) and PKCα (E) in control and PMA- or insulin-stimulated AML-12 liver cells. Cells were transfected with GFP-labeled PKC for 16–20 h and then stimulated with either PMA (upper photographs) or insulin (lower photographs). PMA induced accumulation of PKCδ at the plasma and nuclear membranes, and PKCα at the plasma membrane; insulin induced cytoplasmic accumulation of PKCδ (arrows), but had no detectable effect on distribution of PKCα. The results are representative of those obtained in three independent experiments. The calibration line in the upper left-hand photomicrograph in D represents 20 μm and applies to all photomicrographs.

Figure 3A,B, PKCδ appeared to be rapidly phosphorylated on tyrosine in both liver cell preparations. In contrast, the tyrosine phosphorylation state of PKCα was not changed by insulin treatment in either AML-12 cells (Fig. 3C) or primary hepatocytes (not shown). As we earlier showed that insulin induces a rapid (within 5–10 min) increase in PKCδ protein levels (Fig. 1) as occurs in skeletal muscle [Horovitz-Fried et al., 2006ab], this might account for the increased amount of tyrosine phosphorylated PKCδ, even though equal amounts of immunoprecipitated PKCδ were loaded for SDS/PAGE. Therefore, in order to verify that insulin does indeed induce tyrosine phosphorylation of PKCδ, we performed Western blotting with site-specific phosphotyrosine PKCδ antibodies. Examples of results obtained with antibodies specific for tyrosine sites 332, 187, and 311 are shown in Figure 3D and quantified Figure 3E. While the kinetics of insulin-induced phosphorylation differed among the three tyrosine sites, the results clearly show that the phosphorylation state of each of the three tyrosine sites was significantly increased in response to insulin. The effect of insulin began as early as 5 min after addition and continued for at least 30 min (the longest time examined).

INSULIN INCREASES ASSOCIATION BETWEEN IR AND PKCδ

One of the earliest effects of insulin on PKCδ in skeletal muscle is the rapid induction of its physical association with insulin receptor [IR;

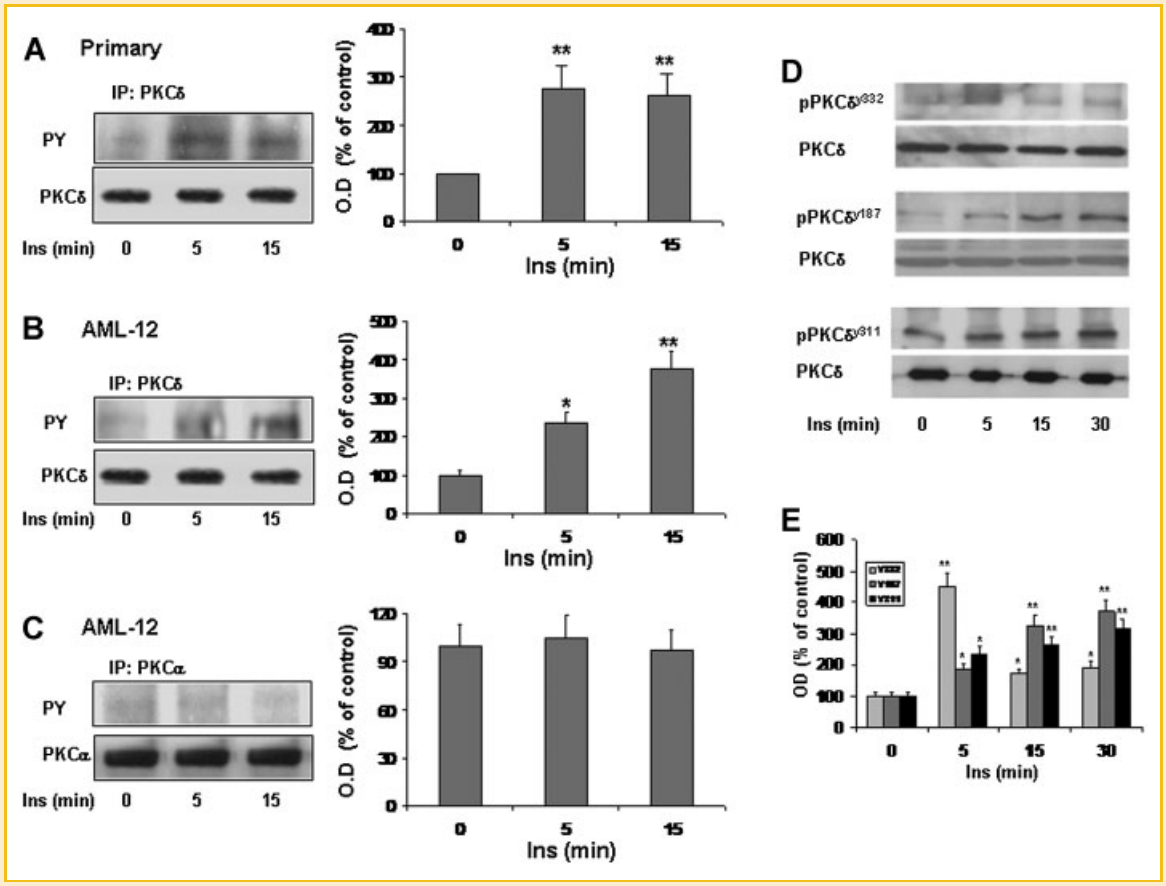

Fig. 3. Insulin induces rapid tyrosine phosphorylation of PKCδ but not PKCα in hepatic cells. Cells were grown in culture and stimulated with insulin for the times indicated, as in Figure 1. A–C: Following insulin stimulation, cell lysates were immunoprecipitated with specific anti-PKCδ (A,B) or PKCα (C) antibodies. The immunoprecipitates were subject to SDS–PAGE and immunoblotting with specific phosphotyrosine antibodies. The graphs represent densitometry measurements of Western blots. Each bar is the mean ± s.e. of measurements made in three experiments ($^{*}P < 0.05$; $^{**}P < 0.01$ compared to time 0). D: Insulin rapidly phosphorylates PKCδ on specific tyrosine sites. Following insulin stimulation, cell lysates were subject to SDS–PAGE and immunoblotting with site-specific anti-phosphotyrosine PKCδ and anti PKCδ antibodies. The graphs represent densitometry measurements of Western blots. Each bar is the mean ± s.e. of measurements made in three experiments. Measurements were normalized to those of PKCδ ($^{*}P < 0.05$; $^{**}P < 0.01$ compared to time 0).

Braiman et al., 2001b]. We investigated the interaction between PKCδ and IR in AML-12 mouse liver cells in two ways. In one, PKCδ was immunoprecipitated from lysates of control and insulin-stimulated AML-12 cells. Following SDS–PAGE and transfer, the immunoprecipitated PKCδ was subjected to Western blotting with anti-IR and anti-PKCδ antibodies. In the second, IR was immunoprecipitated from lysates of control and insulin-stimulated AML-12 cells. Following SDS–PAGE and transfer, the immunoprecipitated IR was subjected to Western blotting with anti-PKCδ and anti-IR antibodies. As can be seen in Figure 4A,C, PKCδ and IR are constitutively associated with one another, and this association is increased by stimulation with insulin. The quantitative data from at least three separate experiments on each preparation are shown in the densitometry graphs (B and D). In contrast, we were unable to detect association of PKCα with either IR or IRS-1 (as occurs in skeletal muscle—see [Rosenzweig et al., 2002; Cipok et al., 2006]) in either the basal or insulin-stimulated state (not shown).

The findings presented so far suggest that PKCδ, but not PKCα, may be involved in insulin signaling in hepatocytes. Accordingly, in order to investigate this further, we compared effects of inhibition or overexpression of PKCδ with those of PKCα on the insulin-induced signaling cascade. Inhibition of PKCδ was achieved by knock-down with small RNAi and overexpression of kinase dead, dominant negative PKCδ utilizing adenovirus constructs [see Braiman et al., 1999a, 2001b]. In addition, in some experiments on primary hepatocytes, which are not readily receptive to transfection or infection, we treated the cells with rottlerin. Whereas rottlerin has been shown to have a number of non-PKCδ-related effects [see Soltoff, 2001], it has been used to confirm the possible role of this isoform in several signaling pathways [Pieper et al., 1999; Braiman et al., 1999a; Caruso et al., 2001; Kayali et al., 2002; Khamaisi et al., 2009; di Giacomo et al., 2010; Bhavanasi et al., 2011; Chew et al., 2011; Kanno and Nishizaki, 2011]. Rottlerin reportedly blocks PKCδ at a concentration of 3–6 μM, one-tenth of that required to block the other PKC isoforms (50–100 μM) in cultured skeletal muscle [Braiman et al., 1999a]. Thus, whereas effects of rottlerin may not necessarily be conclusive for a specific role of PKCδ in various signaling processes, the

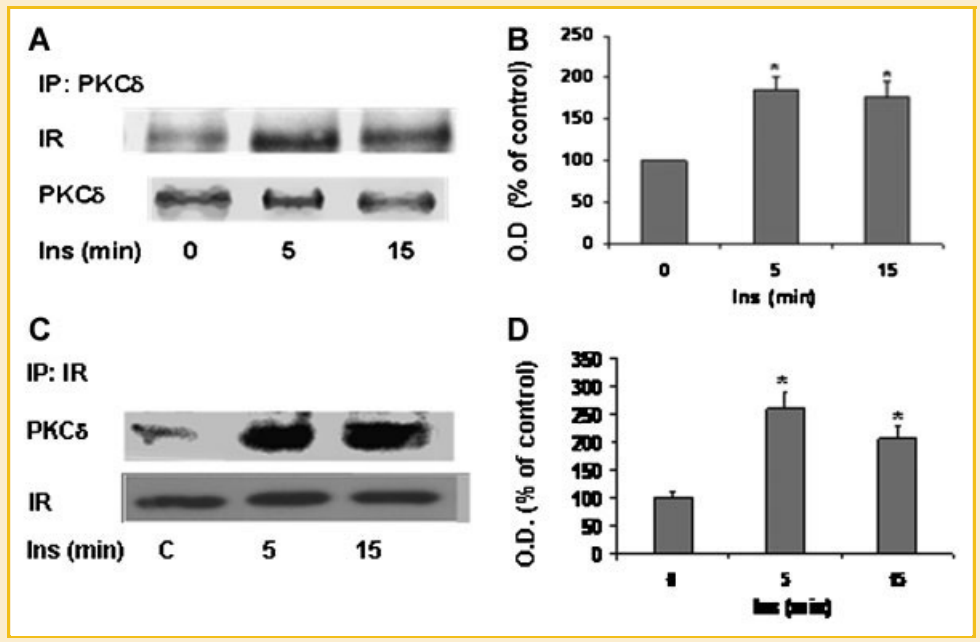

Fig. 4. Insulin increases IR-PKCδ association. AML-12 liver cells were grown as described in Figure 1. Whole cell lysates were immunoprecipitated with specific anti-PKCδ (A) or anti-IR (C) antibodies and the immunoprecipitates subjected to SDS–PAGE and immunoblotting with specific anti-IR and anti-PKCδ antibodies. The graphs (B,D) represent OD measurements of the Western blots. Each bar is the mean ± s.e of measurements from three independent experiments ($^{*}P < 0.05$ compared to time 0).

findings obtained with this pharmacologic blocker may be considered supportive.

INHIBITION OF PKCδ REDUCES INSULIN-INDUCED PKB PHOSPHORYLATION

PKB/Akt is a major enzyme in the insulin signaling cascade and in response to insulin is activated via phosphorylation on threonine and serine residues. We next compared the potential role of PKCδ in insulin-induced phosphorylation of PKB/Akt. In one series of experiments, PKCδ was inhibited by knock down following transfection of AML-12 cells with RNAiδ (Fig. 5A). RNAiδ had no detectable effects on levels of PKCα, PKCβII, PKCε, or PKCζ. Figure 5B,C shows that insulin-stimulated increase in Thr308 phosphorylation of PKB was completely abrogated in PKCδ-knock down cells; similar results were obtained with inhibition by pre-treatment with rottlerin (not shown). In contrast, the insulin-stimulated increase in Ser473 phosphorylation was not reduced by inhibition of PKCδ using PKCδRNAi (Fig. 5D,E).

INHIBITION OF PKCδ REDUCES INSULIN-INDUCED PHOSPHORYLATION OF GSK3

The immediate downstream target of PKB is GSK3, which inhibits glycogen synthase. Inhibition of GSK3 is mediated by phosphorylation of Ser9, and this inhibition thus leads to activation of glycogen synthase. We next examined the possible involvement PKCδ in insulin-induced GSK phosphorylation. We utilized specific phosphoGSK3 antibodies to assess the role of this isoforms in insulin effects, and the results are shown in Figure 5F,G. Inhibition of PKCδ by knock down with RNAiδ, nearly completely abrogated insulin-induced phosphorylation of $GSK3^{ser9}$.

PKCα DOES NOT AFFECT INSULIN-INDUCED SIGNALING TO PKB AND GSK3

Although our initial studies did not provide any indication that PKCα might be involved in insulin-induced signaling, it was important to examine this possibility in more detail. Accordingly, we overexpressed both wild-type and kinase inactive (dominant negative) PKCα in hepatocytes and studied their effects on insulin-induced phosphorylation of PKB and GSK3. The results are summarized in Figures 6 and 7. It was earlier shown that GO6976 a relatively selective inhibitor of the classical and novel PKC isoforms α and β1 [Wenzel-Seifert et al., 1994] selectively inhibits PKCα in primary cultures of rat skeletal muscle and mouse skeletal muscle in vivo [Rosenzweig et al., 2002; Cipok et al., 2006]. Since PKCβ1 is not detected in AML-12 cells, GO6976 may be regarded as selective against PKCα in this system. Therefore, we also treated AML-12 cells with GO6976 (10 nM for 20 min) prior to addition of insulin. As can be seen, neither overexpression of WTPKCα (Fig. 6A,B) nor inhibition of PKCα by treatment with GO6976 (Fig. 6C,D) appeared to alter insulin-induced phosphorylation of PKB/Akt on Ser473 or Thr308. Similarly, neither overexpression of PKCα nor inhibition by overexpression of DNPKCα, nor pre-treatment with GO6976 had any detectable effect on phosphorylation of $GSK3^{ser9}$ in response to insulin (Fig. 7).

PKCδ REGULATES INSULIN-INDUCED GLUCOSE UPTAKE

An essential component of hepatic glucose regulation by insulin is stimulation of glucose uptake into liver cells. We have already shown that PKCδ plays an important role in insulin-induced glucose uptake by skeletal muscle [Braiman et al., 1999ab]. We next examined the possible role of PKCδ in hepatic glucose uptake. To

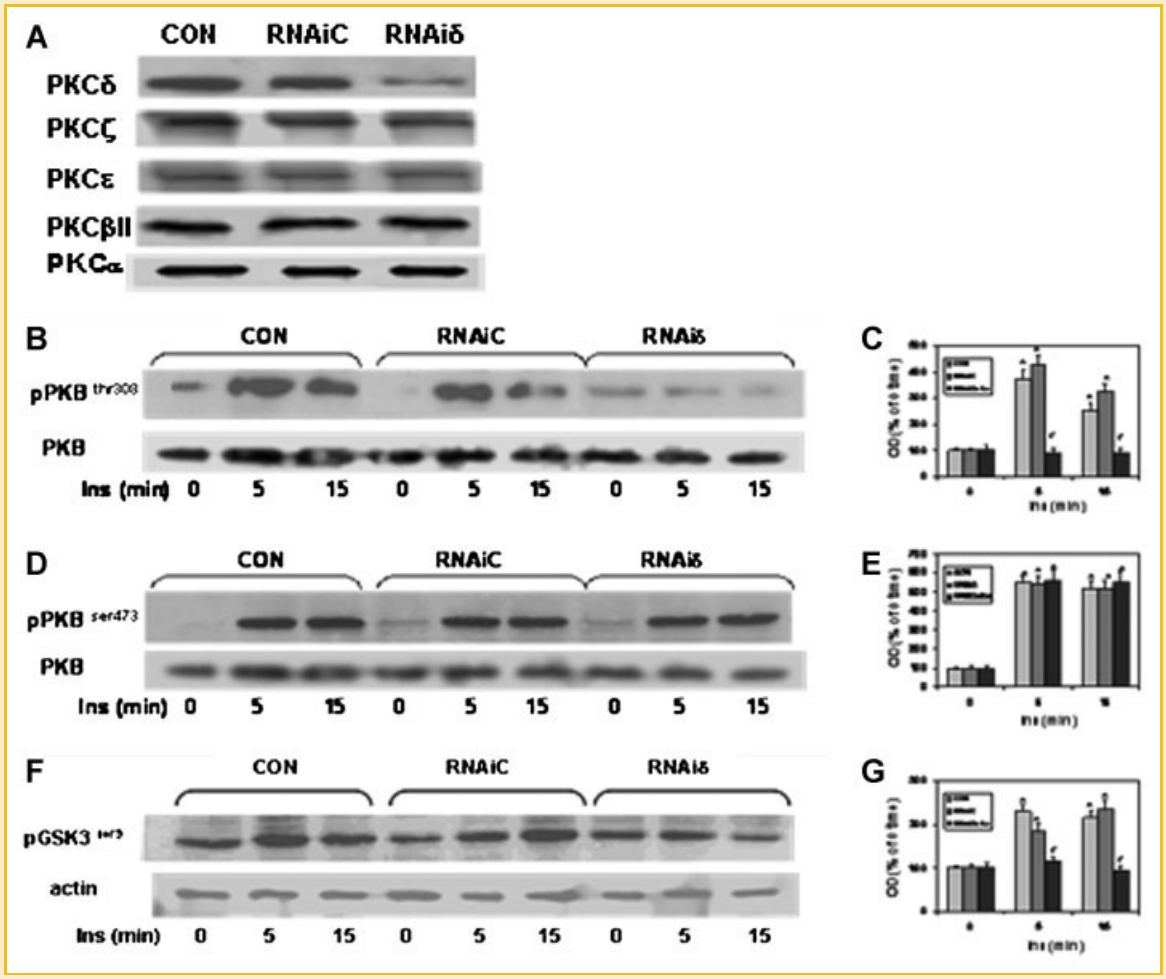

Fig. 5. Inhibition of PKCδ abrogates insulin-induced threonine phosphorylation of PKB/Akt and serine phosphorylation of GSK3. AML-12 liver cells were grown and treated as described in Figure 1. Cells were transfected with scrambled control RNA (RNAiC) or small inhibitory/interference PKCδ RNA (RNAiδ) 16–20 h prior to stimulation with insulin. The levels of PKCδ and other PKC isoforms in control, RNAIC-, and RNAIδ-transfected cells are shown in A. Whole cell lysates were subjected to SDS–PAGE and immunoblotting with specific anti-phosphoPKB^{thre408} (B) or anti-phosphoPKB^{ser423} (D) and anti-PKB antibodies, or specific anti-phosphoGSK^{ser9} (F) and anti-actin antibodies The graphs (C,E,G) represent OD measurements of the corresponding Western blots. Each bar is the mean ± s.e of measurements from three independent experiments ($^{**}P < 0.01$, $^{\#}$not significant, compared to time 0).

accomplish this, we transfected AML-12 cells with either control RNAi sequence or with RNAi PKCδ sequence. Cells were transferred to serum-free, low glucose medium and subsequently stimulated or not with insulin for 40 min. The uptake of [^{3}H]2-deoxyglucose (2-DG) into whole cell cultures in response to insulin was determined as described in the Materials and Methods Section. As shown in Figure 8A, the insulin-induced increase in glucose uptake by cells transfected with PKCδRNAi was significantly lower than that in cells transfected with RNAiC. In another set of experiments, we overexpressed WT PKCδ in AML-12 cells utilizing adenovirus containing PKCδ cDNA, and 24 h later the uptake of 2-DG was studied. As shown in Figure 8B, insulin-stimulated 2-DG uptake in cells overexpressing PKCδ was significantly higher than that in cells expressing the control (empty) vector (C.V.). Consistent with the results regarding PKCα and insulin-induced signaling to PKB and GSK3, insulin-stimulated glucose uptake in cells overexpressing either wild-type PKCα (WTPKCα) or kinase dead, dominant negative PKCα (DNPKCα) was unchanged from that in cells expressing the control vector (Fig. 8C,D).

INHIBITION OF PKCδ REDUCES INSULIN-INDUCED GLYCOGENESIS

A major mechanism by which blood glucose levels are regulated by insulin is the induction of hepatic glycogenesis. To determine if PKCδ might be involved in this phenomenon, we next performed experiments on ^{14}C-glucose incorporation into glycogen as a measure of glycogen synthesis by hepatocytes in primary culture derived from adult rat liver (see the Materials and Methods Section). Because these cells are not readily amenable to transfection or adenovirus infection, they were treated with 5 μM Rottlerin for 7 min and then incubated in a medium containing ^{14}C-glucose without (control) or with insulin for 2 h. The cells were digested and an aliquot was taken for the determination of glycogen and protein as well as determination of radioactivity in a liquid scintillation counter. The results (Fig. 8E) show that treatment rottlerin Cδ

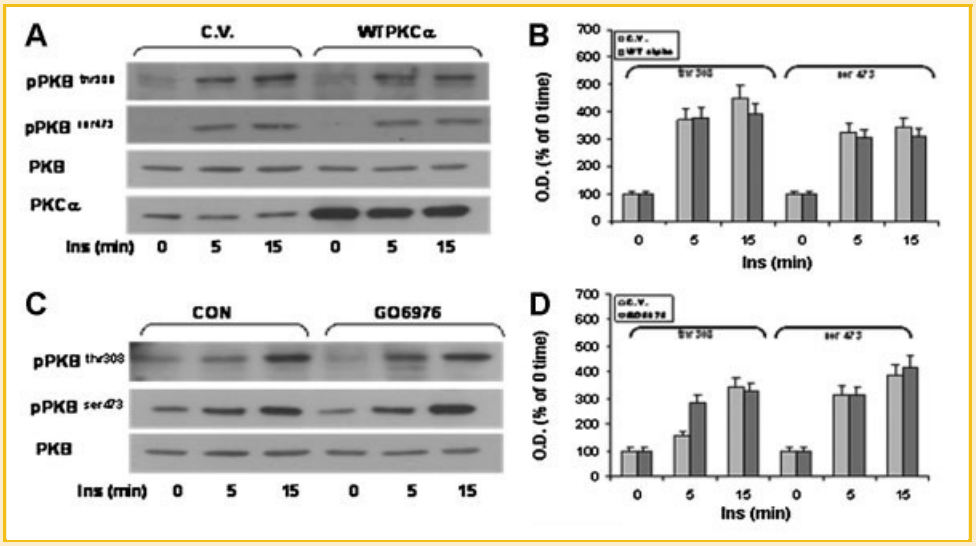

Fig. 6. PKCα is not involved in insulin-induced phosphorylation of PKB. AML-12 liver cells were grown and treated as described in Figure 1. A,B: Overexpression of PKCα does not affect insulin-induced phosphorylation of PKB. Cells were infected with adenovirus constructs of wild-type (WT) PKCα 16–20 h prior to stimulation with insulin. C,D: Inhibition of PKCα by GO6976 does not alter insulin-induced phosphorylation of PKB. Cells were pre-treated with GO6976 (20 mM; 20 min) prior to stimulation with insulin. At the designated times, whole cell lysates were subjected to SDS–PAGE and immunoblotted with specific anti-phosphoPKB^{thre408} or (C,D) anti-phosphoPKB^{ser423} and anti-PKB and anti-PKCα antibodies. The graphs in B and D represent OD measurements of the Western blots. Each bar is the mean ± s.e. of measurements from three independent experiments. Neither overexpression nor inhibition of PKCα affected insulin-induced PKB phosphorylation.

significantly reduces basal glycogenesis and nearly completely abrogates the effect of insulin.

DISCUSSION

In this study we have shown that PKCδ plays an integral role in insulin regulation of glucose uptake and glycogenesis in hepatic cells, whereas PKCα appears not to be involved. We found that insulin rapidly increases kinase activity of PKCδ, but not PKCα, and this effect is accompanied by elevation in its tyrosine phosphorylation state. The differences in time course of PKCδ activation between primary rat hepatocytes and AML-12 cells could reflect both species variation and differences between cell lines and primary cultures, as seen in skeletal muscle [see, e.g., Horovitz-Fried et al., 2006a]. Moreover, whereas PMA causes translocation of both PKCα and δ to the plasma membrane, insulin has no detectable effect on location of PKCα but induces PKCδ appearance in intracytoplasmic locations. It is long recognized that stimulation of PKC isoforms need not occur exclusively by translocation to the plasma membrane. Activation of certain PKC isoenzymes can be detected, in addition by tyrosine phosphorylation [Steinberg, 2004], by translocation to the nuclear zone, as well as by association with cytoskeletal components [see Quest, 1996. In addition, clear translocation of PKCδ to mitochondria by insulin has been shown [Caruso et al., 2001]. Thus, the intracellular appearance of insulin-stimulated PKCδ could result from these phenomena as well as, perhaps, by the rapid increase in PKCδ protein levels that occur in response to insulin [Horovitz-Fried et al., 2006ab; Brand et al., 2010].

In addition, we found that insulin induces a rapid association between IR and PKCδ and appears to be involved in IR tyrosine phosphorylation. In these respects, insulin stimulation of PKCδ in hepatocytes strongly resembles that in a number of preparations of skeletal muscle as we have shown repeatedly [Braiman et al., 1999a, 2001b; Rosenzweig et al., 2002; Heled et al., 2003; Jacob et al., 2010]. Moreover, utilizing pharmacological, adenovirus and sRNAi techniques to overexpress or reduce PKCδ activity, we could show that PKCδ is important in mediation of insulin-induced activation of PKB/Act and phosphorylation of GSK-3. Finally, blockade of PKCδ nearly completely abrogated insulin-induced glucose uptake and glycogenesis by hepatocytes. For pharmacologic blockade of PKCδ in primary hepatocytes, we treated cells with rottlerin because we were not able to successfully utilize sRNAi and adenovirus techniques in these cells. Whereas rottlerin has been shown to have effects to directly uncouple mitochondrial respiration from oxidative phosphorylation, it has been used in several studies to demonstrate involvement of PKCδ in certain signaling pathways [see Pieper et al., 1999; Braiman et al., 1999a; Caruso et al., 2001; Kayali et al., 2002; Khamaisi et al., 2009; di Giacomo et al., 2010; Bhavanasi et al., 2011; Chew et al., 2011; Kanno and Nishizaki, 2011]. Moreover, the results we obtained with rottlerin were compatible with those we obtained utilizing the more selective approaches to inhibit PKCδ. Taken together, these results strongly indicate that PKCδ is a positive regulator of insulin signaling in hepatocytes. Utilizing similar pharmacological and adenoviral expression approaches with PKCα, we found no evidence whatsoever for any role in insulin signaling to glucose uptake and glycogen synthesis in liver cells.

We used two liver cell model systems—epatocytes in primary culture derived from adult rat liver and the AML-12 mouse liver cell line. The AML-12 cell line is one of several non-cancer liver cell lines and may provide a more reliable model of normal liver cells

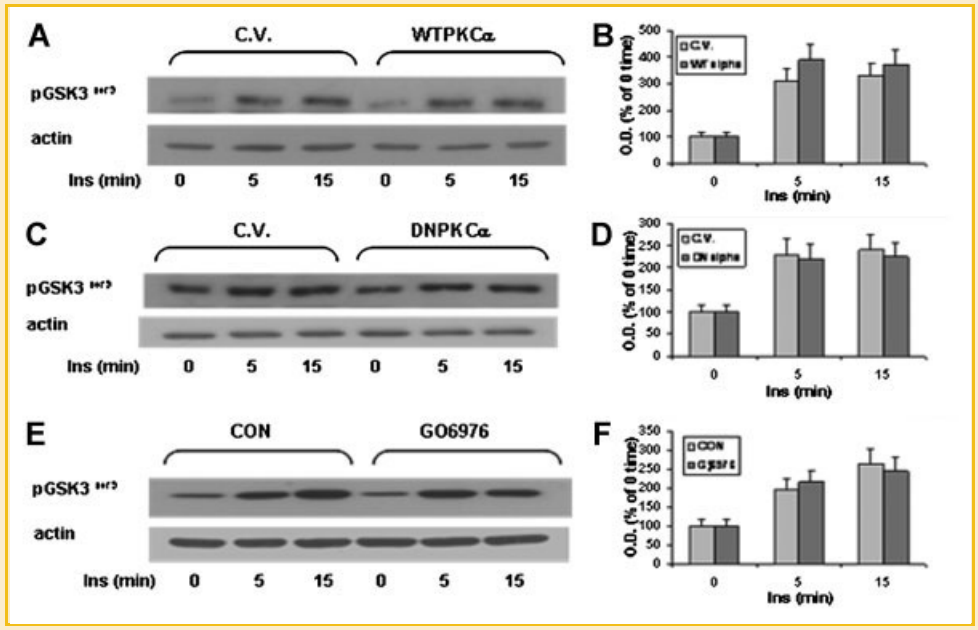

Fig. 7. PKCα is not involved in insulin-induced phosphorylation of GSK3. AML-12 liver cells were grown and treated as described in Figure 1. Following treatment with insulin for the times indicated, whole cell lysates were subjected to SDS–PAGE and immunoblotted with specific anti-phosphoGSK^{ser9} and anti-actin antibodies. overexpression of (A,B) wild-type (WT) or (C,D) kinase dead (DN) PKCα does not alter insulin-induced phosphorylation of GSK^{ser9}. Cells were infected with adenovirus constructs of wild-type WTPKCα (A,B) or DN PKCα (C,D) 16–20 h prior to stimulation with insulin as in Figure 6. E,F: Inhibition of PKCα by GO6976 does not alter insulin-induced phosphorylation of GSK^{ser9}. Cells were pre-treated with GO6976 (20 mM; 20 min) prior to stimulation with insulin. At the designated times, whole cell lysates were subjected to SDS–PAGE and immunoblotted with specific anti-phospho GSK^{ser9} or anti-actin antibodies. The Western blots are representative of results obtained in three independent experiments. The graphs in B, D, and F represent OD measurements of the Western blots. Each bar is the mean ± s.e. of measurements from three independent experiments. Neither overexpression nor or inhibition of PKCα affected insulin-induced GSK^{ser9} phosphorylation.

than do various hepatoma cell lines. In every read-out studied, results on the two model systems were similar, if not identical, to one another. These factors would appear to validate the assumption that the cells used in this study reflect fairly accurately physiological properties of liver cells in vivo.

The findings in this study present an apparent paradox. On the one hand, PKCδ is a serine–threonine kinase which should act to down regulate IR signaling, whereas, on the other hand, PKCδ acts to upregulate IR signaling. This is a situation similar to that in skeletal muscle, in which it was shown that effects of PKCδ involve its interaction with Src tyrosine kinase [Rosenzweig et al., 2004; Jacob et al., 2010]. Src appears to be involved in tyrosine phosphorylation of both IR and PKCδ, and is itself stimulated by insulin. Whether or not a similar situation occurs in hepatic cells remains to be determined. Src was also shown to mediate insulin-induced tyrosine phosphorylation of PKCα in skeletal muscle [Cipok et al., 2006], but we were unable to detect an increase in tyrosine phosphorylation of PKCα in response to insulin.

It was earlier reported [Probst et al., 2003] that insulin did not stimulate PKCδ (or α, ε, or ζ isoforms) in preparations of adult rat hepatocytes. Whereas the results regarding PKCα are in agreement with our findings, those regarding PKCδ contrast sharply with the results we report here. One possible reason for the difference between the two studies might be that Probst et al. relied on the induction of translocation of PKC isoforms by insulin from the cytosol to the plasma membrane as a measure of activation. As pointed out earlier, translocation of PKCδ to the plasma membrane is not the sole or even major indication of its activation [see Quest, 1996; Steinberg, 2004]. Activation of PKCδ can be identified by other means, such as translocation to other cytoplasmic organelles or phosphorylation on tyrosine residues [Steinberg, 2004; our results]. In this respect, Caruso et al. [2001] showed clear translocation of PKCδ to mitochondria induced by insulin. Moreover, direct measurements of activity in immunoprecipitated PKCδ were not reported by Probst et al. The importance of PKCδ in insulin-induced pyruvate dehydrogenase complex activity in liver cells was conclusively demonstrated [Caruso et al., 2001], as well as in other diabetes-related effects on liver cells [Chen et al., 2006].

The precise mechanisms by which PKCδ is involved in insulin-induced glucose regulation in hepatocytes are not entirely clear. On the one hand, our findings that insulin induces a rapid association between PKCδ and IR would indicate that PKCδ, perhaps together with Src tyrosine kinase, may act upstream and influence the tyrosine phosphorylation state and activity of IR, as described for skeletal muscle cells [Braiman et al., 2001b; Rosenzweig et al., 2004]. This notion would appear to be supported by the findings that PKCδ appears to be a major player in regulation of glucose uptake in skeletal muscle, which expresses GLUT4 as well as liver cells, which express GLUT2 as well as several other transporters [Levitsky et al., 1994; Metzger et al., 2004; Takanaga et al., 2008]. It has been shown that overexpression of PKCδ increased insulin-stimulated glucose uptake in NIH3T3 cells, apparently by increasing recruitment of GLUT1 to the plasma membrane [Cooper et al., 1999]. These various findings are consistent with a potential upstream action of PKCδ. On

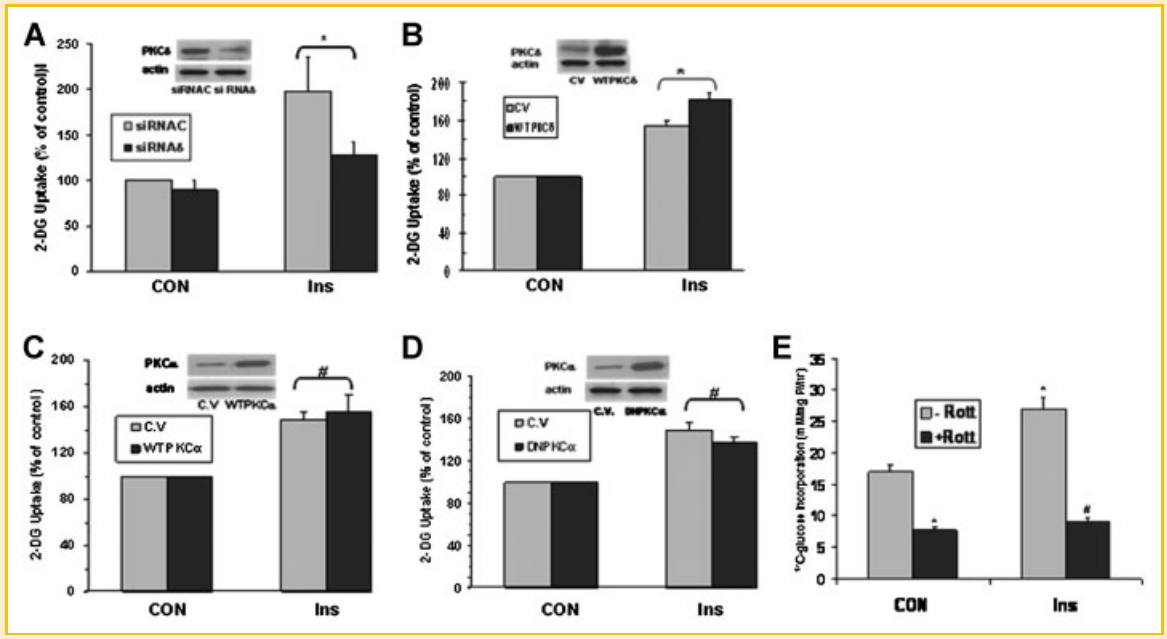

Fig. 8. PKCδ, but not PKCα, is involved in insulin induced increase in glucose uptake and glycogen synthesis by hepatic cells. AML-12 mouse liver cells (A–D) or hepatic cells in primary culture (E) were grown in culture and transferred to low glucose serum-free medium for 18 h. Uptake of 2-deoxyglucose was determined as described in the Materials and Methods Section. A: Inhibition of PKCδ reduces insulin stimulation of 2-deoxyglucose uptake in AML-12 liver cells. AML-12 liver cells were transfected with scrambled control RNA (siRNAC) or small inhibitory PKCδ RNA (siRNAδ) 16 h prior to stimulation with insulin as described in the Materials and Methods Section. B: Overexpression of WT PKCδ increases insulin-stimulated 2-DG glucose uptake in AML-12 liver cells. AML-12 liver cells were infected with adenovirus constructs of WT PKCδ 16–20 h prior to stimulation with insulin. C,D: Overexpression or inhibition of PKCα does not alter insulin stimulation of glucose uptake in hepatic cells. AML-12 liver cells were infected with adenovirus constructs of (C) wild-type (WT) PKCα or (D) kinase dead, dominant negative (DN) PKCα 16–20 h prior to stimulation with insulin as described above. Uptake of 2-deoxyglucose was determined as described in the Materials and Methods Section. In each graph, the bars represent the mean ± s.e. of triplicate measurements in at least three independent experiments ($^{*}P < 0.05$; $^{\#}$not significant). E: Inhibition of PKCδ abrogates insulin-induced glycogen synthesis in hepatic cells in primary culture. Hepatocytes were treated with 5 mM Rottlerin for 7 min and then stimulated with insulin as in Figure 1. ^{14}C-incorporation into glycogen was measured as described in the Materials and Methods Section. The bars represent the mean ± s.e. of triplicate measurements in at least three independent experiments ($^{*}P < 0.05$; $^{\#}$not significant compared to untreated cells).

the other hand, the expression of several GLUT transporters in liver cells leaves open the possibility that one or more might be an appropriate target for activated PKCδ, as GLUT4 is for PKCζ [see Bandyopadhyay et al., 1997; Braiman et al., 2001a]. This requires additional study. Another possible mechanism by which PKCδ may positively influence insulin signaling might be via regulation of PKB through interaction with PDK1. It was reported that that insulin-activated PKCδ interacts with PDK1 to regulate PKB in skeletal muscle [Brand et al., 2006]. PKB has been shown to regulate GLUT4 in skeletal muscle cells [Wang et al., 1999], and it is possible that PKB may also regulate glucose uptake by the GLUT2 transporter in liver cells. This point needs further study.

As mentioned earlier, our findings demonstrate that insulin activates PKCδ and further indicate that this isoform plays a positive regulatory role in hepatic cells, similar to that proposed for skeletal muscle [Braiman et al., 1999ab, 2001b]. This stands in contradiction to results of a recent study on mice [Bezy et al., 2011] in which PKCδ was selectively knocked out either globally or in liver cells, or was overexpressed in the liver. PKCδKO mice (either global or liver-specific) displayed increased hepatic insulin signaling and reduced expression of gluconeogenic and lipogenic enzymes. This resulted in increased insulin-induced suppression of hepatic gluconeogenesis, improved glucose tolerance, and reduced hepatosteatosis with aging. In contrast, mice with liver-specific overexpression of PKCδ developed hepatic insulin resistance characterized by decreased insulin signaling, enhanced lipogenic gene expression, and hepatosteatosis. We have no clear, unequivocal explanation for the different proposed roles for PKCδ. It might be argued that studies in vivo provide the best evidence to understand the various functions of PKCδ. On the other hand, there are certain questions that should be resolved. One relates to the possibility that recently identified PKCδ splice variants [Ueyama et al., 2000; Sakurai et al., 2001; Apostolatos et al., 2010] may have important influences on overall biological effects. Thus, it is not certain that deletion of the PKCδ gene may knock out all the potential splice variants. In addition, there is the possibility that knock out or overexpression of PKCδ might alter expression of other PKC isoforms in liver as well as in other tissues such as fat cells, particularly in animals on a high fat diet. One should also recall that selective deletion of the P85 subunit of PI3 kinase, considered a positive element in insulin regulation of blood glucose, resulted in hypoglycemia rather than expected hyperglycemia presumably due to the involvement of regulatory subunit splice variants [Terauchi et al., 1999; Fruman et al., 2000; Mauvais-Jarvis et al., 2002]. The potential physiological role of splice variants of the PKCδ gene on insulin signaling mechanisms remains to be determined.

A clear role for PKCα in regulation of insulin signaling in hepatocytes has not been described, although this is not the case for

other insulin-sensitive tissues [see Sampson and Cooper, 2006]. The few studies that have been reported are in accord with our results that did not show any effect of either overexpression or blockade of PKCα on insulin-induced glucose uptake and glycogenesis. Although pharmacologic blockade of PKCα by GO6976 may not be selective, the lack of effect of this agent supports the additional evidence for non-involvement of PKCα in insulin-induced glucose transport provided by experiments in which this isoform was either overexpressed or blocked by utilization of appropriate adenovirus constructs. GO6976 can also inhibit PKCβ1 [Martiny-Baron et al., 1993], and insulin was shown to regulate alternative splicing of PKCβ11 in HepG2 (liver) cells [Patel et al., 2004]. However, we did not detect an effect of GO6976 on insulin-induced effects in either AML-12 or primary hepatocytes. Thus, PKCβ appears not be involved in insulin signaling in the model systems we used. This could reflect differences between non-malignant (AML-12, primary hepatocytes) and malignant cell models (HepG2), a possibility that requires additional study. Alternatively, PKCβII could be involved in other effects of insulin; PKCα was shown to mediate H_2O_2-induced necrosis in cultured hepatocytes [Saberi et al., 2008].

In conclusion, we have presented evidence that PKCδ is a positive regulator of insulin-induced glucose uptake and glycogen synthesis in hepatocytes similar to its role in skeletal muscle. In contrast, no role whatsoever could be identified for PKCα in these insulin-induced effects in hepatocytes, contrary to its involvement in skeletal muscle cells. Thus, on the one hand, this study contributes additional information regarding certain PKC isoforms in insulin signaling. On the other hand, the findings indicate that particular roles for each of the isoforms are likely to be tissue specific.

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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