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Activation and Mitochondrial Translocation of Protein Kinase Cδ Are Necessary for Insulin Stimulation of Pyruvate Dehydrogenase Complex Activity in Muscle and Liver Cells*

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Matilde Caruso‡, Maria Alessandra Maitan‡§, Giuseppe Bifulco¶ Claudia Miele, Giovanni Vigliotta§, Francesco Oriente, Pietro Formisano, and Francesco Beguinot‖

From the Dipartimento di Biologia e Patologia Cellulare e Molecolare and Centro di Endocrinologia ed Oncologia Sperimentale del CNR, Federico II University of Naples, 80131 Naples, Italy

In L6 skeletal muscle cells and immortalized hepatocytes, insulin induced a 2-fold increase in the activity of the pyruvate dehydrogenase (PDH) complex. This effect was almost completely blocked by the protein kinase C (PKC) δ inhibitor Rottlerin and by PKCδ antisense oligonucleotides. At variance, overexpression of wild-type PKCδ or of an active PKCδ mutant induced PDH complex activity in both L6 and liver cells. Insulin stimulation of the activity of the PDH complex was accompanied by a 2.5-fold increase in PDH phosphatases 1 and 2 (PDP1/2) activity with no change in the activity of PDH kinase. PKCδ antisense blocked insulin activation of PDP1/2, the same as with PDH. In insulin-exposed cells, PDP1/2 activation was paralleled by activation and mitochondrial translocation of PKCδ, as revealed by cell subfractionation and confocal microscopy studies. The mitochondrial translocation of PKCδ, like its activation, was prevented by Rottlerin. In extracts from insulin-stimulated cells, PKCδ co-precipitated with PDP1/2. PKCδ also bound to PDP1/2 in overlay blots, suggesting that direct PKCδ-PDP interaction may occur *in vivo* as well. In intact cells, insulin exposure determined PDP1/2 phosphorylation, which was specifically prevented by PKCδ antisense. PKCδ also phosphorylated PDP *in vitro*, followed by PDP1/2 activation. Thus, in muscle and liver cells, insulin causes activation and mitochondrial translocation of PKCδ, accompanied by PDP phosphorylation and activation. These events are necessary for insulin activation of the PDH complex in these cells.

Glucose oxidation plays a major role in energy metabolism and survival of eukaryotic cells (1, 2). The first irreversible reaction in glucose oxidation is catalyzed by the pyruvate dehydrogenase (PDH)[1] complex, inside mitochondria (2, 3). In the mitochondria, PDH is present in an active dephosphorylated form and an inactive phosphorylated form (3, 4). *In vivo*, regulation of the PDH complex is largely accomplished by changes in the phosphorylation state and represents a predominant mechanism controlling glucose oxidation (3–5). The PDH complex is inactivated by phosphorylation accomplished by a PDH kinase (4, 6, 7). PDH phosphatases dephosphorylate the PDH complex and reactivate the complex (8, 9). The relative activities of PDH kinase and phosphatase determine the proportion of PDH in the active dephosphorylated form. Insulin has been known to increase PDH activity in tissues, thereby regulating glucose oxidation (9). There is evidence that the acute effect of insulin on PDH depends on insulin activation of PDH phosphatase rather than inactivation of PDH kinase (10). However, the intracellular signaling events involved in insulin regulation of the PDH complex have not been elucidated yet.

The protein kinase C (PKC) family of serine/threonine kinases is involved in intracellular signals that regulate growth and metabolism, differentiation, and apoptosis (11, 12). At least 12 PKC isoforms have been described (12) as follows: (i) conventional PKCs (α, β, and γ), which are dependent on calcium and activated by diacylglycerol and phorbol esters; (ii) novel PKCs (δ, ϵ, θ, and η), which are calcium-independent and activated by diacylglycerol and phorbol esters; and (iii) atypical PKCs (ζ and λ), which are calcium-independent and not activated by diacylglycerol and phorbol esters. Several PKC isoforms have also been reported to be necessary for insulin control of receptor intracellular routing (13), mitogenesis (14), glucose transport (15,16), and glycogen synthesis (17). In addition, there is evidence that insulin-dependent activation of the PDH complex may be mediated by a PKC-dependent pathway (18). Which PKC isoform is necessary for insulin to induce PDH activity, which molecular events lead PKC to activate PDH in the insulin-stimulated cell, and whether other major insulin signaling pathways contribute to insulin stimulation of PDH are unknown.

In the present report, we have addressed these issues in skeletal muscle and liver cells, two major targets of insulin action. We show that insulin specifically induces PKCδ translocation to mitochondria accompanied by phosphorylation of PDH phosphatase and activation of the PDH complex.

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‡ Both authors contributed equally to this work.

§ Recipient of fellowship from the Federazione Italiana per la Ricerca sul Cancro.

¶ Current address: Dept. di Ginecologia, Ostetricia e Fisiopatologia della Riproduzione Umana, Federico II University of Naples Medical School.

‖ To whom correspondence should be addressed: Dept. di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II, Via S. Pansini, 5, 80131 Naples, Italy. Tel.: 39 081 7463248; Fax: 39 081 7463235; E-mail: beguino@unina.it.

[1] The abbreviations used are: PDH, pyruvate dehydrogenase; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; PDK, PDH kinase; PAGE, polyacrylamide gel electrophoresis; PDP, PDH phosphatase.

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EXPERIMENTAL PROCEDURES

Materials—Media, sera, and antibiotics for cell culture, the LipofectAMINE reagent, rabbit polyclonal antibodies toward specific PKC isoforms, and the PKC assay system (catalog number 13161-013) were from Life Technologies, Inc. PDK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies for the PDP1 H-Ala-Ser-Thr-Pro-Gln-Lys-Phe-Tyr-Leu-Thr-Pro-Pro-Gln-Val-Asn-OH and the PDP2 H-Thr-Ser-Thr-Glu-Glu-Glu-Asp-Phe-His-Leu-Gln-Leu-Ser-Pro-Glu-OH sequences were generated by PRIMM S.R.L. (Milan, Italy). The PDH α-subunit peptides H-Tyr-His-Gly-His-Ser-Met-Ser-Asn-Pro-Gly-Val-Ser-Tyr-Arg-OH and H-Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser(P)-Tyr-Arg-OH used for the pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase assays, respectively, have been previously described (19) and were generated by PRIMM S.R.L. (Milan, Italy). The phosphorothioate PKCα, PKCβ, PKCδ, and PKCζ antisense and control oligonucleotides have been described previously (14, 15). The LY379196 inhibitor was a generous gift from Lilly, and PD98059 and V1-2 were purchased, respectively, from ICN Biomedicals INC (Costa Mesa, CA) and DBA (Milan, Italy). Recombinant PKCδ and the PKCδ inhibitor Rottlerin were from Calbiochem. The wild-type and constitutively active PKCδ cDNA constructs have been reported previously (20) and were generously donated from Dr. M. S. Marber (St. Thomas's Hospital, London, UK). The cell-permeant mitochondrion-selective dye Mitotracker (CM-H2-TMRos) and fluorescein- and rhodamine-conjugated antibodies were from Molecular Probes Europe (Leiden, The Netherlands). Protein electrophoresis reagents were from Bio-Rad, and Western blotting and ECL reagents were from Amersham Pharmacia Biotech. All other chemicals were from Sigma.

Cell Cultures, Transfection, and Cell Subfractionation—The L6 and the Hep cell clones expressing wild-type human insulin receptors have been previously characterized and reported and were cultured and differentiated (21). Transient transfection experiments were performed by the LipofectAMINE method according to the manufacturer's instructions (14). Briefly, 50–80% confluent cells were washed twice with Opti-MEM and incubated for 8 h with 12 μg of PKC antisense oligonucleotides or with 5 μg of wild-type or active PKCδ cDNAs in the pCAGGS expression vector (20) and 45 μl of LipofectAMINE. The medium was then replaced with DMEM supplemented with 10% fetal calf serum and cells further incubated for 15 h before being assayed. By using pCAGGS-β-gal as a reporter, transfection efficiency was consistently between 65 and 85%, staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Subcellular fractions were obtained as described (22, 23). Briefly, cells were broken in ice-cold 10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 40 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin. Broken cells were centrifuged at 200 × *g* to pellet nuclei. Supernatants were centrifuged at 10,000 × *g* to pellet the heavy membrane fraction containing mitochondria, and the resulting liquid phase was further centrifuged at 150,000 × *g* to pellet plasma membranes. The last supernatant represented the cytosolic fraction (22). Mitochondria were further purified by resuspending heavy membrane pellets in 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, pH 7.4, 0.1% bovine serum albumin and layering on 30% Percoll, 225 mM mannitol, 1 mM EGTA, 25 mM HEPES, pH 7.4, 0.1% bovine serum albumin. After centrifugation at 95,000 × *g*, mitochondria were recovered from the lower phase (24). Mitochondria were then centrifuged, washed, and resuspended in 50 mM potassium phosphate, pH 7.4, and used in the experiments described below. Purity of the mitochondrial fraction was assessed by assaying succinate dehydrogenase and cytochrome *c* oxidase activities (25, 26). Results showed that >99% activity of these enzymes associated with the mitochondrial fractions and <1% of the total activities with the other fractions. Western blotting analysis for the cell surface markers transferrin receptor and 5′-nucleotidase (22) indicated localization to the plasma membrane fraction only.

Immunofluorescence Staining and Co-localization Studies in L6 and Hep Cells—Double labeling experiments with the cell-permeant mitochondrion-selective dye Mitotracker Red CM-H2-TMRos were performed as specified in the manufacturer's instructions. Briefly, L6 and Hep cells were seeded on uncoated 22-mm coverslips and grown for 2 days in DMEM with 10% fetal calf serum, in a humidified atmosphere of 95% air 5% CO_2 at 37 °C. The cells were then deprived of serum for 18 h and further incubated for 30 min in phosphate-buffered saline, pH 7.0, with 150 nM Mitotracker. Labeled cells were subjected to fixation in 2% formaldehyde in Hanks' salt solution containing 20 mM HEPES, pH 7.0, permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and 1% bovine serum albumin for 5 min and incubated with PKCδ antibodies (0.4 mg/ml) as described previously (27). After treatment with the secondary fluorescein isothiocyanate (FITC)-conjugated antibody (1:250), coverslips were embedded in Moviol and viewed with a Leica confocal microscope.

PDH, PDP, PDK, and PKCδ Activities—The activity of the PDH complex was assayed as release of $^{14}CO_2$ from [1-^{14}C]pyruvic acid according to Seals and Jarrett (28). For these assays, 100-mm cell dishes were incubated for 10 min at 37 °C in DMEM supplemented with 10 mM HEPES, pH 7.4, 0.2% BSA, in the absence or the presence of 100 nM insulin. Cells were then solubilized according to Clot *et al.* (29). The addition of 10 mM NaF and 10 mM dichloroacetic acid to the solubilization buffer inhibited PDH phosphatase and kinase, respectively. Under these conditions, the measured PDH activity was designated basal activity and was attributed to the active form of the PDH complex (29). 50 μl of cell extracts were added to 200 μl of 50 mM Tris-HCl, pH 7.4, 50 μM $CaCl_2$, 50 μM $MgCl_2$ for determining the active form of the PDH complex (active PDH complex, PDH_a). In some experiments, the cells were solubilized in the absence of NaF and dichloroacetic acid, and the extracts (50 μl) were added to 200 μl of 50 mM Tris-HCl, pH 7.4, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$ to assay fully activated PDH complexes (total activity, PDH_t). As reported previously by Clot *et al.* (30), this fully induced activity was very similar to that obtained by preincubating samples with purified PDH phosphatase. The assay was initiated by the addition of 1 mM dithiothreitol, 0.1 mM coenzyme A, 0.25 mM pyruvic acid, [1-^{14}C]pyruvic acid (specific activity 9.8 mCi/mmol), 0.5 mM β-NAD, 0.1 mM L-co-carboxylase (final concentrations). The assay tubes were immediately capped with a rubber stopper through which a plastic well was suspended, containing a small roll of filter paper. After 5 min at 37 °C, the reactions were stopped by injecting 0.4 ml of 3 M H_2SO_4 through the rubber stopper into the reaction mixture. 0.2 ml of 1 M hyamine hydroxide were injected onto the filter paper through the stopper, and $^{14}CO_2$ were collected for 1 h. Radioactivity in the paper rolls was quantitated by scintillation counting. Blank values were obtained by using boiled cell extracts and were subtracted from the corresponding data points. Results were expressed as nanomoles of $^{14}CO_2$/min/mg of extract protein. The absolute values of PDH_a and PDH_t measured in this work were about one-third lower than those reported previously in L6 cells (31). These differences might have been generated by a slight increase in the L6 myotube *versus* myoblast ratio (improved differentiation) of the cultures used for the assays. The amount of insulin stimulation of PDH complex and all other activities described in the present study were very similar to or greater than those reported previously (31).

PDH kinase (PDK) activity was assayed by a modification of the method of Stepp *et al.* (32), using the PDH α-subunit peptide H-Tyr-His-Gly-His-Ser-Met-Ser-Asn-Pro-Gly-Val-Ser-Tyr-Arg-OH as substrate (19). For this assay, mitochondria preparations were obtained from L6 and Hep cells as described above. Upon freezing and thawing followed by ultrasonic irradiation, broken mitochondria preparations (70 μg of proteins) were immunoprecipitated with Sepharose-bound PDK antibodies. Immunoprecipitates were equilibrated in 20 mM potassium phosphate, pH 7.0, 0.1 M KCl, 0.1 mM EDTA, 2 mM dithiothreitol and then further resuspended in 80 μl of a reaction mixture containing 50 μM of the substrate peptide, 20 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM dithiothreitol. Upon equilibration at 30 °C for 30 s, 10 mCi/ml [γ-^{32}P]ATP was added, and samples were further incubated for 20 min at room temperature. 40 μl of the reaction mixtures were spotted on 3MM Whatman paper disks and washed with ice-cold 10% trichloroacetic acid, followed by further washes with ethanol and with diethyl ether. The disks were air-dried, and radioactivity was determined by scintillation counting. Results were expressed as picomoles of phosphate incorporated in the substrate peptide/mg protein/min.

PDH phosphatase (PDP) activity was assayed by quantitating phosphate release from the PDH α-subunit phosphopeptide H-Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser(P)-Tyr-Arg-OH (19), using the Non-radioactive Phosphatase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. For these experiments, PDP was isolated from L6 and Hep cells according to Ref. 33. L6 and Hep cell fractions containing PDP1 or PDP2, respectively, were passed on to Sephadex G-25 spin columns to remove free phosphate, and eluates (70 μg of proteins) were immunoprecipitated with Sepharose-bound PDP1 or PDP2 antibodies. The immunoprecipitates were washed with HNT buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 0.05% Triton X-100) and then incubated for 30 min at 30 °C in a reaction mixture (100 μl) containing 50 μM substrate peptide, 20 mM imidazole buffer, pH 7.0, 50 μM $MgCl_2$, 50 μM $CaCl_2$, 2 mM dithiothreitol, and 120 μg of bovine serum albumin. The reaction was stopped by adding the

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TABLE I
Activity of the PDH complex in L6 and Hep cells

100 nM insulin	L6		Hep	
	−	+	−	+
PDH_a (nmol $[^{14}C]O_2$/min/mg of extract protein)[a]	0.030 ± 0.006[b]	0.058 ± 0.004	0.026 ± 0.005	0.049 ± 0.006
PDH_t (nmol $[^{14}C]O_2$/min/mg of extract protein)[a]	0.080 ± 0.005	0.081 ± 0.005	0.074 ± 0.006	0.073 ± 0.009

[a] Determined as described under "Experimental Procedures."
[b] Values are the means ± S.D. of duplicate determinations in four independent experiments.

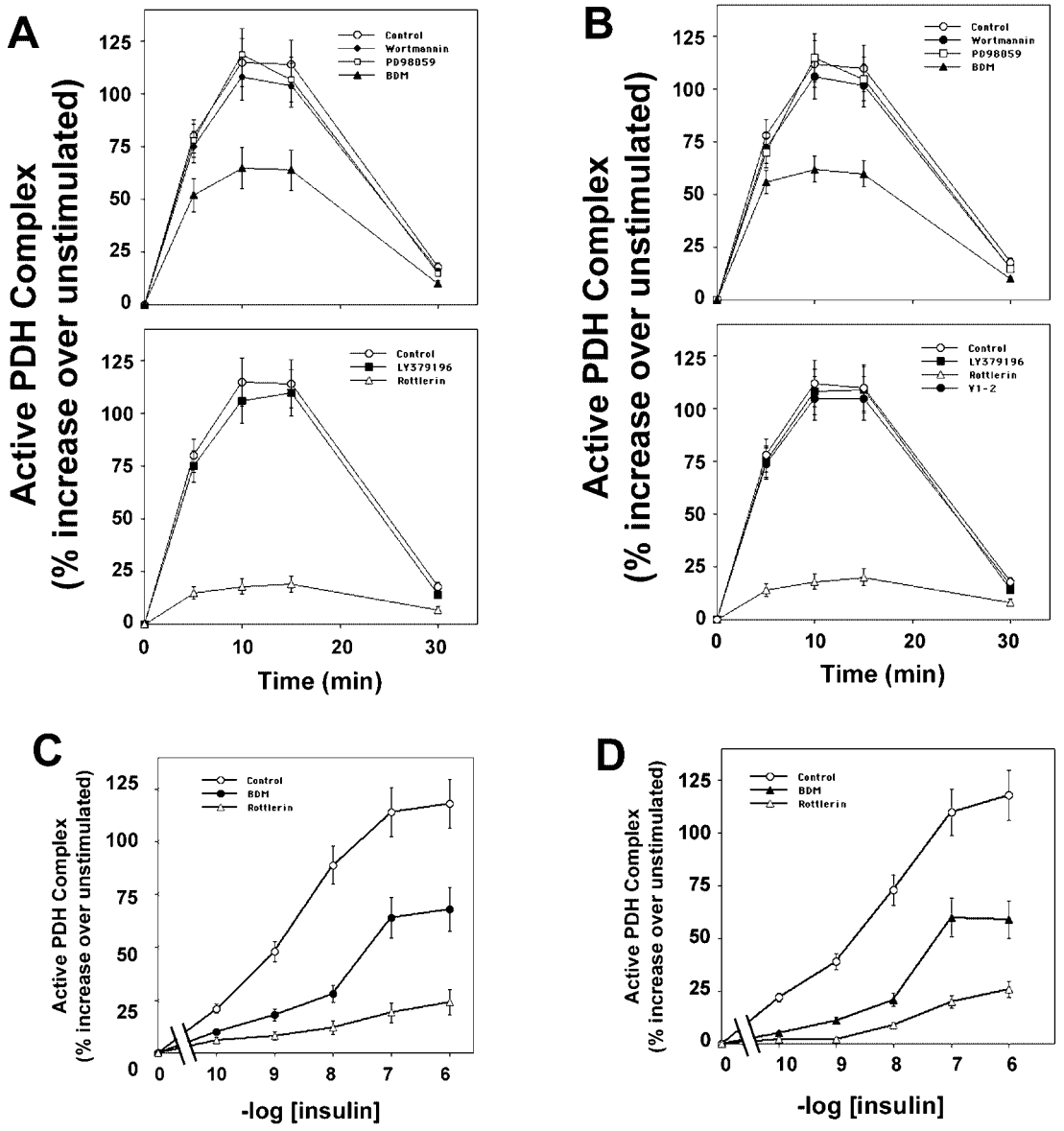

FIG. 1. **Insulin action on the activity of the PDH complex in L6 and Hep cells.** *A* and *B*, L6 and Hep cells were incubated with 100 nM insulin for the indicated times in the absence or the presence of 50 nM wortmannin, 50 μM PD98059, 100 nM bisindolylmaleimide (BDM), 50 nM LY379196, 150 μg/ml V1-2, or 3 μM Rottlerin. PDH complex activity was then assayed as described under "Experimental Procedures." *C* and *D*, the cells were stimulated with the indicated concentrations of insulin for 10 min in the absence or the presence of 100 nM bisindolylmaleimide (BDM) or 3 μM Rottlerin. Cells were then assayed for PDH complex activity as above. Each data point represents the mean ± S.D. of duplicate determinations from four independent experiments.

Molybdate Dye Solution followed by spectrophotometric quantitation of released phosphate at 600 nm. PDP activity was expressed as picomoles of phosphate released per min/mg protein.

PKCδ activity was determined as described (14) using the H-Arg-Phe-Ala-Val-Arg-Asp-Met-Arg-Gln-Thr-Val-Ala-Val-Gly-Val-Ile-Lys-Ala-Val-Asp-Lys-Lys-OH peptide as substrate.

Western Blot Analysis, Immunoprecipitation, and Co-precipitation Studies—For these experiments, cells were solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) for 2 h at 4 °C. Lysates were centrifuged at 5,000 × *g* for 20 min and assayed (34). In some of the experiments Western blot analysis was performed using the mitochondrial or other subcellular fractions. Briefly, solubilized proteins were separated by SDS-PAGE and transferred on 0.45-mm Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with the primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions. Immu-

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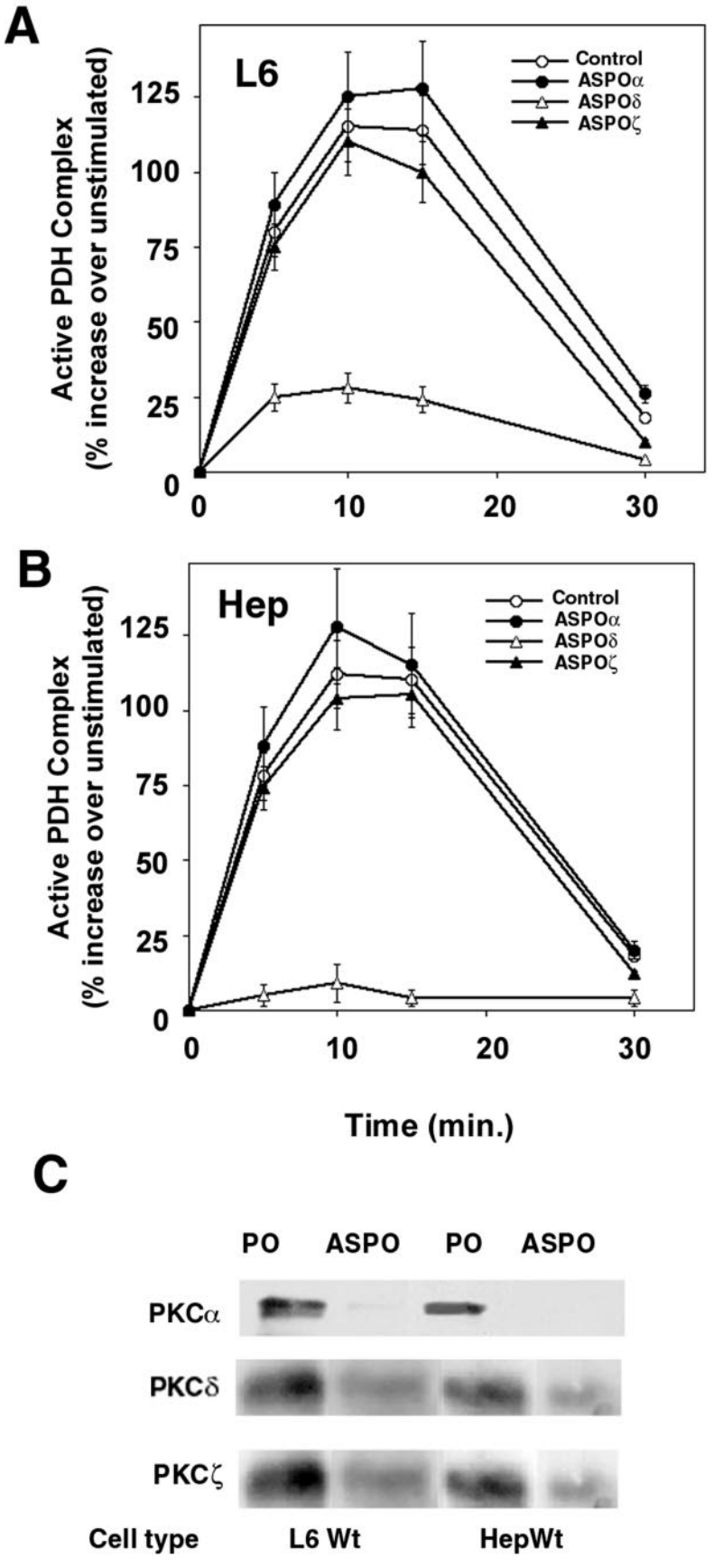

FIG. 2. **Effect of PKCδ, -α, and -ζ antisenses on insulin activation of the PDH complex in L6 and Hep cells.** *A* and *B*, cells were transiently transfected with PKCδ, -α, and -ζ antisense oligonucleotides as described under "Experimental Procedures." 24 h later, the cells were stimulated with 100 nM insulin for the indicated times and assayed for PDH activity. Each data point represents the mean ± S.D. of duplicate determinations from five independent experiments. For control, aliquots of the cell extracts were subjected to Western blotting with PKCδ, -α, and -ζ antibodies (*C*). Filters were revealed by ECL according to the manufacturer's instructions. The autoradiograph shown is representative of five control experiments. *Wt*, wild type.

noprecipitation of specific PKC isoforms and co-localization studies were performed as described previously (15).

PDP1 and PDP2 Phosphorylation and Overlay Blots—PDP phosphorylation in intact L6 and Hep cells was analyzed as described (35). Briefly, the cells were equilibrium labeled with [^{32}P]orthophosphate and then solubilized in 50 mM HEPES, pH 7.4, 1% Triton X-100, 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml aprotinin. Labeled PDP1 and PDP2

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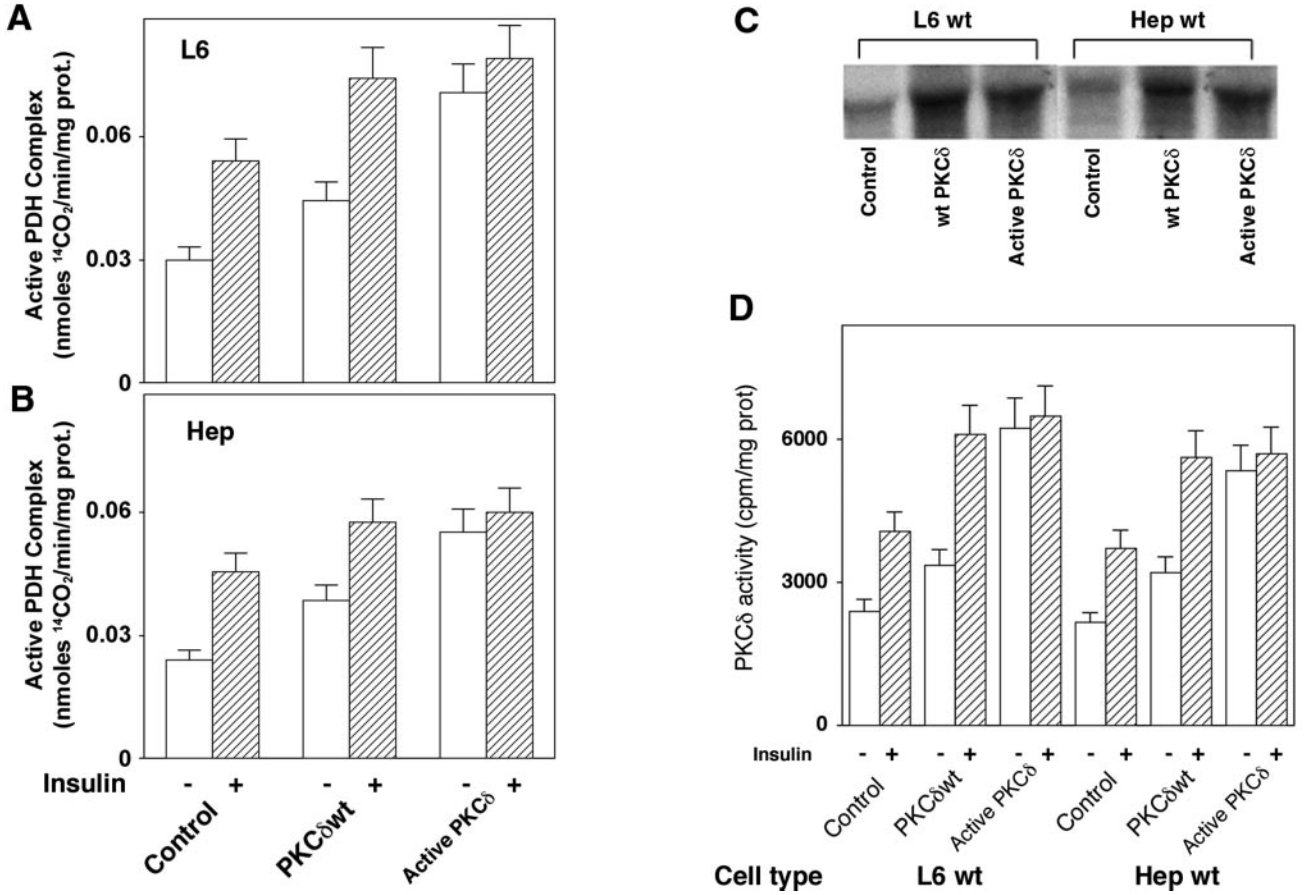

FIG. 3. **Effects of overexpression of wild-type and constitutively active PKCδ on insulin activation of the PDH complex in L6 and Hep cells.** The wild-type (*wt*) and constitutively active PKCδ cDNAs were transiently transfected in L6 and Hep cells as described under "Experimental Procedures." 24 h later, the cells were stimulated with 100 nM insulin for 10 min and assayed for PDH activity (*A* and *B*). For control, aliquots of the cell extracts were subjected to Western blotting with PKCδ antibodies and ECL (*C*) and assayed for PKCδ activity (*D*). Each bar is the mean ± S.D. of duplicate determinations from four (*A* and *B*) and five (*D*) independent control experiments. The autoradiograph shown in *C* is representative of four control experiments.

were precipitated with specific antibodies, respectively, from L6 and Hep cells. PDPs were then separated by reducing PAGE and identified by autoradiography. For investigating *in vitro* phosphorylation of PDP, mitochondrial fractions were first prepared from L6 and Hep cells. Lysates were precipitated with specific PDP antibodies, and precipitated proteins were immobilized on protein A-Sepharose and incubated with recombinant PKCδ in the absence or the presence of PKC activators as described (36). Phosphorylation reactions were initiated by adding 20 μM ATP, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 4 mM Tris, pH 7.5, and 10 μCi of [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) and prolonged for 15 min at room temperature. Phosphoproteins were separated by SDS-PAGE and analyzed by autoradiography. For overlay blotting, mitochondrial preparations were obtained as described above and solubilized and precipitated with PDP1 or PDP2 antibodies. Precipitated proteins were separated by SDS-PAGE and blotted with biotinylated PKCδ. Upon incubation with horseradish peroxidase-steptavidin (15), filters were revealed by ECL according to the manufacturer's instructions.

RESULTS

Insulin Activation of the PDH Complex in L6 and Liver Cells—We addressed the mechanism of insulin action on the PDH complex in L6 skeletal muscle cells and immortalized mouse hepatocytes. The levels of basal (PDH_a) and total (PDH_t) activities of the PDH complex in the absence and the presence of insulin stimulation are shown in Table I. Insulin increased basal activity of the PDH complex in a concentration- and time-dependent fashion. Insulin EC_{50} on PDH complex activity was 2 and 5 nM, respectively, in muscle and liver cells, and maximum insulin effect (2-fold above the insulin-unstimulated state) was achieved at 100 nM (Fig. 1, *C* and *D*). Maximum insulin effect was achieved upon 10 min of incubation and declined thereafter (Fig. 1, *A* and *B*). A block of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities with PD98059 and wortmannin, respectively, caused no change in insulin-stimulated activity of the PDH complex. At variance, maximal insulin stimulation was 50% inhibited ($p < 0.001$) by pretreating the cells with 100 nM bisindolylmaleimide, which simultaneously inhibits different PKC isoforms. This initial finding suggested that PKC activity may be necessary for insulin signaling to the PDH complex. To identify the PKC isoforms involved in PDH activation by insulin, we incubated the cells with LY379196, V1-2, or Rottlerin which selectively block PKCβ, -ε, and -δ, respectively (14, 15). As shown in Fig. 1 (*A* and *B, bottom graphs*), there was no change in insulin-stimulated activity of the PDH complex upon treatment with the V1-2 and LY379196 inhibitors. At variance, Rottlerin almost completely inhibited insulin-stimulated activity of the PDH complex in both the muscle cells and the hepatocytes. Transient transfection of these cells with a specific PKCδ antisense inhibited PKCδ expression by 70%, as compared with control (with 70% transfection efficiency, Fig. 2*C*). Simultaneously, the PKCδ antisense decreased insulin-dependent activation of the PDH complex by 75% both in muscle and in liver cells (Fig. 2, *A* and *B*), with no effect on glycogen synthase (data not shown). Transfection of PKCα and -ζ antisenses also inhibited PKCα and -ζ expression by 80 and 60%, respectively, but caused no change in activation of the PDH complex. To address further the potential role of PKCδ in signaling insulin activa-

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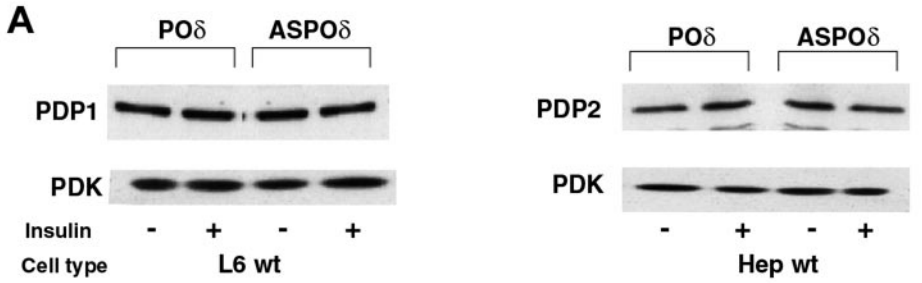

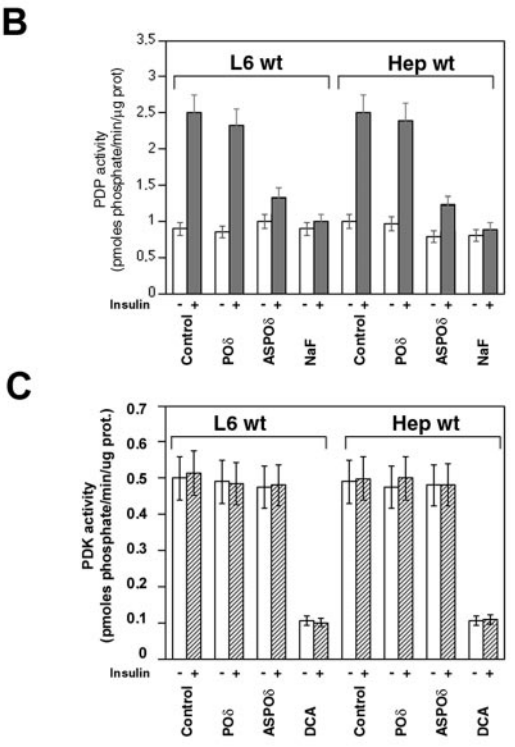

FIG. 4. **Effect of PKCδ inhibition on PDP and PDK expression and activities in L6 and Hep cells.** L6 and Hep cells were transiently transfected with PKCδ or control antisense oligonucleotides (ASPOδ and POδ, respectively). 24 h later, the cells were stimulated with 100 nM insulin for 10 min and assayed for PDH phosphatase (*B*) or PDH kinase (*C*) activity. Part of the cells were also treated with 75 mM NaF or 10 mM dichloroacetic acid (*DCA*) for 30 min before insulin addition, as indicated. Aliquots of the cell extracts were analyzed by Western blotting with PDP1, PDP2, or PDK antibodies and ECL (*A*). Each *bar* is the mean ± S.D. of duplicate determinations in four independent experiments. The autoradiographs shown are also representative of four control experiments. *wt,* wild type.

tion of the PDH complex, we transfected the L6 cells and hepatocytes with either wild-type or constitutively active PKCδ mutant cDNAs. Overexpression of wild-type PKCδ caused a 25 and 30% increase ($p < 0.05$) in basal and insulin-stimulated PKCδ activities in both the muscle and liver cells (Fig. 3, *C* and *D*). These changes were accompanied by 30 and 40% increases in unstimulated and insulin-stimulated activities of the PDH complex, respectively, compared with the untransfected cells (Fig. 3, *A* and *B*, $p < 0.01$). Overexpression of the active PKCδ mutant constitutively increased PKCδ activity in the cells causing no further insulin activation. PDH complex activity was also constitutively induced and was not further stimulable by insulin in cells expressing the active PKCδ mutant. Thus, PKCδ, but not other PKC isoforms, is necessary for insulin action on the PDH complex in the L6 skeletal muscle cells and the mouse hepatocytes.

PKCδ Action on the Activity of the PDH Complex—PDK and PDP are major regulators of the PDH complex. In both L6 and liver cells, insulin stimulated PDP activity. Insulin-induced increase was time- and dose-dependent (data not shown). The maximum effect (2.5-fold above unstimulated) was achieved within 10 min upon insulin exposure of the cells (Fig. 4*B*). In both L6 and liver cells, insulin action on PDP was inhibited by 95%, following treatment with 75 mM sodium fluoride. Interestingly, transfection of PKCδ antisense (but not the POδ control antisense) also inhibited insulin induction of PDP by >70% (Fig. 4*B*). At variance with PDP, insulin elicited no effect on PDK activity, neither in the absence nor in the presence of the PKCδ antisense (Fig. 4*C*). PDK was completely blocked by treatment of the cells with the PDK inhibitor dichloroacetic acid, however. Neither the PKCδ nor the control antisense caused any change in the levels of PDK or in those of the muscle-specific (PDP1) and the liver-specific (PDP2) PDP isoforms (Fig. 4*A*), indicating that PKCδ affected the activity of the PDH complex by inducing PDP activity rather than inactivating PDK.

Subcellular Localization of PKCδ in L6 and Hep Cells—PDPs are intramitochondrial resident enzymes. Thus, to investigate the cellular bases for potential PKC isoform interactions with PDPs, we first performed subcellular fractionation of L6 and Hep cells. We then blotted subcellular protein fractions with isoform-specific PKC antibodies. In basal L6 and Hep cells, PKCα, -β, -δ, -ε, and -ζ were mainly cytosolic (Fig. 5*A*). Insulin treatment of the cells induced a differential redistribution of these PKC isoforms. Whereas all of the isoforms largely associated to the plasma membrane in insulin-stimulated cells, PKCδ also redistributed to the mitochondrial fraction upon insulin exposure of the cells. This suggested that PKCδ translocated to the mitochondria as well as to the cell surface after insulin stimulation. In addition, the specific increase of PKCδ in the mitochondrial fractions from insulin-stimulated L6 and Hep cells was accompanied by a >2-fold increase in PKCδ

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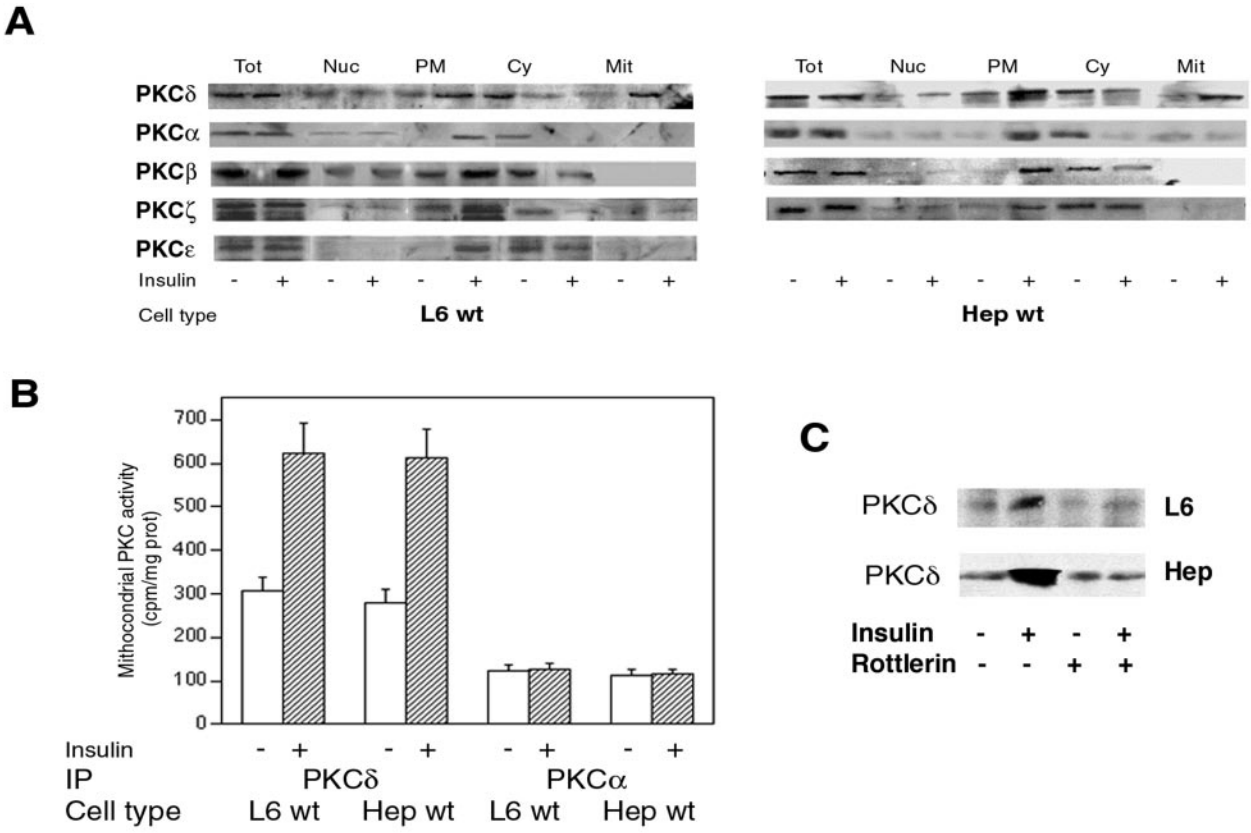

FIG. 5. **Intracellular localization of PKC isoforms in L6 and Hep cells.** *A,* L6 and Hep cells were exposed to 100 nM insulin for 10 min, and nuclear (*Nuc*), plasma membrane (*PM*), cytosolic (*Cy*), and mitochondrial fractions (*Mit*) were obtained as reported under "Experimental Procedures." 80 μg of proteins from total cell lysates (*Tot*) and from each fraction were then subjected to Western blotting with PKCδ, -α, -β, -ζ, or -ε antibodies and revealed by ECL and autoradiography. The autoradiographs shown are representative of three independent experiments. *B,* L6 and Hep cells were stimulated with 100 nM insulin for 10 min. Mitochondrial fractions were prepared, and proteins were immunoprecipitated with PKCδ or PKCα antibodies as indicated. PKC activity was then assayed in the specific immunoprecipitates as described under "Experimental Procedures." *Bars* represent the mean ± S.D. of triplicate determinations in four independent experiments. *C,* cells were exposed to 3 μM Rottlerin for 30 min before insulin stimulation. Mitochondrial fractions were then obtained and Western blotted with PKCδ antibodies as outlined above. The autoradiographs shown are representative of four independent experiments. *wt,* wild type.

activity in those fractions (Fig. 5*B*) and was blocked by Rottlerin (Fig. 5*C*). To confirm further the insulin-dependent translocation of PKCδ to the mitochondria, we performed double labeling experiments using the mitochondria-selective dye MitoTracker Red and FITC-conjugated PKCδ antibodies. Treatment of L6 cells with MitoTracker resulted in a bright red mitochondrial fluorescence at the confocal microscope (Fig. 6*A*). A very similar staining pattern was also obtained using fluorescent antibodies to the mitochondrial protein PDP1 (data not shown). In basal cells, the mitochondrial fluorescence revealed very little co-localization with the PKCδ green fluorescence (Fig. 6, *B* and *C*). However, consistent with mitochondrial translocation of PKCδ in response to insulin, co-localization of PKCδ with the mitochondria became very evident after insulin addition to the cells (Fig. 6*F*). Insulin-dependent PKCδ, although not PKCα, co-localization with mitochondria was also observed with Hep cells (data not shown).

PDP Phosphorylation by PKCδ—To address further the mechanisms conveying insulin signal toward the PDH complex, we investigated potential PKCδ interactions with PDP. As shown in Fig. 7*A*, insulin induced co-precipitation of PKCδ with PDP1 and PDP2 in solubilized mitochondrial preparations from L6 and Hep cells, respectively. Insulin-induced PKCδ-PDP co-precipitation occurred with no change in the total levels of PDP1 or -2 in the cells. No PDP co-precipitation with PKCα or -ζ occurred in these same lysates (data not shown). In addition, in overlay blots, immunoprecipitated PDP1 and PDP2 were revealed by recombinant biotinylated PKCδ (Fig. 7*B*),

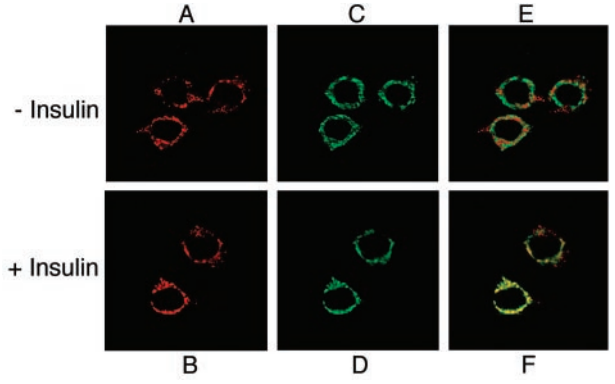

FIG. 6. **Mitochondrial translocation of PKCδ in insulin-stimulated L6 cells.** L6 cells were grown on 22-mm coverslips and exposed to 100 nM insulin for 10 min as indicated. The cells were labeled with the mitochondrion-selective dye MitoTracker red CM-H2-TMRos (*A* and *B*) and further incubated with PKCδ and FITC-conjugated secondary antibodies (*C* and *D*) as described under "Experimental Procedures." Overlays of the two colors are shown in *E* and *F*. Coverslips were viewed with a Leica confocal microscope.

suggesting that PKCδ may directly interact with PDP1 and -2 also in intact cells.

Consensus sites for PKC phosphorylation have been described in PDP. We therefore sought to investigate whether PKCδ phosphorylates PDP1 and PDP2 *in vivo*. In intact L6 and Hep cells insulin increased phosphorylations of PDP1 and PDP2 by 2.2- and 2.5-fold, respectively (Fig. 8*A*). Interestingly,

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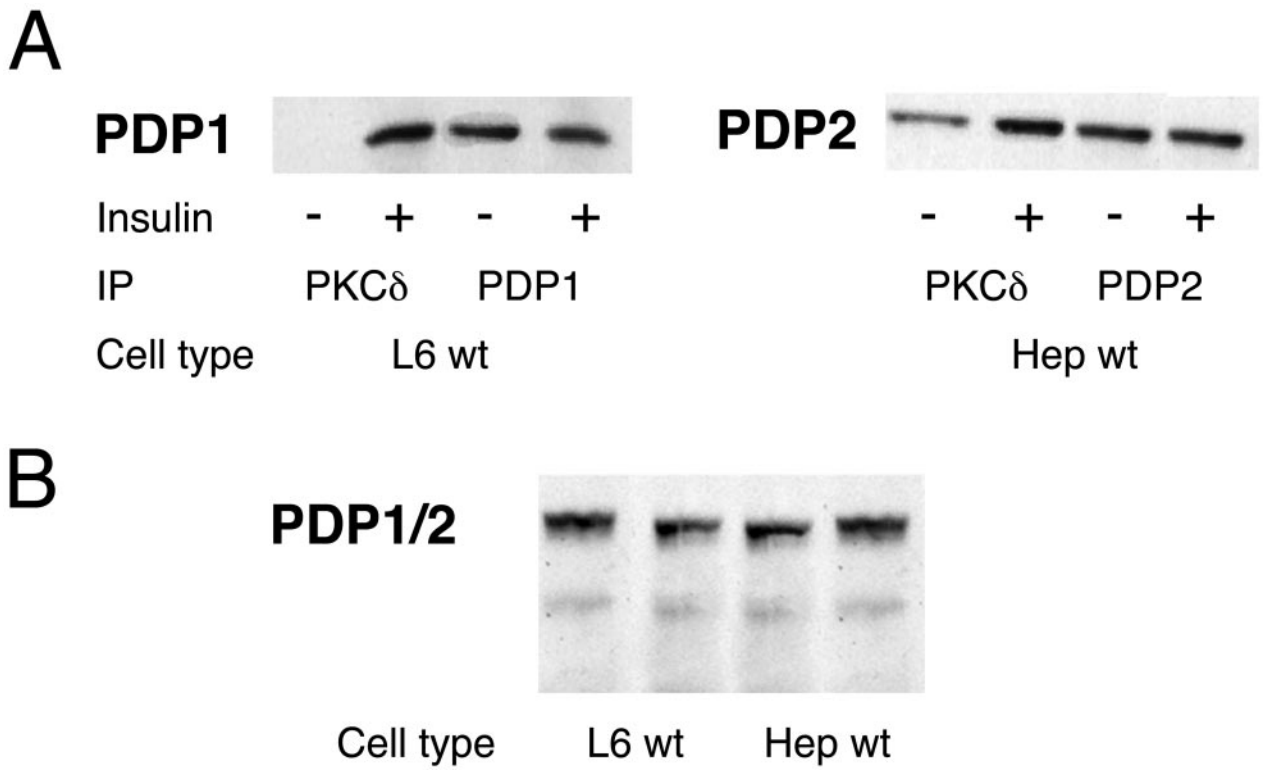

FIG. 7. **PKCδ-PDP interaction in L6 and Hep cells.** *A,* L6 and Hep cells were exposed to 100 nM insulin for 10 min, broken, and fractionated as described under "Experimental Procedures." Equal amounts of proteins from mitochondrial fractions (70 μg) were immunoprecipitated with PKCδ, PDP1, or PDP2 antibodies. Immunoprecipitates were further subjected to immunoblotting with PDP1 or PDP2 antibodies as indicated. Filters were revealed by ECL and autoradiography. *B,* mitochondrial preparations (equal amounts of proteins/lane) were solubilized and immunoprecipitated with PDP1 or PDP2 antibodies as described under "Experimental Procedures." Precipitated proteins (in duplicate) were immunoblotted with biotinylated PKCδ. Upon incubation with horseradish peroxidase-streptavidin, filters were revealed by ECL and autoradiography. The autoradiographs shown are representative of four (*A*) and three (*B*) independent experiments. *IP,* immunoprecipitation; *wt,* wild type.

in both cell types, phosphorylation was inhibited by >70% by transfection of the PKCδ but not the control antisense. In addition, transfection of the constitutively active PKCδ mutant increased PDP1 and PDP2 phosphorylation preventing further insulin-dependent phosphorylation. *In vitro,* recombinant PKCδ also phosphorylated PDP1 and PDP2 purified from L6 and Hep cells (Fig. 8*B*). *In vitro* phosphorylation of PDP1 and PDP2 was accompanied by a 2-fold increase in PDP activity (Fig. 8*C*), suggesting that direct PKCδ phosphorylation and activation of PDP may occur *in vivo* as well.

DISCUSSION

Activation of the PDH complex is a major event regulated by insulin in most cells (1, 2, 33, 37). However, the molecular mechanism of insulin induction of PDH has not been completely elucidated as yet. In the present report, we have investigated the mechanism of insulin regulation of the PDH complex activity in liver and skeletal muscle cells, two models of major insulin target tissues. In these cells, insulin elicited a rapid and transient increase in the activity of the complex. A similarly transient effect has been reported previously (30, 38) in freshly isolated rat hepatocytes, whereas insulin stimulation was more sustained in adipocytes and in fibroblasts (39, 40). These findings suggest that regulation of the PDH complex may feature cell specificity and that diversity in the mechanism of insulin action on the PDH complex may occur in insulin target tissues as well as in isolated cells. Previous studies (18) generated evidence that insulin-dependent activation of the PDH complex is mediated by a PKC-dependent pathway. But which PKC isoform is involved and whether other major insulin-dependent pathways are also involved in activation of the PDH complex is unknown. In this work, we show that pharmacological inhibition of mitogen-activated protein kinase or phosphatidylinositol 3-kinase do not affect insulin induction of PDH complex activity, either in L6 skeletal muscle cells or in mouse hepatocytes. Thus, the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways do not convey insulin signaling toward PDH. At variance, pharmacological inhibition of PKCδ activity as well as antisense block of PKCδ expression almost completely blocked insulin activation of the PDH complex. A block of other PKC isoforms, including PKCα, -β, and -ε did not elicit any effect on PDH complex activity, indicating that PKCδ is specifically involved in transducing activation of the complex by insulin in muscle and liver cells.

The activation of the PDH complex following acute treatment of the cells with insulin could result from decreased PDH kinase (PDK) activity, increased PDH phosphatase (PDP) activity, or both (4, 6–9). However, we found that insulin does not affect PDK activity either in liver or in muscle cells, while strongly inducing that of PDP within the mitochondria. Previous work (13, 15) has demonstrated that PKCδ translocates to the plasma membrane in response to different stimuli. PKCδ has also been shown to translocate to mitochondria in 12-*O*-tetradecanoylphorbol-13-acetate-exposed cells, however (41). The present study demonstrates, for the first time, that insulin stimulation of target cells also induces mitochondrial translocation of PKCδ. This finding has been confirmed by cell fractionation and confocal microscopy immunofluorescence. The functional significance of PKCδ translocation to mitochondria is supported by the finding that this event is accompanied by activation of PKCδ in insulin-stimulated cells, leading to the presence of active PKCδ within mitochondria. In addition, abrogation of PKCδ translocation to mitochondria is accompanied

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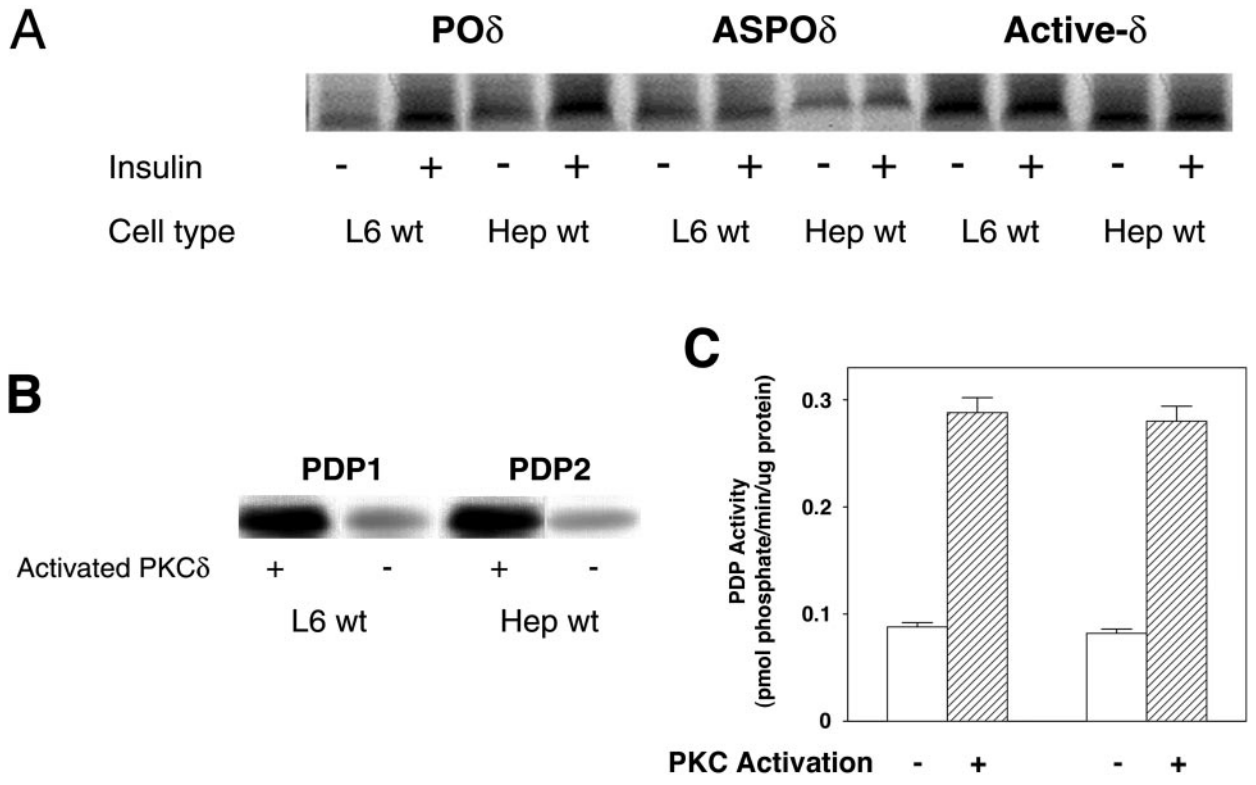

FIG. 8. **PDP phosphorylation and activation by PKCδ in L6 and Hep cells.** *A,* the cells were transiently transfected with PKCδ (*ASPO*δ) or control (*PO*δ) antisenses or with the constitutively active PKCδ mutant cDNA. 18 h later, the cells were equilibrium labeled with [^{32}P]orthophosphate, stimulated with 100 nM insulin for 10 min, and mitochondrial fractions obtained as described under "Experimental Procedures." Equal amounts of mitochondrial proteins (70 μg) were immunoprecipitated with specific PDP antibodies (PDP1 antibody for L6 cells and PDP2 antibody for Hep), and immunoprecipitated proteins were separated by SDS-PAGE and revealed by autoradiography. The autoradiograph shown is representative of three independent experiments. *B* and *C*, mitochondrial fraction lysates (equal amounts of proteins) were immunoprecipitated with specific PDP antibodies. Precipitated proteins were immobilized on protein A-Sepharose and incubated with recombinant PKCδ and [^{32}P]ATP in the absence or the presence of PKC activators as indicated. Upon phosphorylation, phosphoproteins were assayed for PDP activity (*C*) or analyzed for ^{32}P content by SDS-PAGE and autoradiography (*B*). *Bars* represent the mean ± S.D. of duplicate determinations in four independent experiments. The autoradiograph shown in *B* is representative of three independent experiments. *wt*, wild type.

by block of insulin induction of PDP activity. Thus, in muscle and liver cells, PKCδ plays a key role in transducing insulin signal to PDP, thereby activating the PDH complex.

Cell treatment with insulin caused co-precipitation of PKCδ with the major muscle and liver PDP isoforms (PDP1 and PDP2, respectively) in mitochondria lysates. In overlay blots, immunoprecipitated PDP could be revealed by recombinant PKCδ, suggesting that PKCδ may directly interact with PDP *in vivo* as well. In intact cells, insulin induced rapid phosphorylation of PDP, which was prevented by antisense block of PKCδ expression and fostered by expression of an active PKCδ mutant. *In vitro*, activated PKCδ also phosphorylated PDP1 and PDP2, accompanied by PDP1 and -2 activation. Insulin-induced mitochondria translocation of PKCδ may therefore lead to PKCδ binding to PDP followed by phosphorylation and activation. The identification of the key PKC phosphorylation sites of PDP1 and -2 is currently in progress in our laboratory.

Majumder *et al.* (41) have recently reported that 12-*O*-tetradecanoylphorbol-13-acetate-triggered translocation of cytoplasmic PKCδ to mitochondria induces release of cytochrome *c* and the activation of caspase 3 leading U-937 and MCF-7 cells to apoptosis. While inducing mitochondria translocation of PKCδ, insulin does not induce apoptosis either in the L6 or in the liver cells (data not shown). Because PKC expression and function feature tissue specificity (42), it is possible that PKCδ redistribution may elicit different responses depending on the cell type. It is also possible, however, that translocation of PKCδ to the mitochondria is necessary but not sufficient to trigger activation of apoptotic program in cells. Even more likely, insulin activates survival pathways (43, 44) whose function prevails over the induction of PKCδ translocation in determining cell fate.

The novel PKCs PKCδ and -θ have been shown to convey insulin signal toward glucose transport (16, 45), glycogen synthesis (17), and cell proliferation (46). In addition, these PKCs may down-regulate insulin signaling in response to high glucose concentrations (15) and other stimuli (13). Therefore, it appears that novel PKC isoforms may both mediate insulin stimulatory effects on glucose metabolism and inhibit insulin intracellular signals. Which of these actions prevails may depend on the effector protein with whom the individual PKC interacts and, as shown in the present paper, where, within the cell, the interaction occurs.

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Activation and Mitochondrial Translocation of Protein Kinase Cδ Are Necessary for Insulin Stimulation of Pyruvate Dehydrogenase Complex Activity in Muscle and Liver Cells

Matilde Caruso, Maria Alessandra Maitan, Giuseppe Bifulco, Claudia Miele, Giovanni Vigliotta, Francesco Oriente, Pietro Formisano and Francesco Beguinot

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