Protein Kinase C (PKC)-α Activation Inhibits PKC-ζ and Mediates the Action of PED/PEA-15 on Glucose Transport in the L6 Skeletal Muscle Cells

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Overexpression of the PED/PEA-15 protein in muscle and adipose cells increases glucose transport and impairs further insulin induction. Like glucose transport, protein kinase C (PKC)-α and -β are also constitutively activated and are not further stimulatable by insulin in L6 skeletal muscle cells overexpressing PED ($L6_{PED}$). PKC-ζ features no basal change but completely loses insulin sensitivity in $L6_{PED}$. In these cells, blockage of PKC-α and -β additively returns 2-deoxy-D-glucose (2-DG) uptake to the levels of cells expressing only endogenous PED ($L6_{WT}$). Blockage of PKC-α and -β also restores insulin activation of PKC-ζ in $L6_{PED}$ cells, with that of PKC-α sixfold more effective than PKC-β. Similar effects on 2-DG uptake and PKC-ζ were also achieved by 50-fold overexpression of PKC-ζ in $L6_{PED}$. In $L6_{WT}$, fivefold overexpression of PKC-α or -β increases basal 2-DG uptake and impairs further insulin induction with no effect on insulin receptor or insulin receptor substrate phosphorylation. In these cells, overexpression of PKC-α blocks insulin induction of PKC-ζ activity. PKC-β is 10-fold less effective than PKC-α in inhibiting PKC-ζ stimulation. Expression of the dominant-negative $K^{281}\rightarrow W$ PKC-ζ mutant simultaneously inhibits insulin activation of PKC-ζ and 2-DG uptake in the $L6_{WT}$ cells. We conclude that activation of classic PKCs, mainly PKC-α, inhibits PKC-ζ and may mediate the action of PED on glucose uptake in L6 skeletal muscle cells. *Diabetes* 50:1244–1252, 2001

PED/PEA-15 is a 15-kDa cytosolic protein whose gene has been shown to be overexpressed in insulin-resistant type 2 diabetic subjects (1). PED protein has also been found to be overexpressed in skeletal muscle, adipose tissue, and fibroblasts from type 2 diabetic individuals (1). Muscle and adipose cells overexpressing PED to levels comparable to those occurring in type 2 diabetes feature slightly increased basal glucose uptake with a lack of further insulin-dependent uptake (1). The changes in glucose uptake in muscle and adipose cells overexpressing PED are accompanied by inhibition of insulin-dependent translocation of GLUT4, the major insulin-responsive glucose transporter (1). PED overexpression also impairs GLUT1 translocation in response to insulin, but increases GLUT1 content in the plasma membranes of basal cells (1). Antisense inhibition of endogenous PED expression in muscle cells and adipocytes significantly expands insulin sensitivity of glucose uptake (2). Thus, PED may represent a physiological regulator of glucose transporter trafficking and glucose transport in the major insulin-responsive skeletal muscle and adipose tissues. Furthermore, defective expression of the PED gene may contribute to insulin resistance in glucose transport in type 2 diabetes.

Protein kinase C (PKC) comprises a multigene family that encodes at least 12 distinct isoforms differing in catalytic and regulatory properties (3–6). These PKC (1) isoforms can be divided into the following three subgroups based on cofactor requirements: conventional PKCs (α, β, and γ), which are dependent on Ca^{2+} and diacylglycerol (DAG) for activity; novel PKCs (δ, ϵ, η, and θ), which are not dependent on Ca^{2+} but are activated by DAG; and atypical PKCs (ζ, λ, and ι), which are not dependent on Ca^{2+} and are not stimulated by DAG (7). PKC plays a pivotal role in controlling numerous cellular functions, including glucose transport (8). Indeed, current evidence indicates that DAG-dependent PKCs may control insulin-independent glucose transport in several different cell types (9,10). More recent work also indicates that different PKC isoforms have an important role in signaling insulin action on glucose transport (9–13). However, at the molecular level, the role of specific PKC isoforms in controlling glucose transport as well as their regulatory mechanisms have been only partially elucidated.

PED is a substrate of PKC (1,14). In previous work, we found that pharmacological inhibition of PKC reduces the high basal glucose uptake of muscle and adipose cells overexpressing PED and rescues most of the insulin effect in glucose uptake (1). These findings implied an important role of the PKC system in PED function, but whether PED is controlled by PKC phosphorylation or whether PKC is

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2-DG, 2-deoxy-D-glucose; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; IRS, insulin receptor substrate; PI, phosphatidylinositol; PKB, protein kinase B; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

downstream of PED and mediates its action on glucose uptake is unknown. In the present work, we have addressed this issue and found that PED affects the activity of multiple PKC isoforms in the L6 skeletal muscle cells. In these cells, PKC-α, -β, and -ζ have a major role in controlling glucose uptake, with the α isoform restraining the activity of PKC-ζ.

RESEARCH DESIGN AND METHODS

Materials. Media, sera, and antibiotics for cell culture; the lipofectamine reagent; rabbit polyclonal antibodies toward PKC-α, -β (recognizing both the β_1 and the β_2 isoforms), and -ζ; and the PKC assay system were purchased from Life Technologies (Grand Island, NY). The PED antibody has been previously described (1). Phosphorothioate PKC-α and PKC-β antisense and control oligonucleotides have also been previously reported (15,16). PKC-α, -β_1, -β_2, -ζ, and the K^{281}→W PKC-ζ mutant cDNAs were a gift from Dr. S. Gutkind (National Cancer Institute, National Institutes of Health, Bethesda, MD). The LY379196 inhibitor was also donated by Eli Lilly (Indianapolis, IN), and its use in L6 cells has been characterized previously (16). Protein electrophoresis reagents were from Bio-Rad (Richmond, VA). Western blot as well as enhanced chemiluminescence (ECL) reagents and radioisotopes were from Amersham (Arlington Heights, IL). All other chemicals were from Sigma (St. Louis, MO).

Cell culture and transfection. The L6 muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 2% L-glutamine in a humidified CO_2 incubator. The $L6_{PED}$ myotubes have been previously reported (1). Transfection of the PKC cDNAs and the phosphorothioate oligonucleotides was performed with the lipofectamine method (17) according to the manufacturer's instructions. In these studies, 100-mm dishes of 80% confluent cells were washed in serum-free DMEM and then incubated with 12 μg PKC cDNAs or antisenses and 30 μl lipofectamine. After 5 h, an equal volume of DMEM supplemented with 20% fetal calf serum was added and the incubation was prolonged for an additional 19 h. The medium was subsequently replaced with DMEM supplemented with 10% fetal calf serum, and cells were assayed 24 h later. 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.

Generation of the phosphorylation-defective PED mutants. The Ser^{104}→Gly PED mutant was generated by amplifying PED wild-type cDNA with the following two sets of primers: PED5′ (5′-GGCCGGTACCGCCAGAGCGCGCGGGGCAGTGTG-3′) and 3′S^{104}PEDmut (5′-TGTACTTCTTGGCACCGGGGATACGGGT-3′), and 5′S^{104}PEDmut (5′-ACCCGTATCCCCGGTGCCAAGAAGTACA-3′) and PED3′Myc (5′CGCGGATCCTCACAGATCCTCTTCTGAGATGAGATGAGTTTTTGTTCGGCCTTCTTCGGTGGGGGAGCCAATTTGATGATGTGTT-3′). Ser^{104}→Gly substitution was achieved through the GGT/ACC (underlined) for AGT/TAC codon replacement. The two fragments obtained were amplified again with the PED5′ and the PED3′Myc primers, carrying a *Kpn*I restriction site and a *Bam*HI restriction site, respectively. The $PED_{S104\rightarrow G}$ mutant was finally cloned in the PcDNA3 plasmid and sequence verified by the Sanger method (18), using the ^{T7}Sequencing Kit by Pharmacia Biotech (Uppsala, Sweden). The triple mutant of PED ($Ser_{33,70,104\rightarrow Gly}$) was generated as above using the $PED_{S104\rightarrow G}$ mutant and the following two sets of primers: 5′S^{33}mut (5′-GGAAGACATCCCCGGCGAAAAGAGTGAG-3′) and 3′S^{33}mut (5′-CTCACTCTTT TCGCCGGGGATGTCTTCC-3′), and 5′S^{70}mut (5′-CATCTTTGAGATCGGC-CGCCGTCCTGACCT-3′) and 3′S^{70}mut (5′-AGGTCAGGACGGCGGCCGATCTCAAAGATG-3′). The Gly^{33}→Ser and Gly^{70}→Ser substitutions were obtained through the GGC/CCG for AGC/TCG and the GGC/CCG for TCC/AGG codon replacements, respectively. Transfection of the mutant cDNAs in the L6 cells was achieved as reported previously (1). For each of the two mutants, several clones were isolated and three of these were fully characterized, together with the cell pool. Four characterized clones overexpressing either the $Ser_{33,70,104}$→Gly or the Ser_{104}→Gly mutant are described in the present report.

PED phosphorylation in intact cells. For these experiments, cells were grown in 100-mm tissue culture dishes. Monolayers were rinsed with 150 mmol/l NaCl, incubated in phosphate-free culture medium for 16 h at 37°C, and further incubated in 6 ml phosphate-free medium supplemented with 50 μCi/ml [^{32}P]orthophosphate for 8 h. 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 1 μmol/l final concentration) was subsequently added for 30 min and cells were rapidly rinsed with ice-cold saline and solubilized with 1 ml/dish lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 2 mmol/l Na_3VO_4, 100 mmol/l NaF, 10% glycerol, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml aprotinin) for 1 h at 4°C. Lysates were centrifuged at 5,000*g* for 20 min and assayed as described previously (15). Briefly, solubilized proteins were precipitated with PED antibodies and separated by SDS-PAGE, and the dried gels were exposed for the indicated times.

Western blot analysis. For these studies, the cells were solubilized in lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 2 mmol/l Na_3VO_4, 100 mmol/l NaF, 10% glycerol, 1% Triton X-100, 1 mmol/l PMSF, 10 mg/ml aprotinin) for 2 h at 4°C. Lysates were centrifuged at 5,000*g* for 20 min and assayed as described by Miele et al. (19). 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.

PKC assay. PKC activity was measured as previously described (20). Briefly, for these assays, cells were solubilized in 20 mmol/l Tris, pH 7.5, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 25 μg/ml aprotinin, 25 μg/ml leupeptin (extraction buffer), and then clarified by centrifugation at 5,000*g* for 20 min. Supernatants were further centrifuged at 60,000*g* for 2 h and pellets solubilized with extraction buffer containing 0.5% Triton X-100. Soluble pellets were supplemented with the lipid activators (10 mmol/l phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidyl serine, and 4 mg/ml dioleine, final concentrations) and phosphorylation reactions initiated by addition of the substrate solution (50 μmol/l Ac-MBP [4–14], 20 μmol/l ATP, 1 mmol/l $CaCl_2$, 20 mmol/l $MgCl_2$, 4 mmol/l Tris, pH 7.5, and 10 μCi/ml [3,000 Ci/mmol] [γ-^{32}P]ATP, final concentrations). The reaction mixtures were further incubated for 10 min at room temperature, rapidly cooled on ice, and spotted on phosphocellulose discs. Disc-bound radioactivity was quantitated by liquid scintillation counting. Activity of the specific PKC isoforms was assayed as above but with use of precipitates with specific antibodies (16). Determinations of PKC-ζ activity using the Ac-MBP (4–14) peptide as substrate or the pseudosubstrate region of PKC-ε (specific for PKC-ζ) provided consistent results.

Glucose transporter detection and 2-deoxy-D-glucose (2-DG) uptake. Detection of glucose transporters at the cell surface was accomplished as previously reported (21). Briefly, L6 cells were preincubated in serum-free DMEM supplemented with 0.25% bovine serum albumin for 24 h and then further incubated in the absence or presence of 100 nmol/l insulin for 30 min. The cells were rinsed and then treated with 0.5 mg/ml *N*-hydroxysuccinimide long-chain biotin in phosphate-buffered saline containing 0.1 mmol/l $CaCl_2$ and 1 mmol/l $MgCl_2$, pH 7.4 (biotinylation buffer) for 30 min at 4°C. The biotinylation reaction was stopped by adding to the cells 15 mmol/l glycine (final concentration). The cells were lysed in 1% Triton X-100, 50 mmol/l HEPES, pH 7.4, 10 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 4 mmol/l EDTA, 2 mmol/l Na_3VO_4, 2 mmol/l PMSF, and 0.2 mg/ml aprotinin, and lysates were precipitated with GLUT1 or GLUT4 antibodies. Precipitated proteins were separated by 10% SDS-PAGE and blotted on nitrocellulose filters. The biotinylated transporters were revealed by incubation with peroxidated streptavidin followed by detection of chemiluminescence by autoradiography. For detecting the total transporter content, cell lysates prepared as described above were subjected to 10% SDS-PAGE and Western blotting with GLUT1 or GLUT4 antibodies, followed by ECL detection. 2-DG uptake was measured as reported previously (22). Briefly, confluent cells were incubated in DMEM supplemented with 0.25% albumin for 18 h at 37°C. The medium was aspirated and cells were further incubated for 30 min in glucose-free HEPES buffer (5 mmol/l KCl, 120 mmol/l NaCl, 1.2 mmol/l $MgSO_4$, 10 mmol/l $NaHCO_3$, 1.2 mmol/l $KHPO_4$, and 20 mmol/l HEPES, pH 7.8, 2% albumin). The cells were incubated with 100 nmol/l insulin for 30 min, supplemented during the final 10 min with 0.2 mmol/l [^{14}C]2-DG. Cells were then solubilized and the 2-DG uptake was quantitated by liquid scintillation counting.

RESULTS

PKC activity in PED overexpressors. To address the role of PKC in PED function, we first analyzed PKC activity in differentiated L6 skeletal muscle cells overexpressing PED ($L6_{PED}$). The cell clones overexpressing PED by threefold, as in type 2 diabetes, have been previously characterized and reported (1). In plasma membrane extracts from these cells, PKC showed constitutively increased activity as compared with the control cells, either the untransfected myotubes (L6) or those transfected with the empty vector (Fig. 1). However, in $L6_{PED}$ myotubes, insulin was unable to increase membrane PKC activity further, while eliciting an almost threefold stimulation in those

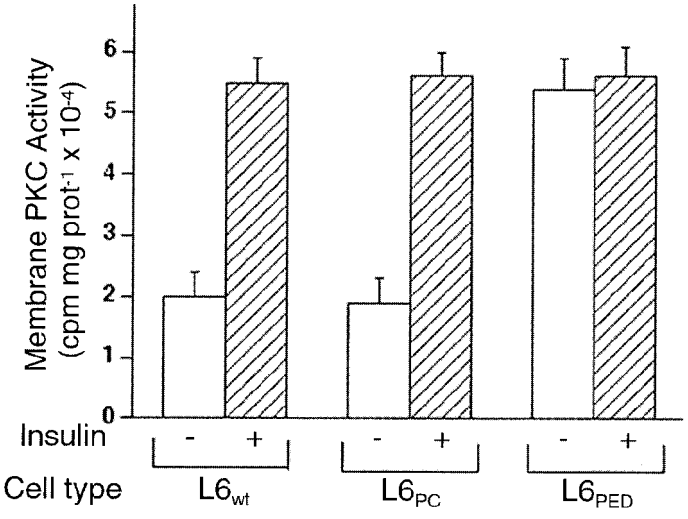


FIG. 1. PKC activity in $L6_{PED}$ cells. L6 myotubes, the wild-type clone ($L6_{WT}$) or those transfected with PED ($L6_{PED}$) or with the empty vector ($L6_{PC}$), were exposed to 100 nmol/l insulin for 30 min as indicated. Cells were then collected and cell membrane preparations were solubilized and assayed for PKC as described under RESEARCH DESIGN AND METHODS. PKC activity is plotted as counts per minute incorporated into the MBP(4–14) substrate, with equal amounts of membrane proteins analyzed per assay. Each bar represents the mean ± SD of triplicate determinations in four independent experiments. Based on *t* test analysis, different basal PKC activity in $L6_{PED}$ and control cells was significant at the $P < 0.001$ level.

from parental cells. We then analyzed PKC activity in cell immunoprecipitates with isoform-specific PKC antibodies. PKC activity in PKC-α and -β immunoprecipitates from lysates of $L6_{PED}$ cell membranes—similar to what is seen in total plasma membrane PKC activity—exhibited 70 and 50% increases, respectively, compared with the control cells ($P < 0.001$) with no further insulin response (Fig. 2).

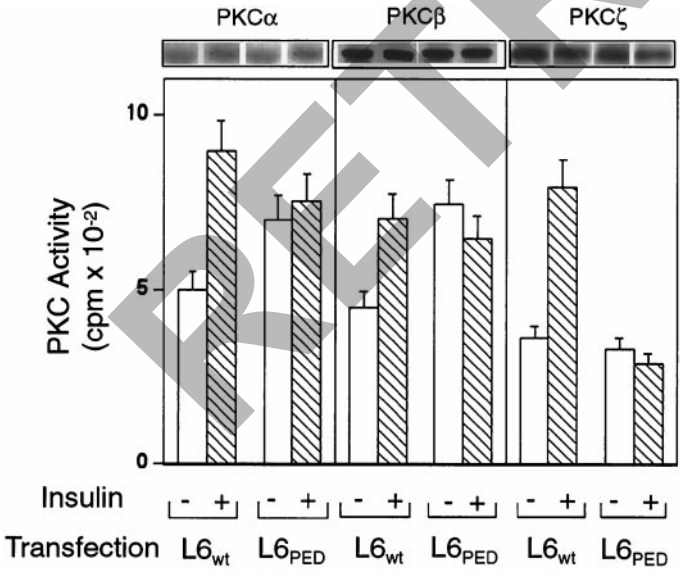


FIG. 2. Activity of specific PKC isoforms in $L6_{PED}$ cells. $L6_{PED}$ and control cells were treated with 100 nmol/l insulin. Plasma membranes were prepared and solubilized and the lysates (200 μg protein/sample) were immunoprecipitated with antibodies to PKC-α, -β (recognizing both the β_1 and β_2 isoforms), and -ζ isoforms, as indicated. PKC activity was then assayed in the immunoprecipitates as described under RESEARCH DESIGN AND METHODS. Bars represent the means ± SD of duplicate determinations in four independent experiments. By *t* test analysis, insulin effect on the activity of PKC-α, -β, and -ζ in $L6_{WT}$ cells was significant ($P < 0.001$).

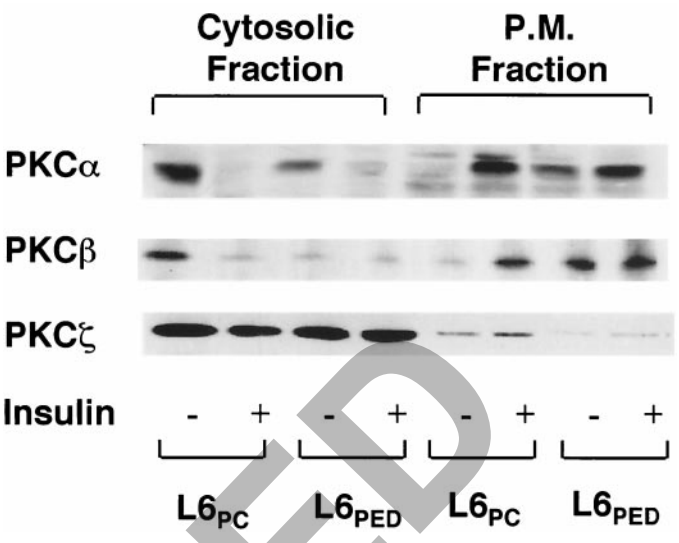


FIG. 3. Localization of PKC-α, -β, and -ζ in $L6_{PED}$ cells. $L6_{PED}$ and control cells were treated with 100 nmol/l insulin, fractionated, and solubilized as described under RESEARCH DESIGN AND METHODS. Plasma membranes (P.M.) and cytosolic proteins (100 μg/sample) were then immunoblotted with the PKC-α, PKC-β, or PKC-ζ antibodies described in legend of Fig. 2. Filters were revealed by ECL. The autoradiograph shown is representative of three independent experiments.

Basal membrane PKC-ζ activity was not different in $L6_{PED}$ and control cells. However, PKC-ζ showed no insulin response in $L6_{PED}$ cells despite a greater than twofold activation in the control cells. The expression levels of all of these PKC isoforms were identical in the $L6_{PED}$ and in control myotubes (Fig. 2 [top]). Western blotting of cytosolic and plasma membrane preparations with isoform-specific antibodies showed that most of PKC-α and -β were cytosolic in parental L6 cells, but almost completely translocated to the plasma membranes upon insulin exposure (Fig. 3). At variance, in basal $L6_{PED}$ cells, 70 and 90% of total PKC-α and -β, respectively, localized in the membranes, with very little in the cytosolic fractions. Insulin addition to these cells caused only a small increase in membrane PKC-α and -β. Unlike PKC-α and -β, PKC-ζ was largely cytosolic both in $L6_{PED}$ and in parental cells. In both cell types, PKC-ζ exhibited little membrane translocation upon insulin exposure. Again, the levels of each of these PKC isoforms were identical in parental and PED-transfected cells (not shown). Thus, the overexpression of PED differentially affected the subcellular localization and the function of PKC-α and -β, and that of PKC-ζ.

Effect of PKC-α and -β activation on glucose uptake. We have previously shown that PED-overexpressing L6 cells feature constitutively increased glucose uptake with no further stimulation by insulin (1). Thus, glucose uptake correlates with the activity of PKC-α and -β in these cells. To address this relationship between the glucose transport alterations and PKC activation, we analyzed glucose uptake in $L6_{PED}$ cells upon blocking of PKC-α and -β by using either PKC-α or -β antisense oligonucleotides or the specific PKC-β inhibitor LY379196 (23). As shown in Fig. 4*A* and *B*, transfection of PKC-α or -β antisense oligonucleotides almost completely inhibited the expression of PKC-α or -β, respectively, in the $L6_{PED}$ myotubes and, simultaneously, returned basal glucose uptake to levels only slightly higher than those of $L6_{PC}$ control cells (difference not statistically significant). These changes were

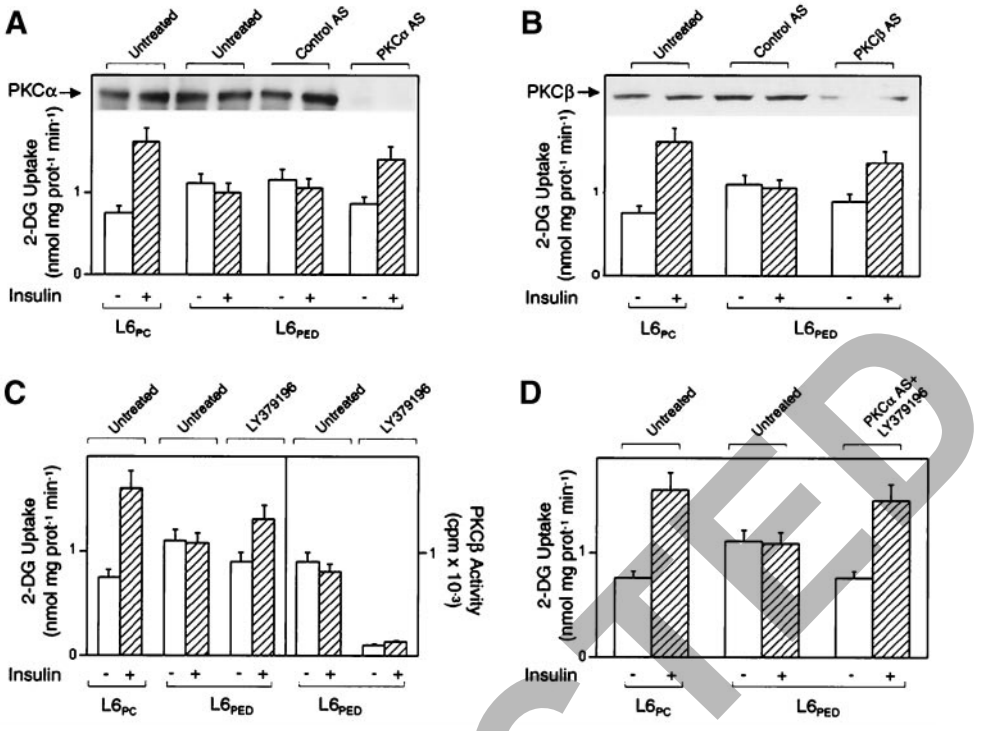


FIG. 4. Effect of inhibition of PKC-α and -β on 2-DG uptake in $L6_{PED}$ cells. *A* and *B*: $L6_{PED}$ cells were transfected with phosphorothioate PKC-α or PKC-β antisense (PKCα AS or PKCβ AS, respectively) or control (control AS) oligonucleotides as reported under RESEARCH DESIGN AND METHODS. The cells were then exposed to insulin as indicated and assayed for 2-DG uptake. The differences in basal 2-DG uptake in $L6_{PED}$ cells transfected with PKC-α or PKC-β AS and in those transfected with the control AS or in the untransfected $L6_{PED}$ cells were statistically significant ($P < 0.001$). For control, aliquots of the cells (50 μg protein/aliquot) were also lysed and blotted with PKC-α or PKC-β antibodies (insets in *A* and *B*). $L6_{PED}$ cells were treated for 30 min with 50 nmol/l LY379196 either alone (*C*) or in combination with PKC-α antisense transfection (*D*). The cells were then assayed for 2-DG uptake as described above. In control experiments, PKC-β activity was also assayed upon cell incubation with LY379196. Bars represent the means ± SD of triplicate determinations in five (*A* and *B*) and four (*C* and *D*) independent experiments.

accompanied by >60% recovery of the insulin-stimulated glucose uptake. Transfection of a control antisense in the $L6_{PED}$ cells had no effect on either the basal or the insulin-stimulated uptake. Treatment of $L6_{PED}$ cells with 50 nmol/l LY379196 inhibited PKC-β activity by 10-fold (Fig. 4*C*). As with the PKC-β antisense, PKC-β inhibition with LY379196 was accompanied by a 20% decrease in basal glucose uptake ($P < 0.05$), and almost 50% recovery of insulin action as compared with $L6_{PC}$ control cells. Simultaneous block of PKC-α and -β completely rescued the low levels of basal glucose uptake of the control cells and restored maximal levels of insulin-stimulated uptake by >85% (Fig. 4*D*).

To further address the role of PKC-α and -β in PED function, we have also analyzed glucose uptake in cells with only the endogenous levels of PED but transfected with either PKC-α or -β_1. Fivefold overexpression of PKC-α and -β_1 in these cells increased basal glucose uptake by 45% ($P < 0.001$), as seen in PED overexpressors (Fig. 5*A*). As in the $L6_{PED}$ cells, insulin stimulation of glucose uptake was abolished in L6 parental cells overexpressing PKC-α and was inhibited by almost 50% in those overexpressing the β_1 isoform. Overexpression of PKC-β_2 affected glucose transport to a degree similar to overexpression of PKC-β_1 (data not shown). These data suggested that the effect of PED overexpression on glucose uptake is caused by induction of PKC-α and -β. Hence, overexpression of these PKC isoforms mimicks the phenotype generated by that of PED gene. As previously reported in PED-overexpressing cells (1), PKC-α and -β, at these low expression levels, did not affect insulin-dependent receptor phosphorylation or phosphorylation of the major substrates insulin receptor substrate (IRS)-1 and IRS-2 (Fig. 5*B*).

The overexpression of PKC-α or -β did not change the total content of either GLUT1 or GLUT4 in L6 cells (Fig. 6). However, as seen in PED-overexpressing cells (1), overexpression of PKC-α or -β in the wild-type L6 myotubes increased basal GLUT1 localization in the plasma membranes by twofold, respectively, as compared with the control cells, impairing subsequent insulin-dependent translocation. GLUT4, the major insulin-dependent glucose transporter, was almost absent in the plasma membrane both in the control cells and in cells overexpressing PED or PKC, under basal conditions. GLUT4 increased its plasma membrane compartmentalization by almost threefold after insulin stimulation of control cells, but the membrane translocation was almost absent in PED and in PKC-α–overexpressing cells, and was reduced by twofold in those overexpressing PKC-β.

Effects of PKC-α and -β on PKC-ζ. Antisense block of PKC-α and LY379196 inhibition of PKC-β in $L6_{PED}$ cells also caused a 70 and 20% recovery of PKC-ζ responsiveness to insulin, respectively (Fig. 7*A*). No significant change occurred in PKC-ζ basal activity. Similar data were obtained using the PKC-β antisense instead of LY379196 (data not shown). This finding suggested that the constitutive activation of PKC-α and -β accompanying the overexpression of PED contributed to the blocking insulin activation of PKC-ζ. To verify this possibility, we analyzed

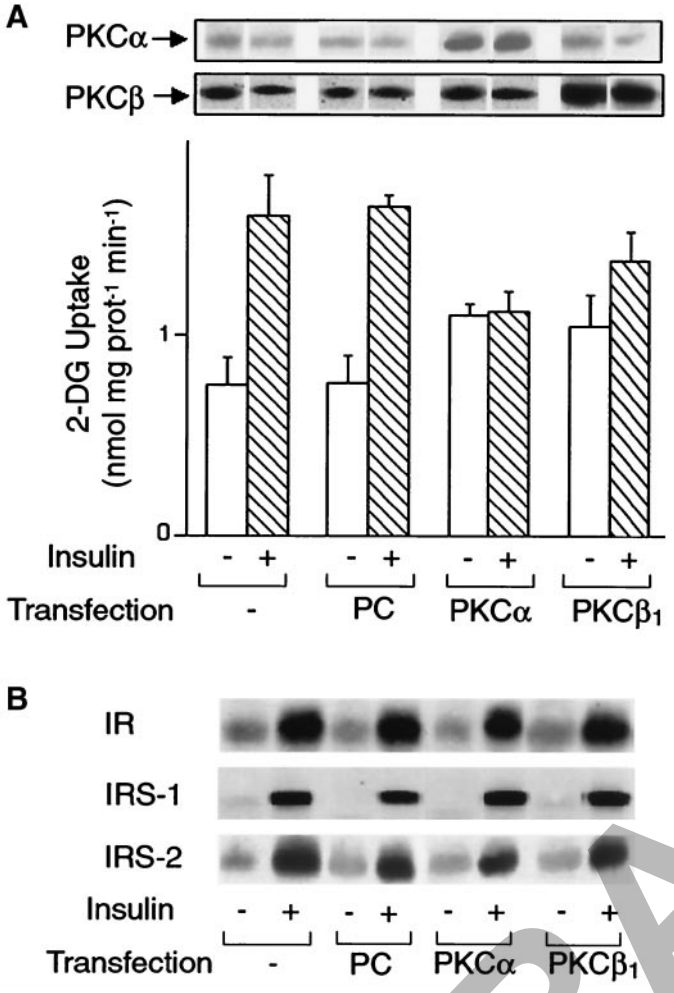


FIG. 5. Effects of overexpression of PKC-α and -β in L6 cells. *A*: Wild-type L6 myotubes were transfected with either PKC-α or PKC-β cDNAs or with the empty vector (PC) and then exposed to 100 nmol/l insulin as indicated. The cells were then assayed for 2-DG uptake as outlined in the legend of Fig. 4. For control, the expression of PKC-α and -β levels was also checked by immunoblotting aliquots of the cells (50 mg protein/aliquot) with specific PKC antibodies (inset). Bars represent the means ± SD of triplicate determinations in four independent experiments. *B*: Cells transfected with PKC-α or PKC-β_1 and control cells were exposed to 100 nmol/l insulin for 10 min, solubilized as described under RESEARCH DESIGN AND METHODS, precipitated with insulin receptor (IR), IRS-1, or IRS-2 antibodies, and then blotted with phosphotyrosine antibodies. The specific bands were revealed by ECL. The autoradiograph shown is representative of three independent experiments.

insulin effect on PKC-ζ in L6 cells expressing only the endogenous PED, but transfected with PKC-α or -β_1. As shown in Fig. 7*B*, overexpression of PKC-α almost completely blocked PKC-ζ induction by insulin with no significant change in its basal activity. Overexpression of PKC-β_1 determined only 40% inhibition of PKC-ζ response to insulin. Overexpression of PKC-β_2 inhibited insulin stimulation of PKC-ζ in a fashion similar to overexpression of PKC-β_1 (data not shown). No significant change in PKC-ζ activity occurred in cells transfected with the vector alone, either in the absence or presence of insulin.

Insulin activation of PKC-ζ has been previously suggested to mediate insulin stimulation of glucose uptake in the L6 skeletal muscle cells (11). We therefore tested the hypothesis that the blocking of PKC-ζ activation caused by the increased function of PKC-α and -β in $L6_{PED}$ cells is responsible for the lack of insulin-dependent increase in glucose uptake. To this end, we have used a dominant-negative PKC-ζ mutant featuring $K^{281}\rightarrow W$ substitution in the kinase domain (PKC-ζ DN). Expression of this mutant inhibited PKC-ζ activation by insulin by >80% in $L6_{WT}$ cells (Fig. 8*A*), while eliciting no measurable effect on activation of PKC-α or -β (data not shown). In parallel with the inhibition of PKC-ζ, expression of the dominant-negative mutant also decreased insulin stimulation of glucose uptake by 80% in these cells (Fig. 8*C*). In addition, 30-fold overexpression of PKC-ζ in the $L6_{PED}$ cells allowed >50% recovery of PKC-ζ sensitivity to insulin (Fig. 8*B*). This recovery was accompanied by almost complete rescue of the insulin-stimulated glucose uptake by the PED overexpressors (Fig. 8*D*). Their high levels of basal glucose uptake remained unchanged, however. Thus, the block of PKC-ζ appeared to play an important role in altering insulin-stimulated glucose uptake—though not the basal glucose uptake—in the $L6_{PED}$ cells.

Mutagenesis of PKC phosphorylation sites of PED. PED possesses three consensus serine phosphorylation sites for PKC (Ser^{33}, Ser^{70}, and Ser^{104}). Of these, Ser^{104} represents a major PKC phosphorylation site in vivo (14); this led us to hypothesize that PED phosphorylation by PKC may also affect its glucose transport regulatory function. To address this possibility, we generated two mutants of PED featuring either the $Ser^{104}\rightarrow Gly$ substitution ($PED_{S1\rightarrow G}$) or the simultaneous substitution of Ser^{33}, Ser^{70}, and $Ser^{104}\rightarrow Gly$ ($PED_{S3\rightarrow G}$). We obtained stable L6 transfectants overexpressing similar levels of either the wild-type or the mutant PED cDNAs (Fig. 9*A*). In these cells, phosphorylation of the $PED_{S1\rightarrow G}$ mutant in response to TPA was reduced by 80% as compared with the wild-type PED, whereas phosphorylation of the $PED_{S3\rightarrow G}$ mutant was completely abolished (Fig. 9B). However, glucose uptake was similarly altered by expression of the mutant and the wild-type PED, either in the absence or presence of insulin (Fig. 10, top panel). Similar results were obtained using the cell pools instead of the mutant clones (data not shown). PKC activity was also constitutively increased in cells transfected with the mutated PED

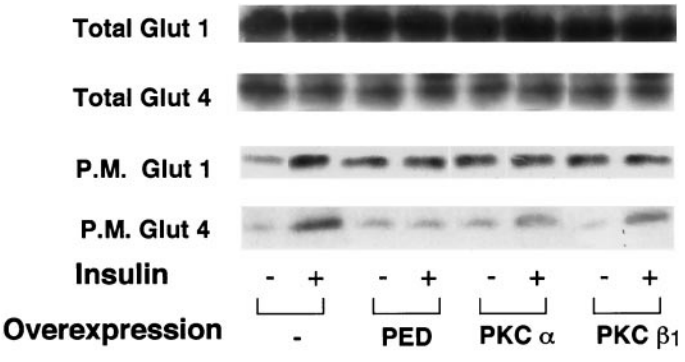
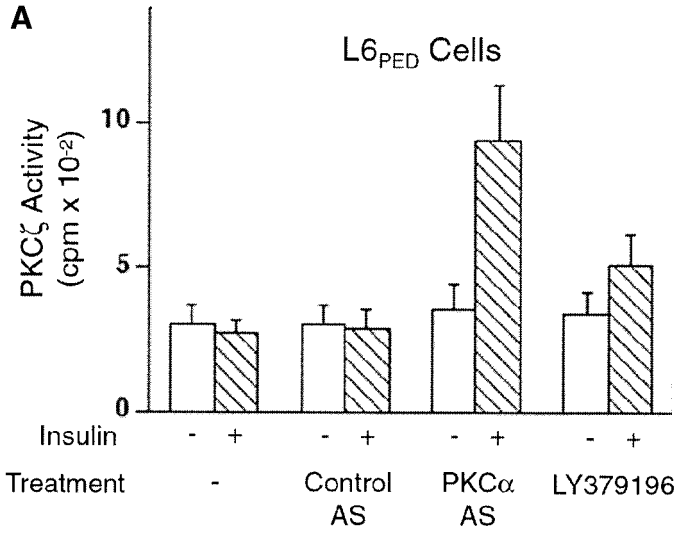


FIG. 6. Glucose transporter localization in L6 cells overexpressing PKC-α and -β. For determining the total levels of transporter, cells transfected with PED, PKC-α, or PKC-β_1 and control cells were exposed to 100 nmol/l insulin for 30 min, solubilized, and then blotted with GLUT1 or GLUT4 antibodies. Alternatively, for quantitating the membrane levels of transporter, plasma membranes (P.M.) were prepared from the cells as described under RESEARCH DESIGN AND METHODS. The membranes were then solubilized and blotted with GLUT1 or GLUT4 antibodies as outlined above. Filters were revealed by ECL. The autoradiograph shown is representative of three (GLUT1) and four (GLUT4) independent experiments.



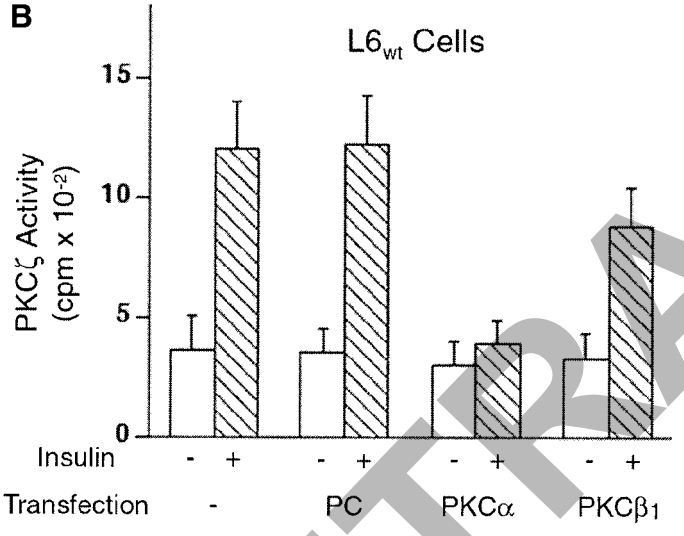


FIG. 7. Effect of PKC-α and -β activities on PKC-ζ. *A*: $L6_{PED}$ cells were either incubated with 50 nmol/l LY379196 or transfected with control or PKC-α antisense (AS) oligonucleotides. The cells were then exposed to 100 nmol/l insulin and assayed for PKC-ζ as outlined in the legend to Fig. 2. *B*: Wild-type L6 cells were transfected with PKC-α or -β or with the empty vector (PC), as indicated. The cells were then stimulated with insulin and assayed for PKC-ζ as above. Bars represent the means ± SD of triplicate determinations in five (*A*) and four (*B*) independent experiments. Insulin activation of PKC-ζ in cells transfected with PKC-$β_1$ was significant ($P < 0.001$); that of PKC-α was not statistically significant.

cDNAs—same as in those transfected with wild-type PED (Fig. 10, bottom panel), indicating that PED is not controlled by PKC phosphorylation, at least in its glucose uptake regulatory function.

DISCUSSION

PED is overexpressed by two- to threefold in tissues from type 2 diabetic subjects (1). A comparable overexpression in muscle and adipose cells increases the plasma membrane content of GLUT1 and basal glucose transport (1). These changes are accompanied by impaired insulin stimulation of glucose transport and GLUT4 translocation. We have also shown that inhibition of PKC activity may reverse these effects in PED overexpressors (1), implying an important role of PKC in the function of PED. However, the mechanism by which PED regulates glucose transport is still undefined. In the present work, we showed that L6 myotubes overexpressing PED ($L6_{PED}$) feature constitutive activation of PKC-α and -β. In these same cells, antisense block of PKC-α and β expression as well as pharmacological inhibition of PKC-β restores most of the defect in both basal and insulin-stimulated glucose uptake. A three- to fivefold overexpression of PKC-α or -β in wild-type L6 cells increases the plasma membrane content of GLUT1 and basal glucose transport, and impairs insulin stimulation of glucose transport and transporter translocation, mimicking the effects of PED overexpression. In addition, mutation of Ser^{104}, the major PKC phosphorylation site in PED, does not change the effects of PED overexpression, indicating that PED is not itself controlled by PKC phosphorylation in its glucose uptake regulatory function. Thus, PKC-α and -β play a major role in controlling glucose transporter trafficking and glucose transport in L6 cells and appear to serve as downstream mediators

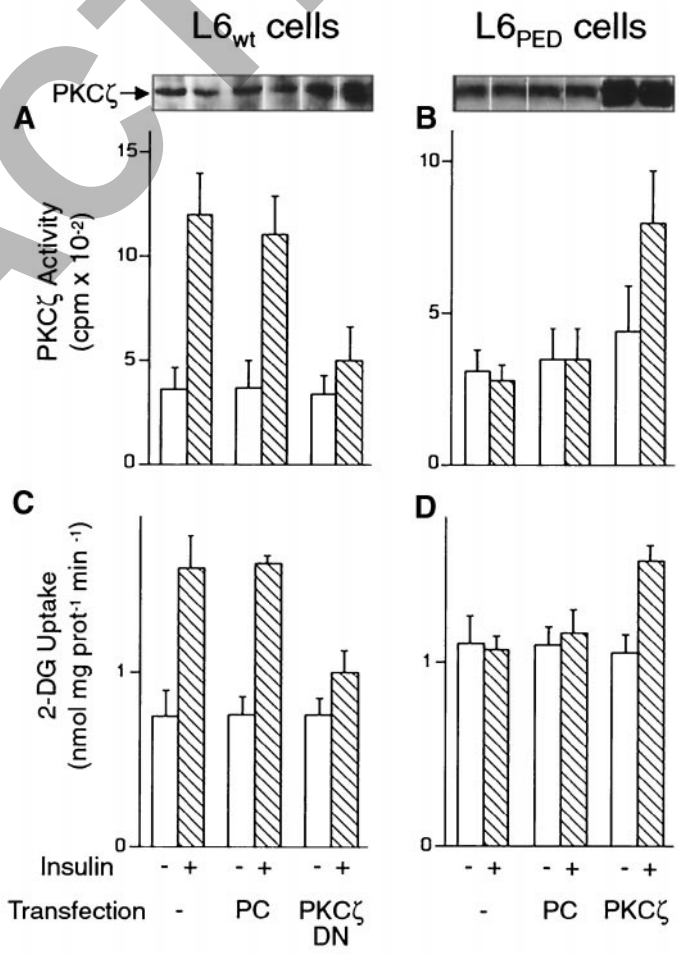


FIG. 8. PKC-ζ action on 2-DG uptake in L6 cells. *A* and *C*: Wild-type L6 myotubes were transfected with the $K^{281} \rightarrow W$ negative-dominant PKC-ζ mutant (PKCζ DN) or with the vector alone (PC) as indicated. The cells were then stimulated with 100 nmol/l insulin and assayed for either PKC-ζ activity (*A* and *B*) or 2-DG uptake (*C* and *D*) as outlined in the legends to Figs. 2 and 4, respectively. *B* and *D*: $L6_{PED}$ cells were transfected with either PKC-ζ cDNA or the plasmid DNA alone (PC), as indicated. The cells were then stimulated with insulin and assayed for PKC-ζ activity (*A* and *B*) or 2-DG uptake (*C* and *D*), as above. Bars represent the means ± SD of triplicate determinations in three (*A* and *B*) or four (*C* and *D*) independent experiments.

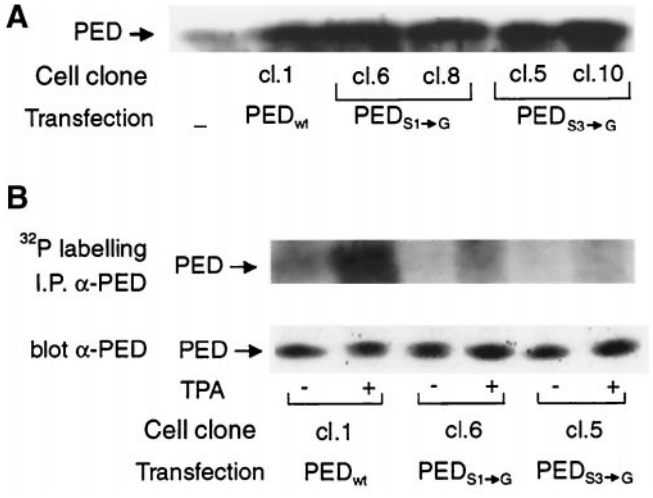


FIG. 9. Function of phosphorylation-defective PED mutants in L6 cells. *A*: Parental (untransfected) L6 cells or cells overexpressing wild-type PED or the phosphorylation-defective mutant PED$_{S1→G}$ (cell clones 6, 8) and PED$_{S3→G}$ mutants (cell clones 5, 10) were lysed as described under RESEARCH DESIGN AND METHODS. Lysed proteins were then subjected to Western blotting analysis using PED antibodies and ECL. *B*: Cells were labeled with [^{32}P]orthophosphate as described under RESEARCH DESIGN AND METHODS and then exposed to 1 μmol/l TPA. Cell lysates were then precipitated (I.P.) with PED antibodies, separated by SDS-PAGE, and revealed by autoradiography. The same gel was subsequently rehydrated, immunoblotted with PED antibodies, as indicated, and revealed by ECL. The autoradiographs shown are representative of four (*A*) and three (*B*) independent experiments with different clones of cells expressing the PED mutants.

of PED action rather than upstream modulators of its function.

Previous studies in other laboratories (24–26) have shown that high-level overexpression of classic PKCs, including PKC-α and -β, may impair insulin responses by inhibiting early steps in the insulin signal transduction system. In L6$_{PED}$ cells (1), as well as in the cells transfected with PKC-α and -β described in the present report, insulin receptor autophosphorylation, phosphorylation of IRS-1 and IRS-2, and phosphatidylinositol (PI) 3-kinase activation occur normally. The modest increase in PKC expression achieved in our experiments likely accounts for the difference with those previous studies in which PKC was overexpressed at 20-fold higher levels. In fact, 20- to 40-fold PKC-α overexpression also inhibits phosphorylation of insulin receptor and IRS-1 in the L6 muscle cells (data not shown). Thus, impairment of early signaling events is not the major mechanism through which activated PKC-α and -β in PED overexpressors interfere with insulin action on glucose uptake. Interestingly, however, we found that L6$_{PED}$ cells feature complete blocking of PKC-ζ induction by insulin. Impaired activation of PKC-ζ also occurs in the L6 cells overexpressing PKC-α and, less evidently, in those overexpressing PKC-β. In addition, antisense block of PKC-α expression in L6$_{PED}$ cells almost completely rescues insulin induction of PKC-ζ activation, and recovery of PKC-ζ activation was also achieved by pharmacological inhibition of PKC-β, though less effectively than by blocking PKC-α expression. Thus, in L6$_{PED}$ cells, PKC-ζ is controlled by PKC-α and, to a lesser extent, by PKC-β. Their chronic activation caused by the overexpression of PED appears to downregulate insulin induction of PKC-ζ activity. To our knowledge, this is the first evidence indicating inhibition of PKC-ζ by a distinct PKC isoform. The significance of this finding to in vivo glucose metabolism deserves further analysis. The use of some PKC inhibitors does not appear to improve insulin sensitivity in vivo (27). However, data from our own and other laboratories (28) indicate that insulin induces PKC-α and -β translocation and activation in rat skeletal muscle in vivo. In addition, consistent with the present work, ablation of the PKC-β gene appears to enhance insulin action on glucose metabolism in mice (29).

The mechanism of PKC-ζ inhibition by PKC-α and -β is under investigation in our laboratory. It might involve a direct interaction of PKC-α and -β with PKC-ζ. In fact, there is evidence that PKC-α coprecipitates with PKC-ζ in lysates from HEK293 cells (data not shown). More likely, PKC-α and -β inhibit PKC-ζ through an indirect mechanism, at least in the L6 muscle cells. Consistent with this possibility, we show in this report that PED induction of PKC-α and -β results in increased membrane localization of these PKCs. At variance, both in PED overexpressors and in control cells, PKC-ζ remains largely cytosolic (i.e., in a compartment different from that where most active PKC-α and -β localize). PI 3-kinase and Akt/protein kinase B (PKB) are major molecules implicated in transducing

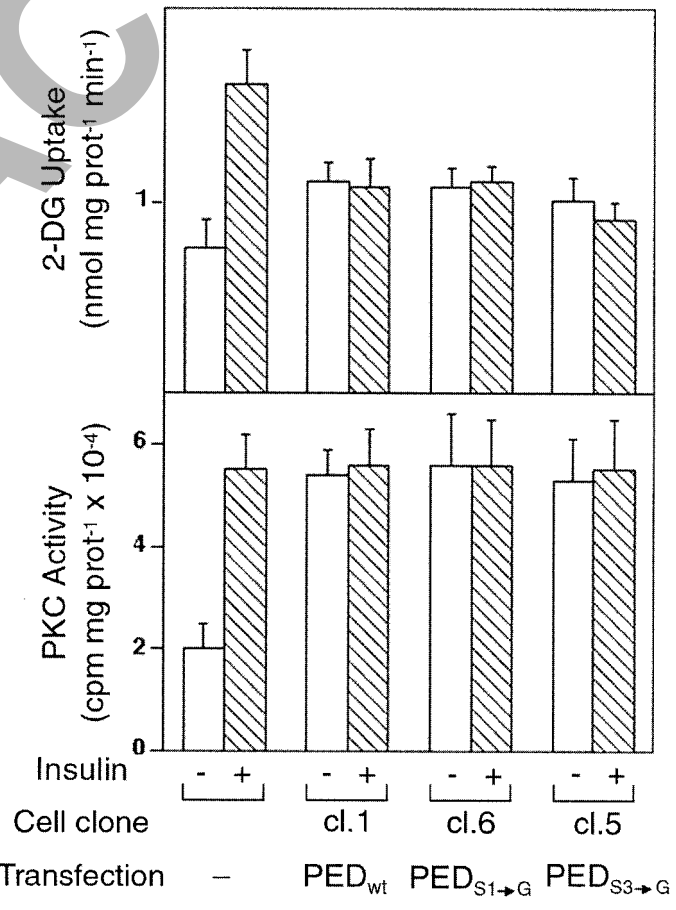


FIG. 10. 2-DG uptake and PKC activity in L6 cells expressing phosphorylation-defective PED mutants. The cells, either the untransfected cells or those expressing the wild-type PED (PED$_{WT}$) or the mutant PED cDNAs, were incubated in the absence or presence of 100 nmol/l insulin and assayed for 2-DG uptake or PKC activity as outlined in legends to Figs. 4 and 1, respectively. Bars represent the means ± SD of triplicate determinations in four (top panel) and five (bottom panel) independent experiments. Identical results were obtained with the other clones of PED overexpressors.

insulin signal to the glucose transporters (30,31). However, insulin activation of PI 3-kinase and Akt/PKB occurs normally in PED-overexpressing cells (1 and data not shown), suggesting that PED interference with glucose transport regulation does not occur at the level of PI 3-kinase or Akt/PKB. There is also evidence indicating that induction of PKC-ζ function is required for the physiological activation of glucose transport by insulin in adipocytes and muscle cells (11–13). In fact, similar to previous studies by Bandyopadhyay et al. (11), we show that a negative-dominant PKC-ζ mutant inhibits insulin activation of glucose uptake in wild-type L6 skeletal muscle cells. Also, in $L6_{PED}$ cells, overexpression of PKC-ζ to levels enabling recovery of its activation by insulin leaves the high basal glucose uptake unchanged but allows further insulin-dependent uptake. It therefore appears that the altered glucose transport caused by PED overexpression in L6 cells is contributed by changes in both PKC-α and -β and in PKC-ζ activities. The activation of PKC-α and -β in $L6_{PED}$ cells induces GLUT1 membrane translocation and increases non-insulin-dependent glucose uptake. Activation of PKC-α and, to a lesser extent, PKC-β also downregulates insulin induction of PKC-ζ function, additively impairing insulin-stimulated glucose transport and GLUT4 translocation.

Insulin activation of PKC-α and -β in the L6 muscle cells and in rat skeletal muscle has been previously observed in several laboratories, including ours (10,28,32,33). However, the role of PKC-β in insulin-stimulated glucose transport has been controversial. On the basis of expression studies with a COOH-terminal truncated mutant of PKC-β_2, Chalfant et al. (9) provided early evidence suggesting a potential role of PKC-β_2 in insulin-stimulated glucose transport in L6 cells. Subsequent investigations in these same cells (11) and in PKC-β knockout mice (29) indicated that DAG-sensitive PKC, including PKC-β_2, is not required for insulin stimulation of glucose uptake, and may exert an inhibitory role. The same result is obtained in the present study in $L6_{PED}$ cells. Similar to the role it plays in the L6 muscle cells, PKC-ζ has been reported to play a major role in transducing insulin activation of glucose transport in several other cell types (12,13). Recently, in primary cultures of rat skeletal muscle cells, Braiman et al. (34) have reported that selective blocking of PKC-δ with rottlerin prevents insulin-induced GLUT4 translocation and reduces insulin-stimulated glucose uptake. It has been concluded from these findings that PKC-δ has a major role in mediating glucose transport in these cells. This is not the case with the L6 cells, however. Hence, antisense blocking of PKC-δ expression in L6 cells does not affect either the basal or the insulin-stimulated glucose uptake, whereas antisense inhibition of PKC-ζ impairs GLUT4 translocation (data not shown). The regulation of the glucose transport apparatus may feature cell specificity so that different PKC isoforms are involved in different tissues. The finding that in the primary skeletal muscle cells, at variance from the L6 cells, PKC-α does not affect the basal glucose transport (10) is consistent with this possibility.

In a previous publication, we reported that PED inhibits the apoptotic responses elicited by FAS ligand and tumor necrosis factor–α in different cell types (35). PED antisense studies and subsequent work in PED knockout mice indicated that protection from apoptosis is a physiological function of PED (36). Its relationships with the effects on glucose transport are still under investigation in our laboratory. At variance with PED effects on glucose transport, however, PED antiapoptotic effect is controlled by PKC phosphorylation (37). Hence, mutation of the PKC phosphorylation site in PED destroys its apoptosis-protective effect, indicating independent regulation of PED antiapoptotic and glucose uptake regulatory functions. Thus, PED appears to play a multifaceted role in cell survival, simultaneously controlling at least two major functions involved in survival (i.e., glucose utilization and programmed cell death).

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