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Protein Kinase C-Independent Effects of Protein Kinase D3 in Glucose Transport in L6 Myotubes

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ABSTRACT

Protein kinase C (PKC) and protein kinase D (PKD) coordinate and regulate many fundamental cellular processes. In this study, we evaluate the role of classic and novel PKC (c/nPKC) and PKD in glucose transport in L6 myotubes. c/nPKC is either activated by short-term phorbol 12-myristate 13-acetate (PMA) treatment or down-regulated by prolonged PMA treatment at a high dose in L6 myotubes. Our results indicate that PMA treatments have little impact on basal and insulin-stimulated glucose uptake and insulin-induced Akt activation. In contrast, the PKC inhibitors Go6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*] carbazole], Go6983 [2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide], GF 109203X [bisindolylmaleimide I; 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(1*H*-indol-3-yl)maleimide], and Ro 31-8220 [bisindolylmaleimide IX; 2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)maleimide] block basal and insulin-stimulated glucose uptake, and their inhibitory effects persist upon down-regulation of c/nPKC by PMA, implying the presence of PKC-independent effectors in mediating their inhibition of glucose uptake. Go6976, the potent cPKC inhibitor that also effectively inhibits PKD, dose-dependently blocks basal glucose uptake in L6 myotubes, whereas Go6983, the nonselective PKC inhibitor that is ineffective for PKD, has little effect on basal glucose uptake, implying the involvement of PKD in this process. Most prominently, adenoviral gene expression of a dominant-negative PKD isoform, PKD3, primarily inhibits basal glucose uptake and, to a lesser extent, insulin-stimulated glucose uptake, whereas overexpression of wild-type PKD3 significantly enhances basal glucose uptake. Moreover, expression of a PKD3-targeted siRNA significantly inhibits basal glucose uptake. Taken together, our results indicate that PKD, specifically PKD3, directly contributes to insulin-independent basal glucose uptake in L6 skeletal muscle cells.

Glucose transport is a fundamental process of the cell. Insulin-independent glucose transport accounts for most of the basal glucose uptake, whereas insulin-dependent glucose transport is mainly responsible for insulin-stimulated glucose uptake. Although basal glucose uptake is a constitutive process, insulin-induced glucose uptake requires the signaling cascade evoked by insulin. This involves the sequential phosphorylation and activation of insulin receptor substrate 1, phosphoinositol 3-kinase (PI3K), phosphoinositol-dependent kinase 1, and its two downstream targets, protein kinase B (PKB)/Akt and protein kinase Cζ (PKCζ) (Nystrom and Quon, 1999; Saltiel and Pessin, 2002).

Glucose uptake is mediated by a group of facilitative glucose transporters (GLUTs), in which GLUT1, a ubiquitously-distributed transporter, is implicated in basal glucose uptake, and GLUT4, a muscle- and fat-distributed transporter, mediates insulin-stimulated glucose uptake (Kraegen et al., 1993; Robinson et al., 1993). In the case of insulin-dependent transport, the activation of PKB/Akt and PKCζ induces glucose uptake by recruiting the internally sequestered insulin-sensitive GLUT4 to the plasma membrane (Saltiel and Pessin, 2002; Rudich and Klip, 2003).

Skeletal muscle accounts for the majority of insulin-stimulated glucose uptake in rodents and humans. Reduction of glucose uptake in muscles in the state of insulin resistance is the principal factor that accounts for reduced systemic glu-

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ABBREVIATIONS: PI3K, phosphoinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; c/nPKC, classic and novel PKC; GLUT, glucose transporter; DAG, diacylglycerol; PKD, protein kinase D; MAPK, mitogen-activated protein kinase; PH, pleckstrin homology; Ro 31-8220, bisindolylmaleimide IX, 2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)maleimide; GSK, glycogen synthase kinase; Go6983, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide; PMA, phorbol 12-myristate 13-acetate; GF 109203X, bisindolylmaleimide I, 2-[1-(3-dimethylamino-propyl)indol-3-yl]-3-(1 *H*-indol-3-yl)maleimide; Go6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*] carbazole; wt, wild-type; DN, dominant-negative; Adv, adenovirus; MEM, minimal essential medium; FBS, fetal bovine serum; [^{3}H]2-DG, [^{3}H-1,2]2-deoxy-D-glucose; MOI, multiplicity of infection(s); GFP, green fluorescent protein; RFP, red fluorescent protein.

cose utilization. In this regard, skeletal muscle has long been regarded as a critical organ/cellular system for the investigation of insulin resistance and the pathogenesis of type II diabetes. L6 myoblasts are derived from rat skeletal muscle, and this cell line is one of the most frequently used cellular model systems to investigate insulin-stimulated glucose transport. These cells are unique among other muscle cell lines in that, upon differentiation to myotubes, insulin induces significant glucose uptake to these cells, reflecting their expression of the muscle/fat-specific GLUT4 glucose transporters. In addition, the GLUT1 glucose transporter responsible for basal glucose uptake is also expressed. Thus, this cell line serves as an ideal model system to investigate both insulin-dependent and -independent glucose transport.

The PKC family comprises 11 isoforms that are classified as cPKC [α, βI/βII, and γ; Ca^{2+}- and diacylglycerol (DAG)-dependent], nPKC (δ, ϵ, η, and θ; Ca^{2+}-independent but DAG-dependent), and aPKC (ζ and ι/λ; Ca^{2+}- and DAG-independent) (Newton, 2001). Although PKCζ mediates insulin-stimulated glucose transport (Bandyopadhyay et al., 1997, 1999), the role of c/nPKC in glucose transport is rather obscure.

Protein kinase D (PKD) is a newly identified serine/threonine kinase family. It shares higher homology to the Calcium-Calmodulin kinases and is more distant to the AGC family of kinases such as PKC. It has now been classified as a subfamily of the Calcium-Calmodulin kinase superfamily (Manning et al., 2002). Four isoforms have been identified for the PKD family, including PKD1 (mouse PKD and its human homolog PKCμ), PKD2, and PKD3 (human PKCν) (Valverde et al., 1994; Hayashi et al., 1999; Sturany et al., 2001). Among members of the PKD family, PKD1 is the best characterized. PKD has been implicated in regulating protein trafficking and possibly apoptosis. It also modulates a number of signaling pathways such as MAPK and c-Jun NH_2-terminal kinase signaling pathways (Van Lint et al., 2002; Rykx et al., 2003). Most notably, PKD1 has been demonstrated to regulate the trafficking of transport carriers from the *trans*-Golgi network to the cell surface (Jamora et al., 1999; Liljedahl et al., 2001; Baron and Malhotra, 2002). Similar effects have been reported for PKD2 and PKD3 (Yeaman et al., 2004). Many reagents are known to activate PKD1 in vivo. These include pharmacological agents (phorbol esters and DAG analogs) and many physiological stimuli including G protein-coupled receptor agonists, growth factors, and antigen-receptor engagement (Zugaza et al., 1996, 1997; Waldron and Rozengurt, 2000; Van Lint et al., 2002). In all cases, PKD has been shown to be activated through a PKC-dependent signaling pathway that involves the phosphorylation of $Ser^{744/748}$ in the activation loop of the catalytic domain of PKD1 (Zugaza et al., 1996; Iglesias et al., 1998; Waldron et al., 2001). Later, novel PKCϵ and -η have been shown to phosphorylate $Ser^{744/748}$ in the activation loop of PKD1 and activate PKD1 by releasing the autoinhibiton of the PH domain (Waldron and Rozengurt, 2002). Oxidative stress also activates PKD1, and Src, phospholipase C, and PKC inhibitors can block this activation. It has been suggested that oxidative stress leads to the activation of Src and phospholipase C, which activate several PKCs and in turn PKD1 (Waldron and Rozengurt, 2000). In addition to the PKC-dependent activation, recent studies indicate that PKD can also be regulated through mechanisms that are independent of PKC (Jamora et al., 1999; Endo et al., 2000; Storz et al., 2003; Lemonnier et al., 2004).

The PKC inhibitors developed in recent years show much improved selectivity toward different PKC isoforms; however, some also exert actions on targets that are unrelated to and independent of PKC (Davies et al., 2000). For example, the bisindolylmaleimide derivative Ro 31-8220 exhibits varying potencies against MAPK-activated protein kinase 1, mitogen- and stress-activated protein kinase 1, serum- and glucocorticoid-induced kinase, p70 ribosomal protein S6 kinase, glycogen synthase kinase 3 (GSK-3), and other kinases (Alessi, 1997; Davies et al., 2000). Likewise, other than inhibiting classic PKCs, Go6976 also potently inhibits PKD (Gschwendt et al., 1996; Lemonnier et al., 2004). Thus, these studies indicate the need for caution when using PKC inhibitors and the necessity of using multiple approaches to confirm PKC-relevant signaling mechanisms. Here we use adenoviruses carrying wild-type (wt) and dominant-negative (DN) PKD3 and PKD3-targeted siRNA to further examine and validate our conclusions derived from the studies using PKC/PKD inhibitors.

In summary, we have sought to dissect the role of PKD—specifically PKD3—in glucose uptake in L6 myotubes using PKC/PKD-selective inhibitors as well as adenoviruses carrying wild-type and dominant-negative PKD3. We find that blocking the activity of endogenous PKD3 by PKD inhibitor or DN-PKD3 as well as knocking down the endogenous expression of PKD3 primarily inhibits basal glucose uptake. These findings imply that PKD3 predominantly regulates basal glucose uptake and, to a lesser extent, insulin-stimulated glucose uptake in L6 myotubes.

Materials and Methods

Materials. Go6983 and wortmannin were purchased from EMD Biosciences (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA), GF 109203X, Ro 31-8220, and Go6976 were obtained from LC Laboratories (Woburn, MA). Insulin, α-minimal essential medium (α-MEM), fetal bovine serum (FBS), and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Penicillin/streptomycin was purchased from Cambrex Bio Science Baltimore, Inc. (Baltimore, MD). 2-Deoxy-D-glucose and cytochalasin B were obtained from Sigma-Aldrich (St. Louis, MO). [^{3}H-1,2]2-Deoxy-D-glucose was purchased from MP Biomedicals (Irvine, CA).

Cell Culture. The rat L6 skeletal muscle cell line was obtained from Dr. Amira Klip (The Hospital for Sick Children, Toronto, ON, Canada). L6 myoblasts were grown and maintained in α-MEM supplemented with 10% FBS, 1000 units/l penicillin, and 1 mg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C. For differentiation, L6 myoblasts were plated at 4×10^4 cells/ml in α-MEM supplemented with 2% FBS to induce spontaneous fusion of myoblasts to myotubes. The formation of myotubes was monitored by phase contrast microscopy. The differentiation medium was replenished every 48 h. Mature myotubes formed within 7 to 8 days after cell seeding.

[^{3}H-1,2]2-Deoxy-D-glucose Uptake Assay in L6 Myotubes. [^{3}H-1,2]2-Deoxy-D-glucose ([^{3}H]2-DG) uptake assay was carried out in 24-well plates as described by Klip and Ramlal (1987), with minor modifications. In brief, L6 myotubes were serum-starved for 3 h before experimentation. Cells were treated with PMA or insulin in the presence or absence of various PKC inhibitors at specified concentrations, as indicated in the text for the final portion of the 3 h. After treatment, myotubes were washed once with HEPES-buffered saline (140 mM NaCl, 20 mM Na-HEPES, 5 mM KCl, 2.5 mM $MgSO_4$, and 1.0 mM $CaCl_2$, pH 7.4) at room temperature, followed by incubation for 10 min in HEPES-buffered saline containing 10 μM of unlabeled 2-deoxy-D-glucose and 0.5 μCi/ml [^{3}H-1,2]2-deoxy-D-glucose. Nonspecific uptake was determined in the presence of 10 μM

cytochalasin B. Uptake was stopped by two rapid washes of ice-cold 0.9% NaCl (w/v). Cells were lysed with 0.05 N NaOH at room temperature for 30 min with continuous shaking. Four hundred microliters of lysate from each well was transferred to a scintillation vial, and the associated radioactivity was determined for 5 min in an LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

PKD3 Antibody and Western Blot Analysis. A custom-made PKD3-specific antibody was raised in rabbit against a PKD3 C-terminal peptide conjugated to ovalbumin (Covance Research Products, Berkeley, CA). Antisera showing immunoreactivity to PKD3 were affinity-purified and concentrated for immunoblotting, immunoprecipitation, and immunostaining.

Cells in six-well plates were lysed using lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 μM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM $NaVO_3$, 10 mM NaF, and one tablet of protease inhibitor]. Protein concentration was determined by a microprotein assay using the bicinchoninic acid protein assay kit according to manufacturer's instructions (Pierce, Rockford, IL). Approximately 40 μg of lysates were mixed with equal volumes of 2× SDS sample loading buffer (60 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, and 2% SDS) and size-fractionated by electrophoresis on 7.5% SDS-polyacrylamide gel electrophoresis at 100 V for 1 h followed by electrotransfer onto a nitrocellulose membrane at 100 V for 1 h. The membrane was preblotted with 5% dry milk in Tris-buffered saline (50 mM Tris-HCl and 150 mM NaCl, pH 7.5) at room temperature for 1 h. The blots were probed with rabbit or mouse antisera raised against all isoforms of PKC (Invitrogen), PKD1 (Santa Cruz Biotechnology. Inc., Santa Cruz, CA), PKD2 (EMD Biosciences), PKD3 (as described above), Akt, and phospho-Akt (Cell Signaling Technology Inc., Beverly, MA) antibodies. Goat anti-rabbit IgG or goat anti-mouse IgG, coupled to horseradish peroxidase (1:1000; Bio-Rad, Hercules, CA), were used as the secondary antibodies. Bands were visualized by the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences Inc., Piscataway, NJ).

Immunoprecipitation and PKCζ Kinase Assay. Cells were harvested in lysis buffer. PKCζ was immunoprecipitated by incubating 200 μg of total cellular protein with protein A/G Sepharose beads (Santa Cruz Biotechnology, Inc.) preconjugated with anti-PKCζ antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C with constant rotation. The immunocomplexes were then pelleted and washed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, and 1% Triton X-100). Equal amounts of immunocomplex were then subjected to PKCζ kinase assay, as described previously (Bandyopadhyay et al., 1997). In brief, the assay was carried out by coincubating 20 μl of immunoprecipitated PKCζ with 50 μM ATP, 5 μg of phosphatidylserine, and 40 μM PKCε substrate peptide, a preferred substrate of PKCζ (Upstate Biotechnology, Lake Placid, NY), 0.2 μl of [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) in a final volume of 50 μl. The reaction was allowed to proceed at 30°C for 10 min. An aliquot of the reaction mixture was then spotted on p81 paper, washed in 5% acetic acid, and counted.

Construction of DN-PKD3 and Generation of Adenoviruses Carrying wt- and DN-PKD3. The wt-PKD3 cDNA cloned in the pGEM-Teasy vector was described previously (Hayashi et al., 1999). The kinase inactive D720A PKD3 was prepared by mutating an aspartic acid at position 720 (corresponds to Asp733 in PKD and Asp727 in PKCμ) to an alanine using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primers are GTGAAGCTGTGTGCCTTTGGATTTGCACGC (sense) and GCGTGCAAATCCAAAGGCACACAGCTTCAC (antisense).

The polymerase chain reaction fragments of PKD3 and DN-PKD3 were inserted into an XhoI/XbaI site of pShuttle-CMV, an AdEasy adenovirus shuttle vector with cytomegalovirus promoter (obtained from the Vogelstein laboratory at the Johns Hopkins Oncology Center, Baltimore, MD). The recombinant adenovirus construct carrying these genes was generated by homologous recombination in *Escherichia coli* BJ5183 cells between the shuttle vector and an adenoviral backbone vector, pAdEasy-1, as described previously (He et al., 1998). Recombinant plasmid was then transfected into 293 cells to generate the replication-deficient adenoviruses. Large-scale virus production was obtained by amplification and purification using cesium chloride gradient banding. Titers of the viral stocks were normally higher than 1×10^9 plaque-forming units/ml. An adenovirus containing the *lacZ* gene was used as a control.

Infection with Adenovirus. Subconfluent cultures were infected with PKD3 adenoviruses at different multiplicities of infection (MOI) in a minimum volume of serum-free medium. Plates with cultures incubated with the adenoviruses were gently rocked a few times during incubation to maximize contact with cells. After 90 min of incubation at 37°C, more growth medium was added, and the cultures were continued for 24 h before harvesting for assays.

Indirect Immunofluorescence and Confocal Microscopy. A previously characterized pcDNA3-GLUT4-eGFP (GFP-GLUT4) construct was kindly provided by Dr. Jeff Pessin (Department of Physiology, the University of Iowa, Iowa City, IA). To generate the dominant-negative red fluorescent protein (RFP) fusion construct (RFP-DN-PKD3), the kinase-inactive D720A PKD3 was amplified by polymerase chain reaction and subcloned into a SacI/SalI site of a pDsRed1-C1 vector (BD Biosciences Clontech, Palo Alto, CA) to generate an N-terminal fusion of PKD3.

To image GLUT4, GFP-GLUT4 was cotransfected with pDsRed1-C1 empty vector or RFP-DN-PKD3. Cells were imaged live 2 days after transfection using a Zeiss LSM 5 inverted confocal fluorescent microscope (Carl Zeiss Optical Inc., Chester, VA). To image GLUT1, L6 myoblasts with or without transfection were fixed with 3.7% paraformaldehyde at room temperature for 30 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline plus 1 mM EGTA for 5 min at room temperature. After preblocking with 2% bovine serum albumin in phosphate-buffered saline for 30 min, cells were incubated with an anti-GLUT1 antibody (EMD Biosciences) at 1:100 for 1 h, washed, and then incubated with the secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG; 1:100) for 1 h. The slide was mounted using DAKO fluorescent mounting medium (DakoCytomation California Inc., Carpinteria, CA) and imaged under a confocal fluorescent microscope.

Statistical Analysis. All experiments were carried out independently at least three times. Triplicate determinations were obtained for each of the [^{3}H]2-DG uptake assays. Results are expressed as the mean ± S.E.M. Comparison of means was performed using a two-tailed Student's t test. $p < 0.05$ is considered to be statistically significant.

Results

Expression of PKC and PKD Isoforms in L6 Myoblasts and Myotubes. We first examined the levels of endogenous PKC and PKD in L6 myoblasts and differentiated myotubes. Among PKC isoforms, only PKCα, -δ, -ε, and -ζ were detected, whereas all PKDs, namely PKD1–3, were detected and expressed at similar levels (Fig. 1). Meanwhile, the levels of PKC and PKD before and after differentiation were also relatively constant.

To evaluate the contribution of c/nPKC in glucose uptake in L6 myotubes, we sought to down-regulate the endogenous c/nPKC by prolonged PMA treatment (Klip and Ramlal, 1987). PMA at 1 μM for 24 h completely down-regulated the endogenous PKCα and -δ and >90% of the endogenous level of PKCε in L6 myotubes (Fig. 1). In contrast, the levels of PMA-insensitive atypical PKCζ and the endogenous PKD1–3 were not affected by this treatment. In addition, short-term

treatment with PMA and insulin at 100 nM for 10 to 20 min did not alter the levels of endogenous PKC and PKD in L6 myotubes (Fig. 1). We noted the appearance of higher molecular weight bands of PKD1 and PKD2 upon 1 μM PMA treatment for 24 h that were absent when each underwent short-term treatment with PMA and insulin. These bands may correspond to the hyperphosphorylated forms of PKD1 and PKD2.

c/nPKC Was Not Involved in Basal and Insulin-Stimulated Glucose Uptake in L6 Myotubes. The L6 myoblasts used in our study have been described previously by Mitsumoto et al. (1991). Upon differentiation to myotubes, these cells typically show an 80 to 100% increase in glucose uptake in response to a prior 20-min stimulation with 100 nM insulin. In line with this and as shown in Figs. 2 and 4, we obtained comparable levels of glucose uptake in response to insulin stimulation in L6 myotubes.

To assess the contributions of c/nPKC isoforms in basal and insulin-stimulated glucose uptake, we altered the levels and activities of c/nPKC by short- and long-term PMA treatments and evaluated their impact on basal and insulin-stimulated glucose uptake. As shown in Fig. 2, short- and long-term PMA treatments neither significantly altered basal glucose uptake ($p > 0.05$ versus non-PMA-treated cells) (Fig. 2a, uptake at 0 nM insulin) nor affected the insulin-stimulated glucose uptake at 1 to 100 nM insulin ($p > 0.05$ versus non-PMA-treated cells) (Fig. 2, a and b). Lower (0.03–1 nM) or higher (100 nM–1 μM) insulin concentrations yielded similar results (data not shown). Our findings indicate that c/nPKC is not involved in basal and insulin-stimulated glucose uptake in L6 myotubes.

Akt regulates insulin-stimulated GLUT4 translocation and glucose uptake in insulin-sensitive muscle and fat cells (Wang et al., 1999). Therefore, we next determined if short- and long-term PMA treatments affected insulin-induced Akt activation using a phospho-specific antibody, phospho-S473-Akt, which recognizes the active form of Akt. As shown in Fig. 3, activation of c/nPKC by 100 nM PMA for 10 min did not alter insulin-induced Akt activation, and down-regulating c/nPKC by 1 μM PMA for 24 h only slightly reduced it. Accordingly, PMA treatment did not affect the activation status of a downstream target of Akt, GSK-3β. Together, these results demonstrate that c/nPKC has little impact on the level of Akt activation induced by insulin.

Selective PKC/PKD Inhibitors Blocked Glucose Uptake in L6 Myotubes Independently of PKC. To further delineate the roles of PKC and PKD in basal and insulin-stimulated glucose uptake in L6 myotubes, we used a set of inhibitors—Go6976, Go6983, GF 109203X, and Ro 31-8220—that show variable selectivity and potency toward classic, novel, and atypical PKC isoforms, as well as PKD (see Table 1 for inhibitor selectivity and potency) (Toullec et al., 1991; Martiny-Baron et al., 1993; Wilkinson et al., 1993; Gschwendt et al., 1996; Standaert et al., 1997). It should be noted that the affinities listed in Table 1 are determined in a cell-free assay with purified PKC proteins. The inhibitory activities of these inhibitors show competitive kinetics versus ATP; because ATP levels are generally very high in cells, the potency of the inhibitors is substantially reduced in cell-based assays. In general, a 10- to 100-fold drop of activity is expected in whole-cell assays. In this study, L6 myotubes were treated with increasing concentrations of the inhibitors for 1 h, and insulin was added for the last 20 min of incubation. As shown in Fig. 4a, Go6976 dose-dependently inhibited basal glucose uptake (approximately 50% inhibition at 10 μM) and, to a lesser degree, insulin-stimulated glucose uptake (70% uptake dropped to 48%). (Fig. 4a, top). Go6983 inhibited insulin-stimulated glucose uptake in a dose-dependent manner (50% inhibition at 10 μM) but had no effect on basal glucose uptake (Fig. 4a, middle). Although after prolonged PMA treatment as shown in Fig. 4b (middle) the mean glucose uptake values seemed lower in Go6983-treated cells, they are not significantly different from those of the controls without the inhibitor, implying that PKCζ does not play a major role in regulating basal glucose uptake ($p > 0.05$ versus non-Go6983-treated cells). GF 109203X at 2 μM and Ro 31-8220 at 1 μM inhibited approximately 15% of basal glucose uptake and 8% of insulin-stimulated glucose uptake (Fig. 4a, bottom).

To assess whether c/nPKC mediates the inhibitory effects of these inhibitors, we down-regulated c/nPKC by prolonged PMA treatment before the addition of the inhibitors. Strik-

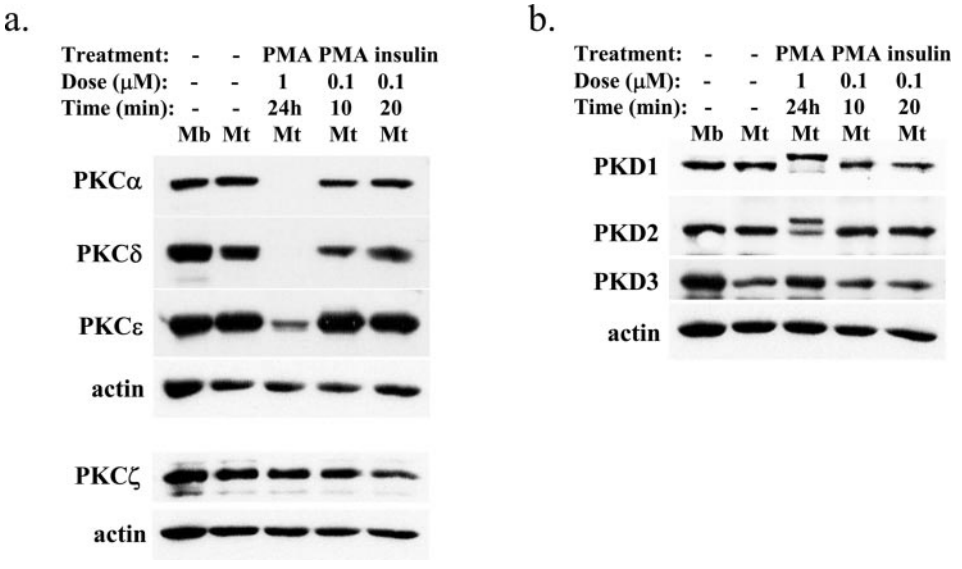

Fig. 1. Expression of PKC and PKD isoforms in L6 myoblasts (Mb) and myotubes (Mt). Lysates from L6 myoblasts and L6 myotubes treated with or without 1 μM PMA for 24 h, 100 nM PMA for 10 min, and 100 nM insulin for 20 min were subjected to immunoblotting using antibodies to PKC isoforms (a) and PKD isoforms (b) as described under *Materials and Methods*. Equal protein loading was confirmed by blotting with an anti-actin antibody.

ingly, the lack of c/nPKC did not alter the inhibition of basal and insulin-stimulated glucose uptake by the PKC/PKD inhibitors but rather slightly potentiated those of Go6976 and Go6983 (Fig. 4b). These results support the noninvolvement of c/nPKC isoforms in glucose transport, because prolonged PMA treatment effectively down-regulated c/nPKC isoforms, as illustrated in Fig. 1. The endogenous PKCϵ, although not completely down-regulated like PKCδ, was reduced by >90% in levels of expression, and this did not in turn reverse/abolish the inhibition of glucose uptake by PKC inhibitors, suggesting no major involvement of PKCϵ. Together, our data in Fig. 4 point to the potential role of non-phorbol ester-responsive targets in the control of basal and insulin-stimulated glucose uptake in L6 myotubes.

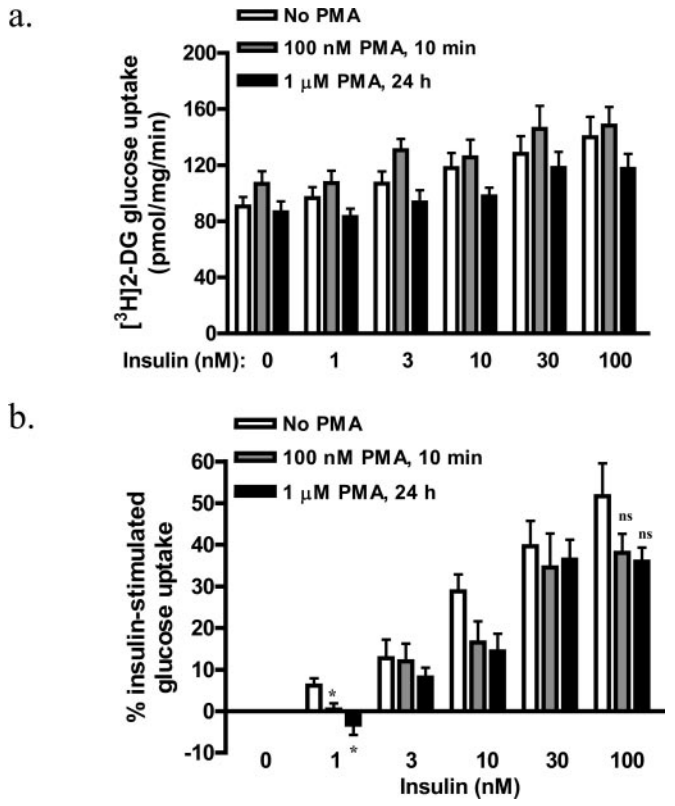

Fig. 2. c/nPKC is not involved in basal and insulin-stimulated glucose uptake in L6 myotubes. L6 myotubes were pretreated with 100 nM PMA for 10 min or 1 μM PMA for 24 h. Glucose uptake in the absence and presence of increasing concentrations of insulin were assessed by [^{3}H]2-DG uptake assay as described under *Materials and Methods*. a, [^{3}H]2-DG uptake expressed as picomoles per milligram per minute. b, net insulin-stimulated [^{3}H]2-DG uptake expressed as a percentage of stimulation above basal uptake. Values represent the mean ± S.E.M. of at least seven to nine independent experiments of triplicate determinations. $p > 0.05$ versus non-PMA-treated cells.

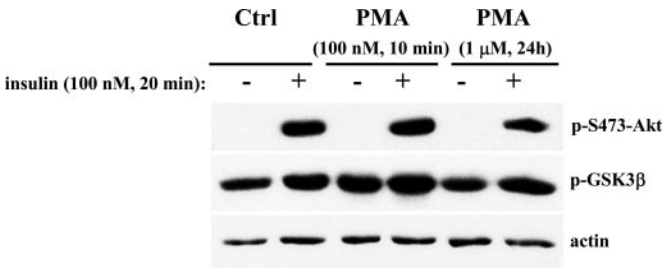

Fig. 3. PMA treatments do not alter insulin-induced Akt activity. L6 myotubes were pretreated with or without either 100 nM PMA for 10 min and 1 μM PMA for 24 h in the absence (−) and presence (+) of insulin. The lysates were subjected to immunoblotting using phospho-specific antibodies to active Akt(S473) and GSK-3β(S9) as described under *Materials and Methods*. Equal protein loading was confirmed by blotting with an anti-actin antibody.

Because PKD and the atypical PKCζ are expressed in L6 myotubes and both are irresponsive to phorbol esters, they may account for some of the c/nPKC-independent effects of PKC inhibitors. Go6983 is known to effectively inhibit PKCζ but not PKD. On the contrary, Go6976 effectively inhibits PKD but is not effective for atypical PKCs (Table 1) (Gschwendt et al., 1996). Accordingly, as shown in Fig. 4b, top and middle, Go6983 inhibited insulin-stimulated glucose uptake but had little effect on basal uptake. Go6976 predominantly inhibited basal glucose uptake and had less effect on insulin-stimulated uptake. These results imply a role of PKD in regulating basal glucose uptake and PKCζ in insulin-stimulated glucose uptake. To confirm that PKCζ is not a target of Go6976, we determined PKCζ activities in cells pretreated with 10 μM Go6976 in the presence or absence of insulin and PMA. As shown in Fig. 4c, PKCζ kinase activities were not significantly altered in cells pretreated with Go6976 ($p > 0.05$), implying that PKD is more likely the target of Go6976 in inhibiting glucose uptake.

The Effects of PKC/PKD Inhibitors on Glucose Uptake Are Not Mediated through PKB/Akt. PKB/Akt is one of the key regulators of glucose uptake; here we sought to examine whether selective PKC/PKD inhibitors also affect Akt activity. As shown in Fig. 5, we found that in the absence of PMA treatment, 10 μM Go6976, 10 μM Go6983, 2 μM GF 109203X, and 1 μM Ro 31-8220 did not inhibit insulin-induced Akt activation, whereas a PI3K inhibitor, wortmannin (100 nM, 30 min), completely abolished insulin-induced Akt activation. Similar results were obtained in the presence of 1 μM PMA for 24 h (Fig. 5b). As control, the treatments did not alter the levels of endogenous Akt. Our data suggest that the inhibition of glucose uptake by Go6976, Go6983, GF 109203X, and Ro 31-8220 is not mediated through Akt.

Adenoviral Gene Delivery of Wild-Type and Dominant-Negative PKD3 on Glucose Uptake. To confirm our hypothesis that PKD regulates glucose transport, we generated adenoviruses carrying empty vector (Adv-null), wt-PKD3, and DN-PKD3. L6 myotubes were infected with increasing concentrations of these adenoviruses. Endogenous PKD3 as well as overexpressed wt- and DN-PKD3 were detected by Western blotting. The levels of gene expression were quantitated by laser scanning densitometry, and the -fold increase in protein levels above control was calculated (Fig. 6, a–c, insets). As shown in Fig. 6a, increasing levels of DN-PKD3 inhibited both basal and total insulin-stimulated glucose uptake to a similar extent, indicating a predominant effect on basal glucose uptake. Specifically, when expressed approximately 8.5-fold above noninfected control (corresponding to cells infected at the 100 MOI unit of Adv-DN-PKD3), DN-PKD3 caused an approximately 40% drop in basal glucose uptake (Fig. 6a, last two bars). As shown in Fig. 6b, when expressed approximately 3.3-fold above noninfected control (corresponding to cells infected at the 10 MOI unit of wt-PKD3), wt-PKD3 caused a 35% increase in basal glucose uptake for noninsulin-treated cells and a 12% increase in total insulin-stimulated uptake for insulin-treated cells (Fig. 6b, seventh and eighth bars). Therefore, our results indicate a significant effect of PKD3 in glucose uptake. It was noted

that, when overexpressed, wt-PKD3 had less effects on the glucose uptake of insulin-treated cells (Fig. 6b, black bars) compared with its effects on control cells without insulin treatment (Fig. 6b, white bars). The maximal glucose uptake may have been reached in this case; thus, the net insulin-stimulated glucose uptake (the difference between basal and total insulin-stimulated uptake) in the case of overexpressed wt-PKD3 may not reflect the actual contribution of PKD3 in this process. As controls, cells infected with Adv-null showed no significant changes in basal and insulin-stimulated glucose uptake (Fig. 6c). Together, these results demonstrate that PKD3 primarily regulates basal glucose uptake and, to a lesser extent, insulin-stimulated glucose uptake.

PKD3-Targeted siRNA Inhibited Glucose Uptake in L6 Myotubes. To further evaluate the contribution of PKD3 to glucose transport, we developed a PKD3-targeted siRNA. As shown in Fig. 7b, after transiently transfected into L6 myotubes, the PKD3 siRNA caused a 77% knockdown of endogenous PKD3 at 100 nM. When glucose uptakes were determined, as shown in Fig. 7a, both basal and total insulin-stimulated glucose uptakes were reduced by 57% at 100 nM siRNA versus the control without siRNA, although the net insulin-stimulated glucose uptake was not significantly altered. Together, our results again indicate a significant role of PKD3 in regulating basal glucose uptake.

DN-PKD3 Did Not Alter the Intracellular Localization of GLUT4 and GLUT1. GLUT1 and GLUT4 are the main glucose transporters that mediate basal and insulin-stimulated glucose uptake in insulin-sensitive cells, and their localization at the plasma membrane is a prerequisite for transporting glucose inside the cells. In this study, to explore the mechanisms through which PKD3 regulates glucose transport, we sought to evaluate whether DN-PKD3 affects the intracellular distribution of GLUT1 and GLUT4 in L6 myoblasts. To do this, we generated RFP-DN-PKD3. When overexpressed alone in L6 myoblasts, RFP-DN-PKD3 showed patchy cytoplasmic distribution, whereas RFP alone distributed evenly in the cytoplasm and the nucleus (data not shown). The intracellular localization of GLUT1 was examined by immunocytochemistry. Endogenous GLUT1 was found in discreet vesicular structures throughout the cytoplasm in L6 myoblasts. RFP-DN-PKD3, upon overexpression in L6 myoblasts, did not affect the overall distribution of GLUT1, as illustrated in Fig. 8a. However, GLUT1 was found to be partially colocalized with DN-PKD3 in cells (regions indicated by arrows in Fig. 8a), implying a potential role of PKD3 in regulating GLUT1 activity. The localization of GLUT4 was visualized using GFP-GLUT4 in L6 myoblasts. GFP-GLUT4 expressed alone was primarily located in the perinuclear regions, with little on the plasma membrane (data not shown). As shown in Fig. 8b, when coexpressed with RFP-DN-PKD3, GFP-GLUT4 was similarly distributed as when expressed alone in cells. In addition, RFP-DN-PKD3 and GFP-GLUT4 are located in separate subcellular compartments, and no colocalization was observed. Together, these results suggest that the inhibitory effect of DN-PKD3 on glucose uptake is not mediated through altering intracellular localizations of GLUT1 and GLUT4.

Discussion

In this report, we investigated the potential role of a novel serine/threonine kinase family, PKD, in basal and insulin-stimulated glucose transport in L6 myotubes. This is the first report on the functional input of PKD in glucose metabolism. The study was carried out using a panel of selective inhibitors for PKD and PKC and adenoviruses carrying wild-type and dominant-negative PKD3 to modulate the endogenous PKD activity and the levels of PKD3 gene expression. Our data indicate that PKD3 predominantly regulates basal glucose uptake and, to a lesser extent, insulin-stimulated glucose uptake. Blocking PKD activity by using inhibitors or dominant-negative PKD3 potently inhibited basal glucose transport in L6 myotubes.

Members of the PKC family are implicated in insulin-induced signaling events and glucose transport. The atypical PKCζ is activated downstream of PI3K and is directly involved in the regulation of insulin-stimulated glucose transport in muscle and fat cells (Bandyopadhyay et al., 1997, 1999). c/nPKC, on the other hand, features more prominently in modulating the insulin signaling pathway. It has been shown that 1) insulin stimulates diacylglycerol production and activates c/nPKC to some degree (Standaert et al., 1996); 2) c/nPKC isoforms negatively modulate the activities of the

TABLE 1

Potency and selectivity of PKC/PKD inhibitors

IC_{50} of PKC/PKD inhibitors to classic, novel, and atypical PKC isoforms as well as PKD are shown. Values are obtained in cell-free assays. References for the IC_{50} values are indicated in parentheses after each inhibitor.

	Selectivity for PKC Isoforms	IC_{50} (Measured in Vitro)
Go6976 (Martiny-Baron et al., 1993; Gschwendt et al., 1996)	Inhibits cPKC	PKCα (2.3 nM)
		PKCβI (6.2 nM)
	Not effective for n/aPKC	PKCδ, -ϵ, -ζ (> μM)
	Selectively for PKD	PKD1 (20 nM)
Go6983 (Gschwendt et al., 1996)	Inhibits cPKC	PKCα, β (7 nM)
		PKCγ (6 nM)
	Inhibits nPKC	PKCδ (10 nM)
	Inhibits aPKC	PKCζ (60 nM)
	Not effective for PKD	PKD (20 μM)
GF 109203X (Toullec et al., 1991; Martiny-Baron et al., 1993)	Inhibits cPKC	8–20 nM
	Inhibits nPKC	210–700 nM
	Not effective for aPKC	PKCζ (5.8 μM)
	Not effective for PKD	PKD1 (2 μM)
Ro 31-8220 (Wilkinson et al., 1993; Standaert et al., 1997)	Inhibits cPKC	PKCα, -β, -γ (20–40 nM)
	Inhibits nPKC	PKCδ, -ϵ, -η (20–100 nM)
	Not effective for aPKC	PKCζ (1–4 μM)
	Not effective for PKD	PKD1 (>2 μM)

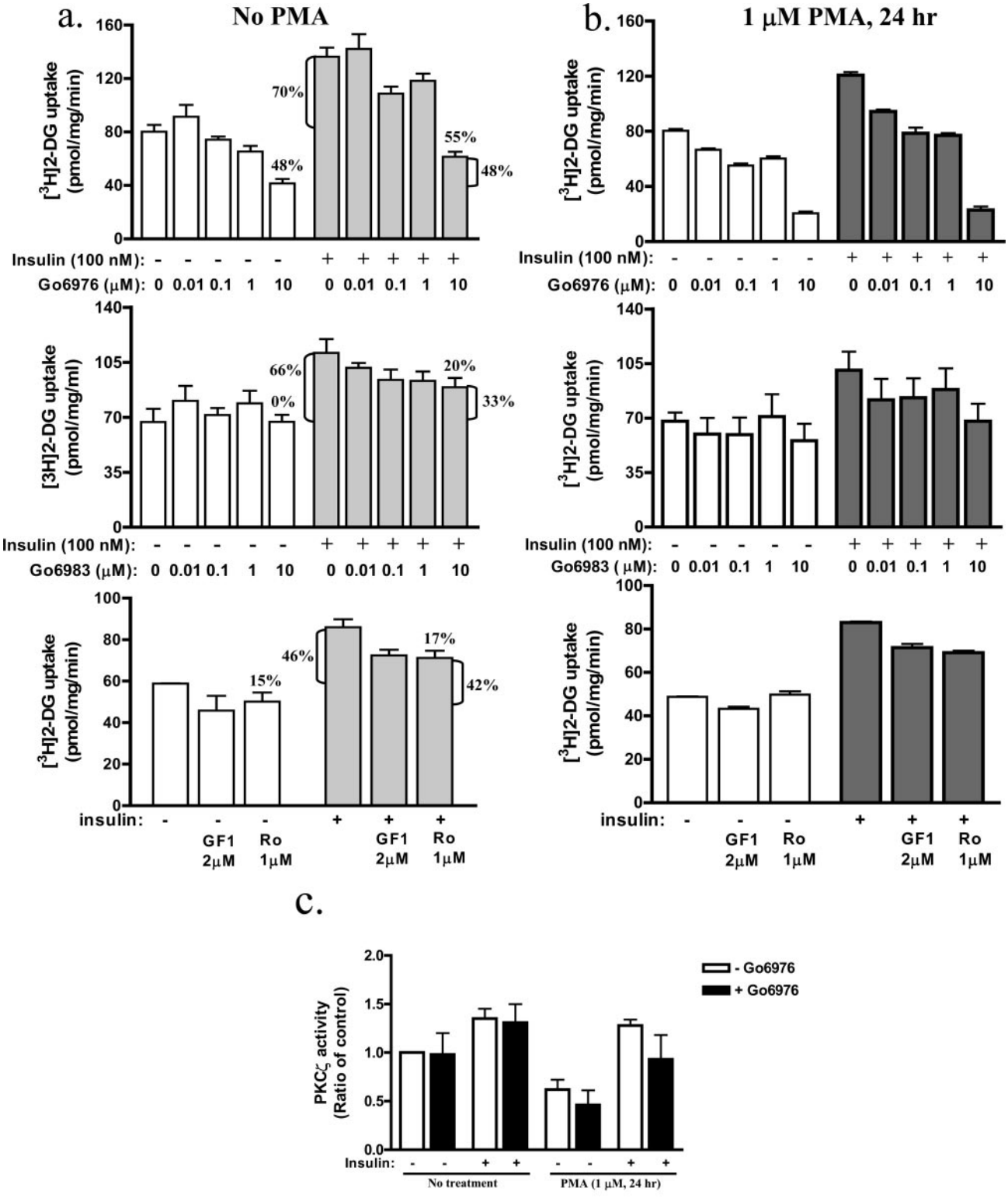

Fig. 4. Selective PKC/PKD inhibitors block glucose uptake in L6 myotubes independently of PKC but correspond to the inhibition of PKD. a, PKC/PKD inhibitors block glucose uptake in the absence of PMA. L6 myotubes pretreated with different doses of Go6976, Go6983, GF 109203X, and Ro 31-8220 were assessed for [^{3}H]2-DG uptake in the absence or presence of 100 nM insulin for 20 min. Values represent the mean ± S.E.M. of triplicate determinations of one of three such similar experiments. b, PKC/PKD inhibitors block glucose uptake after prolonged PMA treatment. L6 myotubes were pretreated with 1 μM PMA for 24 h before the addition of Go6976, Go6983, GF 109203X, and Ro 31-8220. [^{3}H]2-DG uptake was assessed in the absence (−) or presence (+) of 100 nM insulin for 20 min. Values represent the mean ± S.E.M. of triplicate determinations of one of three such similar experiments. Numbers on top of the bars represent the percentage of reduction of glucose uptake compared with controls in the absence of inhibitor treatment. Numbers to the side of the bars represent the percentage of insulin-stimulated uptake above basal. c, PKCζ activity was not altered in cells pretreated with Go6976. L6 myotubes before and after prolonged PMA treatment were preincubated with or without 10 μM Go6976 (1 h) followed by insulin stimulation (100 nM, 20 min). PKCζ was then immunoprecipitated, and kinase activity was determined by in vitro kinase assay. Results were expressed as ratios of control kinase activity without treatments. Values represent the mean ± S.E.M. of four independent experiments.

components of the insulin signaling pathway (Schmitz-Peiffer, 2002); and 3) elevated c/nPKC expression and activity couple to the pathogenesis of insulin resistance and type II diabetes (Chalfant et al., 2000). Direct involvement of c/nPKC in glucose transport has also been demonstrated; however, their effects differ widely depending on PKC isoforms, cell/tissue types, and the methods employed (Chalfant et al., 1996; Bandyopadhyay et al., 1997, 1999; Braiman et al., 1999). Here we show that the activation and down-regulation of c/nPKC by PMA did not alter basal and insulin-stimulated glucose uptake in L6 myotubes, indicating little involvement of c/nPKC in these processes. Our findings are consistent with several reports in which L6 myotubes were investigated (Klip and Ramlal, 1987; Bandyopadhyay et al., 1997).

Although PMA treatments suggest the noninvolvement of c/nPKC in glucose uptake, selective c/nPKC inhibitors, on the other hand, dose-dependently inhibited basal and/or insulin-stimulated glucose uptake in L6 myotubes; however, these inhibitory effects persisted after down-regulating c/nPKC, implying the involvement of c/nPKC-independent protein kinases. Effects of PKC inhibitors on glucose uptake have also been reported in several studies. In general, the required concentrations for these inhibitors to inhibit the insulin effects have exceeded those required to inhibit c/nPKC isoforms (Nishimura and Simpson, 1994; Standaert et al., 1997). This has been the case for the bisindolylmaleimide derivative Ro 31-8220, which inhibits c/nPKC at a nanomolar range but inhibits PKCζ only at a relatively high micromolar concentration range. This latter range closely matches the dosage required for the inhibition of insulin-stimulated glucose uptake (Standaert et al., 1997, 1999). In our study, PKCζ-sensitive Go6983 significantly inhibited the insulin-stimulated glucose uptake (Fig. 4, a–b, middle), whereas lower doses of Ro 31-8220 (1 μM) and GF 109203X (2 μM) had little effects (Fig. 4). These findings are consistent with the reports of Standaert et al. (1997 and 1999) that imply a role of PKCζ in insulin-stimulated glucose uptake. It is also worth noting that the inhibitory effects of the PKC inhibitors on glucose uptake were, in general, enhanced upon down-regulating c/nPKC. It is possible that c/nPKC may indirectly contribute to glucose transport and that the lack of c/nPKC alters the cells' sensitivity to insulin. It also cannot be ruled out that long-term PMA treatment might also affect other protein targets required for glucose transport.

The implication of PKD in glucose transport is derived from the analysis of Go6976 and Go6983, the paired inhibitors with opposite sensitivity to PKD. Go6976 potently inhibited the basal glucose uptake while affecting, to a lesser degree, insulin-stimulated glucose uptake. Accordingly, Go6983—a nonselective PKC inhibitor that does not effectively inhibit PKD—did not affect basal glucose uptake but inhibited only insulin-stimulated uptake, presumably as a result of inhibiting PKCζ. Meanwhile, down-regulation of c/nPKC did not abolish the inhibition of glucose transport by these inhibitors, suggesting that the effects of PKD in glucose transport can be dissociated from PKC and implying that the constitutive basal activity of PKD is required for basal glucose uptake. It has been reported that Go6976 did not inhibit insulin-stimulated glucose transport at concentrations up to 100 μM (Standaert et al., 1997; Bandyopadhyay et al., 1999). These findings diverge from ours, in which insulin-stimulated glucose uptake was inhibited significantly at micromolar concentrations. It should be noted that these reports showed only the effect of a single dose of Go6976 on insulin-stimulated glucose uptake without accompanying data on basal uptake. In addition, rat adipocytes were used instead of myotubes. Nevertheless, the basis for the apparent discrep-

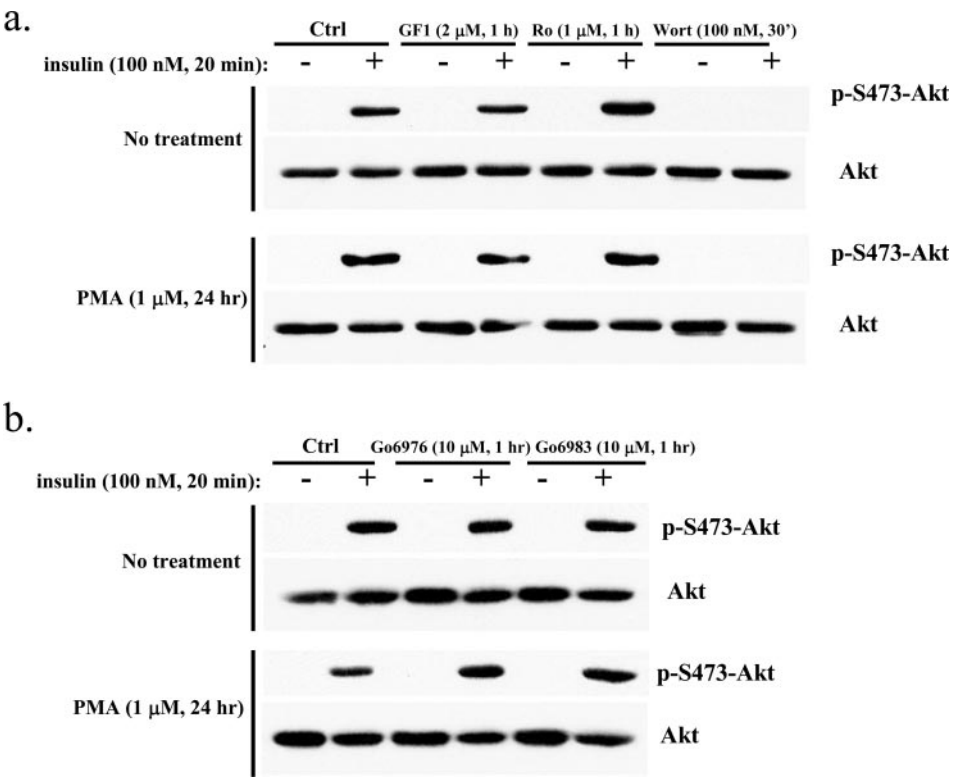

Fig. 5. The effects of PKC/PKD inhibitors on glucose uptake are not mediated through inhibition of Akt. L6 myotubes pretreated with (a) 10 μM Go6976, 10 μM Go6983, (b) 2 μM GF 109203X, 1 μM Ro 31-8220, and 100 nM wortmannin (30 min) for 1 h. Insulin (100 nM) was added for the last 20 min. The lysates were subjected to immunoblotting for native and phospho-S473-Akt as described under *Materials and Methods*. Equal protein loading was confirmed by blotting with an anti-actin antibody.

ancy deserves further investigation. The involvement of PKD in basal glucose uptake is strongly supported by ectopically expressed wild-type and dominant-negative PKD3 using adenoviruses. In this regard, an 8.5-fold DN-PKD3 overexpression inhibited approximately 40% of the basal uptake. A comparable level of wild-type PKD3 (3.3 fold overexpression) promoted a similar percentage of increase in basal uptake. In addition, knocking down endogenous PKD3 by >70% caused a >50% reduction in basal glucose uptake. Overall, these results strongly indicate that PKD3 actively contributes to basal glucose transport. It should also be noted that L6 myotubes additionally contain PKD1 and PKD2, and our results do not rule out the involvement of PKD1 and PKD2 in glucose transport. It remains possible that these isoforms may also contribute to the glucose transport process.

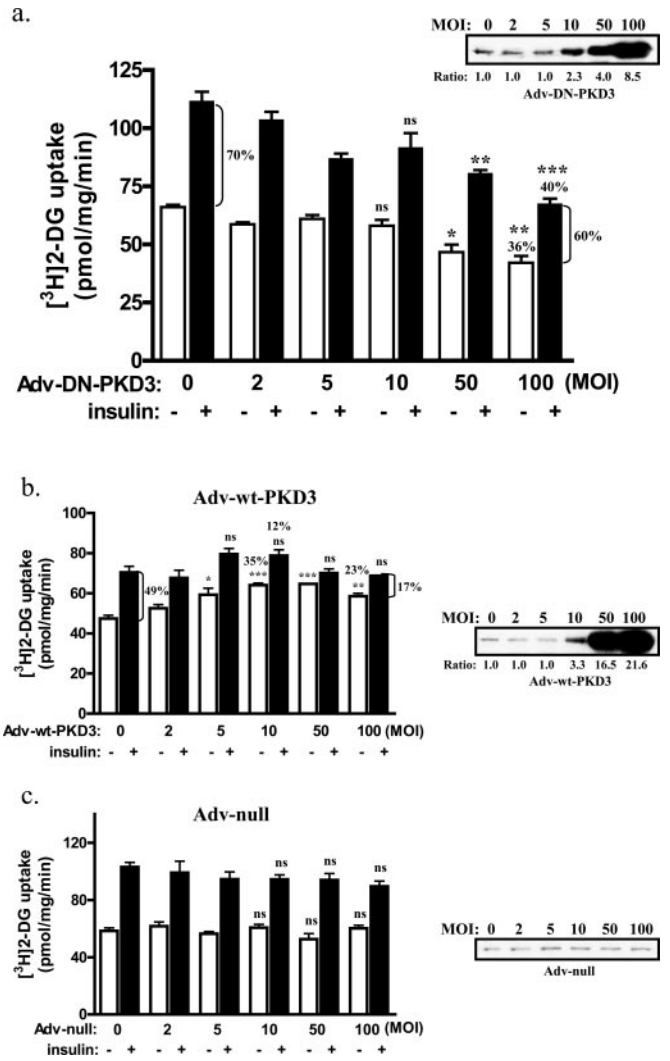

Fig. 6. Adenoviral gene delivery of wild-type and dominant-negative PKD3 on glucose uptake. L6 myotubes were first infected with increasing MOI of Adv-DN-PKD3 (a), Adv-wt-PKD3 (b), and Adv-null (c) as described under *Materials and Methods*. [^{3}H]2-DG uptake was then assessed in the absence (−) and presence (+) of 100 nM insulin for 20 min. Insets, levels of protein expression detected by the PKD3 antibody. Ratios of gene expression at different MOI versus noninfected controls are indicated. Values represent the mean ± S.E.M. of triplicate determinations of one of three such similar experiments. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus noninsulin-treated cells. Numbers on top of the bars represent the percentage of reduction/increase of total glucose uptake compared with noninfected controls. Numbers to the side of the bars represent the percentage of net insulin-stimulated uptake above basal.

Our findings support the notion that the effect of PKD3 on glucose uptake is independent of PKC. This is in line with several of the following recent reports showing that PKD can also be regulated by PKC-independent mechanisms. 1) G$\beta\gamma$ subunits can activate PKD1 through direct interaction with the PKD1 PH domain (Jamora et al., 1999). 2) Oxidative stress can also activate PKD1 via a PKC-independent mechanism. Reactive oxygen species activate the Src-Abl signaling pathway that causes tyrosine phosphorylation of PKD1 in the PH domain and subsequent PKD1 activation (Storz et al., 2003). 3) PKD1 is cleaved in a caspase-mediated reaction during the induction of apoptosis by genotoxic drugs. The cleaved catalytic fragment when stably expressed sensitizes cells to apoptosis induced by genotoxic stress (Endo et al., 2000). The cleavage of PKD1 by apoptotic agents is PKC-independent, although it is uncertain whether cleavage of PKD1 represents catalytic activation or down-regulation. 4) Bone morphogenetic protein 2-induced activation of c-Jun NH_2-terminal kinase and p38 is mediated through the activation of PKD by a PKC-independent mechanism in osteoblastic cells (Lemonnier et al., 2004). Thus, these reports and ours indicate that PKD can function independently of PKC, and the biological processes regulated by PKD may be dissociated from those controlled by PKC.

PKD1 has been shown to regulate the fission of transport carriers from the *trans*-Golgi network going to the plasma membrane (Liljedahl et al., 2001; Baron and Malhotra, 2002). PKD1 localizes in part to the Golgi (Prestle et al., 1996) and acts as the downstream target of the trimeric G protein subunit $\beta\gamma$ in regulating Golgi structure and function (Jamora et al., 1999; Liljedahl et al., 2001). In addition, the most recent report shows that PKD1–3 activity is required for the transport of basolateral, but not apical, proteins to the plasma membrane, although the identity of the cargo regulated by PKD is unknown (Yeaman et al., 2004). Glucose transport is mediated by a group of facilitative glucose transporters in cells. Although it is not known at present how PKD regulates glucose transport, it is tempting to speculate that PKD regulates the trafficking of glucose transporters that mediate basal glucose uptake. In support of this view, our colocalization studies indicate that PKD3 is partially colocalized with GLUT1 but not GLUT4 in L6 myoblasts. Although the patterns of intracellular distribution of GLUT1 and GLUT4 are not altered by the overexpressed DN-PKD3, it remains possible that PKD3 regulates the activities of glucose transporters such as GLUT1 through direct interaction or phosphorylation. These possibilities are currently under investigation.

In summary, we used the specific activators and inhibitors of PKC and PKD as well as the adenoviral gene transfer and siRNA approaches to examine the contribution of PKD3 in glucose transport in L6 myotubes. Our findings indicate that c/nPKC is unlikely to be involved in glucose transport and that the effects of PKD on glucose transport can be dissociated from PKC. Our study strongly supports our hypothesis that PKD, specifically PKD3, contributes directly to the insulin-independent basal glucose uptake in L6 skeletal muscle cells.

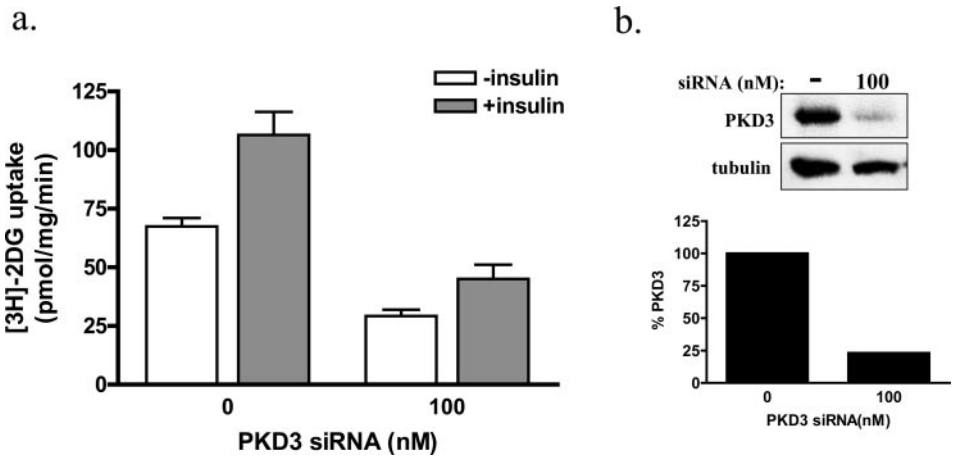

Fig. 7. PKD3 siRNA inhibited glucose uptake in L6 myotubes. a, effect of siRNA on basal and insulin-stimulated glucose uptake. L6 myotubes were transiently transfected with PKD3 siRNA at 100 nM. Glucose uptake was determined 2 days after the transfection. Values represent the mean ± S.E.M. of three independent experiments of triplicate determinations. b, PKD3 siRNA on the levels of endogenous PKD3. Lysates from L6 myotubes transfected with or without PKD3 siRNA were subjected to immunoblotting using anti-PKD3 antibody. Equal protein loading was confirmed by blotting with an anti-tubulin antibody. The bands were quantitated and expressed as ratios of PKD3 level in control cells without siRNA treatment (bottom bar chart).

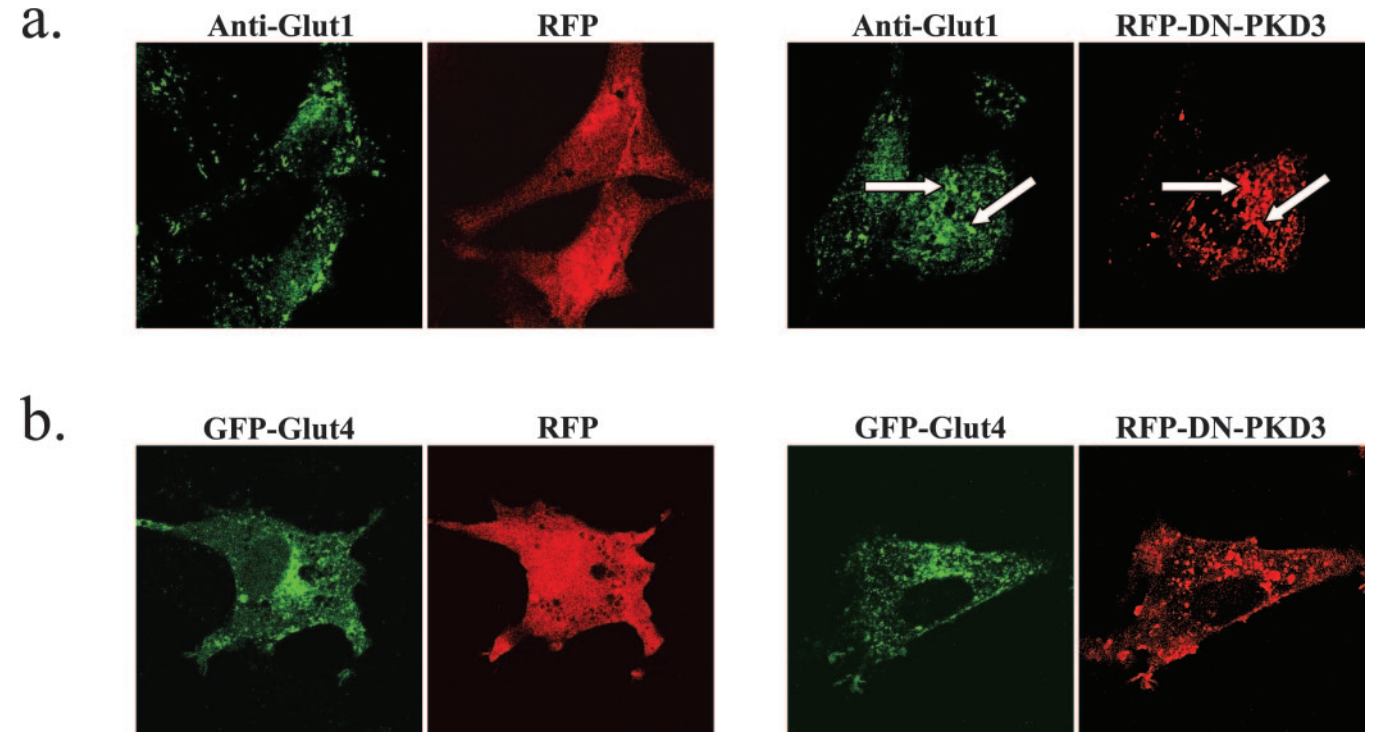

Fig. 8. The effects of DN-PKD3 on the intracellular localization of GLUT1 and GLUT4. a**,** DN-PKD3 on GLUT1 localization. L6 myoblasts transiently transfected with RFP and RFP-DN-PKD3 were stained for GLUT1 as described under *Materials and Methods*. Cells with red fluorescent signal were imaged. Representative fields from three independent experiments are shown. Arrows indicate regions of colocalization. b, DN-PKD3 on GLUT4 localization. GFP-GLUT4 was transiently transfected into L6 myoblasts with either RFP or RFP-DN-PKD3. One day after transfection, cells positive for red and green fluorescence were imaged live. Representative fields from three independent experiments are shown.

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