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

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Running title: Protein kinase D3 regulates glucose transport

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**Abbreviations:** c/nPKC, classical and novel PKC; 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); Ro 31-8220, Bisindolylmaleimide IX (2-[1-[3-(Aimidinothio)propyl]-1H-indol-3-yl]-3-(1-methylindol-3-yl)maleimide); Go6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c] carbazole; Go69863, 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide; GLUTs, glucose transporters; GSK-3β, glycogen synthase kinase-3β.
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 classical and novel PKC (c/nPKC) and PKD in glucose transport in L6 myotubes. c/nPKC is either activated by short-term PMA treatment or down-regulated by prolonged PMA treatment at high dose in L6 myotubes. Our results indicate that PMA treatments have little impact on basal and insulin-stimulated glucose uptake and on insulin-induced Akt activation. In contrast, the PKC inhibitors Go6976, Go6983, GF 109203X, and Ro 31-8220 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 effecters 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, while Go6983, the non-selective 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 (DN-PKD3) inhibits primarily basal glucose uptake and, to a small extent, insulin-stimulated glucose uptake, while 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.
INTRODUCTION

Glucose transport is a fundamental process of the cell. Insulin-independent glucose transport accounts for most of the basal glucose uptake, while 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 (IRS-1), phosphoinositol 3-kinase (PI3K), phosphoinositol-dependent kinase 1 (PDK1) 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 (Rudich and Klip, 2003; Saltiel and Pessin, 2002).

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 accounting for reduced systemic glucose 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.

Protein kinase C (PKC) family comprises 11 isoforms that are classified as cPKC (α, βI/βII, γ; Ca\(^{2+}\) and diacylglycerol (DAG)-dependent), nPKC (δ, ε, η, θ; Ca\(^{2+}\)-independent but DAG-dependent), and aPKC (ζ, ι/λ; Ca\(^{2+}\)- and DAG-independent) (Newton, 2001). Although PKCζ mediates insulin-stimulated glucose transport (Bandyopadhyay et al., 1997; Bandyopadhyay et al., 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 Ca-Calmodulin kinases (CAMK) and is more distant to the AGC family of kinases such as PKC. It has now been classified as a subfamily of the CAMK superfamily (Manning et al., 2002). Four isoforms have been identified for PKD family including PKD1 (mouse PKD and its human homologue PKCµ), PKD2, and PKD3 (human PKCv) (Hayashi et al., 1999; Sturany et al., 2001; Valverde et al., 1994). 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 JNK signaling pathways (Rykx et al., 2003; Van Lint et al., 2002). Most notably, PKD1 has been demonstrated to regulate the trafficking of transport carriers from the trans-Golgi network to the cell surface (Baron and Malhotra, 2002; Jamora et al., 1999; Liljedahl et al., 2001). Similar effects are reported for PKD2 and PKD3 (Yeaman et al., 2004). Many regents are known to activate PKD1 \textit{in vivo}. These include the pharmacological agents phorbol esters and DAG
analogues and many physiological stimuli including GPCR agonists, growth factors and antigen-receptor engagement (Van Lint et al., 2002; Waldron and Rozengurt, 2000; Zugaza et al., 1996; Zugaza et al., 1997). In all cases, PKD has been shown to be activated through a PKC-dependent signaling pathway that involves the phosphorylation of Ser \textsuperscript{744/748} in the activation of loop of the catalytic domain of PKD1 (Iglesias et al., 1998; Waldron et al., 2001; Zugaza et al., 1996). Later, novel PKC\(\varepsilon\) and \(\eta\) have been shown to phosphorylate Ser \textsuperscript{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, PLC and PKC inhibitors can block this activation. It has been suggested that oxidative stress leads to activation of Src and PLC which activate several PKCs and in turn activate 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 (Endo et al., 2000; Jamora et al., 1999; Lemonnier et al., 2004; Storz et al., 2003).

The PKC inhibitors developed in recent years show much improved selectivity towards different PKC isoforms. However, some of them 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 (MAPKAP-K1), mitogen- and stress-activated protein kinase-1 (MSK-1), serum- and glucocorticoid-induced kinase (SGK), p70 ribosomal protein S6 kinase (S6K), glycogen synthase kinase 3 (GSK3), and other kinases (Alessi, 1997; Davies et al., 2000). Similarly, Go6976, other than inhibiting classical PKCs, 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 and dominant-negative 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 - and 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 dominant-negative PKD3 as well as knocking down the endogenous expression of PKD3 inhibits primarily 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 Calbiochem (EMD Biosciences, Inc., San Diego, CA). Phorbol 12-Myristate 13-Acetate (PMA), GF 109203X, Ro 31-8220 and Go6976 were obtained from LC Laboratories (Woburn, MA). Insulin, Alpha-Minimal Essential Medium (α-MEM), fetal bovine serum (FBS) and other cell culture reagents were purchased from Invitrogen (Grand Island, NY). Penicillin/Streptomycin was purchased from BioWhittaker (Walkersville, MD). 2-Deoxy-D-glucose and cytochalasin B were obtained from Sigma (St. Louis, MO). [3H-1,2]-deoxy-D-glucose was purchased from MP Biomedicals (Irvine, CA).

Cell Culture

Rat L6 skeletal muscle cell line was obtained from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Canada). L6 myoblasts were grown and maintained in α-MEM supplemented with 10% FBS, 1,000 units/L Penicillin and 1 mg/ml Streptomycin in an atmosphere of 5% CO2 at 37°C. For differentiation, L6 myoblasts were plated at 4 x 10⁴ 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 hrs. Mature myotubes formed within 7-8 days after cell seeding.

[3H-1,2]2-Deoxy-D-Glucose Uptake Assay in L6 myotubes

[3H-1,2]2-Deoxy-D-glucose ([3H]2-DG) uptake assay was carried out in 24-well plates as described by Klip and Ramlal with minor modifications (Klip and Ramlal, 1987). Briefly, L6 myotubes were serum-starved for 3 hrs prior to 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 three hours. After treatment, myotubes were washed once with HEPES-buffered saline (HBS) (140 mM NaCl, 20 mM Na-HEPES, 5 mM KCl, 2.5 mM MgSO₄, 1.0 mM CaCl₂, pH 7.4) at room temperature, followed by incubating for 10 min in HBS containing 10 µM unlabeled 2-deoxy-D-glucose and 0.5 µCi/ml [³H-1,2]2-deoxy-D-glucose. Non-specific 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. 400 µl lysate from each well was transferred to a scintillation vial and the associated radioactivity was determined for 5 min in a scintillation counter (LS 6500 Multi-Purpose 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). Anti-sera showing immunoreactivity to PKD3 were affinity purified and concentrated for immunoblotting, immunoprecipitation, and immunostaining.

Cells in 6-well plates were lysed using lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 10 % glycerol, 1 % Triton X-100, 5 mM EGTA, 20 µM Leupeptin, 1 mM AEBSF, 1 mM NaVO₃, 10 mM NaF, and 1 tablet of protease inhibitor). Protein concentration was determined by a micro-protein assay using the BCA protein assay kit according to manufacture’s instruction (Pierce, Rockford, IL). Approximately 40 µg of lysates were mixed with equal volumes of 2 x 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-PAGE at 100 volts for 1 h followed by electrotransfer onto a nitrocellulose
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membrane at 100 volts for 1 h. The membrane was pre-blotted with 5% dry milk in TBS (50 mM Tris-HCl, 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, San Diego, CA), PKD1 (Santa Cruz Biotech. Inc., Santa Cruz, CA), PKD2 (provided by EMD Biosciences Inc.), PKD3 (as above), Akt, and phospho-Akt (Cell Signaling Technology, Beverly, MA) antibodies. Goat anti-rabbit IgG or goat anti-mouse IgG, coupled to horseradish peroxidase (1:1000, Bio-Rad Laboratories, Hercules, CA), were used as the secondary antibodies. Bands were visualized by the enhanced chemiluminescence (ECL) western blotting detection system (Amersham Pharmacia Biotech, Arlington Heights, IL).

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, Santa Cruz, CA) pre-conjugated with anti-PKCζ antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA) overnight at 4°C with constant rotation. The immunocomplexes were then pelleted and washed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% Triton X-100). Equal amounts of immunocomplex were then subjected to PKCζ kinase assay, as described (Bandyopadhyay et al., 1997). Briefly, the assay was carried out by co-incubating 20 µl immunoprecipitated PKCζ with 50 µM ATP, 5 µg phosphatidyserine (PS), and 40 µM PKCε substrate peptide, a preferred substrate of PKCζ (Upstate Biotechnology, Inc., Lake Placid, NY), 0.2 µl γ-32P-ATP (PerkinElmer, 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 dominant negative (DN) PKD3 and generation of adenoviruses carrying wild-type (wt) and DN PKD3

The wt-PKD3 cDNA cloned in the pGEM-Teasy vector was described previously (21). The kinase inactive D720A PKD3 was prepared by mutating an aspartic acid (D) at position 720 (corresponds to D733 in PKD and D727 in PKC\(\mu\)) to an alanine (A) using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutagenic primers are: GTGAAGCTGTGTGCCTTTGGATTTGCACGC (sense) and GCGTGCAAATCCAAAGGCACACAGCTTCAC (antisense).

The PCR fragments of PKD3 and DN-PKD3 were inserted into a XhoI/XbaI site of pShuttle-CMV, an AdEasy adenovirus shuttle vector with cytomegalovirus (CMV) promoter (obtained from Vogelstein laboratory at the Johns Hopkins Oncology Center, Baltimore, MD). Recombinant adenovirus construct carrying these genes were generated by homologous recombination in E. 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. Titors of the viral stocks were normally higher than 1 \(\times\) 10\(^9\) pfu/ml. An adenovirus containing the lacZ gene was used as a control.

Infection with Adenovirus

Subconfluent cultures were infected with PKD3 adenoviruses at different Multiplicity of Infection (MOI) in 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 incubation at 37°C, more growth medium was added and the cultures were continued for 24 hr 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 (Dept. 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 PCR and subcloned into a SacI/SalI site of a pDsRed1-C1 vector (Clontech) to generate a N-terminal fusion of PKD3.

To image Glut4, GFP-Glut4 was co-transfected with pDsRed1-C1 empty vector or RFP-DN-PKD3. Cells were imaged live two days after transfection using a Zeiss LSM 5 inverted confocal fluorescent microscope (Zeiss, Chester, VA). To image Glut1, L6 myoblasts with or without transfection were fixed with 3.7% paraformaldehyde at room temperature for 30 min, permeabilized with 0.5% Triton X-100 in PBS plus 1 mM EGTA for 5 min at room temperature. After pre-blocking with 2% BSA in PBS for 30 min, cells were incubated with an anti-Glut1 antibody (Calbiochem, EMD Biosciences, Inc., San Diego, CA) at 1:100 for 1 hour, washed, and then incubated with the secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:100) for 1 hour. The slide was mounted using DAKO fluorescent mounting medium (DAKO Corp., 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 [3H]2-DG uptake assays. Results are expressed as the mean ± SEM. Comparison of means was performed using a two-tailed Student’s t-test. P-value <0.05 is considered to be statistically significant.
RESULTS

Expression of PKC and PKD isoforms in L6 myoblasts and myotubes.

To examine the roles of PKC and PKD in glucose transport, we used L6 skeletal muscle cell line, a physiologically relevant cellular model system that exhibits active insulin-dependent and -independent glucose uptake (Klip and Ramlal, 1987; Mitsumoto et al., 1991).

We first examined the levels of endogenous PKC and PKD in L6 myoblasts and differentiated myotubes. Among PKC isoforms, only PKC\(\alpha\), \(\delta\), \(\epsilon\), and \(\zeta\) were detected, while all PKDs, namely PKD1-3, were detected and expressed at similar levels (Figure 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 \(\mu\)M for 24 hrs completely down-regulated the endogenous PKC\(\alpha\) and \(\delta\), and >90% of the endogenous level of PKC\(\epsilon\) in L6 myotubes (Figure 1). In contrast, the levels of PMA-insensitive atypical PKC\(\zeta\) and the endogenous PKD1-3 were not affected by this treatment. In addition, short-term treatment with PMA and insulin at 100 nM for 10-20 min did not alter the levels of endogenous PKC and PKD in L6 myotubes (Figure 1). We noted the appearance of higher molecular weight bands of PKD1 and PKD2 upon 1 \(\mu\)M PMA treatment for 24 hrs, which were absent when each was treated for short-term 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 a 80-100% increase in glucose uptake in response to a prior 20 min stimulation with 100 nM insulin. In line with this
and as shown in Figures 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 Figure 2, short- and long-term PMA treatments neither significantly altered basal glucose uptake (nsP>0.05 versus non-PMA-treated cells) (Fig. 2a, uptake at 0 nM insulin), nor affected the insulin-stimulated glucose uptake at 1-100 nM insulin (nsP>0.05 versus non-PMA-treated cells) (Fig. 2a, b). Lower (0.03-1 nM) or higher (100 nM-1 µM) insulin concentrations gave 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 Figure 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 hrs only slightly reduced it. Accordingly, PMA treatment did not affect the activation status of a downstream target of Akt, glycogen synthase kinase-3β (GSK-3β). Taken 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-
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8220 - that show variable selectivity and potency towards classical, novel, and atypical PKC isoforms, as well as PKD (see Table 1 for inhibitor selectivity and potency) (Gschwendt et al., 1996; Martiny-Baron et al., 1993; Standaert et al., 1997; Toullec et al., 1991; Wilkinson et al., 1993). It should be noted that the affinities listed in Table I are determined in a cell-free assay with purified PKC proteins. The inhibitory activities of these inhibitors show competitive kinetics versus ATP; since 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 hr and insulin was added for the last 20 min of incubation. As shown in Figure 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%). (Figure 4a, upper panel). Go6983 inhibited insulin-stimulated glucose uptake in a dose-dependent manner (50% inhibition at 10 µM), but had no effect on basal glucose uptake (Figure 4a, middle panel). Although, after prolonged PMA treatment as shown in Figure 4b (middle panel), the mean glucose uptake values appeared 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 (NSP>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 (Figure 4a, lower panel).

To assess whether c/nPKC mediates the inhibitory effects of these inhibitors, we down-regulated c/nPKC by prolonged PMA treatment prior to the addition of the inhibitors. Strikingly, 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 (Figure 4b). These results support the non-involvement of c/nPKC isoforms in glucose transport since prolonged PMA treatment effectively down-regulated c/nPKC isoforms, as illustrated in Figure 1. The endogenous PKCe, although was not completely down-regulated like PKCδ, was reduced >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 PKCe. Taken together, our data in Figure 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.

Since PKD and the atypical PKCζ are expressed in L6 myotubes and both are irresponsible 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 Figure 4b (upper and middle panels), Go6983 inhibited insulin-stimulated glucose uptake but had little effect on basal uptake. Go6976 predominant 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 kinase activities in cells pretreated with 10 µM Go6976 in the presence or absence of insulin and PMA. As shown in Figure 4c, PKCζ kinase activities were not significantly altered in cells pre-treated 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 if selective PKC/PKD inhibitors also affect Akt activity. As shown in Figure 5, we found that in
the absence of PMA treatment, Go6976 (10 μM), Go6983 (10 μM), GF 109203X (2 μM), and Ro 31-8220 (1 μM) did not inhibit insulin-induced Akt activation, while, 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 hrs (Figure 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), wild-type (wt-PKD3), and dominant-negative PKD3 (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 of increase in protein levels above control was calculated (Figure 6a-c, insets). As shown in Figure 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 non-infected control (corresponding to cells infected at 100 MOI unit of Adv-DN-PKD3), DN-PKD3 caused an approximately 40% drop of basal glucose uptake (Figure 6a, the last two bars). In Figure 6b, when expressed approximately 3.3-fold above non-infected control (corresponding to cells infected at 10 MOI unit of wt-PKD3), wt-PKD3 caused a 35% increase in basal glucose uptake for non-insulin-treated cells and 12% increase of total insulin-stimulated uptake for insulin-treated cells (Figure 6b, the 7th and 8th bars). Therefore, our results indicate a significant effect of PKD3 in glucose uptake. It was noted that wt-PKD3 when overexpressed had less effects on
the glucose uptake of insulin-treated cells (Figure 6b, black bars) compared to its effects on control cells without insulin treatment (Figure 6b, white bars). The maximal glucose uptake may have been reached in this case and 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 (Figure 6c). Taken 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 Figure 7b, after transiently transfected into L6 myotubes, the PKD3 siRNA caused a 77% knock-down of endogenous PKD3 at 100 nM. When glucose uptakes were determined, as shown in Figure 7a, both basal and total insulin-stimulated glucose uptakes were reduced 57% at 100 nM siRNA vs. the control without siRNA, although the net insulin-stimulated glucose uptake was not significantly altered. Taken 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. Here, to explore the mechanisms through which PKD3 regulates glucose transport, we sought to evaluate if DN-PKD3 affects the intracellular distribution of Glut1 and Glut4 in L6 myoblasts. To do this, we had generated a red fluorescent protein (RFP)-tagged dominant negative PKD3 (RFP-DN-
PKD3). When overexpressed alone in L6 myoblasts, RFP-DN-PKD3 showed patchy cytoplasmic distribution, while 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 Figure 8a. However, Glut1 was found to be partially colocalized with DN-PKD3 in cells (regions indicated by arrows in Figure 8a), implying a potential role of PKD3 in regulating Glut1 activity. The localization of Glut4 was visualized using a GFP-tagged Glut4 (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 Figure 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-Glu4 are located in separate subcellular compartments and no colocalization was observed. Taken 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.
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DISCUSSION

In this report, we investigated the potential role of a PKC-related 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 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 in glucose transport. The atypical PKC\(\zeta\) 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; Bandyopadhyay et al., 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 components of the insulin signaling pathway (Schmitz-Peiffer, 2002); 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 (Bandyopadhyay et al., 1997; Bandyopadhyay et al., 1999; Braiman et al., 1999; Chalfant et al., 1996). 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 (Bandyopadhyay et al., 1997; Klip and Ramlal, 1987).

Although PMA treatments suggest the non-involvement 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 that inhibits c/nPKC at nM range, but inhibits PKCζ only at relatively high µM concentration range. This latter range closely matches the dosage required for the inhibition of insulin-stimulated glucose uptake (Standaert et al., 1999; Standaert et al., 1997). In our study, PKCζ-sensitive Go6983 significantly inhibited the insulin-stimulated glucose uptake (Figure 4a-b, middle panels), while lower doses of Ro 31-8220 (1µM) and GF 109203X (2 µM) had little effects (Figure 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 non-selective 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 (Bandyopadhyay et al., 1999; Standaert et al., 1997). These findings diverge from ours, in which insulin-stimulated glucose uptake was inhibited significantly at µM 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. Also, rat adipocytes were used instead of myotubes. Nevertheless, the basis for the apparent discrepancy 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, a 8.5-fold of 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. Additionally, knocking down endogenous PKD3 by >70% caused an >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 contain additionally PKD1 and
PKD2 and our results do not rule out the involvement of PKD1 and PKD2 in glucose transport, and it remains possible that these isoforms may also contribute to the glucose transport process.

Our findings support the notion that the effect of PKD3 on glucose uptake is independent of PKC. This is in line with several recent reports showing that PKD can also be regulated by PKC-independent mechanisms: 1) Gβγ subunits can activate PKD1 through direct interaction with PKD1 PH domain (Jamora et al., 1999); 2) A recent report shows that oxidative stress can also activate PKD1 via a PKC-independent mechanism. Reactive oxygen species activate 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 sensitize 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 if cleavage of PKD1 represents catalytic activation or down-regulation; 4) bone morphogenetic protein 2 (BMP-2)-induced activation of JNK 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 (Baron and Malhotra, 2002; Liljedahl et al., 2001). PKD1 localizes in part to the Golgi (Prestle et al., 1996) and acts as the downstream target of the trimeric G protein subunit βγ in regulating Golgi structure and function (Jamora et al., 1999; Liljedahl et al., 2001). In addition, a 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 direction 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 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.
ACKNOWLEDGEMENTS

We thank Dr. Michael J. Quon and Dr. Hui Chen at the Diabetes Unit, Laboratory of Clinical Investigation, NCCAM, NIH, for insightful discussions and suggestions in the processes of the study. We thank Dr. Amira Klip (Hospital for Sick Children, Toronto, Ontario) for providing the L6 myoblasts. We are grateful to EMD Biosciences, Inc. for providing the anti-PKD2 antibody.
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Alessi DR (1997) The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1β (Rsk-2) and p70 S6 kinase. FEBS Lett 402:121-3.


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and -λ by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* **274**:25308-16.


FOOTNOTES

§This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant 1 RO1 DK066168-01.
FIGURE LEGENDS

Figure 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 hr, 100 nM PMA for 10 min, and 100 nM insulin for 20 min were subjected to immunoblotting using antibodies to (a) PKC isoforms and (b) PKD isoforms as described in Materials and Methods. Equal protein loading was confirmed by blotting with an anti-actin antibody.

Figure 2. c/nPKC is not involved in basal and insulin-stimulated glucose uptake in L6 myotubes.
L6 myotubes were pre-treated with 100 nM PMA for 10 min or with 1 µM PMA for 24 hrs. Glucose uptake in the absence and presence of increasing concentrations of insulin were assessed by [3H]2-DG uptake assay as described in Materials and Methods. (a) [3H]2-DG uptake expressed as pmol/mg/min. (b) the net insulin-stimulated [3H]2-DG uptake expressed as % of stimulation above basal uptake. Values represent the mean ± SEM of at least 7 - 9 independent experiments of triplicate determinations. *p>0.05 versus non-PMA-treated cells.

Figure 3. PMA treatments do not alter insulin-induced Akt activity.
L6 myotubes were pre-treated without or with either 100nM PMA for 10 min and 1 µM PMA for 24 hrs in the absence (-) and in the presence (+) of insulin. The lysates were subjected to immunoblotting using phospho-specific antibodies to active Akt(S473) and GSK-3β(S9) as described in Materials and Methods. Equal protein loading was confirmed by blotting with an anti-actin antibody.
Figure 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 pre-treated 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 ± SEM of triplicate determinations of one of three such similar experiments. (b) PKC/PKD inhibitors block glucose uptake after prolonged PMA treatment. L6 myotubes were pre-treated with 1 µM PMA for 24 hrs prior to 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 ± SEM of triplicate determinations of one of three such similar experiments. Numbers on top of the bars represent the percent of reduction of glucose uptake compared to controls in the absence of inhibitor treatment. Numbers on side of the bars represent the percent 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 pre-incubated with or without Go6976 (10 µM, 1hr) followed by insulin stimulation (100 nM, 20min). 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 ± SEM of four independent experiments.

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

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

**Figure 7. PKD3 siRNA inhibited glucose uptake in L6 myotubes.** (a) The 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 two days after the transfection. Values represent the mean ± SEM 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 ratio of PKD3 level in control cells without siRNA treatment (lower bar chart).
Figure 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 in 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 in combination 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.
Table 1. Potency and selectivity of PKC/PKD inhibitors.

IC$_{50}$ of PKC/PKD inhibitors to classical, 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 the parenthesis following each name of the inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Selectivity for PKC isoforms</th>
<th>IC$_{50}$ (measured in vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Go6976</td>
<td>Inhibits cPKC</td>
<td>PKC$\alpha$ (2.3 nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PKC$\beta$I (6.2 nM)</td>
</tr>
<tr>
<td></td>
<td>Not effective for n/aPKC</td>
<td>PKC$\delta$, $\epsilon$, $\zeta$ (&gt; $\mu$M)</td>
</tr>
<tr>
<td></td>
<td>Selectively for PKD</td>
<td>PKD1 (20 nM)</td>
</tr>
<tr>
<td>Go6983</td>
<td>Inhibits cPKC</td>
<td>PKC$\alpha$, $\beta$ (7 nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PKC$\gamma$ (6 nM)</td>
</tr>
<tr>
<td></td>
<td>Inhibits nPKC</td>
<td>PKC$\delta$ (10 nM)</td>
</tr>
<tr>
<td></td>
<td>Inhibits aPKC</td>
<td>PKC$\zeta$ (60 nM)</td>
</tr>
<tr>
<td></td>
<td>Not effective for PKD</td>
<td>PKD (20 $\mu$M)</td>
</tr>
<tr>
<td>GF 109203X</td>
<td>Inhibits cPKC</td>
<td>8-20 nM</td>
</tr>
<tr>
<td></td>
<td>Inhibits nPKC</td>
<td>210-700 nM</td>
</tr>
<tr>
<td></td>
<td>Not effective for aPKC</td>
<td>PKC$\zeta$ (5.8 $\mu$M)</td>
</tr>
<tr>
<td></td>
<td>Not effective for PKD</td>
<td>PKD1 (2 $\mu$M)</td>
</tr>
<tr>
<td>Ro 31-8220</td>
<td>Inhibits cPKC</td>
<td>PKC$\alpha$, $\beta$, $\gamma$ (20-40 nM)</td>
</tr>
<tr>
<td></td>
<td>Inhibits nPKC</td>
<td>PKC$\delta$, $\epsilon$, $\eta$ (20-100 nM)</td>
</tr>
<tr>
<td></td>
<td>Not effective for aPKC</td>
<td>PKC$\zeta$ (1-4 $\mu$M)</td>
</tr>
<tr>
<td></td>
<td>Not effective for PKD</td>
<td>PKD1 (&gt;2 $\mu$M)</td>
</tr>
</tbody>
</table>
Figure 1

| Treatment: | - | PMA | PMA insulin |
| Dose (µM): | - | 1   | 0.1         | 0.1         |
| Time (min): | - | 24h | 10          | 20          |

 Mb Mt Mt Mt Mt

PKCα
PKCδ
PKCe
PKCζ
actin

PKD1
PKD2
PKD3
actin
Figure 2

a. [Graph showing % insulin-stimulated glucose uptake vs. Insulin (nM) for different conditions: No PMA, 100 nM PMA, 10 min, and 1 μM PMA, 24 h.]

b. [Graph showing % insulin-stimulated glucose uptake vs. Insulin (nM) for different conditions: No PMA, 100 nM PMA, 10 min, and 1 μM PMA, 24 h.]

* ns

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Figure 3

<table>
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<tr>
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<th>PMA (100 nM, 10 min)</th>
<th>PMA (1 µM, 24h)</th>
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<tbody>
<tr>
<td>insulin (100 nM, 20 min):</td>
<td>- +</td>
<td>- +</td>
<td>- +</td>
</tr>
</tbody>
</table>

- p-S473-Akt
- p-GSK3β
- actin
Figure 4  

a. **No PMA**

- [3H]2-DG uptake (pmol/mg/min)
  - Insulin (100 nM): - - - - + + + + +
  - Go6976 (µM): 0 0.01 0.1 1 10 0 0.01 0.1 1 10

- [3H]2-DG uptake (pmol/mg/ml)
  - Insulin (100 nM): - - - - + + + + +
  - Go6983 (µM): 0 0.01 0.1 1 10 0 0.01 0.1 1 10

b. **1 µM PMA, 24 hr**

- [3H]2-DG uptake (pmol/mg/min)
  - Insulin (100 nM): - - - - + + + + +
  - Go6976 (µM): 0 0.01 0.1 1 10 0 0.01 0.1 1 10

- [3H]2-DG uptake (pmol/mg/ml)
  - Insulin (100 nM): - - - - + + + + +
  - Go6983 (µM): 0 0.01 0.1 1 10 0 0.01 0.1 1 10
c.

Figure 4

PKCζ activity (Ratio of control)

Insulin: - - + + - - + +

No treatment PMA (1 µM, 24 hr)

- Go6976 + Go6976
Figure 5

a.  

<table>
<thead>
<tr>
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<th>GF1 (2 µM, 1 h)</th>
<th>Ro (1 µM, 1 h)</th>
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<tbody>
<tr>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PMA (1 µM, 24 hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-S473-Akt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

b.  

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>Go6976 (10 µM, 1 hr)</th>
<th>Go6983 (10 µM, 1 hr)</th>
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<tr>
<td>insulin (100 nM, 20 min)</td>
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<td>PMA (1 µM, 24 hr)</td>
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<td>p-S473-Akt</td>
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</tr>
<tr>
<td>Akt</td>
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</tbody>
</table>
Figure 6

a.

**MOI:** 0 2 5 10 50 100

**Ratio:** 1.0 1.0 1.0 2.3 4.0 8.5

Adv-DN-PKD3

**[^H]2-DG uptake (pmol/mg/min)**

- **ns**
- *****
- ****

<table>
<thead>
<tr>
<th>MOI</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
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</thead>
<tbody>
<tr>
<td>Ratio</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.3</td>
<td>4.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Adv-DN-PKD3</td>
<td>*</td>
<td>**</td>
<td>***</td>
<td>70%</td>
<td>36%</td>
<td>60%</td>
</tr>
</tbody>
</table>

**Adv-DN-PKD3:**

- 0
- 2
- 5
- 10
- 50
- 100 (MOI)

**insulin:**

- -
- +
- -
- +
- -
- +
- -
- +

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Figure 6

b. Adv-wt-PKD3

![Bar graph showing [3H]2-DG uptake (pmol/mg/min) for Adv-wt-PKD3 at different MOIs (0, 2, 5, 10, 50, 100) and insulin treatments (-, +).]

- MOI: 0 2 5 10 50 100 (MOI)
- [3H]2-DG uptake (pmol/mg/min)
- Adv-wt-PKD3: insulin: - + - + - + - + - + - +
- 35% 23%
- MOI: 0 2 5 10 50 100
- Ratio: 1.0 1.0 1.0 3.3 16.5 21.6
- Adv-wt-PKD3

C. Adv-null

![Bar graph showing [3H]2-DG uptake (pmol/mg/min) for Adv-null at different MOIs (0, 2, 5, 10, 50, 100) and insulin treatments (-, +).]

- MOI: 0 2 5 10 50 100 (MOI)
- [3H]2-DG uptake (pmol/mg/min)
- Adv-null: insulin: - + - + - + - + - + - +
- ns

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Figure 7

a. [3H]-2DG uptake (pmol/mg/min) with PKD3 siRNA (nM)

b. % PKD3 with PKD3 siRNA (nM)
Figure 8

a. Anti-Glut1  RFP  Anti-Glut1  RFP-DN-PKD3

b. GFP-Glut4  RFP  GFP-Glut4  RFP-DN-PKD3