

Glucose Regulates Diacylglycerol Intracellular Levels and Protein Kinase C Activity by Modulating Diacylglycerol Kinase Subcellular Localization*

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Although chronic hyperglycemia reduces insulin sensitivity and leads to impaired glucose utilization, short term exposure to high glucose causes cellular responses positively regulating its own metabolism. We show that exposure of L6 myotubes over-expressing human insulin receptors to 25 mM glucose for 5 min decreased the intracellular levels of diacylglycerol (DAG). This was paralleled by transient activation of diacylglycerol kinase (DGK) and of insulin receptor signaling. Following 30-min exposure, however, both DAG levels and DGK activity returned close to basal levels. Moreover, the acute effect of glucose on DAG removal was inhibited by >85% by the DGK inhibitor R59949. DGK inhibition was also accompanied by increased protein kinase C- α (PKC α) activity, reduced glucose-induced insulin receptor activation, and GLUT4 translocation. Glucose exposure transiently redistributed DGK isoforms α and δ , from the prevalent cytosolic localization to the plasma membrane fraction. However, antisense silencing of DGK δ , but not of DGK α expression, was sufficient to prevent the effect of high glucose on PKC α activity, insulin receptor signaling, and glucose uptake. Thus, the short term exposure of skeletal muscle cells to glucose causes a rapid induction of DGK, followed by a reduction of PKC α activity and transactivation of the insulin receptor signaling. The latter may mediate, at least in part, glucose induction of its own metabolism.

Prolonged hyperglycemia is a key contributor in development and progression of diabetic complications (1). It has also been established that chronically elevated glucose levels may worsen insulin sensitivity and impair insulin control of glucose metabolism (2). This is due, at least in part, to persistent activation of conventional isoforms of the protein kinase C (PKC),³

which in turn, down-regulate insulin signaling either by direct phosphorylation of insulin receptor (IR) and insulin receptor substrates (IRSs) or by indirect mechanisms (3–5). The latter include regulation of gene expression and stress-related responses (6, 7). Increased activity of the conventional PKC and elevated levels of diacylglycerol (DAG), the main endogenous activator, have also been documented in several tissues from animal models and diabetic individuals (4, 8–10). On the other hand, evidence exists indicating that glucose acutely activates its own utilization, both *in vitro* and *in vivo* (11–13). *In vivo*, these regulatory actions have been attributed to the mass action effect of glucose (14). Subsequently, it has been shown that acute hyperglycemia *per se* may increase muscle membrane content of GLUT4 (12, 15).

Although several studies have investigated the chronic effect of high glucose concentrations on PKC activity (2–4), the molecular mechanisms of the short term autoregulatory effect have been only partially elucidated. In a previous work we have shown that, in a skeletal muscle cell model, acute exposure to increasing glucose concentrations determined a parallel increase in glucose uptake and its intracellular metabolism (13). Glucose autoregulation involves PKC α retrotranslocation from the membrane to the cytoplasm and dissociation from the IR. This is followed by the transient trans-activation of the IR tyrosine kinase and a consequent induction of glucose uptake (13). However, how glucose acutely modulates PKC α remains to be investigated. The regulation of DAG intracellular levels by acute hyperglycemia represents an attractive hypothesis. DAG is a lipid second messenger with important signaling functions (3, 15). Generation and removal of diacylglycerol are indeed critical for different intracellular signaling pathways (16–18). In response to many extracellular stimuli DAG is generated through the action of phospholipases (mainly PLC and PLD) (17). The removal of DAG is largely operated by specific enzymes of the diacylglycerol kinase (DGK) family. DGK phosphorylates DAG to produce phosphatidic acid (PA) and plays an important role in signal transduction by modulating the balance between these two lipids (17). By controlling the cellular levels of DAG, DGK can serve as a negative regulator of PKC (16). To date, ten DGK isozymes have been identified in mammals and divided into five classes based on their primary structure (19, 20). DGKs feature a conserved catalytic domain at the C terminus and, within the regulatory domain at the N termi-

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³ The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; DGK, diacylglycerol kinase; PA, phosphatidic acid; 2-DG, 2-deoxyglucose; IR, insulin receptor; IRS, IR substrate.

DGK and Glucose Signaling

nus, possess two or three cysteine-rich regions homologous to the C1A and C1B motifs of PKC (21). Moreover, these enzymes share other conserved motifs that are likely to play a role in lipid-protein and protein-protein interactions in various signaling pathways dependent on DAG and/or PA production (22).

The differences in the regulatory domains of the various subtypes, together with the differential tissue expression pattern of the different isoforms suggest that the regulation of DGKs varies among cell types and/or in response to different stimuli and that the DGK isoforms serve distinct although related functions (23, 24). DGK may serve as an off switch controlling PKC activation, thereby mediating the acute glucose regulation of its own metabolism. In the present work we have investigated the mechanism involved in acute regulation of PKC α activity by glucose. We have found that the acute exposure to high glucose concentration leads to a decrease in DAG levels and concomitantly impairs the enzymatic activity and translocation of PKC α . This effect is mediated by glucose action on DGK activity and subcellular localization.

EXPERIMENTAL PROCEDURES

Materials—Media and sera for tissue culture and the transfection reagent, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride/dioleoylphosphatidylethanolamine, were purchased from Invitrogen. Electrophoresis reagents were from Bio-Rad. Protein A-Sepharose beads was from Pierce. Radiochemicals, Western blot, ECL reagents, and the DAG quantification test kit and the PKC enzyme assay system were purchased from Amersham Biosciences. Polyclonal GLUT4 antibody was from Biogenesis (Sandown, NH). Polyclonal antibody toward PKC was from Invitrogen. Polyclonal antibodies toward DGK α and DGK δ have been previously generated and characterized (25, 26). DGK ζ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents, including the DGK inhibitor R59949, were from Sigma.

Cell Culture—The L6 cell clones expressing the wild-type human insulin receptors have been previously characterized and described (13). Cells were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose and supplemented with 2% fetal bovine serum as described previously (13) and used at the myotube stage of differentiation.

Determination of DAG Cellular Content—DAG content was quantified radioenzymatically by incubating aliquots of the lipid extract with DAG kinase and [32 P]ATP, as described by Preiss *et al.* (27). The manufacturer's instructions for the commercially available DAG test kit were followed. The 32 P-labeled PA was purified using chloroform/methanol/acetic acid (65:15:5, v/v) as a solvent system and quantified with a Storm 860 PhosphorImager (Amersham Biosciences).

Western Blot Analysis—Western blot analysis was performed as previously reported (12, 28). Briefly, cells were rinsed and incubated in glucose-free buffer (20 mM HEPES, pH 7.8, 120 mM NaCl, 5 mM KCl, 2.5 mM MgSO $_4$, 10 mM NaHCO $_3$, 1.3 mM CaCl $_2$, 1.2 mM KH $_2$ PO $_4$, 0.25% bovine serum albumin) for 3 h. The cells were subsequently incubated for 5 and 30 min in the same buffer supplemented with the indicated concentrations of glucose or R59949, as indicated. Then the cells were solubilized

in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM Na $_2$ P $_2$ O $_7$, 2 mM Na $_3$ VO $_4$, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin) for 2 h at 4 $^{\circ}$ C. Cell lysates were clarified by centrifugation at 5000 \times *g* for 20 min, separated by SDS-PAGE, and transferred into 0.45- μ m Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

Purified Plasma Membrane Preparations—Purified plasma membrane preparations were obtained as in Caruso *et al.* (13) with slight modifications. Briefly, the cells, after exposure to glucose or R59949 for the indicated times, were further incubated for 10 min in glucose-free buffer, washed in ice-cold phosphate-buffered saline, and homogenized in 500 μ l of ice-cold fractionation buffer (20 mM HEPES-NaOH, pH 7.4, 250 mM sucrose, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 2 μ M microcystin LR, 1 mM benzamide) by passing them 10 times through a 22-gauge needle. The cell lysates were centrifuged at 800 \times *g* for 5 min at 4 $^{\circ}$ C. Supernatants were further centrifuged at 100,000 \times *g* for 20 min at 4 $^{\circ}$ C. The final supernatants were collected and used as the cytosolic fraction. The membrane pellet was solubilized in Buffer A containing 1% Triton X-100 by bath sonication and centrifuged at 12,000 \times *g* for 10 min at 4 $^{\circ}$ C, and the supernatant was used as the membrane fraction. Cytosolic and membrane fractions were then analyzed by Western blot. Purity (>90%) of the subcellular fractions was assessed by immunoblot with antibody against the β -subunit of the insulin-like growth factor-1 receptor (as control of membrane fraction) and against β -actin (as control of cytosolic fraction).

Determinations of 2-Deoxy-D-glucose Uptake—For 2-deoxyglucose (2-DG) uptake studies, cells were rinsed and incubated in glucose-free buffer (20 mM HEPES, pH 7.8, 120 mM NaCl, 5 mM KCl, 2.5 mM MgSO $_4$, 10 mM NaHCO $_3$, 1.3 mM CaCl $_2$, 1.2 mM KH $_2$ PO $_4$, 0.25% bovine serum albumin) for 3 h. The cells were subsequently incubated for 3 min in the same buffer supplemented with the indicated concentrations of glucose or/and R59949, washed again, and incubated for further 10 min in glucose-free buffer containing 2-DG (final concentration, 0.15 mM) and 0.5 μ Ci/assay [14 C]2-DG (13). The cells were finally lysed, and 2-DG uptake was determined by liquid scintillation counting.

Protein Kinase C Assays—Determination of PKC activity was achieved with a commercially available kit (Invitrogen, catalogue number 13161-013). This assay kit is based on measurement of phosphorylation of the synthetic peptide from myelin basic protein Ac-MBP (4–14) by PKC (in the presence of activators) as described by Yasuda *et al.* (29). PKC specificity is confirmed by using the PKC pseudosubstrate inhibitor peptide. For analyzing PKC activity, L6 cells were deprived from serum and glucose as described above and then exposed to 25 mM glucose as indicated. PKC activity was then quantitated in total cell lysates or cell fractions or in immunoprecipitates as previously reported (30) and according to the manufacturer's instructions. Briefly, cells were solubilized in the extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 mg/ml aprotinin, and 25 mg/ml leupeptin)

and then clarified by centrifugation at $10,000 \times g$ for 15 min at 4 °C. Upon protein quantitation, equal aliquots of the extract were added to the lipid activators (10 μM phorbol 12-myristate,13-acetate, 0.28 mg/ml phosphatidylserine, and 4 mg/ml dioleine, final concentrations) and the ^{32}P -substrate solution (50 mM Ac-MBP (4–14), 20 μM ATP, 1 mM CaCl_2 , 20 mM MgCl_2 , 4 mM Tris, pH 7.5, and 10 mCi/ml (3,000 Ci/mM) [γ - ^{32}P]ATP), in the presence or the absence of the substrate peptide and/or of the inhibitor. The samples were incubated for 20 min at room temperature and rapidly cooled on ice, and 20- μl aliquots were spotted on phosphocellulose disc papers (Invitrogen). Discs were washed twice with 1% H_3PO_4 , followed by two additional washes in water, and the disc-bound radioactivity was quantitated by liquid scintillation counting.

DGK Antisense silencing—For antisense studies, a phosphorothioate DGK α oligodeoxynucleotide was generated with the following sequence, 5'-TACCGGTTTCTGTTTTCACA-3' and a phosphorothioate DGK δ oligodeoxynucleotide was generated with the following sequence, 5'-TACCTGGTAAAGAGTCCCTG-3'. For control, scrambled oligodeoxynucleotides (POs) with the sequences 5'-CTTGATATTCCCGTCGGACC-3' and 5'-GGTCCACGTGCCACTTGGAC-3' were also obtained. L6hIR cells were grown in 6-well plates. The cells were then rinsed with 3 ml of serum-free Dulbecco's modified minimum essential medium and 3 ml of medium containing 2 $\mu\text{g}/\text{ml}$ N-[1-(2,3-diomeoyloxy)propyl]-N,N,N-trimethylammonium chloride/dioleoylphosphatidylethanolamine transfection reagent, and 4 $\mu\text{g}/\text{ml}$ antisense were added for 16 h. The cells were washed with serum-free Dulbecco's modified minimum essential medium and incubated for 18 h in the same medium supplemented with 0.25% bovine serum albumin. Transfected cells were exposed to 25 mM glucose as indicated and assayed for DAG levels and PKC α activation as described above.

DGK Enzymatic Assays—DGK activity was assayed *in vitro* as previously described (31). Briefly, octylglucoside/DAG-mixed micelles were prepared as follows: a mixture of 0.25 mM DAG, 55 mM octylglucoside, and phosphatidylserine (either 1 mM, resulting in 1.8 mol% in micelles, or 5 mM, resulting in 8.3 mol% in micelles) was resuspended in 1 mM diethylenetriaminepentaacetic acid, pH 7.4, by vortex-mixing and sonication until the suspension appeared clear. 20 μl of mixed micelles was added to 70 μl of reaction mix (final concentration: 100 μM diethylenetriaminepentaacetic acid, pH 7.4, 50 mM imidazole-HCl, 50 mM NaCl, 12.5 mM MgCl_2 , 1 mM EGTA, 1 mM dithiothreitol, 1 mM [γ - ^{32}P]ATP). 10 μl of total homogenate was added to 90 μl of mixed-micelles reaction mix solution. The reaction was started by vortex-mixing for 3 s and sonication for 5 s. After 30-min incubation at 25 °C, the reaction was terminated by the addition of chloroform/methanol/1% perchloric acid (1:2:0.75, v/v), then vortex-mixed. 1% perchloric acid and chloroform (1:1, v/v) were added, and the mixture was centrifuged for 5 min at 2,000 rpm in a tabletop Sorval centrifuge at 25 °C. The organic phase was washed twice in 1% perchloric acid, and an aliquot was dried under a stream of nitrogen and spotted onto a silica gel 60 TLC plate. PA was separated by chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v). The amount

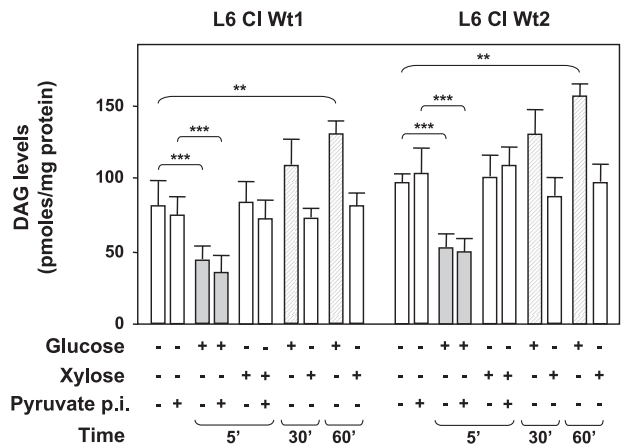


FIGURE 1. **Glucose effect on diacylglycerol levels in L6 myotubes.** Determination of DAG levels was performed as described under "Experimental Procedures." Two independent clones of L6hIR cells (L6 CI Wt1 and Wt2) were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose, and then incubated for 3 h in glucose-free buffer, in the absence or in the presence of 25 mM pyruvate, and further incubated with 25 mM glucose or xylose for 5, 30, and 60 min, as indicated. [^{32}P]Phosphatidic acid was separated by TLC and quantitated by densitometric analysis. Bars represent the mean \pm S.D. of values obtained from three independent experiments in duplicate. Asterisks denote statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

of [γ - ^{32}P]PA was measured by using a liquid scintillation spectrophotometer.

RESULTS

Glucose Effect on DAG Levels in L6 Skeletal Muscle Cells—We investigated whether the intracellular levels of DAG were changed by exposure of L6hIR myotubes to high glucose concentrations. The L6hIR (L6 CI Wt1 and Wt2) are clones of L6 cells overexpressing human insulin receptors and have been previously generated and characterized (13). In both clones of L6hIR myotubes, incubation with 25 mM glucose for 5 min induced a decrease in DAG levels of ~ 2 -fold as compared with untreated cells ($p < 0.001$). Prolonging glucose exposure at 30 and 60 min, DAG levels were increased by 1.4- and 1.6-fold over basal levels, respectively (Fig. 1). To evaluate the specificity of glucose effect, DAG levels were measured in the same cells treated with 25 mM xylose. No variation occurred in DAG levels after xylose treatment (Fig. 1). In addition, acute glucose exposure of the cells following preincubation with 25 mM pyruvate in the glucose-free medium elicited similar effects (Fig. 1), indicating that glucose effect was not due to changes in osmolarity or to energy depletion.

Glucose Effect on DGK Activity in L6 Myotubes—To investigate the mechanism by which glucose regulates DAG levels, we measured cellular DGK activity in L6hIR (L6 CI Wt1 and Wt2) myotubes after exposure to 25 mM glucose for 5 and 30 min. After 5 min of glucose stimulation, the levels of PA were increased by 12-fold ($p < 0.001$) (Fig. 2A). However, PA levels returned close to basal levels by prolonging the incubation with high glucose concentrations for up to 30 min. In addition, pharmacological inhibition of DGK with a well characterized non-isofom specific inhibitor, R59949 (1 mM), prevented the acute glucose-dependent increase in PA levels, suggesting that this increase was due to DGK activation. We next analyzed the

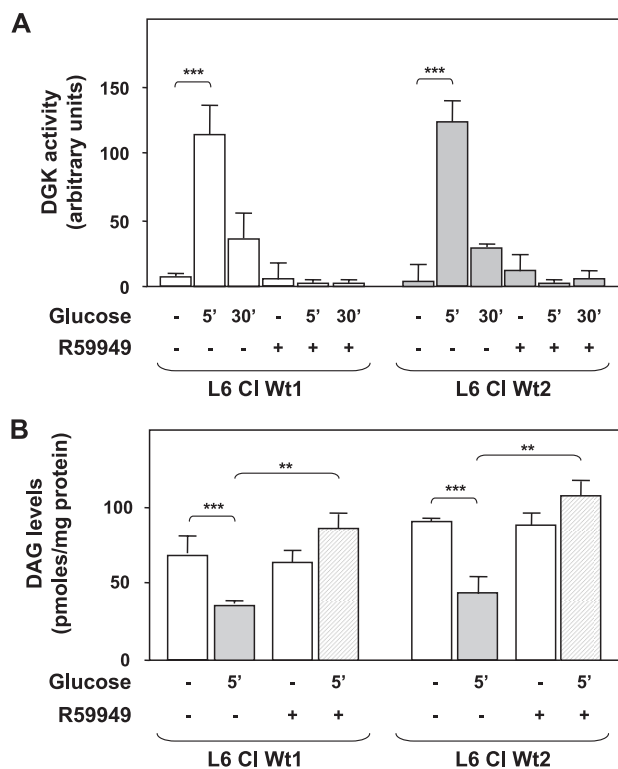


FIGURE 2. Glucose effect on DGK activity in L6 myotubes. Two independent clones of L6hIR cells (L6 CI Wt1 and Wt2) were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and pre-treated with DGK inhibitor R59949 (1 mM) where indicated, then incubated with 25 mM glucose for 5 and 30 min. Total homogenates of the cells were assayed for DGK activity. [³²P]Phosphatidic acid was separated by TLC and quantitated by densitometric analysis (A). Bars represent the mean ± S.D. of values obtained from three independent experiments. DAG level were measured as described in the legend of Fig. 1 (B). Bars represent the mean ± S.D. of values obtained from four independent experiments in duplicate. Asterisks denote statistically significant differences (**, $p < 0.01$; ***, $p < 0.001$).

effect of R59949 on the glucose-induced decrease in DAG levels (Fig. 2B). Interestingly, in the presence of the DGK inhibitor, DAG levels were slightly increased by glucose stimulation for 5 min, compared with basal levels. Thus, glucose-induced reciprocal changes of PA and DAG were largely prevented by DGK inhibition.

Effect of DGK Inhibition on Glucose-induced PKC α Activity and Translocation—To investigate the role of DGK in glucose regulation of the DAG/PKC pathway, we evaluated the effect of DGK inhibition on PKC α activity and translocation to the plasma membrane after exposure to 25 mM glucose. As previously shown (13), incubation of L6hIR myotubes with 25 mM glucose for 5 min induced a 2-fold decrease in PKC α activity (Fig. 3A). Inhibition of DGK activity with R59949 prevented the negative effect of glucose on PKC α activity, which, instead, was increased by 1.5-fold over basal levels after 5 min of high glucose stimulation. Incubation with PA did not affect PKC α activity in *in vitro* assays, suggesting that its accumulation was not responsible for PKC inhibition (data not shown). Treatment with R59949 also prevented glucose-induced cytosolic retrotranslocation of PKC α (Fig. 3B), indicating that DGK is involved in acute glucose regulation of PKC α activity and translocation.

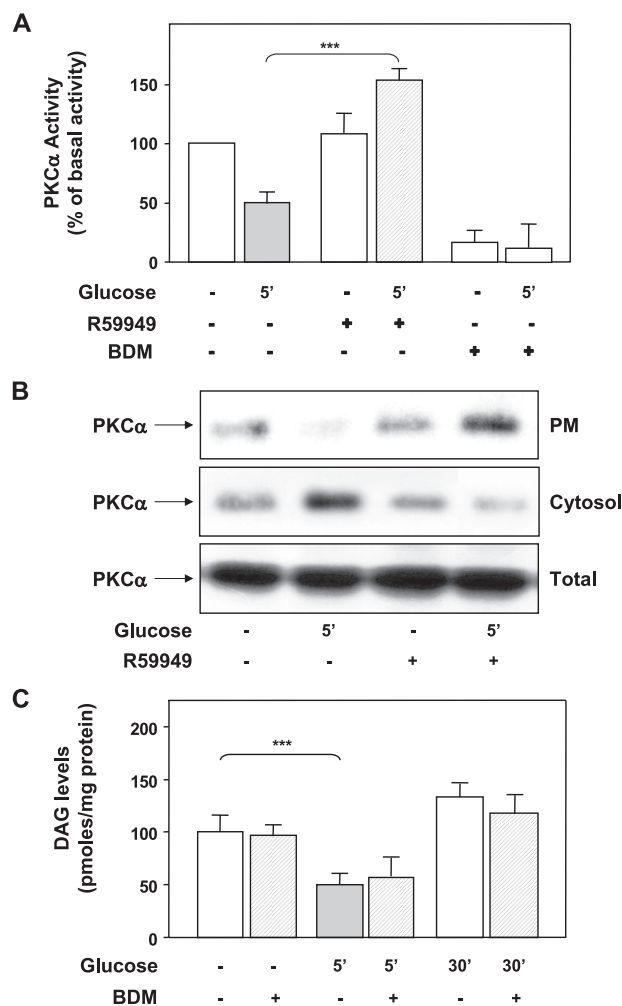


FIGURE 3. Effect of DGK inhibition on glucose-induced PKC α activity. L6hIR cells were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and incubated with 25 mM glucose for 5 min in absence or presence of 1 mM R59949 or 100 nM bisindolylmaleimide (BDM) as indicated. Cell lysates were either immunoprecipitated with anti-PKC α antibody (A) or subjected to subcellular fractionation as described under "Experimental Procedures" (B). A, PKC α activity was measured in anti-PKC α immunoprecipitates as described under "Experimental Procedures." Bars represent the mean ± S.D. of values obtained from three independent experiments in triplicate. B, PKC α was immunoblotted from total lysates, plasma membranes (PM), and cytosolic preparations from the cells, as indicated. Detection of the blots shown in the figure was achieved by chemiluminescence. The autoradiographs shown are representative of three independent experiments. C, L6hIR cells were incubated with 25 mM glucose for the indicated times in absence or presence of bisindolylmaleimide. Determination of DAG levels was performed as described in the legend of Fig. 1. Bars represent the mean ± S.D. of values obtained from three independent experiments in duplicate. Asterisks denote statistically significant differences (***, $p < 0.001$).

PKC may directly regulate DGK activity and modify intracellular DAG concentration (29). We therefore measured DAG levels in L6hIR cells after treatment with 100 nM bisindolylmaleimide, an inhibitor of the conventional PKC isoforms. In L6hIR myotubes, bisindolylmaleimide, although strongly reducing PKC α activity (Fig. 3A), did not alter the glucose-induced decrease in DAG levels (Fig. 3C). Similarly, antisense inhibition of PKC α did not significantly change the detection of cellular DAG (data not shown).

Effect of DGK Inhibition on Glucose Uptake and GLUT4 Recruitment on the Plasma Membrane—To investigate the role of DGK in regulation of glucose disposal, we analyzed glucose

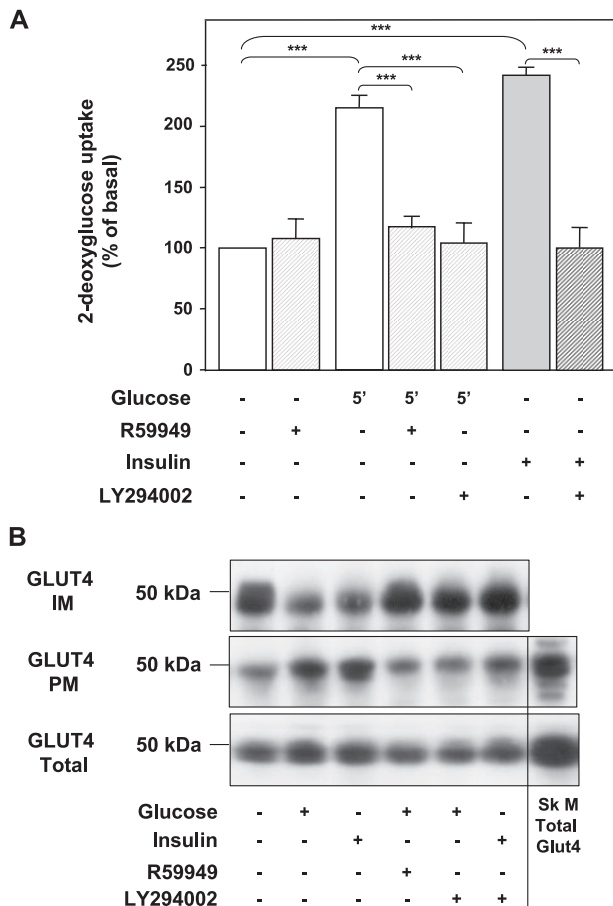


FIGURE 4. Effect of DGK inhibition on 2-DG uptake and GLUT4 localization. L6hIR cells were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and incubated with 25 mM glucose for 5 min or with 10 nM insulin for 15 min in absence or presence of 1 mM R59949 or 100 μ M LY294002 as indicated. Determination of 2-deoxyglucose uptake (A) and membrane preparation procedures (B) were performed after additional 10 min to allow glucose transporter translocation as described under "Experimental Procedures" (see also Ref. 13). Error bars represent mean \pm S.D. of values obtained in three independent experiments in triplicate. Asterisks denote statistically significant different (***, $p < 0.001$). B, plasma membranes (PM) and intracellular membranes (IM) were obtained as previously described. The membranes were solubilized and blotted with GLUT4 antibodies. Alternatively, for determining the total levels of transporter, cells were solubilized and blotted with GLUT4 antibodies. Filters were revealed by ECL. The autoradiographs shown are representative of three independent experiments.

uptake in L6 myotubes upon blocking DGK with R59949 (Fig. 4A). Exposure of L6 cells to 25 mM glucose for 5 min was paralleled by 2.3-fold increase in 2-deoxy-D-glucose (2-DG) uptake ($p < 0.001$). Preincubation of the cells with R59949 or with the PI3K inhibitor LY294002 (100 μ M) resulted in a >80% decrease in glucose-stimulated 2-DG uptake. In parallel with glucose uptake, the exposure to high glucose for 5 min increased GLUT4 content on the plasma membrane by ~5-fold above the basal levels ($p < 0.01$). A similarly sized decrease of GLUT4 in the intracellular membrane fraction was also observed. Upon treatment with either the DGK inhibitor, or with LY294002, however, GLUT4 was mainly localized in the intracellular compartment compared with the plasma membrane fraction (Fig. 4B).

Glucose Activation of DGK Isoforms—Several DGK isoforms have been identified to date (19–21). To identify that responsi-

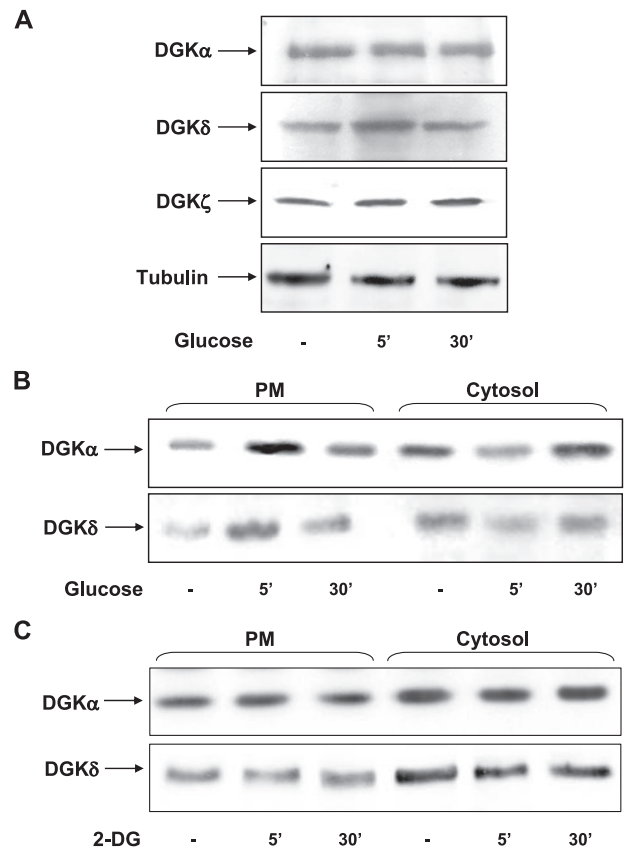


FIGURE 5. Glucose effect on DGK isoform expression and localization. L6hIR cells were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and incubated with 25 mM glucose (A and B) or 2-DG (C) for 5 and 30 min as indicated. Subcellular fractions were obtained and analyzed as described under "Experimental Procedures." A, total lysates were blotted with anti-DGK α , -DGK δ , and -DGK ζ antibodies, as indicated. Tubulin was used as standard to ascertain equal loading. B, plasma membranes (PM) and cytosolic proteins (Cytosol) were blotted with anti-DGK α and -DGK δ antibodies as outlined above. Filters were revealed by ECL. The autoradiographs shown are representative of four independent experiments.

ble for acute glucose effect on DAG/PKC α pathway, protein lysates of L6hIR myotubes were analyzed by Western blotting with isoform-specific DGK antibodies. We found that three isoforms of DGK (DGK- α , - δ , and - ζ) were preferentially expressed in these cells (Fig. 5A). Translocation to the plasma membrane regulates DGK activity and isoform-specific functions (19). Thus, L6hIR cells were stimulated with 25 mM glucose for 5 and 30 min, and subcellular fractionation experiments were performed (Fig. 5B). In unstimulated myotubes, DGK α and - δ were mainly cytosolic. After acute glucose exposure, DGK α and - δ isoforms were rapidly (5 min) recruited to the plasma membrane. Membrane expression of both isoforms was then reduced after 30 min, returning to basal levels. Moreover, glucose effect was neither mimicked by 2-DG (Fig. 5C), nor by pyruvate (data not shown). At variance with the other isoforms, DGK ζ remained largely cytosolic both in basal conditions and after glucose exposure (data not shown). Moreover, stimulation with 25 mM glucose for 5 min induced a 6-fold increase of DGK δ -specific activity (Fig. 6). The activity of DGK δ was still higher than basal after 30 min (difference not statistically significant), but ~3-fold reduced, compared with the earlier time.

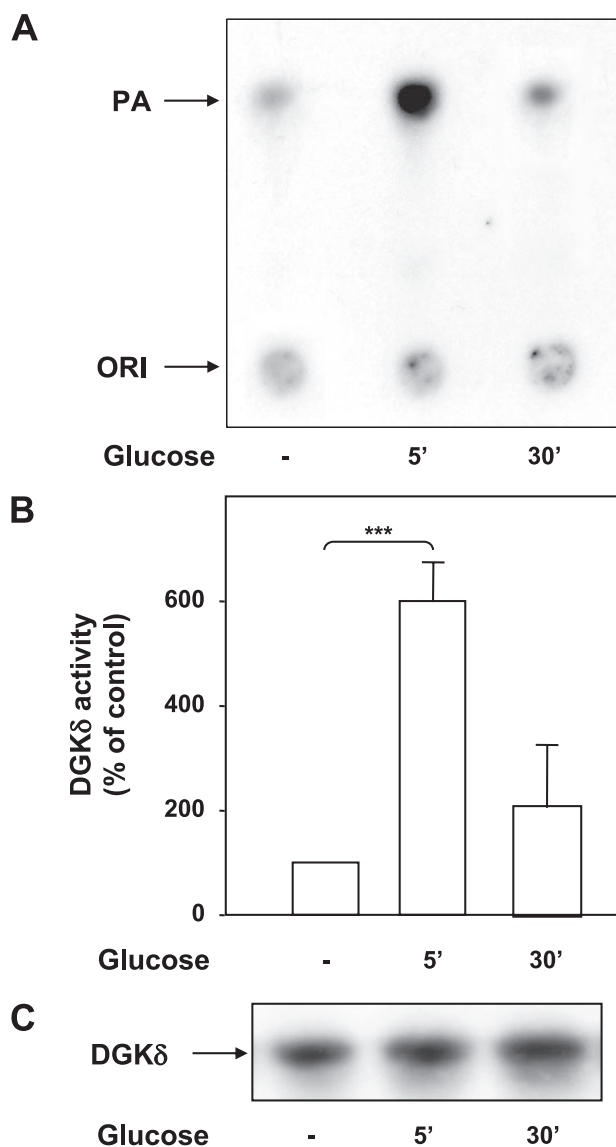


FIGURE 6. Glucose effect on DGKδ activity. L6hIR cells were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and incubated with 25 mM glucose for 5 and 30 min. Total homogenates of the cells were immunoprecipitated with anti-DGKδ antibodies and then assayed for DGK activity (A and B) or analyzed by immunoblot (C). [³²P]Phosphatidic acid was separated by TLC, analyzed by autoradiography (A), and quantitated by densitometric analysis (B). Bars represent the mean ± S.D. of values obtained from four independent experiments. Asterisks denote statistically significant differences (***, *p* < 0.001). Immunoprecipitates were also blotted with anti-DGKδ antibodies to ensure the recovery of equal amounts of protein (C). A representative blot is shown.

Effect of DGKδ and DGKα Antisense on Glucose-regulated DAG Levels and PKCα Activation—Next, we used specific DGKα and DGKδ phosphorothioate antisense oligonucleotides (DGKASα and DGKASδ). Treatment of L6hIR myotubes with DGKASα or DGKASδ led to a selective 70% reduction of the cellular content of DGKα and DGKδ expression, respectively (Fig. 7A). The expression levels of both DGK isoforms were not affected by transfecting the nonspecific scrambled phosphorothioate oligonucleotides (POα and POδ). Interestingly, inhibition of DGKδ with the specific antisense oligonucleotides prevented the negative effect of glucose on PKCα activation, which was actually increased by 3-fold over basal levels

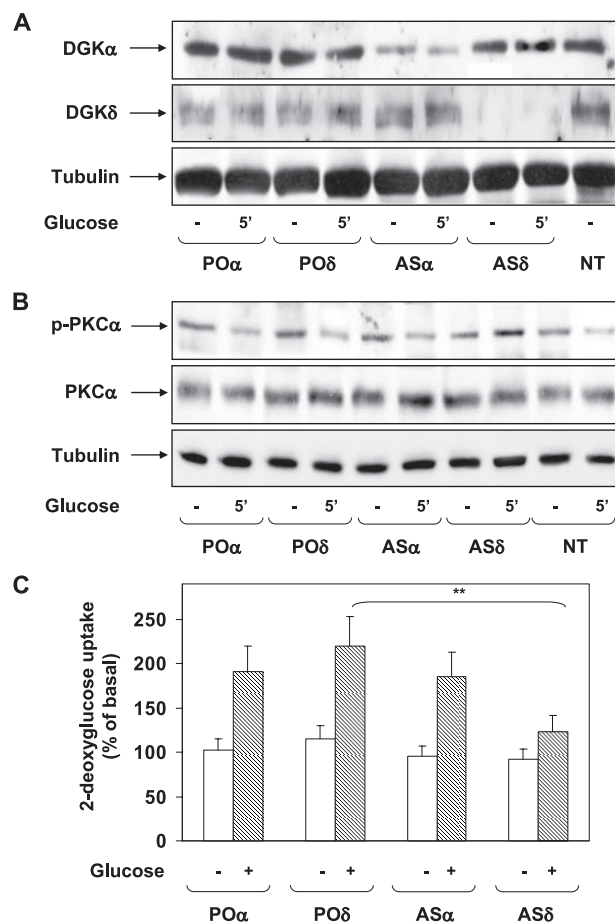


FIGURE 7. Effect of DGKδ and DGKα antisense on glucose-regulated DAG levels and PKCα activation. L6hIR cells were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and treated with either DGKα (ASα) or DGKδ (ASδ) antisense oligonucleotide or with DGKα (POα) or DGKδ (POδ) scrambled oligonucleotide, and then incubated with 25 mM glucose for 5 min, as indicated. A, DGKα and DGKδ total levels were measured by blotting with specific anti-DGKα and -DGKδ antibodies. Tubulin was used as standard to ascertain equal loading. Filters were revealed by ECL. B, for determining PKCα activation and total levels, cell were solubilized, and then blotted with specific anti-phospho-PKCα and PKCα antibodies, respectively. Tubulin was used as standard to ascertain equal loading. Filters were revealed by ECL. The autoradiographs shown are representative of five independent experiments. C, L6hIR cells, treated with either antisense or scrambled oligonucleotides as previously described, were incubated with 25 mM glucose for 5 min, as indicated, and the determination of 2-DG uptake was performed as described under "Experimental Procedures." Error bars represent mean ± S.D. of values obtained in three independent experiments in triplicate. Asterisks denote statistically significant different (**, *p* < 0.01).

after 5 min of high glucose stimulation (Fig. 7B). At variance, inhibition of DGKα had no effect on PKCα activation. No changes of PKCα expression were observed, however. Glucose-stimulated 2-DG uptake was also reduced by ~80%, upon antisense inhibition of DGKδ, but not of DGKα (Fig. 7C). Moreover, consistent with a negative regulation of insulin signaling, glucose-induced activation of insulin receptor, IRS2 tyrosine phosphorylation and protein kinase B/Akt activation were strongly reduced upon R59949 and DGK-ASδ treatment of the cells (Fig. 8).

DISCUSSION

The ability of glucose to regulate its own metabolism has been extensively investigated (1–15). Although chronic expo-

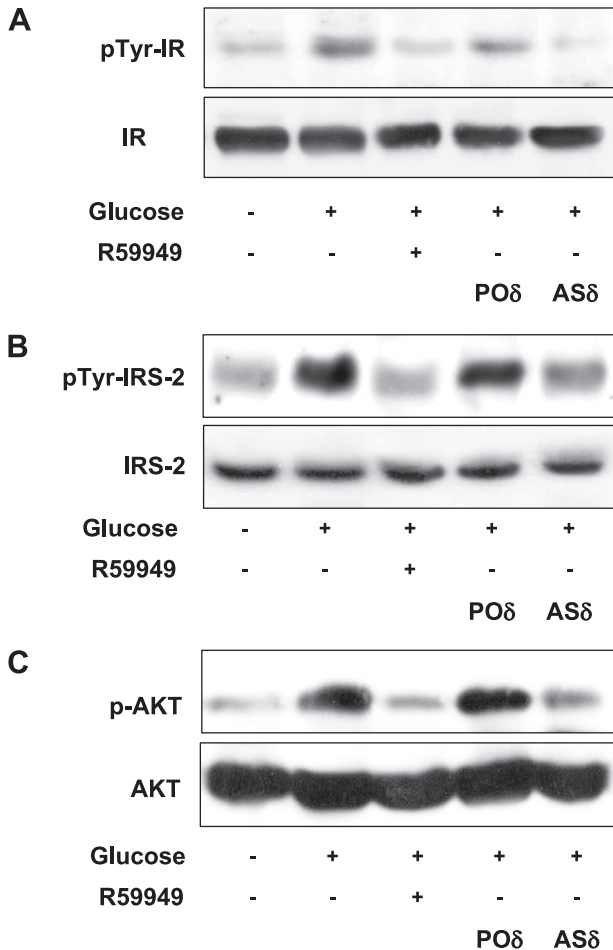


FIGURE 8. Effect of DGK inhibition on insulin receptor signaling. L6H1R cells were grown as described under "Experimental Procedures" and in the legend to Fig. 1 and incubated with 25 mM glucose for 5 min in the absence or in the presence of R59949 and DGK δ (AS δ) antisense oligonucleotide. Total homogenates of the cells were immunoprecipitated with anti-insulin receptor (A) or IRS2 (B) antibodies and then analyzed by immunoblot with anti-phosphotyrosine antibodies (A and B). Alternatively, total homogenates were directly blotted with anti-phosphoSer-Akt antibodies (C). Filters were also re-probed with IR (A), IRS2 (B), and Akt (C) antibodies. All the filters were revealed by ECL. The autoradiographs shown are representative of four independent experiments.

sure to high glucose concentrations, *in vivo* and in isolated cells, leads to inhibition of insulin sensitivity (2, 3) and glucotoxicity (32, 33), short term hyperglycemia induces glucose utilization (11–13) and may affect the utilization of other energy sources as well (14, 15, 34). For instance, hyperglycemia very rapidly induces insulin secretion by pancreatic β cells (35). This plays an important role in regulating whole body glucose utilization (36). Nevertheless, peripheral tissues, including skeletal muscle, may contribute to glucose disposal by insulin-independent mechanisms (11–13). However, the molecular events responsible for the latter effect have been only partially defined. Our previous studies provided evidence that, in cultured skeletal muscle cells, high glucose concentrations transiently transactivate the insulin receptor kinase (13). In particular, we have shown that PKC α is constitutively associated to the insulin receptor. Acute exposure of the cells to high glucose causes cytosolic translocation of PKC α and induces its dissociation from the insulin receptor. This is followed by the removal of a

tonic inhibitory constraint on the receptor tyrosine kinase, thereby promoting activation of downstream molecules and stimulation of the glucose uptake (13). In the present work we have further investigated the molecular mechanism by which glucose regulates PKC α activity. DAG is the key lipid physiologically regulating PKC α (14). We report that, at variance with chronic hyperglycemia (3), glucose treatment acutely reduces DAG levels. DAG metabolism is generally controlled by distinct pathways: (i) DAG lipase-mediated hydrolysis of fatty acyl chain to generate a monoacylglycerol and a free fatty acid; (ii) addition of CDP-choline or -ethanolamine to form phosphatidylcholine or phosphatidylethanolamine, or (iii) DGK-mediated phosphorylation of the free hydroxyl group to produce PA (22). Although we cannot exclude the former two possibilities, we show that acute glucose exposure induces DGK activation concomitantly to a reduction in PKC α activity and its cytosolic translocation. The finding that the nonspecific DGK inhibitor R59949 prevents glucose effect on its own uptake further supports the hypothesis that DGK activity plays a crucial role in glucose autoregulatory functions. Recently, another inhibitory compound, R59022, has been shown to stimulate glucose uptake in C2C12 skeletal muscle cells via a p38-mediated pathway (37). However, this stimulatory effect occurs upon 24 h incubation with the compound and may reflect cellular adaptation. Alternatively, different DGK isoforms might be independently involved.

Ten mammalian DGK isoforms have been identified to date (19, 20, 22). Except for DGK δ and $-\epsilon$, all mammalian isoforms are highly expressed in the brain. DGKs are also highly expressed in muscle, with DGK δ and $-\zeta$ mainly expressed in striated muscle and DGK β and $-\epsilon$ in cardiac muscle (38). Here we show that DGK α , $-\delta$, and $-\zeta$ are expressed in L6 cells, and their total content is not regulated by glucose. At variance, acute glucose exposure induces DGK α and $-\delta$ to selectively translocate to the plasma membrane and induces a specific increase of DGK- δ activity. Stronger evidence indicates that DGK δ , rather than DGK α , is necessary for glucose to exert its function on PKC α activity and cytosolic translocation. Indeed, selective silencing of DGK δ is sufficient to prevent glucose effect on PKC α . These data are also in agreement with recent evidence indicating that DGK δ null mice feature increased PKC α activity (39). Other groups have demonstrated that DGK α activation is regulated by insulin, at least in the brain (40, 41), and Src-mediated phosphorylation at Tyr-334 is responsible for membrane translocation (40). In our experimental conditions, we failed to measure DGK α activity, possibly due to technical limitation of the precipitating antibody. However, antisense inhibition of DGK α in L6 myotubes did not produce effects on glucose-mediated PKC α activation. Collectively, our data suggest that DGK δ is the major candidate for mediating glucose regulation of its own uptake. In this regard, lower doses of DGK δ antisense oligonucleotides reduced DGK δ intracellular levels to a lesser extent and were still capable of reducing PKC α activation (data not shown). In addition, inhibition of DGK δ leads to a paradoxical glucose-induced increase of DAG levels (data not shown) and PKC α activity (Fig. 7). Thus, the rapid and transient translocation of DGK δ on the plasma membrane may mediate the acute removal of DAG, preceding the

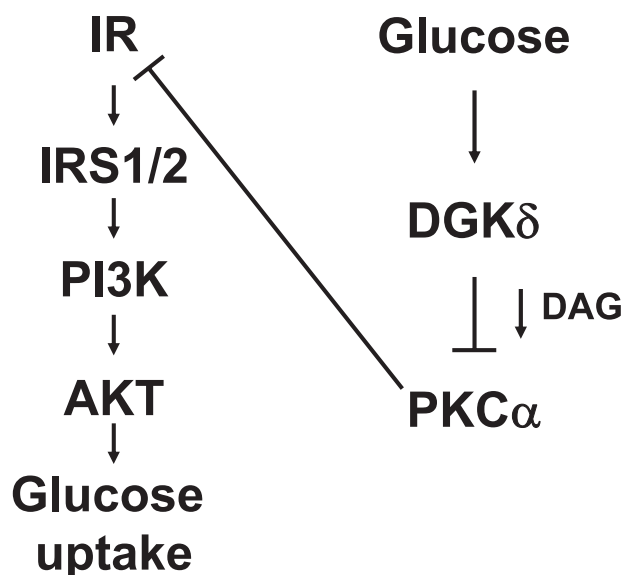


FIGURE 9. Schematic diagram of glucose action on insulin receptor signaling. A proposed schematic model by which glucose acutely activates DGK, followed by reduction in intracellular DAG levels and inhibition of PKC α activity. This, in turn, removes the tonic inhibition exerted on the insulin receptor signaling pathways, causing transactivation of the cascade leading to glucose uptake in muscle cell models.

sustained increase of DAG intracellular concentration which occurs after prolonged exposure to glucose.

It is possible that intermediate products of glucose metabolism or changes in lipid composition may lead to plasma membrane recruitment of DGK δ . However, our data indicate that neither 2-DG, a glucose analogue that is phosphorylated and not further metabolized, nor pyruvate mimic glucose action on DGK δ . It was reported that DGK δ is translocated from the cytoplasm to the plasma membrane by phorbol ester (DAG analogue) (42). Thus, it is possible that DAG concentration is quickly (within seconds) increased upon acute glucose load, but its detection is negligible because DGK δ consumes DAG immediately after its translocation.

We and others have previously shown that inhibition of PKC increases the activity of several tyrosine kinase receptors, including the insulin receptor (13, 39, 43). The reduction of PKC α activity, consequent to glucose-induced activation of DGK, may directly up-regulate insulin receptor signaling and induce GLUT4 translocation and glucose uptake. We have previously shown that glucose increases GLUT1 plasma membrane expression, as well, in the L6 cells (13). Thus, similar to GLUT4, GLUT1 translocation may occur following up-regulation of insulin receptor signaling. Alternatively, the increase of PA, following DGK activation, may directly enhance the activity of the atypical PKC ζ , a kinase with a pivotal role in facilitating GLUT-4 membrane translocation (44, 45). Indeed, DGK-produced PA, which is largely composed of polyunsaturated fatty acid, is a stronger activator for PKC ζ , compared with phospholipase-D-produced PA, which is composed of saturated fatty acids (46). A direct effect on PKC ζ represents a less likely possibility, because glucose effect on its own uptake is blunted in L6 cells expressing non-functional insulin receptors (13), indicating that receptor transactivation due to PKC α down-regulation is required.

In conclusion, our data indicate that glucose activates DGK, followed by reduction in intracellular DAG levels and removal of PKC α from the plasma membrane (Fig. 9). It therefore removes the tonic inhibition exerted by PKC α on insulin receptor signaling and induces the transactivation of the insulin receptor cascade leading to GLUT4 membrane translocation and glucose uptake.

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Glucose Regulates Diacylglycerol Intracellular Levels and Protein Kinase C Activity by Modulating Diacylglycerol Kinase Subcellular Localization

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