

Protein Kinase C δ Mediates Insulin-Induced Glucose Transport in Primary Cultures of Rat Skeletal Muscle

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Insulin activates certain protein kinase C (PKC) isoforms that are involved in insulin-induced glucose transport. In this study, we investigated the possibility that activation of PKC δ by insulin participates in the mediation of insulin effects on glucose transport in skeletal muscle. Studies were performed on primary cultures of rat skeletal myotubes. The role of PKC δ in insulin-induced glucose uptake was evaluated both by selective pharmacological blockade and by overexpression of wild-type and point-mutated inactive PKC δ isoforms in skeletal myotubes. We found that insulin induces tyrosine phosphorylation and translocation of PKC δ to the plasma membrane and increases the activity of this isoform. Insulin-induced effects on translocation and phosphorylation of PKC δ were blocked by a low concentration of rottlerin, whereas the effects of insulin on other PKC isoforms were not. This selective blockade of PKC δ by rottlerin also inhibited insulin-induced translocation of glucose transporter 4 (GLUT4), but not glucose transporter 3 (GLUT3), and significantly reduced the stimulation of glucose uptake by insulin. When overexpressed in skeletal muscle, PKC δ and PKC α were both active. Overexpression of PKC δ induced the translocation of GLUT4 to the plasma membrane and increased basal glucose uptake to levels attained by insulin. Moreover, insulin did not increase glucose uptake further in cells overexpressing PKC δ . Overexpression of PKC α did not affect basal glucose uptake or GLUT4 location. Stimulation of glucose uptake

by insulin in cells overexpressing PKC α was similar to that in untransfected cells. Transfection of skeletal myotubes with dominant negative mutant PKC δ did not alter basal glucose uptake but blocked insulin-induced GLUT4 translocation and glucose transport. These results demonstrate that insulin activates PKC δ and that activated PKC δ is a major signaling molecule in insulin-induced glucose transport. (Molecular Endocrinology 13: 2002–2012, 1999)

INTRODUCTION

The binding of insulin to the α -subunit of the insulin receptor (IR) activates the IR tyrosine kinase, inducing a cascade of events leading to stimulation of glucose uptake into several tissues (1). Glucose uptake is preceded by translocation of the GLUT3 and GLUT4 glucose transporters (GLUTs) from internal membranes to the plasma membrane (2). While several of the key molecules participating in this cascade have been identified, the precise steps between IR activation and GLUT translocation have not been entirely delineated. Activation of the IR leads to phosphorylation of the IR substrate family of proteins. This leads to activation of a number of downstream signaling pathways including mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3K) (1). One component of this cascade is protein kinase C (PKC), which has been shown to be activated by insulin in some systems (3–5). PKC comprises a family of serine-threonine kinases that play an important regulatory role in a variety of biological phenomena (6, 7). The family is composed of a number of individual isoforms that are categorized according to their mechanisms of activa-

tion. It is generally believed that the enzymes, when quiescent, are located in the cytoplasm and that upon activation they are translocated to the plasma membrane (7). The pattern of PKC isoform distribution varies among different tissue (8).

Although PKC isoforms are activated by substances released intracellularly by PI3K activity, the involvement of specific PKC isoforms in insulin-induced glucose uptake has not been definitively established. This is primarily because of the failure of phorbol ester-induced down-regulation of certain PKC isoforms to alter either basal or insulin-stimulated glucose transport (9, 10). However, recent studies implicate certain PKC isoforms, including α , β_2 , λ , and ζ , in the insulin-signaling cascade (11–13). We recently reported that rat skeletal muscle in primary culture expresses six isoforms, α , θ , ϵ , β_2 , ζ , and δ , and that insulin-stimulated glucose uptake involves activation of PKCs β_2 , δ , and ζ (14). Although PKC λ has been shown to participate in insulin signaling in some systems (15), this isoform was barely detectable in skeletal muscle in primary culture. The activation of PKCs β_2 , δ , and ζ was associated with a specific increase in tyrosine phosphorylation and translocation of the three isoforms. The insulin-induced effects on glucose transport, and on PKC β_2 and ζ , were blocked by selective inhibitors of phosphatidylinositol-3-kinase (PI3K), whereas those on PKC δ were not. These findings raise a question as to whether or not PKC δ is likely to be involved in insulin-induced glucose uptake.

Although several roles have been ascribed to PKC δ in a variety of systems (16–20), the possible involvement of this isoform in insulin-induced glucose transport has not been reported. The potential participation of this isoform in insulin signaling is suggested by the *in vitro* findings that coincubation of PKC δ with IR resulted in tyrosine phosphorylation of purified PKC δ , accompanied by an increase in its activity (21). Similarly, it was recently reported that insulin could induce coprecipitation of PKCs α , δ , and ζ with IR in NIH-3T3 cells expressing the human IR (22).

Skeletal muscle is the major target organ for insulin regulation of blood glucose levels. The preparation of primary skeletal muscle cultures obtained from neonatal rat pups is a useful model for the study of regulation of glucose uptake by insulin. These cells, plated initially as individual myoblasts, align and fuse into multinucleated muscle fibers by day 3–4 *in vitro*. The mature fibers display resting membrane and action potentials that are nearly identical to those seen *in vivo*, and the physiological expression of a number of membrane proteins in this preparation, in contrast to muscle cell lines such as L6, resembles closely that observed *in vivo* (23–25). An earlier report from this laboratory suggested that activation of PKC might be a fundamental early signal in stimulation of the Na⁺/K⁺ pump by insulin in cultured skeletal muscle, but the specific isoforms involved were not considered (26).

In this study, we have used the primary muscle culture system to specifically investigate the possibil-

ity that insulin activation of endogenous PKC δ may play a role in insulin stimulation of glucose transport in skeletal muscle. We have found that selective blockade of PKC δ resulted in elimination of insulin-induced stimulation of glucose transport and GLUT4 translocation. In addition, overexpression of PKC δ increased glucose transport and translocated GLUT4. Taken together, these findings indicate that PKC δ is a major isoform mediating insulin-induced effects on glucose transport in skeletal muscle.

RESULTS

Effects of Insulin on Translocation, Phosphorylation, and Activity of PKC α and PKC δ

We first investigated the time course of insulin effects on PKCs α and δ . As shown in Fig. 1, insulin treatment resulted in translocation and tyrosine phosphorylation of PKC δ but not PKC α . Translocation of PKC δ to the plasma membrane was detectable within 10 min after addition of insulin to the cultures, and membrane levels remained elevated for at least 30 min (Fig. 1A). Insulin-induced tyrosine phosphorylation of PKC δ (Fig. 1B) could be detected as early as 1 min after insulin stimulation, remained elevated for 10 min, and decreased to near control levels by 30 min. The insulin-induced translocation and tyrosine phosphorylation of PKC δ was accompanied by a significant increase in its kinase activity, which peaked by 1 min and gradually returned to control levels (Fig. 1C). Insulin had no effect on PKC α activity.

The above findings thus confirm that tyrosine phosphorylation and translocation to the plasma membrane do, indeed, participate in insulin-induced activation of PKC δ in a manner similar to that by which insulin activates PKCs β_2 and ζ (14). To evaluate the possibility that this activation of PKC δ may be important to insulin-induced glucose transport, we studied effects of a selective inhibitor of PKC δ , rottlerin, on insulin-induced activation of PKC δ , glucose uptake, and GLUT4 translocation. Rottlerin prevents the binding of ATP to its binding site, thus preventing PKC δ phosphorylation and activation; it reportedly blocks PKC δ at a concentration of 3–6 μM , one-tenth of that required to block the other PKC isoforms (50–100 μM) in cultured skeletal muscle (27). Figure 2A presents a Western blot showing effects of rottlerin (5 and 100 μM) on insulin-induced translocation of PKCs β_2 , ζ , and δ . As expected, 5 μM rottlerin significantly reduced insulin-induced translocation of PKC δ while translocation of PKC β_2 and ζ was essentially unaltered. At a concentration of 100 μM , the translocation of PKC β_2 and ζ was also significantly reduced, thus demonstrating the selectivity of low concentrations of rottlerin for PKC δ . Figure 2B shows that tyrosine phosphorylation of the three PKC isoforms was affected in a similar dose-effect relation. We next examined the effects of rottlerin on insulin-induced glucose uptake and trans-

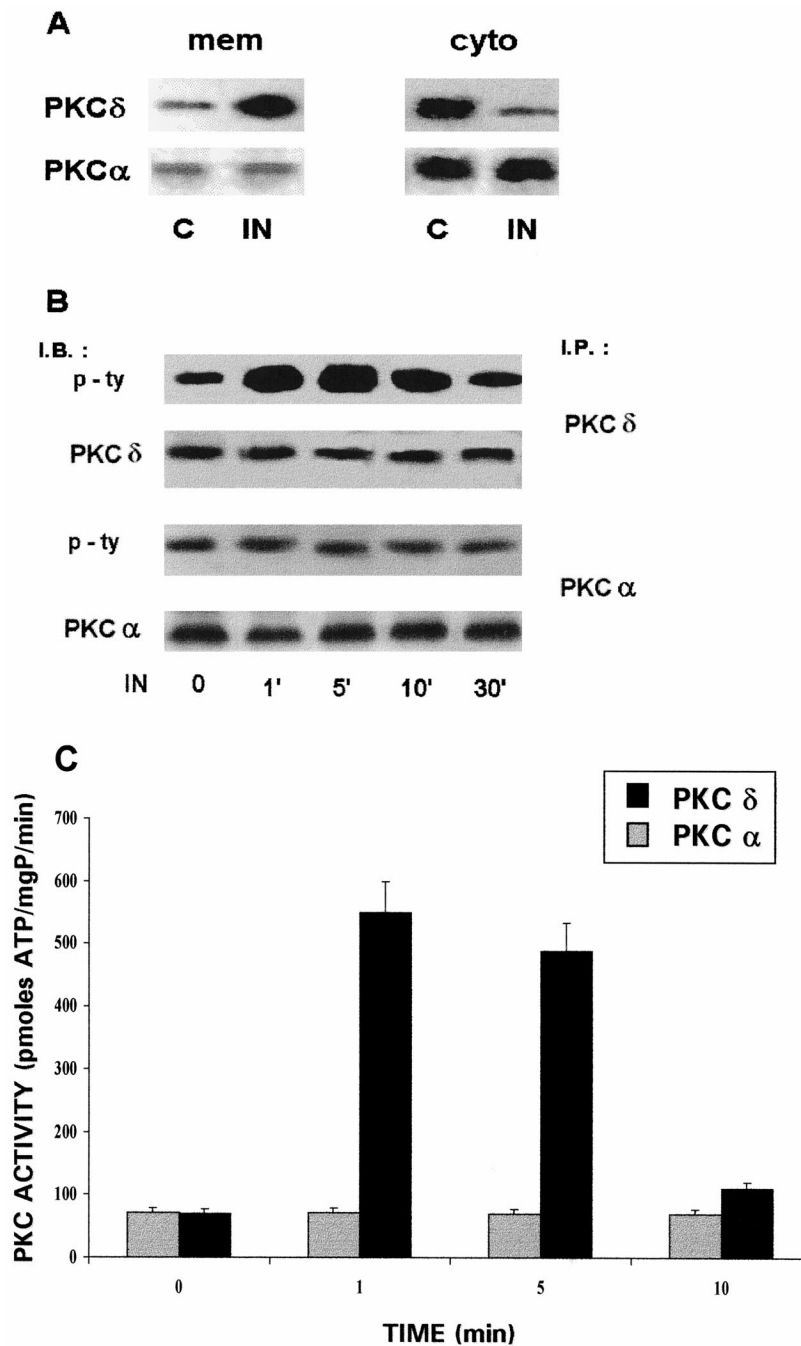


Fig. 1. Effects of Insulin Stimulation on Translocation, Tyrosine Phosphorylation, and Specific Activity of PKC α and PKC δ

A, PKC isoform translocation: myotube cultures were either untreated (C, control) or stimulated with insulin (IN) and were fractionated to membrane (mem) and cytosolic (cyto) fractions, as described in *Materials and Methods*. Equal amounts (20 μ g) of protein were subjected to SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC α or anti-PKC δ antibodies. Insulin stimulation resulted in PKC δ , but not PKC α , translocation from the cytosolic to the plasma membrane fraction. The data presented are representative of three separate experiments. B, Tyrosine phosphorylation of PKC isoforms: protein extracts from untreated cultures (0), or insulin-stimulated cultures treated for different time periods (1, 5, 10, or 30 min) were immunoprecipitated with specific antiphosphotyrosine (p-ty) antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC α , anti-PKC δ , or antiphosphotyrosine (p-ty) antibodies. Tyrosine phosphorylation on PKC δ was induced within 1 min after insulin stimulation, while PKC α was not affected. The data presented are representative of four separate experiments. C, PKC α and PKC δ activity assays: protein extracts from untreated (0) or insulin-stimulated cultures treated for different time periods (1, 5, or 10 min) were immunoprecipitated with specific anti-PKC α or anti-PKC δ antibodies. Immunoprecipitates were analyzed for PKC activity as described in *Materials and Methods*. PKC δ activity was increased within 1 min after insulin stimulation, whereas PKC α activity was not affected by insulin stimulation. Each bar represents the mean \pm SE of three measurements in each of three experiments.

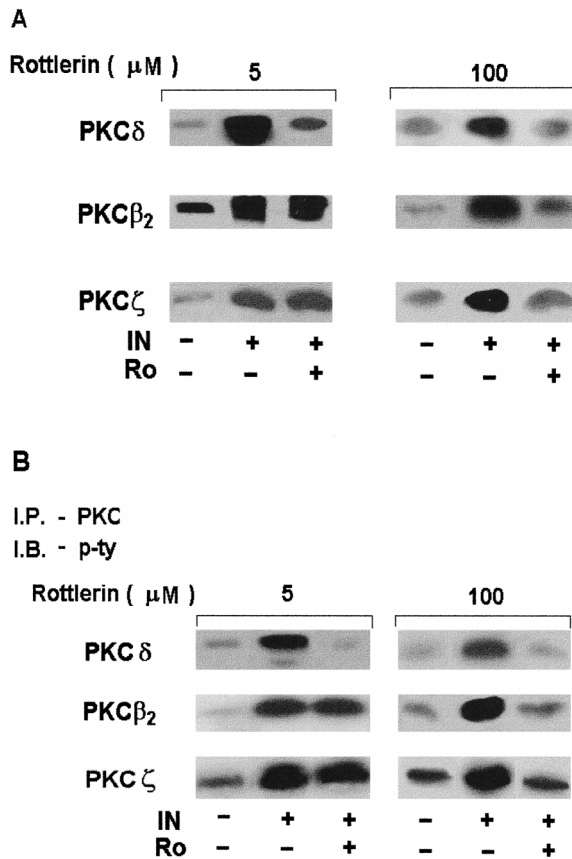


Fig. 2. Effects of Rottlerin on Insulin-Induced PKC Translocation and Tyrosine Phosphorylation

Studies were performed on 6-day-old cultured myotubes, which were transferred to serum-free, low glucose Eagle's medium, 24 h before experiments were conducted. A, PKC translocation to plasma membrane: control cultures or cultures stimulated with insulin, with or without rottlerin pretreatment (5 μM or 100 μM), were fractionated as described in *Materials and Methods*. Equal amounts (20 μg) of membrane proteins were separated on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC δ , anti-PKC β_2 , or anti-PKC ζ antibodies. Results are representative of nine Western blots from three different experiments. Rottlerin (5 μM) blocked PKC δ translocation induced by insulin stimulation. At a concentration of 5 μM , rottlerin did not affect translocation of PKC β_2 or PKC ζ . An increase in rottlerin concentration to 100 μM blocked translocation of all three isoforms. The data presented are representative of three separate experiments. B, Tyrosine phosphorylation of PKC isoforms: control or insulin-stimulated cultures, with or without rottlerin pretreatment (5 μM or 100 μM), were fractionated as described in *Materials and Methods*. The plasma membrane fractions were immunoprecipitated with specific antiphosphotyrosine (p-ty) antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC δ , anti-PKC β_2 , or anti-PKC ζ antibodies. Insulin-induced tyrosine phosphorylation of PKC δ , but not of PKC β_2 or of PKC ζ , was blocked by 5 μM rottlerin. When increased to 100 μM , rottlerin blocked phosphorylation of all three isoforms. The data presented are representative of three separate experiments.

location of GLUTs 3 and 4. The *graph* in Fig. 3A shows that rottlerin, when added to the cultures to a final concentration of 5 μM , selective for inhibition of PKC δ , nearly eliminated the effect of insulin to increase glucose uptake. Finally, as seen in Fig. 3B, this concentration of rottlerin also blocked insulin-induced translocation of GLUT4 but not that of GLUT3.

The results obtained with rottlerin demonstrate that prevention of insulin-induced activation of PKC δ severely impairs the ability of insulin to increase glucose transport and to translocate the GLUT4 glucose transporter. They further indicate that this PKC isoform may play an important role in this phenomenon. However, the pharmacological approach is not specific. Therefore, to test the role of PKC δ more directly, we used an adenovirus expression system to overexpress specific PKC isoforms in skeletal myotubes. As can be seen in Fig. 4A, myotubes overexpressing PKC isoforms α and δ show high protein expression of each isoform compared with endogenous protein levels. Overexpression of each isoform resulted in an increase in activity of that isoform without altering the activity of other isoforms. Thus, overexpression of PKC δ did affect the activity of PKCs α , ζ , and β_2 (data not shown). The overexpression of a point-mutated dominant negative PKC δ resulted in a high level of expression of this protein (Fig. 4C), which, however, was inactive. The basal activity and insulin-induced stimulation of PKCs β_2 and ζ were not altered by the overexpression of dominant negative PKC δ (Fig. 4D).

We next measured glucose uptake in cells overexpressing PKC α and PKC δ . Figure 5C shows that basal glucose uptake in cells overexpressing PKC δ was elevated to levels similar to those attained by addition of insulin to control myotubes. In addition, insulin failed to further increase glucose uptake in cells overexpressing PKC δ . In contrast, overexpression of PKC α in myotubes did not alter either basal or insulin-induced glucose uptake. To validate the contribution of endogenous PKC δ to insulin-induced glucose uptake in myotubes, cells were infected with a dominant negative PKC δ adenovirus construct, which down-regulates endogenous PKC δ activity. Overexpression of this dominant negative PKC δ did not alter basal glucose uptake. However, whereas insulin increased glucose uptake by 250% in control myotubes, it increased glucose uptake by only 50–60% in cells overexpressing dominant negative PKC δ .

As the transport of glucose into cells is accomplished by activation of glucose transporters, we next determined the effects of this overexpression on GLUT4 expression and distribution. In control myotubes, GLUT4 is expressed mainly in the internal membrane fraction, with detectable but lesser amounts in the plasma membrane. Insulin induced translocation of this transporter from the internal membrane fraction to the plasma membrane (Fig. 5A). As further shown in Fig. 5A, concomitant with the increase in glucose uptake, overexpression of PKC δ caused an increase in plasma membrane GLUT4 to levels achieved by insu-

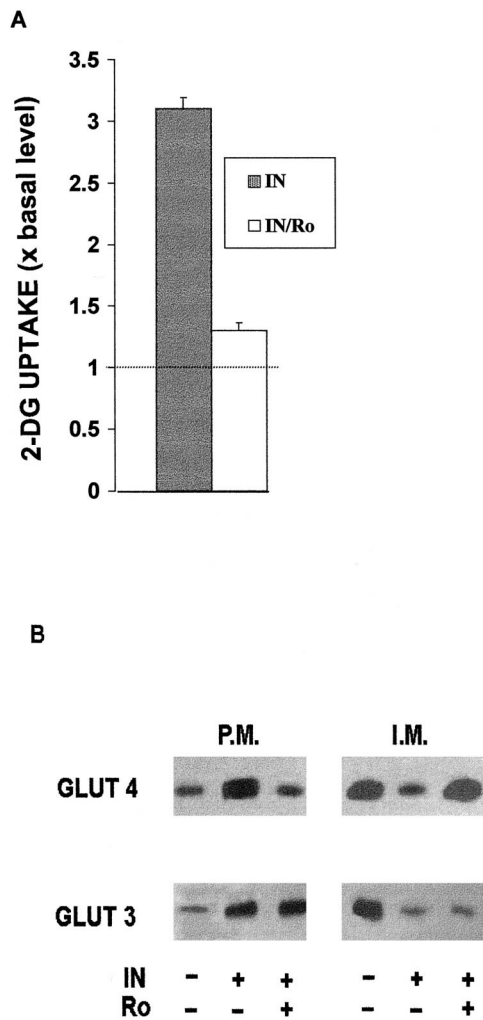


Fig. 3. Rottlerin Effects on Glucose Transport in Skeletal Myotubes

Myotube cultures were transferred to serum-free, low-glucose Eagle's medium, 24 h before experiments were conducted. A, Insulin stimulation of glucose uptake in cells untreated or pretreated with rottlerin: glucose uptake was measured, as described in *Materials and Methods*, in cells stimulated for 30 min by insulin (gray bar) and in cells pretreated for 7 min with rottlerin, followed by 30 min of insulin stimulation (light bar). Data represent the mean \pm SE of triplicate measurements obtained in three different experiments ($n = 9$, $P < 0.005$) and are presented as the fold increase above basal level of glucose uptake in untreated cells. Rottlerin pretreatment resulted in decrease of glucose uptake after insulin stimulation, in comparison to rottlerin untreated but insulin-stimulated cells. B, GLUT3 and GLUT4 distribution in control cells and in cells stimulated with insulin, with or without rottlerin pretreatment: control cultures and cultures stimulated for 30 min by insulin, with or without pretreatment for 7 min with rottlerin, were fractionated into plasma membrane (P.M.) and internal membrane (I.M.), as described in *Materials and Methods*. Equal amounts of protein (20 μ g) were run on SDS-PAGE, transferred to filters, and immunoblotted with anti-GLUT3 or anti-GLUT4 antibodies. GLUT3 and GLUT4 were translocated to the P.M. after 30 min of insulin stimulation. Pretreatment for 7 min with rottlerin blocked GLUT4 but not GLUT3 translocation. The data presented are representative of three separate experiments.

lin in control cells. There was no change in translocation of GLUT 3 by PKC δ overexpression. Moreover, addition of insulin to cells overexpressing PKC δ did not cause a further translocation of additional GLUT4 to the plasma membrane. Indeed, stimulation by insulin of cells overexpressing PKC δ induced a decrease in plasma membrane GLUT4 and an increase in the amount of GLUT4 in the internal membrane fraction. In contrast, overexpression of PKC α neither translocated GLUT4 nor altered the effect of insulin to translocate this transporter to the plasma membrane (Fig. 5A). As shown in Fig. 5B, overexpression of the dominant negative PKC δ completely abrogated insulin-induced translocation of GLUT4. No change in expression of GLUT4 in internal membrane and plasma membrane components could be detected. Overexpression of dominant negative PKC δ altered neither basal distribution nor insulin-induced translocation of GLUT3 (not illustrated).

DISCUSSION

The results of this study show for the first time that insulin activates endogenous PKC δ in primary skeletal muscle cells and that the activated PKC δ is associated with selective translocation of the insulin-sensitive glucose transporter GLUT4 to the plasma membrane and increased glucose uptake. These results were validated by both pharmacological intervention and overexpression of wild-type and dominant negative PKC δ . Pharmacological blockade of PKC δ was accomplished by utilizing rottlerin, which preferentially inhibits PKC δ at low concentrations, presumably by competing with ATP for its binding site (27). At a concentration reportedly selective for PKC δ (5 μ M), we were able to selectively block insulin-induced translocation and tyrosine phosphorylation of this PKC isoform without altering insulin-induced translocation and tyrosine phosphorylation of PKCs β 2 and ζ . We have recently shown that insulin-induced activation of PKCs β 2 and ζ similarly involves tyrosine phosphorylation and translocation, and that this occurs via a PI3K-dependent pathway (14). The kinase responsible for phosphorylation of PKC δ has not yet been identified. It is known, however, that phosphorylation can occur by the Src family of kinases as well as by autophosphorylation (28). Similar inhibition of insulin-induced activation of PKC δ was obtained with overexpression of dominant negative PKC δ . Of special interest is the finding that this selective inhibition of PKC δ was associated with blockade of insulin-induced translocation of the GLUT4 transporter and a significant reduction in insulin-induced glucose uptake. Insulin-induced translocation of GLUT3, on the other hand, was not affected by rottlerin. These results strongly suggest that PKC δ is likely to play a cardinal role in insulin-induced translocation of GLUT4 and accompanying increase in

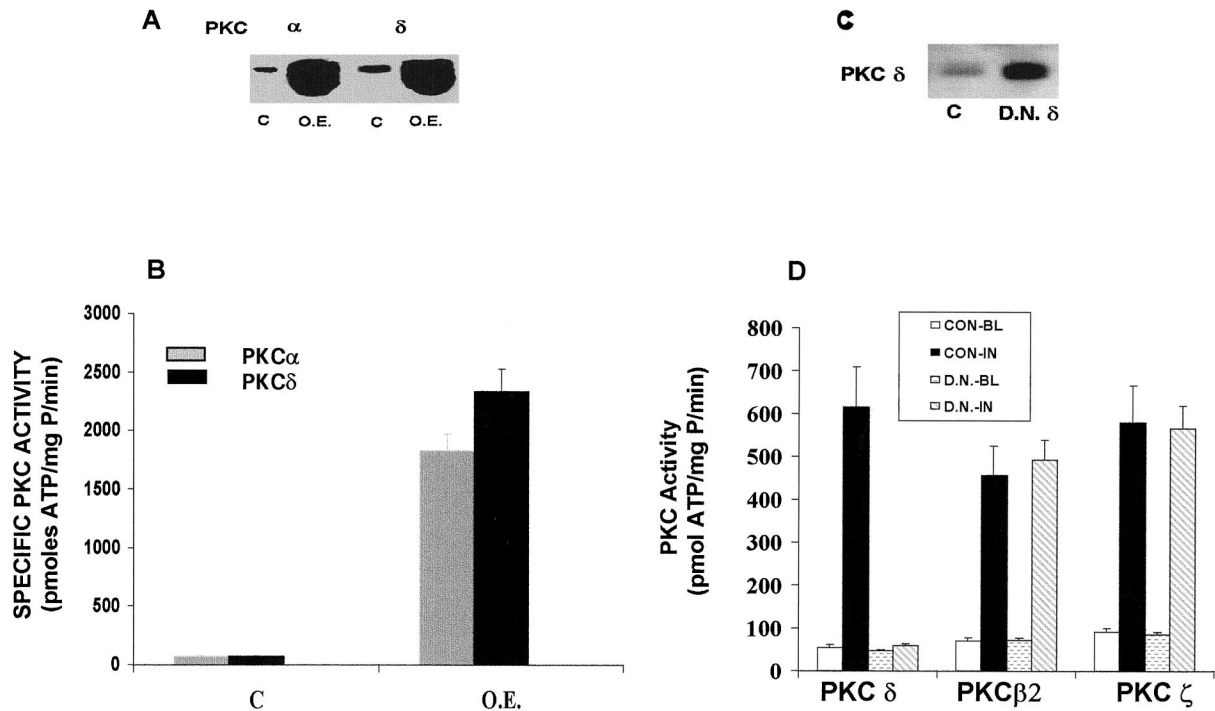


Fig. 4. Overexpression of PKC δ or Its Mutated Form PKC D.N. δ in Cultured Skeletal Myotubes
 Five-day-old myotubes were infected with distinct PKC adenoviruses, as described in *Materials and Methods*. A, Overexpression of PKC isoforms in cultured myotubes: equal amounts of protein extracts (20 μ g) from untreated cultures (C), or cultures overexpressing PKC α or PKC δ isoforms (O.E.), were run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC antibodies. High levels of overexpressed PKC isoforms, in comparison to the endogenous PKC isoforms levels, are clearly seen. The data presented are representative of three separate experiments. B, Activity assays in myotubes overexpressing PKC α and PKC δ isoforms: protein extracts from untreated cultures (C), or cultures overexpressing PKC α or PKC δ isoforms (O.E.), were immunoprecipitated with specific anti-PKC antibodies. Immunoprecipitates were analyzed for PKC activity, as described in *Materials and Methods*. Cultures overexpressing PKC α and PKC δ isoforms displayed significantly higher PKC activity than the untreated cells. The data presented are representative of four separate experiments. C, Overexpression of mutated PKC D.N. δ in cultured myotubes: equal amounts of protein extracts (20 μ g) from untreated cultures (C) or PKC D.N. δ overexpressing cultures (D.N. δ) were run on SDS-PAGE, transferred to filters, and immunoblotted with anti-PKC δ antibodies. The mutant PKC D.N. δ protein could be clearly detected in the infected cells. The data presented are representative of three separate experiments. D, Effects of mutated PKC D.N. δ on basal and insulin-stimulated kinase activity of specific PKC isoforms. PKCs δ , $\beta 2$, and ζ were immunoprecipitated and analyzed for PKC activity as described in *Materials and Methods*. The expression of the dominant negative (D.N.) PKC δ mutant resulted in blockade insulin-induced stimulation of PKC δ but not that of PKC $\beta 2$ or of PKC ζ . No change was detected in basal activity (BL) of any of the PKC isoforms. The results are the mean \pm SE of duplicate values in four experiments. CON-BL, Basal activity of noninfected cells; CON-IN, activity of insulin-stimulated, noninfected cells; D.N.-BL, basal activity of mutant PKC δ -infected cells; D.N.-IN, activity of insulin-stimulated, mutant PKC δ -infected cells.

glucose transport in skeletal muscle. Moreover, these findings, together with those of a recent study from our laboratory (14), indicate that insulin-induced translocation of GLUT3 does not involve PKC δ but may involve the participation of PKCs $\beta 2$ and ζ , which are also activated by insulin.

The notion that PKC δ is involved in mediation of insulin effects (not necessarily exclusively) on glucose transport is strengthened further by the results of experiments on overexpression of this isoform. Thus, overexpression of PKC δ translocated GLUT4 to the plasma membrane, increased basal glucose uptake, and actually induced PKC δ to leave the plasma membrane and increase in the internal membrane fraction. The mechanism of this reverse translocation is not clear and requires further study. One possible expla-

nation may involve mechanisms similar to those that are responsible for glucose-induced down-regulation of plasma membrane GLUT4 in skeletal muscle (29, 30). Clearly, however, overexpression of PKC δ interfered with the ability of insulin to translocate GLUT4 to the plasma membrane and to further increase glucose uptake. The latter finding indicates that PKC δ and insulin share a common pathway. In contrast, overexpression of PKC α was without effect on either GLUT4 translocation, basal glucose uptake, or on insulin-induced translocation of GLUT4 and glucose transport. These findings attest not only to the involvement of PKC δ in mediation of insulin-induced effects on glucose uptake, they also conclusively demonstrate that PKC α is not at all involved in either basal or insulin-stimulated glucose transport in skeletal mus-

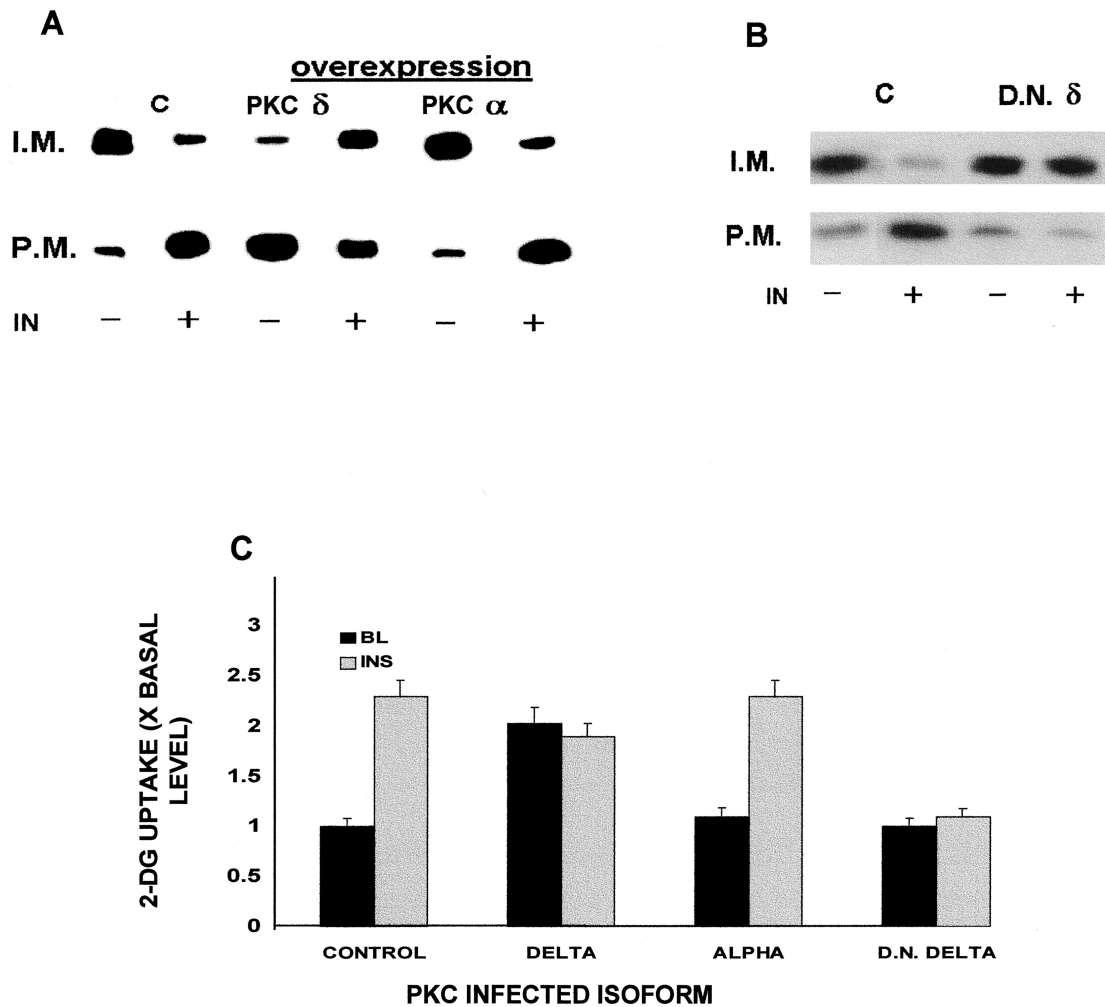


Fig. 5. GLUT4 Distribution and Glucose Uptake in Cultured Myotubes Overexpressing Distinct PKC Isoforms, with or without Insulin Stimulation

A, GLUT4 distribution in untreated or overexpressing PKC α or PKC δ cultures, in the presence or absence of insulin stimulation: control myotube cultures (C) and overexpressing PKC α or PKC δ isoforms (O.E.) were either untreated or stimulated with insulin and were fractionated to plasma membrane (P.M.) and internal membrane (I.M.), as described in *Materials and Methods*. Equal amounts of protein were subjected to SDS-PAGE, transferred to filters, and immunoblotted with anti-GLUT4 antibodies. Overexpression of PKC δ , but not PKC α , resulted in GLUT4 translocation, without insulin treatment. The data presented are representative of three experiments. B, Effect of insulin stimulation on GLUT4 distribution in control or overexpressing PKC D.N. δ cultures: control myotube cultures (C) and myotubes overexpressing PKC D.N. δ isoform (O.E.) were either untreated or stimulated with insulin and were fractionated to plasma membrane (P.M.) and internal membrane (I.M.). Equal amounts of proteins were subjected to SDS-PAGE, transferred to filters, and immunoblotted with anti-GLUT4 antibodies. GLUT4 was not translocated to plasma membrane in myotubes overexpressing PKC D.N. δ , with or without insulin stimulation. The data presented are representative of three separate experiments. C, Glucose uptake in control or PKC overexpressing cultures, with or without insulin stimulation: glucose uptake was measured in cultures of control (C) or in cultures overexpressing PKC α , PKC δ , or PKC D.N. δ , in the absence or presence of insulin stimulation, as described in *Materials and Methods*. In comparison to control or overexpressing PKC α cells, PKC δ overexpression resulted in high levels of glucose uptake in the absence or presence of insulin. Insulin-induced glucose uptake over the basal level was blocked in cells overexpressing PKC D.N. δ . The data presented are representative of three separate experiments.

cle, either directly or indirectly via activation of other signaling proteins.

Our findings are not in accord with the conclusion that diacylglycerol (DAG)-sensitive PKC isoforms are unlikely to participate in insulin-induced glucose transport (10). This conclusion is based on the failure of phorbol ester-induced down-regulation of certain PKC

isoforms to alter either basal or insulin-stimulated glucose transport (9, 10). This down-regulation assumes that PKC activation depends entirely on translocation of a given isoform to the plasma membrane. As recently pointed out, however, there are certain limitations to this notion (7, 21). Many PKC isoforms can be detected in the particulate fraction of cells indepen-

dent of the activation state, and PKC stimulation need not occur exclusively by translocation to the plasma membrane (31, 32). In this regard, translocation to the nucleus, as well as association with cytoskeletal components on activation, is well documented. In addition, products of lipid hydrolysis, such as free *cis*-unsaturated fatty acids, may activate PKC directly in the cytosol (33). Hence, down-regulation of DAG-sensitive isoforms by chronic phorbol ester stimulation would not appear to be a reliable approach for definitive studies implicating or ruling out the participation of these isoforms in insulin signaling. Using specific blockade of PKC δ by pharmacological inhibition and by overexpression of kinase-inactive PKC δ , we have shown that this isoform is important in insulin-induced glucose transport. Moreover, other studies have also shown that DAG-sensitive PKC β 2 is also an important participant in insulin effects on glucose uptake (11, 14).

Our results do not agree with a previous report that PKC δ was not involved in basal or insulin-induced glucose uptake in rat adipocytes (10). One possible explanation for this discrepancy might be related to the difference in tissues studied; adipocytes and skeletal muscle may indeed utilize different PKC isoforms in the signaling of insulin-induced glucose transport. Another possibility for the failure to find a significant role for PKC δ in insulin signaling might be related to the different methods used for overexpression. Using adenovirus constructs, we obtained very high efficiency of protein expression. More than 90% of the cultured cells demonstrated increased protein levels, and cells overexpressing the α and δ PKC isoforms displayed an increase of more than 10-fold in specific PKC activity as compared with wild-type controls. In contrast, a study using electroporation for transfecting the primary adipocyte cultures (10) achieved only an approximately 2-fold increase in PKC ζ activity; activity levels for δ and β 2 were not reported.

The results of our studies on overexpression of PKC δ indicate that this isoform is essential for insulin-induced translocation of GLUT4. Thus, in cells overexpressing the dominant negative PKC δ , not only was insulin unable to increase activity of this enzyme, insulin had no detectable effect on GLUT4 translocation. The stimulation of glucose transport that remains in cells expressing dominant negative PKC δ can be attributed to the effect of insulin to translocate GLUT3. These findings were similar to those we obtained in pharmacological studies utilizing rottlerin; this agent, in concentrations that selectively inhibit insulin effects on PKC δ , blocked translocation of GLUT4 but not of GLUT3.

Whereas this is the first report implicating PKC δ in insulin signaling, PKC δ has been shown to be important in a number of cell functions. Thus, PKC δ has been shown to be involved in stimulation of the Na⁺/K⁺ antiprotein in C₆ glioma cells, in hydrolysis of phosphoinositide and formation of PGE₂, and in keratinocyte and murine erythroleukemia cell differentia-

tion (26, 34–36). PKC δ has also been implicated in Sis-induced transformation of NIH 3T3 cells as well as activation of Na-K-2Cl cotransport (36–38). In addition, PKC δ has been demonstrated to participate in carbachol-induced secretion in parotid acinar cells (40). Finally, changes in activation state of PKC δ are associated with actions of various growth factors and other agents including platelet-derived growth factor, transforming growth factor- α , substance P, ligand of IgE receptor, and extracellular ATP or UTP (41–43).

Our findings of PKC δ involvement in insulin-induced glucose uptake should not be taken as a negation of a role for other PKC isoforms, or other protein kinases such as protein kinase B (44) or MAP kinase (45) in this phenomenon. Indeed, regarding other PKC isoforms, we also reported that insulin stimulation of PKCs β 2 and ζ play an important role in the activation of glucose uptake by insulin. We would suggest that several of the isoforms participate at different steps in the pathway. Ample evidence has been presented in studies on other cell types for the involvement of DAG-sensitive PKC isoforms, especially PKC β 2 (as well as the non-DAG-sensitive PKC ζ) in insulin-induced glucose transport (5, 11–13). In addition, we have recently reported that insulin activates PKCs β 2 and ζ (associated with tyrosine phosphorylation and translocation) in primary cultures of rat skeletal muscle (14). Thus, in primary cultures of skeletal muscle, the DAG-sensitive PKCs β 2 and δ , in addition to ζ , are activated by insulin and appear to be involved in mediation of glucose uptake stimulated by insulin. The determination of the precise steps at which each of these isoforms acts in the insulin-signaling pathway remains to be investigated.

MATERIALS AND METHODS

Materials

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed with a kit purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Antibodies to various proteins were obtained from the following sources: GLUTs 1, 3, and 4 (polyclonal antibodies) were a gift from Dr. S. Cushman, Diabetes Branch, NIDDM, NIH or purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PKC antibodies were purchased from Santa Cruz Biotechnology, Inc. (polyclonal) and Transduction Laboratories (monoclonal; Lexington, KY). Antiphosphotyrosine (mouse monoclonal antirat IgG) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Horseradish peroxidase-antirabbit and antimouse IgG were obtained from Bio-Rad Laboratories, Inc. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), orthovanadate, and pepstatin were purchased from Sigma (St. Louis, MO).

Preparation of Rat Muscle Cell Cultures

Skeletal muscle cultures were prepared from thigh muscles obtained from 1- to 2-day neonatal rats as described previously (23–25). The muscles were removed from the limbs,

washed in PBS to remove excess blood cells, and then transferred to a Ca^{2+} -free, 0.25% trypsin solution containing EDTA (1 mM) for incubation with continuous stirring at 37 C. Cells were collected after serial trypsinization (successive 10-min periods until all tissue was dispersed), centrifuged for 5 min at $500 \times g$, and resuspended in growth medium (83% DMEM-high glucose, 15% horse serum, 2% chick embryo extract), to a concentration of 0.8×10^6 cells/ml for plating in collagen-coated 10-cm plastic tissue culture (10 ml/dish) or 24-well plates (400 μl /well). Cultures were grown in water-saturated atmosphere of 95% air-5% CO_2 at 37 C. On day 5 in culture, myotubes were transferred to low glucose (4.5 mM), serum-free DMEM containing 1% BSA for 24 h before study.

Preparation of Cell Lysates for Immunoprecipitation

Culture dishes (90 mm; Nunc, Roskilde, Denmark) containing the muscle cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and then mechanically detached in RIPA buffer (Tris HCl, pH 7.4, 50 mM; NaCl, 150 mM; EDTA, 1 mM; NaF, 10 mM; Triton X-100, 1%; SDS, 0.1%; Na deoxycholate, 1%) containing a cocktail of protease inhibitors (leupeptin, 20 $\mu\text{g}/\text{ml}$; aprotinin, 10 $\mu\text{g}/\text{ml}$; PMSF, 0.1 mM; DTT, 1 mM) and phosphatase inhibitors (orthovanadate, 200 μM ; pepstatin, 2 $\mu\text{g}/\text{ml}$). After scraping, the preparation was centrifuged at $20,000 \times g$ for 20 min at 4 C. The supernatant was used for immunoprecipitation.

Immunoprecipitation

To 0.3 ml of cell lysate, 25 μl of Protein A/G Sepharose were added and the suspension was rotated continuously for 30 min at 4 C. The preparation was then centrifuged at $20,000 \times g$ at 4 C for 10 min, and 30 μl of A/G Sepharose were added to the supernatant along with specific monoclonal antibodies to the individual PKC isoforms (dilution 1:100). This was rotated overnight at 4 C. The suspension was then centrifuged at $20,000 \times g$ for 10 min at 4 C, and the pellet was washed twice as above with RIPA buffer. The beads were eluted with 25 μl of sample buffer (0.5 M Tris HCl, pH 6.8; 10% SDS; 10% glycerol; 4% 2- β -mercaptoethanol; 0.05% bromophenol blue). The suspension was again centrifuged at $15,000 \times g$ (4 C for 10 min) and washed four times in TBST. Sample buffer was added and the samples were boiled for 5 min and then subjected to SDS-PAGE.

Cell Fractionation

Crude membrane preparations were isolated from muscle cell cultures according to a modification of the method described by Klip and Ramlal (9). Culture dishes (90 mm; Nunc) containing the muscle cells were washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and then mechanically detached in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS containing 2 mM EDTA with a rubber policeman. The cells were pelleted by centrifugation at $500 \times g$ for 10 min at 4 C. The pelleted cells were resuspended in sonication buffer (Tris HCl, pH 7.4, 50 mM; NaCl, 150 mM; EDTA, 2 mM; EGTA, 1 mM; sucrose, 250 mM) containing leupeptin, 20 $\mu\text{g}/\text{ml}$; aprotinin, 10 $\mu\text{g}/\text{ml}$; PMSF, 0.1 mM; DTT, 1 mM; orthovanadate, 200 μM ; and pepstatin, 2 $\mu\text{g}/\text{ml}$. The suspension was homogenized in a Dounce glass homogenizer (30 strokes) and centrifuged at $1100 \times g$ for 5 min. The supernatant was centrifuged at $31,000 \times g$ for 60 min. The supernatant from this centrifugation was centrifuged at $190,000 \times g$ for 60 min to collect the light microsome fraction. The $31,000 \times g$ pellet was resuspended in homogenization buffer to a final volume of 500 μl and placed on a discontinuous sucrose gradient of 500 μl each of 32% (wt/wt), 40% (wt/wt), and 50% (wt/wt) sucrose solution in 5 mM Tris, pH 7.5. This gradient was centrifuged at $210,000 \times g$ for 50 min. The plasma mem-

branes banded above the 32% layer, and the 32/40% and 40/50% interfaces were collected by puncture with a syringe. These fractions were diluted in homogenization buffer containing 1% Triton X-100, freeze-thawed four times, and centrifuged at $30,000 \times g$ for 30 min, and the supernatant was designated the membrane protein. All membrane fractions were stored at -70 C until use.

Western Blot Analysis

Protein (20–25 μg) was electrophoresed through SDS-polyacrylamide gels (7.5 or 10%) and electrophoretically transferred onto Immobilon-P (Millipore Corp., Bedford, MA) membranes. After transfer, the membranes were subjected to standard blocking and incubation procedures and were incubated with monoclonal antibodies to specific PKC isoforms and phosphotyrosine, and polyclonal antibodies to glucose transporters. The membranes were washed four times for 15 min in Tris-buffered saline-Tween 20 [TBST] and then further incubated for 20 min at room temperature with horseradish peroxidase-labeled secondary antibody (goat antirabbit or mouse IgG) diluted 1:10,000 in blocking buffer. After three washes (1 \times 15 min and 2 \times 5 min) in TBST, the membranes were treated with ECL reagent for 1 min, and then exposed on (Eastman Kodak Co., Rochester, NY) x-ray film for the required times (5–30 sec) and developed.

Adenovirus Constructs

The recombinant adenoviruses were constructed in three steps. Initially the coding sequence for PKC α and δ in the form of cDNA was inserted into the cassette cosmid. The cassette cosmid for constructing recombinant Ad of the E1-substitution type, pAdex1, was an 11-kb charomid vector bearing an Ad5 genome spanning 0–99.3 map units (μm) with deletions of E1 (μm 1.3–9.3) and E3 (μm 79.6–84.8), in which a unique Swal site was created by linker insertion at the E1 deletion. The expression unit was excised with the appropriate restriction enzymes, blunt ended with Klenow fragment of DNA polymerase I, and purified by gel electrophoresis. Thereafter the fragment was ligated with Swal-linearized pAxCawt (46).

After overnight ligation, the DNA sample was digested with Swal, to exclude empty re-ligated cosmids lacking a coding sequence, and an aliquot was packaged *in vitro* using Gigapack (Stratagene, La Jolla, CA). Colonies were obtained after plating the transduced *Escherichia coli* DH5 α , and the majority of the clones contained the desired insert. Ad5-dIX, which has an E3 deletion (μm 79.6–84.8) was used as the parent virus for recombinant Ad construction. The DNA-terminal protein complex (DNA-TPC) of the parent Ad was prepared and purified utilizing CsCl density gradient with guanidine hydrochloride. The DNA-TPC was digested with EcoT22I and was gel filtered through a Sephadex G-50 spin column. The EcoT22I-digested adenovirus DNA-TPC was mixed with the cassette cosmid bearing the desired expression unit, and human embryonic kidney 293 cells were transfected with the mixed DNA by the calcium phosphate method using CellPfect Transfection kit (Pharmacia Biotech). One day later, the cells were dispensed in 96-well plates in 10-fold serial dilutions and mixed with untransfected 293 cells. After being maintained in culture for 10–15 days, virus-containing supernatants were isolated and propagated further to assess restriction analysis and expression of inserted genes.

Mutated PKC δ Adenovirus Construct

The dominant negative mutant of mouse PKC δ was generated by substitution of the lysine residue at the ATP binding site with alanine (47). The mutant δ cDNA was cut from SRD expression vector with EcoRI and ligated into the pAxCawt

cosmid cassette to construct Ax vector. Its kinase-negative nature was demonstrated by abrogation of autophosphorylation activity (48).

PKC Isoform Viral Infection

After differentiation of cultured rat muscle into myotubes, the culture medium was aspirated and cultures were infected with the viral medium containing PKC α or δ recombinant adenoviruses for 1 h. The cultures were then washed twice with DMEM and refed. Cells 10 h postinfection were transferred to serum-free DMEM containing 4.5 mM for 24 h. Control and insulin-treated cultures were used for glucose uptake experiments, or extracted and fractionated into cytosolic and membrane fractions. The fractions were electrophoretically separated and blotted with appropriate antibodies.

PKC Activity

Specific PKC activity was determined in freshly prepared immunoprecipitates from mature muscle cultures after appropriate treatments. These lysates were prepared in RIPA buffer without NaF. Activity was measured with the use of the SignaTECT Protein Kinase C Assay System (Promega Corp., Madison, WI) according to the manufacturer's instruction. PKC biotinylated pseudosubstrate was used as the substrate in these studies.

Glucose Uptake

The total and nonspecific rates of glucose transport were measured in triplicate samples in 24-well plates with the use of [3 H]2-DG (13). After appropriate treatment, cells were washed three times with 0.5 ml PBS, the final wash being replaced immediately with 0.5 ml PBS containing 1 μ Ci/ml of [3 H]2-DG in glucose at a concentration of 2 mM. Cells were then incubated for 15 min at 37 C, after which time they were washed four times with 0.5 ml cold (4–6 C) PBS and then lysed by addition of 300 μ l Triton X-100 (1%) and incubation for 30 min. The contents of each well were transferred to counting vials and 3.5 ml scintillation fluid were added to each vial. Samples were counted in the 3 H window of a Tricarb scintillation counter. Nonspecific uptake was determined in the presence of excess (100 mM) D-glucose. Net specific uptake was then calculated as the difference between the total and nonspecific values. Baseline glucose uptake values under control conditions ranged from 14–20 nmol/min/mg protein.

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