

Insulin Induces Specific Interaction between Insulin Receptor and Protein Kinase C δ in Primary Cultured Skeletal Muscle

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Certain protein kinase C (PKC) isoforms, in particular PKCs β II, δ , and ζ , are activated by insulin stimulation. In primary cultures of skeletal muscle, PKCs β II and ζ , but not PKC δ , are activated via a phosphatidylinositol 3-kinase (PI3K)-dependent pathway. The purpose of this study was to investigate the possibility that PKC δ may be activated upstream of PI3K by direct interaction with insulin receptor (IR). Experiments were done on primary cultures of newborn rat skeletal muscle, age 5–6 days *in vitro*. The time course of insulin-induced activation of PKC δ closely paralleled that of IR. Insulin stimulation caused a selective coprecipitation of PKC δ with IR, and these IR immunoprecipitates from insulin-stimulated cells displayed a striking induction of PKC activity due specifically to PKC δ . To examine the involvement of PKC δ in the IR signaling cascade, we used recombinant adenovirus constructs of wild-type (W.T.) or dominant negative (D.N.) PKC δ . Overexpression of W.T.PKC δ induced PKC δ activity and coassociation of PKC δ and IR without addition of insulin. Overexpression of D.N.PKC δ abrogated insulin-induced coassociation of PKC δ and IR. Insulin-induced tyrosine phosphorylation of IR was greatly attenuated in cells overexpressing W.T.PKC δ , whereas in myotubes overexpressing D.N.PKC δ , tyrosine phosphorylation occurred without addition of insulin and was sustained longer than that in control myotubes. In control myotubes IR displayed a low level of serine phosphorylation, which was increased by insulin stimulation. In cells overexpressing W.T.PKC δ , serine phosphorylation was

strikingly high under basal conditions and did not increase after insulin stimulation. In contrast, in cells overexpressing D.N.PKC δ , the level of serine phosphorylation was lower than that in nonoverexpressing cells and did not change notably after addition of insulin. Overexpression of W.T.PKC δ caused IR to localize mainly in the internal membrane fractions, and blockade of PKC δ abrogated insulin-induced IR internalization. We conclude that PKC δ is involved in regulation of IR activity and routing, and this regulation may be important in subsequent steps in the IR signaling cascade. (*Molecular Endocrinology* 15: 565–574, 2001)

INTRODUCTION

In the cascade of events leading to the multiple effects of insulin, this hormone activates its receptor tyrosine kinase by autophosphorylation of the β -subunits. This leads to stimulation of a number of downstream signaling factors, among which are several insulin receptor substrates (IRS1, IRS2, IRS3), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein (MAP) kinase and other protein kinases (1). Whereas activation of the insulin receptor (IR) depends on tyrosine phosphorylation, recent studies have shown that IR also undergoes serine phosphorylation. The mechanisms and importance of these serine phosphorylations are presently unknown.

We and others have reported that among the proteins activated by insulin stimulation of skeletal muscle and fat cells are certain members of the protein kinase C (PKC) family of serine-threonine kinases (2–8), in particular PKCs β II, δ , and ζ . PKCs β II and ζ are activated via products of PI3K activity (7). While the

pathway for insulin stimulation of PKC δ in skeletal muscle is not known, it is clearly independent of PI3K activity (6). These PKC isoforms, being activated by insulin, are possible candidates for phosphorylating the receptor on serine residues and affecting its routing. In fact, several studies have been directed toward investigating this possibility, but the findings are inconclusive (9, 10).

We have reported that insulin stimulation of PKC δ is associated with phosphorylation on tyrosine residue (7). It is known that tyrosine phosphorylation of PKC δ occurs only in the presence of an activator and appears to be restricted to the activated form of the enzyme (11). Thus, tyrosine phosphorylation sites may be exposed only upon an activator-induced conformational change of PKC δ . This phosphorylation modifies the activity of PKC δ toward certain substrates. Among the proteins that have been shown to tyrosine phosphorylate PKC δ *in vitro* is IR tyrosine kinase (12).

In an earlier study on insulin action in primary cultures of rat skeletal muscle (7), we reported that insulin-induced activation and tyrosine phosphorylation of PKC δ were readily detectable within 1 min. This, together with the lack of PI3K involvement in insulin-induced PKC δ activation, suggested to us that tyrosine phosphorylation of PKC δ occurs upstream in the insulin signaling pathway, perhaps at the level of IR itself. Accordingly, we have investigated the relation between PKC δ and IR in primary cultures of rat skeletal muscle. 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 and other membrane properties that are nearly identical to those seen *in vivo* (13–16). The results show that insulin specifically induces PKC δ to associate with IR and that this IR-PKC δ association plays an important role in early IR signaling. Our results further indicate that PKC δ induces serine phosphorylation of IR and may be involved in the routing of the receptor.

RESULTS

We have recently shown that in cultured skeletal muscle, insulin induces activation of PKC δ as well as PKCs ζ and β II, and that activation and tyrosine phosphorylation of the latter two isoforms occur via a wortmannin-sensitive pathway (7). The pathway for activation of PKC δ is not yet known. We found, however, that activation as well as tyrosine phosphorylation of PKC δ by insulin occurred sooner than PKCs β II and ζ , *i.e.* 1 min as compared with 10 min, respectively, after insulin stimulation. These results suggested that insulin-induced activation and tyrosine phosphorylation of PKC δ could be occurring upstream and independent of PI3 kinase. Therefore, we initially attempted to clarify the pathway by which resident PKC δ could be related to the initial steps of the insulin-induced signaling pathway in intact muscle cells.

Insulin Induces Association between IR and PKC δ

One possible mechanism for insulin-induced activation and tyrosine phosphorylation of PKC δ , suggested both by the rapidity of the effect and the close association with IR tyrosine phosphorylation, might involve a direct physical linkage of this isoform with IR (12). Several studies on cells transfected either with IR or PKC δ have shown that the two proteins can associate *in vitro* (12, 17). To examine this, we immunoprecipitated IR with anti-IR β antibody from control and insulin-stimulated muscle cultures and probed the blots with specific antibodies to PKC isoforms. Before treatment with insulin, no PKC isoform could be detected in IR immunoprecipitates. By 1 min following insulin stimulation, PKC δ was found to coprecipitate with IR; the amount of IR-PKC δ complex decreased by 10 min, and by 30 min PKC δ could no longer be detected in association with IR (Fig. 1A). This rapid induction of insulin-induced IR-PKC δ coprecipitation was essentially parallel to insulin-induced PKC δ tyrosine phosphorylation and activation (6). Moreover, neither PKC ζ nor PKC β II, which were activated and tyrosine phosphorylated by insulin, nor PKC α , which was not activated or tyrosine phosphorylated by insulin, was found to coprecipitate with IR. Similar results were obtained in studies in which PKCs β II, δ , and ζ were immunoprecipitated and the blots were probed with anti-IR antibody (not shown). Thus, insulin induced IR to selectively associate with PKC δ .

To further demonstrate the specificity of insulin induction of IR-PKC δ association, we treated cells with insulin-like growth factor I (IGF-I), which also activates IR (18–21). Figure 1B shows that IGF-I induces tyrosine phosphorylation of IR in this preparation of cultured myotubes. In spite of this stimulation of IR, IGF-I did not induce PKC δ to associate with IR (Fig. 1C). Figure 1D shows that IGF-I induced coimmunoprecipitation of PKC δ with IGFR within 10 min. Insulin also induced tyrosine phosphorylation of IGFR but did not induce association between IGFR and PKC δ . Finally, insulin did not induce PKC δ to associate with other receptor kinases such as epidermal growth factor receptor (EGFR) or nerve growth factor receptor (NGFR) (data not shown).

Insulin Induces PKC Activity in IR Immunoprecipitates

We next examined whether the PKC δ that is physically associated with IR after insulin stimulation is indeed active. This can be determined by measuring PKC activity in IR immunoprecipitates from nonstimulated and insulin-stimulated cells. Accordingly, we immunoprecipitated IR from untreated and insulin-treated cultures and performed a PKC activity assay on those immunoprecipitates. Figure 2 shows that after 1 min of insulin treatment, IR immunoprecipitates displayed a striking level of PKC activity that was sustained for at

least 5 min. In contrast, IR immunoprecipitates from untreated cultures had no detectable PKC activity. To determine that the increased PKC activity of insulin-stimulated IR was exclusively associated with PKC δ induced by insulin stimulation, we immunoprecipitated different PKC isoforms from nonstimulated and insulin-stimulated cell lysates and then immunoprecipitated IR from the remaining supernatant. We subjected these IR immunoprecipitates to PKC activity assay. The results of these experiments are also shown in Fig. 2. Removal of PKC δ resulted in a loss of PKC activity in IR immunoprecipitates. In contrast, removal of PKC ζ (or of PKC α or of PKC β II—not shown) did not reduce PKC activity of the IR immunoprecipitates. This further confirms that the PKC activity detected in IR immunoprecipitates was specifically due to PKC δ .

Overexpressed W.T.PKC δ Associates with IR

The insulin-induced association of PKC δ and IR raises the possibility that PKC δ may be involved in some aspects of the IR signaling cascade. To examine this possibility, we have used recombinant adenovirus constructs of PKC δ to overexpress either W.T.PKC δ or a kinase-inactive PKC δ (D.N.PKC δ) in mature myotubes, and then to investigate the effects of PKC δ overexpression and blockade on the initial steps in the insulin-induced signaling cascade. Cells infected with W.T.PKC δ or with D.N.PKC δ expressed higher protein levels of these isoforms, and overexpressed W.T.PKC δ was found in an activated state (6). We initially examined the effects of overexpression of W.T.PKC δ on insulin-induced association of PKC δ and IR. Interestingly, overexpression of W.T.PKC δ resulted in an association of PKC δ and IR without addition of insulin (Fig. 3). Treatment of the cultures with insulin could not further increase this association. Indeed, insulin stimulation appeared to decrease IR-PKC δ association. In contrast, the inactive mutant form of PKC δ did not associate with IR. Moreover, overexpression of D.N.PKC δ prevented insulin-induced association of IR with the native PKC δ . We have confirmed our earlier report (6) that expression of D.N.PKC δ prevents insulin-induced activation of PKC δ . These findings thus suggest that activation of PKC δ is necessary and sufficient for the interaction between the IR and PKC δ .

PKC δ Regulates Serine Phosphorylation of IR

The findings so far demonstrate not only that PKC δ is induced by insulin to associate with IR but also that the association between the two proteins may regulate the state of IR phosphorylation itself. The regulation of IR tyrosine phosphorylation and activation is also reported to be associated with changes in phosphorylation on serine residues of the IR (see Ref. 22). Indeed, serine phosphorylation of IR is considered one of the possible mechanisms for regulation of IR function (9).

In view of our results, one possible mechanism for regulation of IR activation and distribution associated with serine phosphorylation of IR could be via activation of PKC δ . Therefore, we investigated effects of overexpression of W.T.PKC δ and D.N.PKC δ on serine phosphorylation of IR and effects of insulin stimulation thereon. Figure 4 shows serine phosphorylation of IR in cultured myotubes under different conditions. In control myotubes, IR displayed a low level of serine phosphorylation, and on stimulation by insulin, serine phosphorylation began to increase by 5 min and reached maximum by 10 min, similar to findings reported for other cell types (10, 23). In cells overexpressing W.T.PKC δ , serine phosphorylation was strikingly high under basal conditions (without insulin stimulation) and was decreased by insulin stimulation (Fig. 4, *lower panel*). In contrast, in cells overexpressing D.N.PKC δ , the level of serine phosphorylation was lower than that in control, and insulin-induced serine phosphorylation was completely abrogated. These findings suggest that PKC δ may be involved in insulin-induced serine phosphorylation of IR.

PKC δ Alters Insulin-Induced Changes in IR Distribution

Serine phosphorylation of IR is associated with changes in IR receptor distribution between the plasma and internal membrane components. To determine whether PKC δ may also play a role in IR distribution, we followed the expression of IR in different cellular compartments after insulin treatment of control skeletal myotubes. This was done indirectly by probing plasma and internal membrane fractions with anti-IR antibody at different times after insulin stimulation. Before insulin stimulation, IR was detected almost exclusively in the plasma membrane, where it remained for at least 5 min after addition of insulin (Fig. 5A). By some 10 min after stimulation, IR had largely left the plasma membrane and could be detected in increased amounts in the internal membrane fraction. Interestingly, in myotubes overexpressing W.T.-PKC δ (Fig. 5B), only a small amount of IR was detected in the plasma membrane before insulin stimulation; most of the IR was found in the internal membrane fractions. Thus, the activated overexpressed PKC δ , which associates with IR, appeared to transfer IR out of the plasma membrane. Moreover, insulin had no effect on amount of IR in the plasma membrane and appeared to decrease the amount of IR in the internal membrane fraction. In contrast, in cells overexpressing D.N.PKC δ , IR was enriched in the plasma membrane before addition of insulin and remained in the plasma membrane for at least 30 min after insulin stimulation. At the same time, under all experimental conditions, there was no significant change in total IR expression as measured by Western blot analysis of total cell lysates. In other words, W.T.PKC δ appeared to induce redistribution of IR, and D.N.PKC δ prevented insulin-induced changes in IR distribution between plasma and internal membrane fractions. This

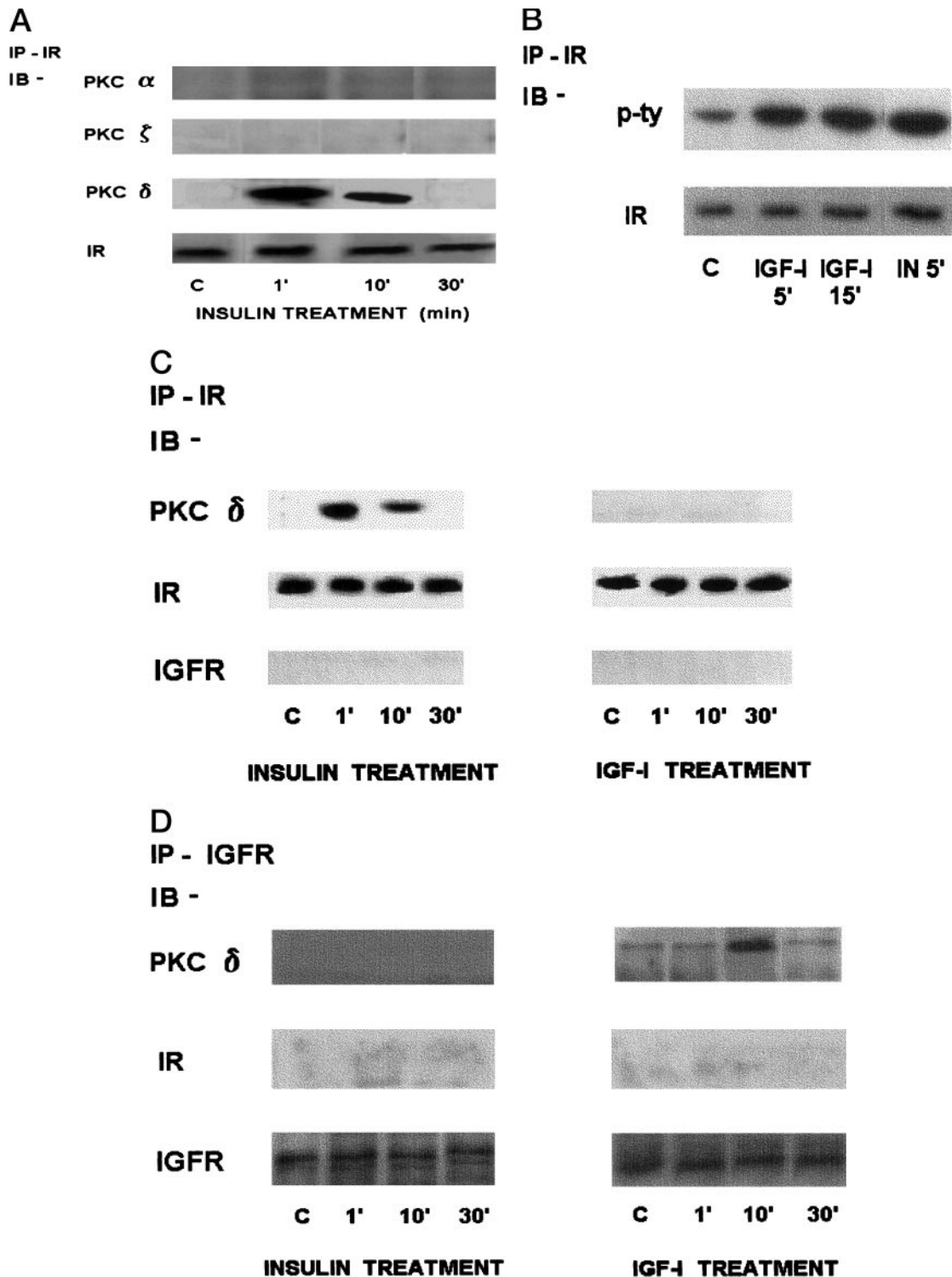


Fig. 1. Insulin Induces Selective Association of PKC δ with IR

A, Western blot of insulin-induced coprecipitation of IR with PKC δ . Studies were performed on 6-day-old cultured myotubes, which were transferred to serum-free, low glucose minimal essential medium (EM), 24 h before experiments were conducted. Protein extracts from untreated cultures (C), or insulin-stimulated cultures treated for different time periods (1, 10, or 30 min) were immunoprecipitated with specific anti-IR antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters and immunoblotted with specific anti-PKC α , anti-PKC δ , or anti-PKC ζ antibodies. Coprecipitation of PKC δ with IR was induced within 1 min following insulin stimulation, while PKC α and PKC ζ did not coprecipitate with IR. The data presented are representative of four separate experiments. B, Western blot of tyrosine phosphorylation of IR induced by IGF-I or insulin (IN). Studies were done on cells as described in panel A. Protein extracts from untreated cultures (C), or cultures stimulated with IGF-I for 5 or 15 min,

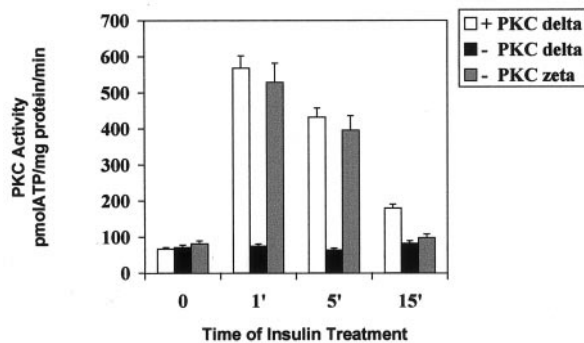


Fig. 2. Effects of Insulin on PKC Activity in IR Immunoprecipitates

Studies were performed on 6-day-old cultured myotubes, which were transferred to serum-free, low glucose EM 24 h before experiments were conducted. Cells were either untreated (time 0) or stimulated with insulin for different time periods (1, 5, or 15 min). After stimulation, lysates from unstimulated and stimulated cells were then either immunoprecipitated directly with anti-IR antibody (*open bars*), or with anti-PKC δ (*black bars*), or anti-PKC ζ (*gray bars*) antibodies followed by anti-IR antibody. IR immunoprecipitates were analyzed for PKC activity as described in *Materials and Methods*. PKC activity was increased within 1 min after insulin stimulation. Immunoprecipitation of PKC δ before IR immunoprecipitation abrogated PKC activity of IR, whereas immunoprecipitation of PKC ζ did not. Each *bar* represents the mean \pm SE of three measurements in each of three experiments.

indicates that insulin-activated PKC δ is required for insulin-induced changes in IR distribution.

We also examined effects of short-term blockade of PKC δ on insulin-induced IR distribution with the use of Rottlerin, which in low concentrations is a selective inhibitor of PKC δ . We have shown that this substance at a concentration of 5 μ M inhibits insulin-induced activation of PKC δ but not that of PKC β II or ζ (6). In the current study, cells were pretreated with 5 μ M Rottlerin for 10 min before addition of insulin. Effects of selective inhibition of PKC δ by Rottlerin were similar to those of overexpression of dominant negative PKC δ (Fig. 5C). In other words, in Rottlerin-treated cells, IR did not move to the intracellular compartment after insulin stimulation. Instead, IR remained in the plasma membrane for at least 30 min after addition of insulin.

or with insulin for 5 min, were immunoprecipitated with specific anti-IR antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific antiphosphotyrosine (p-ty) or anti-IR antibodies. The data presented are representative of three separate experiments. C, Western blot of insulin-induced coprecipitation of IR with PKC δ . Protein extracts from untreated cells (C) or cells stimulated with insulin or IGF-I for different time periods (1, 10, or 30 min) were immunoprecipitated with specific anti-IR antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC δ , anti-IR, or anti-IGFR antibodies. Insulin but not IGF-I induced coprecipitation of IR with PKC δ . D, Western blot of IGF-I-induced coprecipitation of IGFR with PKC δ . Protein extracts from untreated cells (C) or cells stimulated with insulin or IGF-I for different time periods (1, 10, or 30 min) were immunoprecipitated with specific anti-IR antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC δ , anti-IR, or anti-IGFR antibodies. IGF-I but not insulin induced coprecipitation of IGFR with PKC δ by 10 min after stimulation.

PKC δ Regulates the Tyrosine Phosphorylation State of IR

Activation of IR is characterized by tyrosine autophosphorylation. As we have shown that PKC δ is involved in serine phosphorylation and distribution of IR, we considered it important to examine effects of PKC δ overexpression and blockade on insulin-induced tyrosine phosphorylation of IR. To accomplish this, we analyzed insulin-induced changes in tyrosine phosphorylation of IR in plasma membrane and internal membrane fractions prepared from noninfected cells and cells overexpressing either W.T. or D.N.PKC δ . The time course of insulin-induced tyrosine phosphorylation of IR in these fractions from noninfected cells is shown in the *upper blot* in Fig. 6A. In confirmation of our earlier findings, tyrosine phosphorylation of IR in control myotubes reached a maximum 1 min after addition of insulin and returned to near basal levels by 10 min (6). In cells overexpressing W.T.PKC δ (Fig. 6B), the basal level of tyrosine phosphorylation of IR in the plasma membrane was unchanged from control, uninfected cells. However, tyrosine phosphorylated IR was now detected in immunoprecipitates from the internal membrane fraction, even in the absence of insulin stimulation. Insulin had no additional effect on the level of phosphorylation. In myotubes expressing the D.N.PKC δ mutant (Fig. 6B), basal tyrosine phosphorylation of IR in the plasma membrane fraction was higher than that in control, noninfected cells. On stimulation by insulin, IR tyrosine phosphorylation in D.N.PKC δ -expressing cells was higher than that in control uninfected cells treated with insulin. In addition, the high level of tyrosine phosphorylation was sustained for at least 30 min. Moreover, no tyrosine-phosphorylated IR could be detected in IR immunoprecipitates from internal membrane fractions of D.N.PKC δ expressing cells with or without insulin stimulation.

DISCUSSION

In this report we have shown for the first time that insulin induces an immediate physical linkage between IR and PKC δ , and that this occurs with the same time course as tyrosine phosphorylation of both IR and

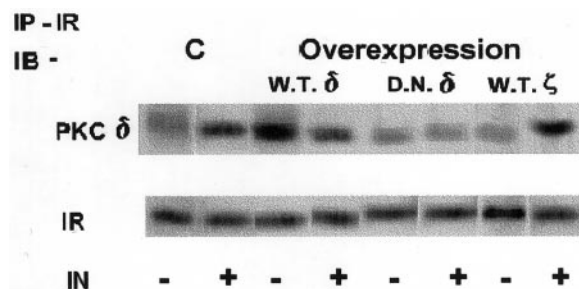


Fig. 3. Western Blot of Coprecipitation of IR with PKC δ in Cells Overexpressing W. T. PKC δ (W.T. δ) or D.N.PKC δ (D.N. δ), or Wild-Type PKC α (W.T. α)

Five-day-old myotubes were infected with PKC adenoviruses, as described in *Materials and Methods*. Studies were performed on cells treated as described in Fig. 1. Protein extracts from uninfected (C) or adenovirus-infected cultures, with or without insulin stimulation, were immunoprecipitated with specific anti-IR antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-PKC δ antibodies. The data presented are representative of four separate experiments. Insulin was added to cells overexpressing W.T. or D.N.PKC δ or W.T.PKC α , for 2 min.

PKC δ . In contrast, PKC isoforms β II and ζ , while also activated and phosphorylated on tyrosine by insulin, were not induced to coprecipitate with IR. We have already shown that tyrosine phosphorylation and translocation of PKCs β II and ζ occur via a wortmanin-sensitive pathway, probably involving PI3 kinase, while PKC δ activation and tyrosine phosphorylation were independent of PI3 kinase. Our findings thus indicate that the change in activation state of PKC δ may be accomplished by direct action of IR tyrosine kinase or another, as yet unidentified, tyrosine kinase upstream in the IR signaling pathway to phosphorylate this isoform on tyrosine residues. Our results on intact muscle cells in primary culture are entirely consistent with the *in vitro* studies of Li *et al.* (12), who reported that cocubation of IR with PKC δ resulted in phosphorylation on tyrosine of this PKC isoform associated with an increase in PKC activity. It is important to emphasize, however, that, in contrast to the results of Li *et al.* (12), our findings were obtained on PKC δ and IR proteins resident in intact skeletal muscle cells in primary culture. This indicates for the first time that the phenomenon may be an important step *in vivo* in the insulin-signaling pathway. Numerous other studies on various cell types have shown that PKC δ is phosphorylated on tyrosine sites in response to activators and growth factors, and that this is sometimes associated with an increase in the serine-threonine kinase activity of PKC δ (12, 24–26).

Insulin-induced coimmunoprecipitation of PKC and IR was also specific for IR. In other words, insulin did not cause association between PKC δ and other receptor tyrosine kinases such as IGF receptor (IGFR), EGFR, and NGFR. Although IGF-I did induce PKC δ to associate with IGFR, this effect occurred only after 10

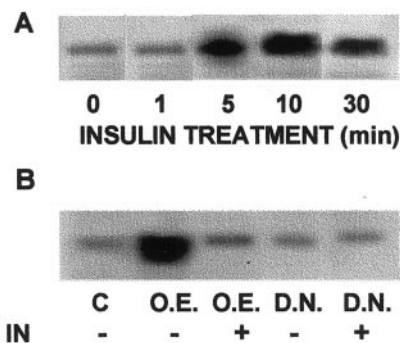


Fig. 4. Effects of Insulin on Serine Phosphorylation of IR in Control Uninfected Cells and in Cells Overexpressing W.T.PKC δ (O.E.) or D.N.PKC δ (D.N.)

Five-day-old myotubes were infected with PKC δ adenoviruses as described in *Materials and Methods*. Studies were performed on cells treated as described in Fig. 1. Protein extracts from uninfected or adenovirus-infected cultures were immunoprecipitated with specific anti-IR antibodies. Immunoprecipitates were run on SDS-PAGE, transferred to filters, and immunoblotted with specific antiphosphoserine antibodies. A, control (uninfected) cells were treated with insulin for the times designated. B, Cells overexpressing W.T.PKC δ (O.E.) or D.N.PKC δ (D.N.) were either unstimulated (–) or treated with insulin for 5 min (O.E. +) or 20 min (D.N. +).

min. This is in contrast to the rapid induction of IR-PKC δ association in response to insulin.

We also showed that the IR-PKC δ complex contained considerable PKC activity, and that this was due specifically to PKC δ . Thus, removal of PKC δ by immunoprecipitation of this protein with specific anti-PKC δ antibodies abrogated the insulin-induced PKC activity of immunoprecipitated IR, whereas immunoprecipitation of other PKC isoforms did not. Results similar to these have not heretofore been reported and indicate that tyrosine phosphorylation of PKC δ may occur as a result of the direct and immediate interaction of PKC δ with IR.

This is the first demonstration of insulin or other growth factor-induced coprecipitation of PKC δ with IR involving resident proteins in intact cells. A recent study by Formisano *et al.* (17) demonstrated that in NIH-3T3 cells expressing human IR (3T3_{hIR}), insulin could induce coprecipitation of IR with PKCs α , δ , and ζ . There are certain other important differences between the studies of Formisano *et al.* and those reported here. First, the insulin-induced coprecipitation in our studies was specific for PKC δ ; neither PKC ζ nor PKC α was induced by insulin to coprecipitate with IR. This specificity for insulin-induced coprecipitation of PKC δ and IR was also observed in skeletal muscle cultures in which PKCs δ and α were transiently overexpressed. Second, insulin-induced association of PKC δ with IR in skeletal muscle occurred within 1 min, whereas that in 3T3_{hIR} cells was observed after 30 min. There was no indication if cells were examined earlier than 30 min after insulin stimulation. Nonetheless, the more rapid time course of insulin effects on

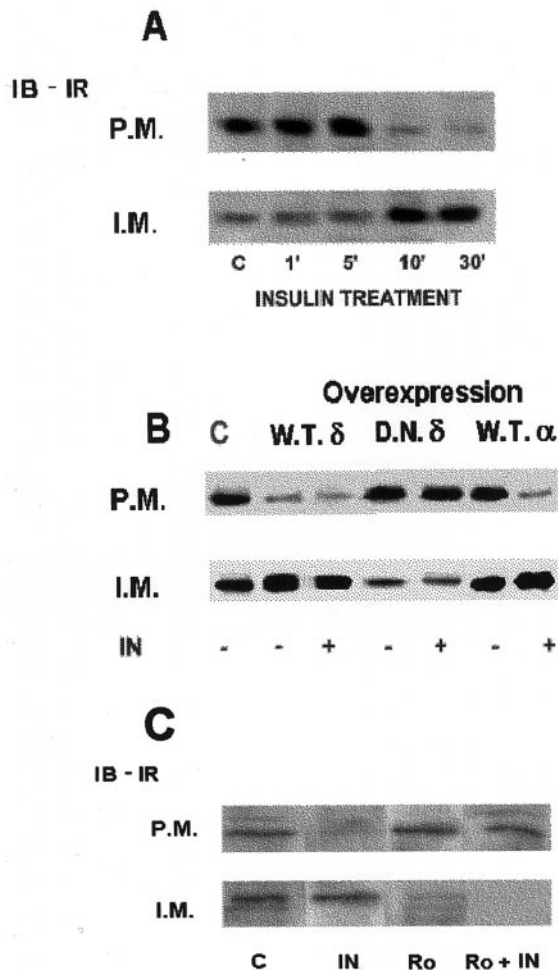


Fig. 5. Effects of Insulin on Distribution of IR in Plasma and Intracellular Membrane Components

A, Control (uninfected) cells treated with insulin for the times designated. B, Cells overexpressing W.T.PKC δ (O.E.) or D.N.PKC δ (D.N.). Five-day-old myotubes were infected with PKC δ adenoviruses as described in *Materials and Methods*. Cells overexpressing W.T.PKC δ (O.E.) or dominant negative (D.N.) PKC δ or W.T.PKC α , were either unstimulated (–) or treated with insulin for 5 min (O.E. +) or 20 min (D.N. +). C, Control (uninfected) cells pretreated or not with the PKC δ inhibitor rottlerin (Ro) for 7 min. All studies were performed on cells treated as described in Fig. 1. After treatment with insulin for the designated times, cell lysates were fractionated into plasma membrane (P.M.) and internal membrane (I.M.) fractions, 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-IR antibodies.

natively expressed PKC δ and IR may be a reflection of physiological as opposed to *in vitro* conditions.

We reported previously that insulin-induced activation of PKC δ occurs via a pathway independent and possibly upstream of PI3 kinase (7). In this study, we found a strong relation among the time courses of insulin-induced tyrosine phosphorylation of PKC δ , PKC δ activation, and the time course of PKC δ -IR coprecipitation. Indeed, insulin-induced tyrosine phosphorylation of

PKC δ and IR-PKC δ coprecipitation paralleled the time course of insulin-induced autophosphorylation of IR as well. This indicates that IR tyrosine kinase may be responsible for tyrosine phosphorylation of PKC δ , as shown by Li *et al.* (12) *in vitro*. This is supported by the additional finding that the PKC δ in these coprecipitates was found to be activated by insulin stimulation. The physical association between IR and PKC δ may indicate that IR is an endogenous substrate for PKC δ in skeletal muscle. As pointed out by Gschwendt (27), tyrosine phosphorylation of PKC δ , possibly by IR tyrosine kinase, is important for the determination of PKC δ substrate specificity. Thus, after the insulin-induced association of IR with PKC δ and its tyrosine phosphorylation, PKC δ proceeds to serine phosphorylate the IR. We are unable, however, to rule out the possibility that either or both tyrosine phosphorylation and activation of PKC δ may occur via some other tyrosine kinase upstream in the IR signaling cascade.

Also of interest are the results implicating PKC δ in serine phosphorylation of IR. Serine phosphorylation has been shown to be an important initial step in IR routing (28–37). We found that IR was phosphorylated on serine residues in cells overexpressing W.T.PKC δ without insulin stimulation, and that IR in these cells was localized primarily in the internal membrane fractions. In addition, when D.N.PKC δ was expressed in

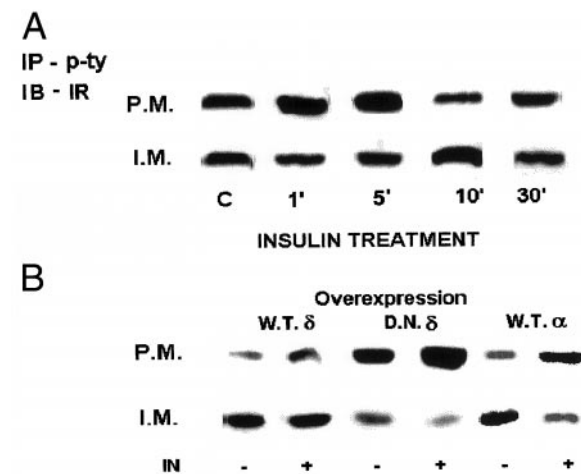


Fig. 6. Effects of Insulin on Tyrosine Phosphorylation of IR

Studies were performed on cells treated as described in Fig. 1. A, Control (uninfected) cells; cells were treated with insulin for the designated times. B, Cells overexpressing W.T.PKC δ (W.T. δ), D.N.PKC δ (D.N. δ), or W.T.PKC α (W.T. α). Five-day-old myotubes were infected with PKC δ or PKC α adenoviruses, as described in *Materials and Methods*. Cells were either unstimulated (–) or treated with insulin for 5 min (W.T. +) or 20 min (D.N. +). After treatment with insulin for the designated times, protein extracts from uninfected or adenovirus-infected cultures were fractionated into plasma membrane (P.M.) and internal membrane (I.M.) fractions as described in *Materials and Methods*. The different fractions were immunoprecipitated with specific antiphosphotyrosine antibodies, run on SDS-PAGE, transferred to filters, and immunoblotted with specific anti-IR antibodies.

the muscle cells, insulin-induced serine phosphorylation was blocked and IR remained in the plasma membrane for at least 30 min after insulin stimulation. These findings, in agreement with earlier *in vitro* studies on several cell types transfected with IR and different PKC isoforms (17, 29, 38, 39), suggest that PKC δ induces serine phosphorylation of IR after insulin stimulation and that this PKC isoform plays a major role in IR routing.

Our results regarding effects of overexpression of W.T. and D.N. PKC δ on tyrosine phosphorylation of IR in plasma and internal membrane fractions indicate that phosphorylation state and routing are very closely coupled. Moreover, our findings support the concept that IR remains tyrosine phosphorylated until after it is routed to internal membranes, as suggested by Di Guglielmo *et al.* (22). We found that PKC δ appeared to act in a manner similar to insulin. Thus, overexpression of PKC δ resulted in tyrosine phosphorylation of IR and routing of phosphorylated IR to the internal membrane fraction, whereas overexpression of inactive PKC δ prevented the internalization of IR where it remained tyrosine phosphorylated.

In conclusion, we propose that PKC δ is essential for IR internalization. We further suggest that serine phosphorylation occurs via PKC δ activity on IR in the plasma membrane, and that serine phosphorylation *per se* does not prevent tyrosine phosphorylation of IR. Rather, phosphorylation of IR on serine residues plays a role in the internalization of tyrosine-phosphorylated IR to internal membranes, where IR is dephosphorylated. The results presented in this report, together with our previous study implicating PKC δ in insulin-induced glucose transport (6), demonstrate a cardinal role of this PKC isoform in the mediation of a multitude of insulin-induced effects in skeletal muscle.

MATERIALS AND METHODS

Materials

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) kit was purchased from Bio-Rad Laboratories, Inc. (Rishon le Zion, Israel). Antibodies for various proteins were obtained from the following sources: monoclonal antibodies to IR β were purchased from Transduction Laboratories, Inc. (Lexington, KY). Polyclonal antiphosphoserine was obtained from Zymed Laboratories, Inc. (South San Francisco, CA), and monoclonal antiphosphotyrosine was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-PKC antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; polyclonal) and from Transduction Laboratories, Inc. (monoclonal). Horseradish peroxidase, and antirabbit and antimouse IgG were obtained from Bio-Rad Laboratories, Inc. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), 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-old neonatal rats as described

previously (7). 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. On the day of study, cells were transferred to PBS (pH 7.36) containing 2 mM glucose for 10 min before addition of insulin (70–100 nM).

Immunoprecipitation

Culture dishes (90 mm; Nunc, Roskilde, Denmark) containing the muscle cells were washed with Ca²⁺/Mg²⁺-free PBS and then mechanically detached in radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors. After scraping, the preparation was centrifuged at 20,000 $\times g$ for 20 min at 4 C. The supernatant was used for immunoprecipitation as described previously (6, 7).

Cell Fractionation

Crude membrane preparations were isolated from muscle cell cultures as described (6, 7). Culture dishes (90 mm; Nunc) containing the muscle cells were washed with Ca²⁺/Mg²⁺-free PBS and then mechanically detached in Ca²⁺/Mg²⁺-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 cells were resuspended in sonication buffer (50 mM Tris HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 1 mM EGTA; sucrose, 25 mM sucrose) containing leupeptin, 20 μ g/ml; aprotinin, 10 μ g/ml; 0.1 mM PMSF; 1 mM dithiothreitol (DTT); 200 μ M orthovanadate; and pepstatin, 2 μ g/ml. The suspension was homogenized in a Dounce glass homogenizer (30 strokes) and centrifuged at 1,100 $\times g$ 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 μ l and placed on a discontinuous sucrose gradient of 500 μ l 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 membranes 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 X100, freeze-thawed four times, centrifuged at 30,000 $\times g$ for 30 min, and the supernatant was designated as the membrane protein. All membrane fractions were stored at -70 C until use.

Western Blot Analysis

Crude and fractionated lysates of control and insulin-stimulated cultures were subjected to SDS-PAGE and electrophoretic transfer to Immobilon-P (Millipore Corp., Bedford, MA) membranes. The membranes were subjected to standard blocking procedures and were incubated with monoclonal antibodies against specific PKC isoforms or phosphotyrosine, and with polyclonal antibodies against glucose transporters as described previously (6, 7).

PKC Recombinant Adenoviruses and Viral Infection of Cultures

The recombinant adenoviruses were constructed as described (40). The dominant negative mutant of mouse PKC δ was generated by substitution of the lysine residue at the ATP binding site with alanine (41). The mutant δ cDNA was cut from SRD expression vector with *EcoRI* and ligated into the pAxCA1w cosmid cassette to construct Ax vector. Its kinase-negative nature was demonstrated by abrogation of autophosphorylation activity (41).

After differentiation of cultured rat myoblasts into myotubes, the culture medium was aspirated and cultures were

infected with medium containing PKC ζ or PKC α recombinant adenoviruses as recently described (6).

PKC Activity

Specific PKC activity was determined in freshly prepared immunoprecipitates from mature muscle cultures after appropriate treatments as described (6, 7). These lysates were prepared in RIPA buffer without NaF. Activity was measured using the SignaTECT Protein Kinase C Assay System (Promega Corp., Madison, WI).

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