The regulatory domain of protein kinase C delta positively regulates insulin receptor signaling

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Abstract

Protein kinase C delta (PKCδ) is induced by insulin to rapidly associate with insulin receptor (IR) and upregulates insulin signaling. We utilized specific JM and CT receptor domains and chimeras of PKCα and PKCδ regulatory and catalytic domains to elucidate which components of PKCδ are responsible for positive regulatory effects of PKCδ on IR signaling. Studies were performed on L6 and L8 skeletal muscle myoblasts and myotubes. PKCδ was preferentially bound to the JM domain of IR, and insulin stimulation increased this binding. Both PKCδ/α and PKCα/δ chimeras (regulatory/catalytic) were bound preferentially to the JM but not to the CT domain of IR. Although IR–PKCδ binding was higher in cells expressing either the PKCα/δ or PKCα/δ chimera than in control cells, upregulation of IR signaling was observed only in PKCα/δ cells. Thus, in response to insulin increases in tyrosine phosphorylation of IR and insulin receptor substrate-1, downstream signaling to protein kinase B and glycogen synthase kinase 3 (GSK3) and glucose uptake were greater in cells overexpressing PKCδ/α and the PKCδ/δ domains than in cells expressing the PKCα/δ domains. Basal binding of Src to PKCδ was higher in both PKCδ/α- and PKCα/δ-expressing cells compared to control. Binding of Src to IR was decreased in PKCα/δ cells but remained elevated in the PKCδ/α cells in response to insulin. Finally, insulin increased Src activity in PKCδ/α-expressing cells but decreased it in PKCα/δ-expressing cells. Thus, the regulatory domain of PKCδ via interaction with Src appears to determine the role of PKCδ as a positive regulator of IR signaling in skeletal muscle.

Introduction

The protein kinase C (PKC) family of serine–threonine kinases plays important roles in many intracellular signaling events, cell growth, and differentiation (Nishizuka 1988, 1989, 1992). It is generally accepted that the enzymes, when quiescent, are located in the cytoplasm and upon activation are translocated to their sites of action (Einspahr et al. 1990). The groups differ from one another in the composition of their regulatory domains.

The insulin receptor (IR) is a heterotetrameric transmembrane glycoprotein composed of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds (White & Kahn 1994). The α-subunits contain the insulin-binding domain, while the transmembrane β-subunits function as a tyrosine-specific protein kinase that undergoes autophosphorylation following insulin binding. This leads to tyrosine phosphorylation of endogenous proteins including the family of insulin receptor substrate (IRS) proteins. Phosphorylated motifs on these proteins serve as binding sites for the recruitment of downstream signaling proteins such as phosphatidylinositol 3-kinase (PI3K) and other protein kinases (White 1997, 1998, Zick 2004a,b, Biddinger & Kahn 2006). It has been reported that IR interacts with its immediate downstream effectors, IRS and Shc, through distinct receptor regions, and that autophosphorylation of Tyr residues located at the CT domain of the IR can modulate these interactions (Paz et al. 2000).

We recently showed that PKCδ plays an important unique role in IR signaling and in the mediation of insulin-induced glucose uptake in skeletal muscle, a major insulin-responsive tissue (Braiman et al. 1999a). PKCδ is activated by insulin stimulation upstream of PI3K and is induced by insulin to associate with IR within 1 min (Braiman et al. 1999b, 2001b). Moreover, overexpression of PKCδ promotes IR tyrosine phosphorylation and internalization and increases basal glucose uptake; blockade of PKCδ has opposite effects. There is an apparent inconsistency in that while PKCδ is a serine–threonine kinase, and that these kinases downregulate IR signaling, PKCδ has a positive effect in IR regulation, although we also reported that PKCδ has a later effect to serine phosphorylate IR, and may thus also downregulate IR signaling.

In this study, we have attempted to resolve this contradiction by examining the molecular interactions between PKCδ and IR. We studied PKCδ interaction...
with individually expressed JM and CT receptor domains. We also utilized chimeras of PKCδ and PKCδ regulatory and catalytic domains to elucidate which component of PKCδ may be responsible for effects of PKCδ on IR signaling. Chimeras have been used to delineate the contributions of individual PKC domains to the specific functions of different PKC isoforms in a number of systems (see Kronfeld et al. 2000, Mandil et al. 2001, Blass et al. 2002). As insulin stimulation induces PKCδ association with Src tyrosine kinase and increases PKCδ tyrosine phosphorylation, perhaps mediated by the Src family of non-receptor tyrosine kinases (Konishi et al. 1997, 2001, Benes & Soltoff 2001, Rosenzweig et al. 2004), we examined effects of insulin stimulation on Src and its interactions with IR and PKCδ in the different PKC chimeras. Src was shown to increase insulin-induced tyrosine phosphorylation of IR (Yu et al. 1985), and it was reported that Src associates with activated fibroblast growth factor receptor (FGFR) in response to FGF2 and has an important role in the regulation of FGFR1 signaling dynamics (Sandilands et al. 2007). In this study, we demonstrate that the regulatory domain of PKCδ is the main determinant of upregulation of IR signaling by PKCδ, most likely via interactions with Src tyrosine kinase.

Materials and methods

Materials

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed with antibodies purchased from Bio-Rad and reagents from Sigma. The following antibodies to various proteins were used: anti-PKCα antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Transduction Laboratories, Lexington, KY, USA); anti-phosphoserine antibodies (Biomedica, Foster City, CA, USA); anti IRS-1 Ser307, anti p-protein kinase B (PKB) Thr308, and anti p-GSK3 Ser9 (Cell Signaling Technology, Beverly, MA, USA); anti-pSrcY529 (Stressgen, Victoria, British Columbia, Canada); HRP-conjugated anti-rabbit and anti-mouse IgG (Bio-Rad). Protease and phosphatase inhibitor cocktails were purchased from Sigma Chemicals. Insulin (Humulin R-recombinant human insulin) was purchased from Lilly France SA (Fegersheim, France).

Cell culture

L8 muscle cells were a gift from Dr D Yaffe (Weizmann Institute of Science, Rehovot, Israel); L6 skeletal muscle cells were obtained from Dr Nava Bashan (Ben-Gurion University, Beersheba, Israel). L6 cells were grown in alpha modified Eagle’s medium supplemented with 10% FCS for 4 days post-confluence, with media changed daily. 3T3 fibroblasts expressing human IR (hIR) or C-terminal (ACT) deleted IR (a kind gift from Prof. Hans Haring, University of Tubingen, Tubingen, Germany) were grown in DMEM supplemented with 10% FCS.

Skeletal muscle cultures were prepared from thigh muscles obtained from 1- to 2-day-old neonatal rats as described. The muscles were removed from the limbs, washed in PBS to remove excess blood cells, and then transferred to a Ca\textsuperscript{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 15-min periods until all tissues were dispersed) and then centrifuged for 5 min at 500 g. Pellets were resuspended in growth medium and preplated for 20–30 min to reduce the number of fibroblasts. The myoblasts were diluted with growth medium to a concentration of 0.8–10\textsuperscript{6} cells/ml for plating in collagen-coated 10-cm plastic tissue culture (10 ml/dish) plates. Cultures were grown in a water-saturated atmosphere of 95% air and 5% CO\textsubscript{2} at 37.0° C. On day 4 in culture, myotubes were transferred to starvation medium for 18 h before study.

Preparation of crude cell lysates

Culture dishes (90 mm; Nunc, Thermo Fisher Scientific, Rochester, NY, USA) containing the muscle cells were washed with Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free PBS and then mechanically detached in NP-40 buffer containing a cocktail of protease inhibitors and phosphatase inhibitors (Sigma). After scraping, the preparation was centrifuged at 20.000 g for 20 min at 4° C. The supernatant was used for immunoprecipitation and binding assays.

Western blot analysis

Western blotting was performed as already described (Brainer et al. 1999b, Rosenzweig et al. 2002, Horovitz-Fried et al. 2006a). Briefly, 20–25 μg protein was electrophoresed through SDS-polyacrylamide gels (7.5 or 10%) and electrophoretically transferred onto Protran nitrocellulose Transfer Membrane (Schleicher & Schuell, Keene, New Hampshire, USA). Following transfer, the membranes were subjected to standard blocking and incubation procedures, and incubated with specific monoclonal and polyclonal antibodies. After washing, membranes were incubated with HRP-labeled secondary antibody, treated with ECL reagent for 1 min, exposed to X-ray film, and developed.
PKC chimeras

PKC chimeras were generated by exchanging the regulatory and catalytic domains of PKCa and PKCδ (Acs et al. 1997a), and were kindly supplied by Dr Peter Blumberg (NCI, NIH, Bethesda, MD, USA). PKCa/δ refers to the chimera with the PKCa regulatory domain and the PKCδ catalytic domain, and PKCδ/α refers to the reciprocal chimera. The PKC cDNAs were subcloned into the metallothionein promoter-driven eukaryotic (episomal MTH) expression rector (MTH) plasmid generating PKC chimeras with a C-terminal ε tag. The expression of these chimeras and their activities were described (Blass et al. 2002). In this study, we used L8 cells stably overexpressing the PKCζ and PKCδ/α chimeras and the control MTH plasmid (Olah et al. 1994). The L8 cell line has a low endogenous expression level of PKCδ protein (M Horovitz-Fried & SR Sampson, unpublished observations). Cells were grown in DMEM supplemented with 15% FCS and G418 (Calbiochem, San Diego, CA, USA).

Peptide expression and purification

(His)₆-tagged fusion peptides corresponding to 41 amino acids (amino acids 943–984) of the juxtamembrane region of IR ((His)₆-JM) or 86 amino acids (amino acids 1245–1331) of the carboxyl-terminal region of IR ((His)₆-CT) were generated in bacteria and purified over ProBond Ni²⁺ beads (Invitrogen) as described by Paz et al. 2000. Peptide conjugated beads were stored at 4 °C for the short term until use.

Precipitation of proteins with IR domain peptides

Crude lysate (300 μg) was incubated with 20 μl peptide-conjugated beads for 2 h. After washing, sample buffer was added and proteins that had been bound to beads were loaded on an SDS/PAGE gel for further analysis.

Immunoprecipitation

Cell lysates containing equal amounts of protein were incubated with the relevant antibody for 1 h, following which protein A/G agarose beads were added and samples incubated overnight at 4 °C. Precipitates were washed 4× with NP-40 buffer and subsequently resuspended in SDS-PAGE sample buffer. Samples were denatured at 90 °C for 4 min and separated by SDS-PAGE.

PKC activity assay

Specific PKC activity was determined in freshly prepared immunoprecipitates (utilizing anti-PKCζ antibodies) from muscle cultures following appropriate treatments, as described (Braiman et al. 1999b, Rosenzweig et al. 2002). The lysates were prepared in RIPA buffer without NaF. Activity was measured using the SignaTECT Protein Kinase C Assay System (Promega). The kit contains phosphatidylinerine and diacylglycerol and utilizes neurogranin as a substrate.

Glucose uptake

Glucose transport was evaluated by measuring 2-deoxy-d-glucose uptake as described (Braiman et al. 1999a, 2001a, Rosenzweig et al. 2004).

Statistical analysis

In all graphs, values are presented as means ± s.e.m. Statistical differences between the treatments and controls were evaluated by the Student’s t-test.

Results

We have shown that activation of PKCζ in skeletal muscle by insulin is associated with a rapid increase in the physical association of PKCζ with IR. This increase in association occurs within 1–5 min in primary cultures of mature rat skeletal myotubes and is associated with regulatory effects on IR internalization and phosphorylation (Braiman et al. 2001b). In the current study, we utilized cells from the L6 and L8 rat skeletal muscle cell lines and have initially confirmed this effect in myoblasts (Fig. 1). PKCζ was immunoprecipitated from mature L6 and L8 myotubes following stimulation with insulin for varying times. As shown, PKCζ is constitutively associated with IR in both cell lines under basal conditions, and insulin stimulation causes an increase in association beginning as early as 1 min and reaching a peak at 5 min (Fig. 1A). Similar results were obtained in studies in which IR was immunoprecipitated from lysates of control and insulin-stimulated cells, and immunoblotting was performed with specific anti-PKCζ antibodies (Fig. 1B). As reported for skeletal muscle in primary culture (Braiman et al. 2001b), PKCs α, βII, ε, and ζ were not found to associate with IR in L6 or L8 myotubes under either basal or insulin-stimulated conditions (not shown, but see Fig. 1D and (Rosenzweig et al. 2002)).

PKCζ preferentially binds to the JM domain of IR

The JM and CT domains of IR have been shown to interact differentially with certain substrates. Thus, Shc was shown to specifically interact with the CT peptide, whereas IRS-1 was found to specifically interact with...
To determine whether PKCδ might also display preferential binding to one or another of the IR domains, we utilized polypeptides of 41 and 86 amino acid lengths, corresponding to the Arg943-Lys984 (JM) and Leu1245-Asn1331 (CT) domains of the IR respectively. These segments were produced as epitope-tagged fusion peptides containing at their amino terminus a His sequence, fused in-frame to an epitope for the T7Tag antibody (Novagen, Madison, WI, USA).

As shown in Fig. 1C, under basal conditions, PKCδ associates primarily with the JM domain of IR, although some PKCδ also associates with the CT domain.

Insulin induces a selective increase in PKCδ binding to the JM domain of IR

We next sought to determine whether the increase in PKCδ-IR binding in response to insulin stimulation occurs in both domains or is selective for either one or the JM polypeptide (Paz et al. 1996, 1997, 1999).

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another. In these studies, mature L6 was stimulated with insulin (100 nM) for 1–5 min, following which PKCδ was immunoprecipitated from whole cell lysates. The stimulated PKCδ was examined for binding to the JM and CT domains. Examples of results of these studies are illustrated in Fig. 1D and E, which shows that PKCδ from insulin-stimulated cells binds selectively to the JM domain. Figure 1D (middle blot) also shows that similar to IR, neither the JM nor CT domain binds detectable amounts of PKCα (or other isoforms – not shown). This confirms for L8 (and L6) myotubes earlier findings obtained in studies on mature skeletal muscle (both rat and mouse) in primary culture that IR selectively binds PKCδ is selective for under both basal and insulin-stimulated conditions (Braiman et al. 2001b, Rosenzweig et al. 2002).

The relative importance of the JM and CT domains of IR in insulin-induced IR–PKC association was further evaluated in 3T3 cells expressing normal hIR or IR in which either the CT (ΔCT) or JM (ΔJM) domains had been deleted (Mosthaf et al. 1995). In these studies, we found that in cells in which the CT domain of IR had been deleted, basal binding of PKCδ to IR was actually higher than in cells expressing normal IR and insulin further increased this association (Fig. 2). In hIR-JM deleted cells, there was a low level of basal association between IR and PKCδ, and this was not increased by insulin (not illustrated).

**IR binds both the PKCδ/α and PKCα/δ chimeras**

In order to further clarify the relationship between IR and PKCδ, we attempted to determine whether one or the other of the regulatory or catalytic domains of PKCδ preferentially binds to IR. For this purpose, special cell lines of L8 skeletal muscle muscle cells were developed in which PKCα–PKCδ chimeras of the respective regulatory and catalytic domains were stably expressed. The preparation and use of these chimeras in other systems have been reported (Acs et al. 1997b, Blass et al. 2002). The assembled proteins are composed of either the PKCα regulatory–PKCδ catalytic chimera (PKCα/δ) or the PKCδ regulatory–PKCα catalytic (PKCδ/α) chimera. In addition, we developed cell lines in which reassembled PKCδ/δ and PKCα/α constructs were stably expressed. The PKC chimeras were tagged with a PKCα epitope, which is recognized by anti-PKCα antibody used for western blotting. We have already determined that IR does not bind PKCε or PKCζ under either basal or insulin-stimulated conditions in these or other skeletal muscle cells (rat and mouse skeletal muscle in primary culture; mouse skeletal muscle in vivo). Western blotting in cells expressing reconstructed PKCs α/α and δ/δ was done with anti-PKCα antibodies. Figure 3A shows the levels of over-expressed PKCα/δ and PKCδ/α chimeras and PKCδ (in the α/α and δ/δ cells). The cells express similar high amounts of each of the chimeras. In the current study, IR was immunoprecipitated from lysates of control and insulin-stimulated cells, and following SDS-PAGE, immunoblotted with anti-PKCα antibodies. Interestingly, as seen in Fig. 3B and C, whereas both the PKCδ/α and the PKCα/δ chimeras display detectable levels of binding to immunoprecipitated IR and increased binding following insulin stimulation, the increase in binding of the PKCα/δ chimera in response to insulin was greater than that of the PKCδ/α chimera. The figure also shows that the association of PKCδ with IR under basal conditions in cells expressing the re-assembled PKCδ/δ was greater than that in either control cells or in cells expressing the reconstructed PKCα/α. This is in agreement with our earlier studies on skeletal muscle cells in primary culture (Braiman et al. 2001b). We next attempted to determine whether the regulatory and catalytic domains might have preferential binding to the JM and CT domains of IR. In these studies, L8 myotubes expressing the PKCδ/α or PKCα/δ chimeras were stimulated with insulin (100 nM) for 1–5 min,
following which PKCε was immunoprecipitated from whole cell lysates. These stimulated PKC chimeras were examined for binding to the JM domain. Examples of results of these studies are illustrated in Fig. 3D and E. Both the PKCα/δ and the PKCδ/α chimeras bind to the JM as detected by immunoblotting with the anti-PKCα antibody. However, the binding of the PKCα/δ chimera to JM was significantly greater than that of the PKCδ/α chimera. Moreover, insulin increased the binding of each to the JM but not to the CT domain, as also was shown in Fig. 1 with regard to PKCδ.

The different responses of the two chimeras with regard to the binding to both IR and to its JM domain suggest that IR signaling in the two types of cells may show fundamental changes. Accordingly, we examined different aspects of IR signaling in the various chimeras.

**Figure 3** Insulin increases the binding of IR to both δ/α and α/δ PKC chimeras. L8 cells stably expressing the designated chimeras were treated as in Fig. 1 (CV, cells transfected with control vector only; δ/α, PKCδ regulatory/PKCα catalytic chimera; α/δ, PKCα regulatory/PKCδ catalytic chimera). (A) Western blot of expression of PKCδ/α and α/δ chimeras tagged with PKCε, and of PKCδ in reconstituted PKCα/δ and δ/δ cells (see Materials and methods). (B) Western blot showing IR–PKCδ binding in control and insulin-stimulated chimeras and cells expressing reconstituted PKCα/δ or PKCδ/δ cells. IR was immunoprecipitated from cell lysates the various cells with specific anti-IRδ) antibodies. Immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-PKCα antibodies (upper blot) or anti-IR antibodies (lower blot). (C) Densitometry measurements made on western blots obtained in experiments in C. Values were normalized to levels of IR. Each bar represents the mean ± S.E.M. of values obtained from three separate experiments. (D) Insulin-induced binding of PKCδ to the JM domain of IR is greater in cells expressing the PKCα/δ chimera than in cells expressing the PKCδ/α chimera. Extracts from cells stimulated or not with insulin were incubated with 10 μg JM purified peptide for 2 h as in Fig. 1. Precipitates were subjected to SDS/PAGE and immunoblotted with anti-PKCδ antibodies. The lower blots show the relative amounts of His-tagged JM domain. The data are representative of five separate experiments. (E) Densitometry measurements made on western blots in D. Values were normalized to levels of JM. Each bar represents the mean ± S.E.M. of values obtained from three separate experiments. (*P < 0.05 versus CV; **P 0.01 versus CV and δ/δ chimera).

**Tyrosine phosphorylation of IR and IRS-1 by insulin is differentially affected in PKCδ/α and PKCα/δ chimeras**

We earlier showed that overexpression of PKCδ increased basal IR tyrosine phosphorylation (Braiman et al. 2001b). We first examined tyrosine phosphorylation of IR and IRS-1 in response to insulin in the cells expressing the different chimeras. The results of the studies on tyrosine phosphorylation of IR in response to insulin are summarized in Fig. 4A and B. IR was immunoprecipitated from control and insulin-stimulated cells and immunoblotted with anti-phosphotyrosine antibodies. As can be seen, IR phosphorylation in the PKCδ/α chimera was increased compared to control cells. In contrast, whereas basal IR phosphorylation was essentially unchanged in the PKCα/δ chimera compared to control, insulin-induced tyrosine phosphorylation was nearly completely abrogated. The pattern of IR tyrosine phosphorylation in

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response to insulin in PKCδ/δ and PKCα/α cells did not appear to differ from that in control and PKCα/δ cells (not shown).

The different effects of insulin on IR tyrosine phosphorylation in cells expressing the different chimeras suggest that downstream insulin signaling might also differ in these cells compared to control. In order to investigate this possibility, we examined possible differences in responses of downstream proteins to insulin among the chimeras. In one series of experiments, we analyzed the effects of insulin on IRS-1. We immunoprecipitated IRS-1 from control and insulin-stimulated cells expressing the different vectors, and immunoblotting was performed with anti-phosphotyrosine antibodies. These results are summarized in Fig. 4C and D. IRS-1 tyrosine phosphorylation in response to insulin in the PKCδ/α chimera was increased over that in control cells, whereas that in cells expressing the PKCα/δ chimera was slightly reduced compared to control. Tyrosine phosphorylation of IRS-1 induced by insulin in the chimeras expressing PKCα/α or PKCδ/δ was not detectably different from that in cells transfected with control vector (CV; not shown).

Serine phosphorylation of IRS-1 in response to insulin is differentially affected in PKCδ/α and PKCα/δ chimeras

The results showing a decrease in insulin signaling to IRS-1 in the PKCα/δ chimera compared to the control or PKCδ/α-expressing cells suggest that an inhibitory effect on IR signaling might be dominant in the PKCα/δ cells and not the others. This effect could also result in part from differences in serine phosphorylation of IRS-1 in response to insulin stimulation. To investigate this possibility, we examined the serine phosphorylation state of IRS-1 in the different cells before and following insulin stimulation. We prepared whole cell lysates of control and insulin-stimulated cultures of each of the clones, as in the foregoing experiments. In order to determine that IRS-1 is indeed phosphorylated, we performed a series of experiments in which immunoblotting with antibodies directed against IRS-1 Ser307 of control and insulin-stimulated chimeras was performed. This serine residue has been suggested to be a specific inhibitory target for PKC isoforms, possibly in response to insulin (Werner et al. 2004, Mussig et al. 2005). Figure 5A and B show that in CV cells, IRS-1 was constitutively phosphorylated on
Ser307, and this phosphorylation was increased by insulin. Interestingly, in cells expressing the PKCδ/α chimera, basal phosphorylation on Ser307 appeared to be reduced, and insulin failed to increase the level of Ser307 phosphorylation. In contrast, Ser307 phosphorylation in PKCα/δ-expressing cells was significantly increased compared to that in cells expressing either the PKCδ/α chimera or the empty vector. The results obtained in PKCδ/δ cells were qualitatively similar to those in the PKCδ/α cells, while those in PKCα/α were similar to those in controls (not shown).

**PKC activity is increased in the PKC chimeras**

The different levels of IRS-1 serine phosphorylation in the chimeras compared to control cells suggest that PKC activities might also differ. Therefore, we measured PKC activity under basal and insulin-stimulated conditions for each of the chimeras. Fresh lysates from insulin-stimulated cells were prepared and submitted to immunoprecipitation with PKCε antibody to specifically bring down the ε-tagged chimeras. The immunoprecipitation complex was then submitted to an activity assay (as described in Materials and methods). Results are summarized in Fig. 5C. In CV cells, insulin caused a fourfold increase in insulin-stimulated PKCε by 5 min. This agrees with unpublished data in our laboratory, and is consistent with the increase obtained in insulin-stimulated PKCδ activation. PKC activity in cells expressing either the PKCδ/α or the PKCα/δ chimeras was increased by insulin. However, both basal and insulin-stimulated activities of the cells expressing the PKCα/δ chimera were greater than that of the cells expressing the PKCδ/α chimera. Activity in PKCα/α did not differ from that in CV cells, whereas that in PKCδ/δ cells was similar to that in PKCδ/α chimera-expressing cells (not shown).

**Activation of downstream proteins by insulin is different in PKCδ/α and PKCδ/δ chimeras**

The different patterns of insulin-induced tyrosine and serine phosphorylations of IRS-1 in the cells expressing the different chimeras suggest that responses of other downstream proteins to insulin might also be altered. An immediate downstream target of PI3K is PKB. One indication of PKB activation is phosphorylation on threonine residues. Therefore, we compared threonine phosphorylation of PKB induced by insulin in cells expressing the PKCδ/α with that in cells expressing the PKCδ/δ chimeras. Whole cell lysates were prepared

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*Figure 5* Phosphorylation of IRS-1 Ser307 and PKC activity are differentially affected in PKCδ/α and PKCα/δ chimeras. LB cells stably expressing the PKCδ/α or the PKCα/δ chimera were treated as in Fig. 1. In all figures, CV, cells transfected with control vector only; δ/α, PKCδ regulatory/PKCα catalytic chimera; and PKCα/δ, PKCα regulatory/PKCδ catalytic chimera. (A) Following insulin treatment and SDS/PAGE, immunoblotting was performed with either anti-phospho-IRS-1 (Ser 307) antibodies (upper blot) or anti-actin antibodies (lower blot). (B) Densitometry measurements made on western blots. Values were normalized to levels of actin. Each bar represents the mean ± S.E.M. of values obtained from three separate experiments (*significantly different from control CV; **P<0.01 versus corresponding CV). (C) Fresh lysates from control and insulin-stimulated cells stably expressing the appropriate chimera were treated as in Fig. 1. Seven hundred and fifty micrograms protein was submitted to IP using anti-PKCε antibodies. Immunoprecipitates were then used in an activity assay, following the manufacturer’s instructions as described in Materials and methods. (*P<0.05 versus control CV; **P<0.05 versus δ/α chimera).
from control and insulin-stimulated cells expressing the different chimeras. The lysates were then subjected to SDS-PAGE and immunoblotting with specific anti-phosphoPKB Thr308 antibodies. As shown in Fig. 6A, protein levels of PKB and GSK3 were similar in each of the PKC chimeras. Figure 6B shows that insulin-induced phosphorylation of PKB and GSK in cells expressing re-assembled PKCδ/δ was slightly higher, and that in cells expressing PKCα/δ was similar to that in control cells. Figure 6C and D show that insulin-induced phosphorylation of PKBThr308 and GSK-3Ser9 was slightly higher in cells expressing the PKCδ/α chimera compared to control. On the other hand, phosphorylation of PKBThr308 and GSK-3Ser9 was strongly and significantly reduced in cells expressing the PKCα/δ chimera.
Insulin-induced 2-DOG uptake is different in PKCδ/α and PKCα/δ chimeras

Our results so far demonstrate that insulin signaling to PKB and GSK3 is increased in PKCδ/α-expressing cells and decreased in PKCα/δ-expressing cells. As PKB is also considered an important component in signaling to glucose uptake, we next measured the uptake of 2-DOG in response to insulin stimulation of L8 myoblasts expressing the different PKC chimeras. Studies were performed on undifferentiated L8 myoblasts expressing the different PKC chimeras. Myoblasts were because cells expressing the different chimeras displayed varying rates of differentiation. This prevented us from performing glucose uptake studies on the same day for each type. Myoblasts have been used as a model for glucose uptake studies (Randhawa et al. 2000, Sweeney et al. 2004, Aga-Mizrachi et al. 2008). The results are summarized in Fig. 6E. For comparison, glucose uptake in non-transfected L8 myoblasts is included. Basal and insulin-stimulated glucose uptake in CV-expressing cells was slightly but significantly lower than that in L8 myoblasts. In myoblasts expressing the CV, insulin induced a slight (20–40%) but significant increase in glucose uptake in CV-expressing cells was slightly but significantly lower than that in L8 myoblasts.

Figure 7 Insulin-induced interactions between PKCδ and Src, between IR and Src, and Src activity are differentially affected in PKCδ/α and PKCα/δ chimeras. L8 cells stably expressing the PKCδ/α or the PKCα/δ chimera were treated as in Fig. 1. In all figures, CV, cells transfected with control vector only; δ/α, PKCδ regulatory/PKCα catalytic chimera; and PKCα/δ, PKCα regulatory/PKCδ catalytic chimera. (A and B) PKC–Src binding. (A) Following insulin treatment and SDS/PAGE, cells were immunoprecipitated with anti-PKCδ antibodies, and immunoblotting was performed with either anti-Src antibodies, and immunoblot was performed with either anti-Src (upper blot) or anti-actin antibodies (lower blot). (C and D) IR–Src binding. Following insulin treatment and SDS/PAGE, cells were immunoprecipitated with anti-IR antibodies, and immunoblotting was performed with either anti-Src (upper blot) or anti-actin antibodies (lower blot). (B) and (D) are graphs of densitometry measurements made on western blots. Values were normalized to levels of the appropriate protein. Each bar represents the mean ± S.E.M. of values obtained from three separate experiments. Src phosphorylation was increased (decreased Src activity) in cells expressing the δ/α and α/δ chimeras. (*P<0.05, **<0.01, versus time 0). Clear bars, time 0; gray bars, insulin 1 min; black bars, insulin 5 min.
uptake. In cells expressing either the PKCδ/δ or the PKCδ/α chimera, basal glucose uptake was significantly higher than that in control, and insulin induced a slight but significant increase in glucose uptake compared to control. In contrast, whereas basal glucose uptake in PKCα/δ-expressing cells was only slightly lower than that in controls, insulin-induced glucose uptake was almost totally abrogated in these cells.

**Interaction of Src with PKCδ and IR is different in PKCδ/α and PKCα/δ chimeras**

The data shown so far demonstrate that insulin signaling from IR tyrosine phosphorylation to glucose uptake is up-regulated in cells expressing the PKCδ/α chimera, whereas it is strongly downregulated in cells expressing the PKCα/δ chimera. The increased IR tyrosine phosphorylation in the δ/α cells is unlikely to be a direct effect of the PKCδ regulatory domain and probably results from involvement of a tyrosine kinase. It was reported that Src tyrosine kinase upregulates IR tyrosine phosphorylation, and we have shown that insulin activates Src and also induces Src–PKC association. Moreover, Src is involved in insulin-induced tyrosine phosphorylation and activation of both PKCζ and PKCα (Rosenzweig et al. 2004, Cipok et al. 2006). We, therefore, examined the possibility that PKCζ–Src and IR–Src interactions may be different in the two chimeras. To this end, we immunoprecipitated PKCζ or IR from the different chimeras before and after treatment with insulin. Then, following SDS-PAGE and transfer, immunoblotting was performed with anti-Src antibodies. As shown in Fig. 7A and B, in control (CV transfected) cells, insulin induced an increase in PKCζ–Src association within 1 min, and this increased further by 5 min, similar to our earlier findings (Rosenzweig et al. 2004). In cells expressing the PKCζ/α chimera, basal PKCζ–Src association was increased over that in CV cells, and insulin increased this association even further at 1 and 5 min. Cells expressing the PKCα/δ chimera also displayed higher basal PKCζ–Src association. This association was also increased after 1 min by insulin but did not increase with time. Insulin-induced PKCζ–Src association after 5 min was higher in the δ/α than in the α/δ chimera. These data suggest that the PKCδ regulatory domain may be important in regulation of PKCζ–Src interactions.

We next examined the effects of insulin on IR–Src association in cells expressing the different chimeras. As shown in Fig. 7C and D, insulin increased IR–Src association in control cells, as we have earlier reported (Rosenzweig et al. 2004). Constitutive IR–Src association in both PKCζ/α- and PKCα/δ-expressing cells was higher than that in control cells; however, effects of insulin on these associations were strikingly different. Thus, elevated IR–Src association in the cells expressing the PKCζ/α chimera remained relatively constant with insulin stimulation, whereas IR–Src association in the cells expressing the PKCα/δ chimeras was decreased. Finally, we examined the effects of insulin on phosphorylation of Src tyrosine 529 (SrcY529). A decrease in phosphorylation is an indication of Src activation (Harder et al. 1998, Bjorge et al. 2000). The results are summarized in Fig. 7E and F. In both CV and PKCδ/α-expressing cells, insulin induced a decrease in SrcY529 phosphorylation. In contrast, in cells expressing the PKCα/δ chimera, SrcY529 phosphorylation was increased, indicating that activity of Src tyrosine kinase actually decreased. Src phosphorylation in PKCα/α-expressing cells was also decreased, although with a different time course, whereas that in PKCδ/δ-expressing cells did not appear to be significantly changed. These findings indicate that the PKCδ and PKCα regulatory domains have different roles in regulation of Src and its effects on IR signaling.

**Discussion**

In an earlier series of studies, we showed that PKCδ is activated by insulin upstream of PI3K (Braiman et al. 1999b) and is tyrosine phosphorylated and activated via a Src tyrosine kinase-dependent mechanism (Rosenzweig et al. 2004). In addition, PKCδ was found to be a positive regulator of insulin signaling and uniquely regulated by insulin. Thus, overexpression of WTPKCδ in primary skeletal muscle cells increased GLUT4 translocation and glucose uptake in the absence of insulin stimulation, and inhibition of PKCδ by pharmacological means or by expression of a kinase-dead dominant-negative construct abrogated insulin-induced GLUT4 translocation and glucose uptake (Braiman et al. 1999a). In addition, PKCδ was found to be involved in the binding of PKCζ to IR in one of the earlier effects of insulin stimulation of skeletal muscle and is unique to PKCζ. Insulin not only stimulates PKCζ but also regulates its expression and protein levels (Horovitz-Fried et al. 2000a,b, 2008, Horovitz-Fried & Sampson 2007). In addition, studies in intact animals have shown that elevated PKCζ activity is involved in the effect of exercise to prevent the development of diabetes in diabetes-prone animals (Heled et al. 2002, 2003). These findings present an apparent paradox. On the one hand, PKCζ is a serine–threonine kinase, which
should act to downregulate IR signaling; on the other hand, PKCδ acts to upregulate IR signaling.

The results of the current study offer a reasonable explanation to resolve this paradox, namely that the regulatory and catalytic domains of PKCδ serve independent functions related to the physical binding between IR and PKCδ and to the effects of PKCδ on IR signaling. Thus, insulin-induced binding of PKCδ to IR was higher in cells expressing the PKCα/PKCδ chimera than in cells expressing any of the other chimeras. In contrast, cells expressing the PKCα/δ chimera displayed higher levels of IR and IRS-1 tyrosine phosphorylation and increased signaling to PKB/Akt, GSK-3, and glucose uptake. PKCα does not bind to IR, either under basal or insulin-stimulated conditions (Fig. 1D and Braiman et al. 2001b, Rosenzweig et al. 2002, Cipok et al. 2006), and this difference could be due to some change imparted to the PKCδ catalytic domain to increase its affinity for IR when activated. Alternatively, this could involve an effect secondary to the lack of the PKC regulatory domain, which might interact with an additional protein such as Src, which binds to both IR and PKCδ (Rosenzweig et al. 2004).

The differential effects of the two chimeras on the insulin signaling cascade lend further support to the proposal that the regulatory domain of PKCδ is responsible for the upregulation of insulin signaling by this isoform. However, as PKCδ is a serine–threonine kinase, it itself cannot increase IR and IRS-1 tyrosine phosphorylation, suggesting that a kinase must be involved; one such possibility is Src tyrosine kinase. In accordance with this, we reported that insulin-activated PKCδ associates with Src tyrosine kinase and increases its activity (Rosenzweig et al. 2004). Furthermore, inhibition of Src reduces insulin-stimulated Src–PKCδ association, PKCδ tyrosine phosphorylation and PKCδ activation. Inhibition of Src also decreases insulin-induced IR tyrosine phosphorylation, IR–PKCδ association, and association of Src with both PKCδ and IR. Moreover, Src tyrosine kinase was earlier shown to catalyze IR tyrosine phosphorylation (Yu et al. 1985). Our current results on interaction of Src tyrosine kinase with PKCδ and IR in the different chimeras support this role for Src, and further provide a plausible explanation for the upregulation of IR signaling in the PKCδ/α-expressing cells compared to the downregulation in the PKCα/δ-expressing cells. Thus, i) PKCδ–Src association was higher in the PKCδ/α-expressing cells than in the PKCα/δ-expressing cells; ii) IR–Src association remained elevated with insulin stimulation in the PKCδ/α-expressing cells, whereas it was decreased by insulin stimulation in the PKCα/δ-expressing cells; and iii) most importantly, insulin decreased phosphorylation of Src (increased activation) in cells expressing the PKCα/δ chimera and increased phosphorylation of Src (decreased activation) in the cells expressing the PKCα/δ chimera. The increased phosphorylation of Src results in inhibition of Src and downregulation of IR signaling, as we have shown here and elsewhere (Rosenzweig et al. 2004). We propose, therefore, that differences in PKCδ–Src and IR–Src interactions between the PKCα/δ- and PKCα/α-expressing cells result in the altered tyrosine phosphorylation and insulin signaling observed in these cells. It was reported that the C2 domain (in the regulatory segment) of PKCδ is a phosphotyrosine-binding domain (Sondermann & Kurian 2005); this mechanism for crosstalk between distinct signaling pathways lends strong support to our findings that PKCδ–Src interactions are an integral component of PKCδ regulation of IR signaling. Other studies have shown that the regulatory domain of PKCδ is responsible for the effects of this isoform on regulation of glutamine synthetase expression and cell proliferation by PKCδ in C6 glioma cells and on U87 cell morphology, proliferation, and GFAP expression (Kronfeld et al. 2000, Mandil et al. 2001, Blass et al. 2002).

The increase in insulin-induced phosphorylation of both PKB and GSK in cells expressing the PKCδ/α chimera, as well as the increase in basal glucose uptake, compared to the PKCα/δ chimera, is consistent with the previously shown positive regulatory role for PKCδ in insulin effects. This is in agreement with other studies in our laboratory in which we found that basal glucose uptake was increased significantly in cells overexpressing PKCδ (Braiman et al. 1999a) and that PKCδ is involved in insulin-induced regulation of PKB activity via interactions with PDK-1 (phosphoinositide-dependent kinase 1; Brand et al. 2006).

We also examined the interaction of PKCδ with specific domains (JM and CT) of IR. Whereas PKCδ did not appear to display any preferential binding to either of these regions under basal non-stimulated conditions, insulin stimulation increased the binding of PKCδ to the JM but not to the CT domain. The JM and CT regions of IR have been shown to be functionally independent entities that contain sufficient structural information to enable independent and direct binding of effector molecules (Paz et al. 1996, 1997, 2000). Thus, the JM region was shown to serve as a binding site for IRS-1, as IRS-1 interacts preferentially with the JM peptide and exhibits no significant binding to the CT peptide, similar to our results regarding PKCδ. The preference of binding of PKCδ to the JM domain was supported further by the findings that deletion of the CT domain of IR did not decrease the association between IR and PKCδ, but even increased it under basal as well as in insulin-stimulated conditions. Finally, the binding to the JM region was specific for PKCδ, as binding of PKCα or other isoforms to either the JM or CT domain, either from unstimulated or from
insulin-stimulated cells, could not be detected. These findings are consistent with earlier reports and confirm and extend our earlier results showing that insulin induces a selective interaction between PKCδ and IR.

Our results regarding PKCδ binding to the JM region are compatible with those of Paz et al. (1997, 2000), who showed that this region in its non-phosphorylated form is sufficient to promote interactions with downstream effectors such as IRS-1. Phosphorylation of the latter most likely potentiates the interactions, but is not an absolute prerequisite. Other studies indicate that Tyr is poorly phosphorylated in vivo (White et al. 1988a,b, Tavare et al. 1988, Torneqvist et al. 1988), although the JM region is a major site for insulin-stimulated Ser phosphorylation (Feener et al. 1993). As we showed, isolated, non-phosphorylated JM regions effectively bind PKCδ in either its inactive or active form.

The results regarding PKCδ-mediated serine phosphorylation of IRS-1 agree with recent studies indicating that this isoform may also be involved in negative regulation of IRS-1, several putative serine sites having been identified (Greene et al. 2004, 2006, Waraich et al. 2008). Phosphorylation of IRS-1 Ser 307 in the cells expressing the different chimeras appears to support findings in recent studies showing that this serine residue may be a target for insulin-activated PKC isoforms, although in CHO-IR cells Ser24 is also specifically implicated. It was reported that Ser307 was one of 18 sites that could be phosphorylated by PKCδ, and that mutation of serine to alanine on this and two other sites completely abrogated the inhibitory effect of PKCδ on IRS-1 tyrosine phosphorylation in vitro (Greene et al. 2004, 2006). It should be emphasized that not only was IRS-1 Ser307 phosphorylation increased in PKCζ/δ-expressing cells, it was completely abrogated in cells expressing the PKCδ/ζ chimera. These observations further support the idea that PKCδ upregulates insulin signaling, and that the regulatory domain plays an important role in this process. Nonetheless, the possibility that PKCζ might have a dual effect similar to PKCζ, see (Liu et al. 2001) and also be involved in IRS-1 serine phosphorylation cannot be ruled out.

Our major findings can be summarized as follows. On stimulation by insulin, PKCδ and Src rapidly associate with each other and with the JM domain of IR, coinciding with increased tyrosine phosphorylation of IR, a process in which Src participates. Overexpression of the PKCδ regulatory domain leads to an increase in IR and IRS-1 tyrosine phosphorylation and in IR signaling. In cells in which the PKCδ regulatory domain is downregulated, such as in those expressing the PKCζ regulatory/PKCδ catalytic domains, Src interaction with IR is reduced (if not abrogated) even though the PKCζ/δ chimera associates with IR, probably via the δ catalytic domain. As a consequence, IR and IRS-1 tyrosine phosphorylations are reduced and serine phosphorylations increased, and IR signaling is downregulated. Thus, our findings strengthen the concept that PKCδ plays an important role in regulation of IR signaling. These effects appear to be dependent on the interactions of the regulatory domain with IR and IRS-1 and involve the participation of additional signaling proteins such as Src tyrosine kinase.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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