Activation of Protein Kinase C (α, β, and γ) by Insulin in 3T3/L1 Cells

TRANSFECTION STUDIES SUGGEST A ROLE FOR PKC-ζ IN GLUCOSE TRANSPORT*

We presently studied (a) insulin effects on protein kinase C (PKC) and (b) effects of transfection-induced, stable expression of PKC isoforms on glucose transport in 3T3/L1 cells. In both fibroblasts and adipocytes, insulin provoked increases in membrane PKC enzyme activity and membrane levels of PKC-α and PKC-β. However, insulin-induced increases in PKC enzyme activity were apparent in both non-down-regulated adipocytes and adipocytes that were down-regulated by overnight treatment with δ µ mol phorbol ester, which largely depletes PKC-α, PKC-β, and PKC-ε, but not PKC-ζ. Moreover, insulin provoked increases in the enzyme activity of immunoprecipitable PKC-ζ. In transfection studies, stable overexpression of wild-type or constitutively active forms of PKC-α, PKC-β₁, and PKC-β₂ failed to influence basal or insulin-stimulated glucose transport (2-deoxyglucose uptake) in fibroblasts and adipocytes, despite inhibiting insulin effects on glycogen synthesis. In contrast, stable overexpression of wild-type PKC-ζ increased, and a dominant-negative mutant form of PKC-ζ decreased, basal and insulin-stimulated glucose transport in fibroblasts and adipocytes. These findings suggested that: (a) insulin activates PKC-ζ, as well as PKC-α and β; and (b) PKC-ζ is required for, and may contribute to, insulin effects on glucose transport in 3T3/L1 cells.

3T3/L1 cells are useful models for studying insulin action, as they offer advantages of a transflectable cultured cell line and contain GLUT1 glucose transporters as fibroblasts, and acquire GLUT4, the major insulin-regulated glucose transporter, during differentiation. The signaling systems that are used by insulin to regulate glucose metabolism and other cellular functions in 3T3/L1 cells are, however, poorly understood. With respect to protein kinase C (PKC), some studies suggested that insulin does not activate this signaling system in either 3T3/L1 fibroblasts (1) or adipocytes (2), although in adipocytes, insulin was found to provoke an increase in cytosolic PKC activity (3), and, in fibroblasts, some insulin effects on the phosphorylation of eukaryotic initiation factors appeared to be PKC-dependent (4). In addition, studies with phorbol esters, as diacetyl glycerol (DAG) analogues that acutely activate and chronically deplete conventional PKCs (cPKCs) and novel PKCs (nPKCs), have suggested that such DAG-sensitive PKCs do not play a major role in insulin-stimulated glucose transport in 3T3/L1 cells (1, 3, 5). On the other hand, atypical PKCs (aPKCs) are not activated or depleted by DAG or phorbol esters, but may nevertheless be activated by other lipid signaling substances, e.g. polyphosphoinositides derived through the activation of phosphatidylinositol (PI) 3-kinase, a process that is activated by insulin.

Because of seemingly conflicting reports on overall PKC activation in 3T3/L1 cells, and because of the paucity of studies on atypical PKCs, such as PKC-ζ, in insulin action, we addressed these questions using several experimental approaches. Accordingly, we found that insulin increased DAG production, and stimulated the translocation of PKC-α and PKC-β from the cytosol to the membrane fraction, in 3T3/L1 cells. Perhaps, more interestingly, we found that insulin increased PKC enzyme activity, not only in membrane preparations from 3T3/L1 cells containing cPKCs, nPKCs, and aPKCs, but also in (a) membrane and cytosolic preparations from adipocytes in which cPKCs and nPKCs were largely depleted by phorbol ester treatment, and (b) in PKC-ζ immunoprecipitates from adipocyte lysates. Moreover, we found in stably transfected 3T3/L1 fibroblasts and adipocytes that: (a) expression of wild-type and constitutively active forms of PKC-α, PKC-β₁, and PKC-β₂ failed to influence basal or insulin-stimulated glucose transport, despite inhibiting insulin effects on glycogen synthesis; and (b) expression of wild-type PKC-ζ enhanced, and dominant-negative PKC-ζ inhibited, basal and insulin-stimulated glucose transport.

EXPERIMENTAL PROCEDURES

General Procedures—3T3/L1 cells were obtained from American Type Culture Collection (Rockville, MD) and were cultured as described (6) to yield fibroblasts and differentiated adipocytes. For adipocytes, insulin was withdrawn 48 h prior to experimentation, and for both fibroblasts and adipocytes, medium was changed to serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 25 mM glucose for 3 h, and then to glucose-free Krebs-Ringer phosphate (KRP) buffer for 30 min prior to experimental use. Cells were treated with vehicle, insulin (Elanco) or 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma) for the indicated times, keeping the total incubation time constant for all samples.

Assays of Total PKC Enzyme Activity—In Method I, PKC enzyme activity was measured in extracts of control and insulin-treated 3T3/L1 cells that were incubated and subsequently assayed in parallel, essentially as described previously in studies of rat adipocytes (7). In brief,
PKC-ζ and Glucose Transport in 3T3/L1 Cells

Cells from two or three 100-mm plates of each treatment group were pooled and homogenized in buffer I containing 0.25 M sucrose, 1.2 M EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, 20 μg/ml β-mercaptoethanol, and 20 μM Tris (pH 7.5). Cytosol and membrane fractions were obtained by centrifugation at 100,000 × g for 60 min, and the membrane fractions were washed with buffer II supplemented with 0.25 M sucrose, 1 mM NaHCO3, 5 mM MgCl2, 1 mM PMSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Membranes and cytosal fractions were obtained by centrifugation at 100,000 × g for 60 min, and the membrane fractions were subsequently immunoblotted with anti-PKC isoform-specific, polyclonal antisera. Antisera for PKC-ζ and PKC-β2, PKC-β3, and PKC-α were obtained for Western analysis of PKC activity. Enzyme activity was measured by phosphorylating PKC substrate derivatives. Approximately 40 μg [Ser159]PKC-α (19–31)-NH2 (Life Technologies, Inc.) or [Ser18]PKC-ε (153–164)-NH2 (Upstate Biotechnology, Inc.) in 100 μl of buffer containing 50 mM Tris/HCl (pH 7.5), 50 μM [γ-32P]ATP (DuPont NEN), 5 mM MgCl2, 100 μg sodium vanadate, 100 μM sodium pyrophosphate, 1 μM CaCl2, 1 mM NaF, and 100 μM EGTA, was incubated for 5 min (or treated with 1 μM calyculin A) in a total volume of 20 μl. In the absence of substrate, the reaction mixture was spotted on P-81 filter papers, washed in 5% acetic acid, and counted for [32P]. In this assay, membrane PKC enzyme activity is dependent upon endogenous lipid and non-lipid co-factors. In experiments in which the cytosal was assayed, PS (40 μg/ml) was added to provide a phospholipid milieu. These assays were conducted in the presence and absence of peptide substrate to define substrate-dependent PKC activity. 32PO4 incorporation in the absence of substrate accounted for only 10–20% of that observed in presence of substrate. In both methods of assay, RO31-8220 (Roche; kindly supplied by Dr. Geoff Lawton), a relatively specific PKC inhibitor, virtually abolished the transfection of PKC into 3T3/L1 cells—Four plasmid eukaryotic expression vectors, pMTH, pMV7, pMV12, and pCDNA3 (Invitrogen), were used to transfected PKC isoforms. pMTH, pMV7, and pCDNA3 vectors contain a neomycin resistance gene, and pMV12 is identical to pMTH, except that the neomycin resistance gene is replaced by a hygromycin resistance gene. PKC-β1, PKC-β2, and PKC-β3, by ribonuclease protection assay have been described previously (12). Transfection of PKC into 3T3/L1 Cells—Four plasmid eukaryotic expression vectors, pMTH, pMV7, pMV12, and pCDNA3 (Invitrogen), were used to transfected PKC isoforms. Glycogen synthesis was assayed by incubating cells for 60 min in KRP buffer containing 5 mM glucose and [U-14C]-glucose and glucose transport and immunoreactive PKC in parallel. [3H]2-Deoxyglucose (DuPont NEN) uptake was determined as described (16). Results in clones expressing significant amounts of transfected PKC (see below) were subsequently pooled and compared as a group to parallel-assayed controls. Glycogen synthesis assays were conducted by incubating cells for 60 min in KRP buffer containing 5 mM glucose and [U-14C]-glucose

In some experiments, we also used β1- and β2-specific antisera kindly supplied by Dr. Susan Jakub. Epitope specificities of antisera were confirmed by showing that signals were lost when assays were conducted in the presence of immunizing peptide, and/or by incubation with recombinant PKCs (see Refs. 9 and 11). Antisera specificities for PKC-α, PKC-β1, PKC-β2, and PKC-β3 were also verified by overexpression of plasmids containing cDNAs that encode each of these isoforms in 3T3/L1 cells (see below). Antisera for GLUT1 and GLUT4 were raised from rabbits immunized with peptides corresponding to the C terminus of GLUT1 and GLUT4, respectively. Immunodetection was accomplished by antibody-associated alkaline phosphatase colorimetric staining as described previously (9) or, in most cases, by chemiluminescence (ECL, Amersham). Blots were scanned and quantified with an LKB laser densitometer or, in most cases, with a Bio-Rad Chemiluminescence Molecular Imaging System, and results were expressed relative to the respective controls, on the same blot, set at 100%.

Assays of PKC mRNA Levels—Methods for measurement of PKC-β1, PKC-β2, and PKC-β3 by ribonuclease protection assay have been described previously (12).

Transfection of PKC into 3T3/L1 Cells—Four plasmid eukaryotic expression vectors, pMTH, pMV7, pMV12, and pCDNA3 (Invitrogen), were used to transfected PKC isoforms. Glycogen synthesis was assayed by incubating cells for 60 min in KRP buffer containing 5 mM glucose and [U-14C]-glucose and glucose transport and immunoreactive PKC in parallel. [3H]2-Deoxyglucose (DuPont NEN) uptake was determined as described (16). Results in clones expressing significant amounts of transfected PKC (see below) were subsequently pooled and compared as a group to parallel-assayed controls. Glycogen synthesis assays were conducted by incubating cells for 60 min in KRP buffer containing 5 mM glucose and [U-14C]-glucose

2 Insulin effects on PKC-ζ activation were found to be inhibited by both wortmannin and LY294002, suggesting dependence upon PI 3-kinase activation (M. L. Standaert, L. Galloway, P. Karnam, G. Bandyopadhyay, and R. V. Farese, submitted for publication).
PKC-ζ and Glucose Transport in 3T3/L1 Cells

(153–164)-NH₂ as the substrate in Method II assays. Method II was not used in fibroblasts.

The incorporation of 32PO₄ in the above assays presumably reflected the sum of enzyme activities of all PKC isoforms, including α, β, ε, and ζ (see below). In order to gain insight into the question of whether insulin activates atypical PKCs, such as PKC-ζ, we took advantage of the fact that overnight treatment with TPA largely depleted PKC-α, β, and ε, but had no effect on 70-kDa PKC-ζ (Fig. 2). (Note: unlike the situation in BCH3-1 myocytes (16), neither TPA nor insulin treatment altered PKC-α, PKC-β₁, PKC-β₂, or PKC-ε mRNA levels in 3T3/L1 adipocytes, and induction or retention of PKC-β₂ was not observed after TPA treatment.) As shown in Table I, in the absence of TPA pretreatment, insulin provoked a 54% increase in membrane-dependent 32PO₄ incorporation into [Ser(153–164)]PKC-ε-(153–164)-NH₂, a preferred substrate for both PKC-ε and PKC-ζ (PKC-α and PKC-β are only 50 and 30% as effective; see Ref. 20). Moreover, after overnight TPA pretreatment, there were 60–70% decreases in overall 32PO₄ incorporation into this substrate, presumably reflecting losses of cPKCs and nPKCs, but insulin-induced increases in PKC activity nevertheless remained apparent in membranes (56% increase) and, somewhat surprisingly, became more clearly evident and statistically significant in cytosol (49% increase) fractions. These results were in keeping with the possibility that insulin activated TPA-resistant PKCs, such as PKC-ζ, which is distributed between cytosol and membrane fractions and is not translocated during agonist treatment.

Immunoprecipitable PKC-ζ Enzyme Activity—In order to test the possibility that insulin activated PKC-ζ, we studied enzyme activity in specific PKC-ζ immunoprecipitates and found that enzyme activity of PKC-ζ immunoprecipitates was increased more than 2-fold after treating adipocytes for 10 min with 100 nM insulin (Fig. 3). As stated under “Experimental Procedures,” there was little or no significant immunoreactive PKC-α, β, or ε in the PKC-ζ immunoprecipitates and preimmune serum did not immunoprecipitate PKC-ζ (Fig. 3). Insulin did not affect the amount of PKC-ζ recovered in these precipitates (approximately 50%), and it may be surmised that insulin increased the specific enzyme activity of PKC-ζ, apparently through a factor or covalent modification that was retained during immunoprecipitation and assay procedures.²

Acute Changes in Immunoreactive PKC—In 3T3/L1 adipocytes, insulin provoked time-dependent decreases in cytosolic, and increases in membrane, PKC-α, and PKC-β (Figs. 1 and 4). The changes in immunoreactive PKC-α and PKC-β were maximal at 2–10 min of insulin treatment and then diminished at 20 min. In 3T3/L1 fibroblasts, the relative effects of insulin on the translocation of PKC-α and PKC-β were approximately one-half of those observed in adipocytes (summarized in Fig. 1). In contrast to PKC-α and PKC-β, insulin had no consistent effect on the cellular distribution of either PKC-ε or the 70-kDa form of PKC-ζ in adipocytes (Fig. 4) and fibroblasts (data not shown). Acute (10 min) TPA treatment, on the other hand, stimulated the translocation of PKC-ε, as well as PKC-α and PKC-β, but not 70-kDa

### Table I

| Pretreatment | Cytosol | Membrane |
|--------------|---------|----------|
|              | Control | Insulin (p vs. control) | Control | Insulin (p vs. control) |
| None         | 123 ± 20 | 164 ± 12 (NS) | 37 ± 4 | 55 ± 5 | 96 ± 2 | 148 ± 12 (p < 0.005) |
| 5 µM TPA (24 h) | 37 ± 4 | 55 ± 5 | 32 ± 5 | 50 ± 5 (p < 0.05) |

*32PO₄ incorporated pmol/mg protein/min*
PKC-\(\zeta\) in adipocytes (Fig. 2). It may be noted in Figs. 2 and 4 that, in addition to the 70-kDa form of PKC-\(\zeta\), the anti-PKC-\(\zeta\) antiserum (from Life Technologies, Inc.) also cross-reacted with an 80-kDa moiety that translocated in response to acute insulin and TPA treatment (Figs. 2 and 4) and down-regulated with overnight, 5 \(\mu\)M TPA treatment. This 80-kDa band is most likely cross-reacting PKC-\(\alpha\), as it mirrored changes in authentic PKC-\(\alpha\) (measured by PKC-\(\alpha\) antiserum), and (b) increased upon transfection of cells with plasmids containing the PKC-\(\alpha\) cDNA insert (data not shown). It may also be noted in Figs. 2 and 4 that the cytosolic PKC-\(\beta\) that translocated better in response to insulin treatment, and down-regulated better in response to overnight TPA treatment, had an apparent mass of 80 kDa on SDS-PAGE. In addition, the cytosol contained a 75-kDa PKC-\(\beta\) moiety that was, in general, more plentiful, but less responsive to acute insulin or TPA treatment, as compared to the 80-kDa moiety. On the basis of overexpression studies (see below), this 75-kDa moiety appeared to be predominately a lower \(M\) form of PKC-\(\beta\), and it is of interest that, in other cell types, a similarly migrating PKC-\(\beta\) has been reported to be poorly activated, as it apparently lacks key prerequisite phosphorylations (see Ref. 21).
Effects of Cellular Differentiation on PKC Isoforms—There were approximately 50% decreases in the cellular concentrations of PKC-α and PKC-β following differentiation of fibroblasts into adipocytes; PKC-ε, on the other hand, increased slightly in adipocytes, and PKC-ζ did not change appreciably during differentiation (data not shown). Immunoreactive PKC-δ was not detectable in either fibroblasts or adipocytes. As shown in Fig. 5, when untransfected cells were blotted with β1- and β2-specific antisera, PKC-β1 (75- and 80-kDa moieties) was readily detectable in fibroblasts and adipocytes, whereas PKC-β2 was poorly detectable, if at all.

Stable Expression of PKC—Stable transfection of cells with PKC-β1, and, even more strikingly, PKC-β2, resulted in sizable overexpression of these isoforms in both fibroblasts and adipocytes (Fig. 5). Transfection-induced increases in immunoreactive PKC-β2 were comparable using a variety of vectors, i.e. pMTH, pMV7, pMV12, and pCDNA3. Transfection-induced expression of PKC-β2 yielded primarily an 80-kDa moiety, whereas expression of PKC-β1 yielded 75-kDa, as well as 80-kDa, moieties (Fig. 5). Stable transfection of cells with cDNAs encoding PKC-α and PKC-ζ increased the levels of these PKCs approximately 2–3-fold (Fig. 6 and Table II), albeit to a lesser relative degree than that observed with PKC-β2 transfection, perhaps reflecting higher basal levels of the PKC-α and PKC-ζ isoforms. In contrast to PKC-α, PKC-β2, and PKC-ζ, we were not able to express PKC-δ or significantly overexpress PKC-ε, as judged by immunoblot analysis (data not shown).

In the case of PKC-β2, we verified that transfection resulted in specific increases in PKC-β2 mRNA (Fig. 5). We also verified that transfected PKC-β2 was down-regulated by overnight 5 μM TPA treatment (Fig. 5), and it may therefore be surmised that transfected PKC-β2 was biologically active (also see below). In some cases, we verified that there were increases in PKC enzyme activity in transfected cells, e.g. as shown in Fig. 7, expression of normal PKC-α and PKC-β2 increased adipocyte membrane and cytosol PKC enzyme activities substantially, and both enzyme activities were further increased in adipocytes transfected with constitutively active forms of these PKCs. As shown in Fig. 6, and as reported by Ways et al. (22), there were concomitant, but variable increases in 80-kDa PKC-α (i.e. the upper band in PKC-ζ blots whose identity as PKC-α was confirmed with anti-PKC-α antisera) in PKC-ζ transfectants, regardless of whether they were wild-type or dominant-negative mutants. As will become apparent (see below), this co-expression of PKC-α could not explain observed changes in glucose transport experiments. In contrast to PKC-α, we did not observe changes in PKC-β or PKC-ε levels in PKC-ζ transfectants.

Effects of PKC Expression on Glucose Transport—As shown in Fig. 8, there was little or no effect of overexpression of wild-type PKC-α, PKC-β1, or PKC-β2, on basal or insulin-stimulated glucose transport in either fibroblasts or adipocytes. Similarly, the expression of other constructs encoding either wild-type or constitutively active (pseudosubstrate-deleted) forms of PKC-α and PKC-β2 failed to influence glucose transport significantly, i.e. relative to untransfected cells or cells transfected with empty vectors, in control or insulin-stimulated fibroblasts and adipocytes (Fig. 9). In other experiments (data not shown), the expression of point-mutated, constitutively active and dominant-negative forms of PKC-α (those obtained from Dr. Peter Parker) also failed to alter glucose transport responses (data not shown).

In contrast to PKC-α and PKC-β, the overexpression of wild-type PKC-ζ increased basal and insulin-stimulated glucose transport in fibroblasts (Figs. 8 and 10). In adipocytes (Fig. 10), overexpression of PKC-ζ increased glucose transport in control fibroblasts

![Fig. 6. Transfection-induced increases in PKC-α (A) and PKC-ζ (B) in 3T3/L1 fibroblasts and adipocytes. - , untransfected cells; +, cells transfected with pMV7-PKC-α or pCDNA3-PKC-ζ.](image_url)

**Table II**

Relative changes in immunoreactive PKC-ζ, GLUT1, and GLUT4 levels in PKC-ζ transfectants

| Transfectant       | Fibroblast (mean ± S.E.) | Adipocyte (mean ± S.E.) |
|--------------------|--------------------------|-------------------------|
|                    | PKC-ζ        | GLUT1     |                  | PKC-ζ        | GLUT1     | GLUT4     |
| Wild-type PKC-ζ    | 169 ± 10   | 107 ± 5   |                  | 213 ± 11   | 98 ± 8    | 93 ± 8    |
| Dominant-negative PKC-ζ | 182 ± 23 | 147 ± 8   |                  | 193 ± 40   | 107 ± 7   | 126 ± 15  |

Clones from Fig. 10 were examined for immunoreactive 70-kDa PKC-ζ, GLUT1, and GLUT4, and levels were quantitated with a Bio-Rad Molecular Imaging System.
FIG. 8. Dose-dependent effects of insulin on \[^{3}H\]2-deoxyglucose uptake in 3T3/L1 fibroblasts (left panels) and adipocytes (right panels) transfected with plasmids containing cDNAs encoding PKC-\(\alpha\) (in PMV7) or PKC-\(\zeta\) (in pCDNA3) (upper panels) and PKC-\(\beta\_2\) or PKC-\(\beta\_1\) (in pMV12, pMV12, or pMTH) (lower panels). Untransfected cells were incubated with CON and \(\bigcirc\). Results with cells transfected with empty vectors were not significantly different from control, and, for simplicity, are not shown. Values are mean \(\pm\) S.E. of \(n\) observations.

PKC-\(\zeta\) and Glucose Transport in 3T3/L1 Cells

and submaximally stimulated cells, but the maximal insulin effect was not changed significantly. Of further interest, a dominant-negative, point-mutated form of PKC-\(\zeta\) inhibited basal and insulin-stimulated glucose transport in both fibroblasts and adipocytes (Fig. 10). As shown in Table II, immunoreactive PKC-\(\zeta\) levels were increased approximately 2-fold in wild-type and dominant negative PKC-\(\zeta\) transfectants, and observed changes in glucose transport in PKC-\(\zeta\) transfectants could not be explained by changes in total GLUT1 and/or GLUT4 levels. As shown in Fig. 11, PKC enzyme activity in TPA-down-regulated adipocytes was 2-fold higher in cytosol fractions of cells transfected with wild-type, but not dominant-negative, PKC-\(\zeta\); this further suggested that TPA-resistant PKC enzyme activity largely reflected PKC-\(\zeta\) activity, as it would be expected to be increased in wild-type overexpressers, but not with expression of catalytically inactive PKC-\(\zeta\). Further, since immunoreactive PKC-\(\zeta\) was increased by approximately 2-fold in both wild-type and dominant-negative transfectants, it may also be surmised that the specific enzyme activity of total PKC-\(\zeta\) was decreased by 50% in dominant-negative transfectants. As shown in Fig. 12, in keeping with increased basal glucose transport activity, both GLUT4 and GLUT1 were more plentiful in plasma membranes and less plentiful in microsomes in wild-type PKC-\(\zeta\) overexpressors, as compared to controls. Also, with insulin treatment, resultant GLUT4 and GLUT1 levels in plasma membranes (approximately 2-fold increases were seen) in wild-type PKC-\(\zeta\) overexpressers were equal to, if not greater than, the levels seen in controls. In contrast, in PKC-\(\zeta\)-dominant-negative transfectants, insulin effects on GLUT4 and GLUT1 appeared to be blunted (Fig. 12).

**Effects of PKC-\(\alpha\) and PKC-\(\beta\) Expression on Glycogen Synthesis**—In contrast to glucose transport, the expression of both wild-type and constitutively active forms of PKC-\(\alpha\) and PKC-\(\beta\_2\) led to inhibition of insulin-induced increases in \[^{14}\text{C}\]glucose incorporation into glycogen (Fig. 7). This confirmed that these transfected PKCs were biologically, as well as enzymatically (also see Fig. 7), active. The greatest inhibition of glycogen synthesis was observed with constitutively active PKC-\(\alpha\). Along these lines, it was of interest to find that overexpression of wild-type PKC-\(\zeta\), and the expression of dominant-negative PKC-\(\zeta\) failed to alter insulin effects on glycogen synthesis (data not shown); this suggested that, in these PKC-\(\zeta\) transfections, initial insulin signaling was intact, and inhibitory effects of PKC on glycogen synthesis were isoform-dependent.

**DISCUSSION**

We presently found that insulin provoked increases in membrane PKC enzyme activity and stimulated the translocation of immunoreactive PKC-\(\alpha\) and PKC-\(\beta\) to membrane fractions in 3T3/L1 adipocytes and fibroblasts. It therefore appeared that increases in membrane PKC enzyme activity, at least partly, reflected increases in PKC-\(\alpha\) and PKC-\(\beta\). However, the enzyme assays of total PKC presently used may also have reflected PKC-\(\zeta\), which is activated by phosphatidic acid, polyphosphoinositides, phosphatidylserine, and certain fatty acids (23–25). Accordingly, insulin is known to activate PI 3-kinase in 3T3/L1 cells (26). In addition, insulin effects on PKC activity were
PKC-ζ and Glucose Transport in 3T3/L1 Cells

![Graphs and images related to PKC-ζ and glucose transport in 3T3/L1 cells.](graph1)

**FIG. 10.** Effects of insulin on [3H]2-deoxyglucose uptake in 3T3/L1 fibroblasts (upper panel) and adipocytes (lower panel) transfected with plasmids containing cDNAs encoding wild-type and dominant-negative forms of PKC-ζ. Cells were not transfected (CONTROL) or transfected with empty vector (pCDNA3) or vector containing cDNAs encoding wild-type (WT) or dominant-negative (DOM-NEG) PKC-ζ. Insulin concentrations (0, 5, and 100 nM) are indicated at bottom of panels. Values are mean ± S.E. of n clones, each assayed in quadruplicate at each insulin concentration. Asterisks indicate p < 0.05 (t test comparison to untransfected controls). See Table I for levels of immunoreactive PKC-ζ, GLUT1, and GLUT4 in these clones.

Evident in both cytosol and membrane fractions of 3T3/L1 adipocytes largely depleted of PKC-α, β, and ε by overnight 5 μM TPA pretreatment. The latter finding suggested that insulin activated PKC-ζ, as well as PKC-α and β, and, indeed, this was confirmed by finding that insulin provoked increases in enzyme activity of immunoprecipitable PKC-ζ.

Although we did not presently study the mechanism of PKC-ζ activation in 3T3/L1 cells, we have found, in rat adipocytes, that PKC-ζ is rapidly phosphorylated during insulin action, and both wortmannin and LY294002 inhibit insulin-induced activation of immunoprecipitable PKC-ζ. It therefore seems likely that PI 3-kinase activation is required for PKC-ζ activation, and we are currently trying to identify the kinase responsible for PKC-ζ phosphorylation.

The failure to observe a significant change in the subcellular distribution of PKC-ε in 3T3/L1 adipocytes during insulin treatment contrasts with observations in rat adipocytes (9). However, it should be noted that: (a) PKC-ε was more prevalent in membrane (relative to cytosol) fractions of 3T3/L1 cells; and (b) insulin activates, but does not translocate, PKC-ε in fetal chick neurons, apparently through a covalent modification (27). Thus, the failure to observe a translocation of PKC-ε does not necessarily mean that this isoform is not activated by insulin in 3T3/L1 cells.

Glucose transport effects of insulin have been reported to be increased by transfection-induced expression of PKC-β2 in NIH3T3 cells that have low levels of endogenous insulin recep-
fore inhibit only certain insulin-sensitive signaling factors, or, alternatively, more distal regulatory factors, or glycogen synthase itself (see Refs. 28–30). Along these lines, it should also be noted that PKC-dependent inhibition of glycogen synthase may occur as a paradoxical restraining mechanism during insulin action, as we have found that the PKC inhibitor, RO 31-8220, increases insulin effects on glycogen synthesis in rat adipocytes and rat skeletal muscle.3

In contrast to PKC-α and PKC-β, the overexpression of PKC-ζ in fibroblasts provoked increases in basal, submaximal, and maximal insulin-stimulated glucose transport. Although not entirely certain, the increase in basal transport may reflect some of the expressed PKC-ζ may have been activated, even in the absence of agonist addition. In adipocytes, although basal and submaximal insulin effects were enhanced by PKC-ζ, the maximal insulin effect was on the average, unchanged, perhaps reflecting a rate limitation caused by factors other than PKC-ζ, e.g., GLUT4 levels. Along the latter lines, in both fibroblasts and adipocytes, it seemed clear that observed alterations in glucose transport in PKC-ζ transfecants could not be explained by changes in total levels of GLUT1 or GLUT4. Moreover, in adipocytes overexpressing wild-type PKC-ζ, the observed changes in glucose transport, both basally and in response to insulin, appeared to reflect changes in the subcellular distribution of both GLUT4 and GLUT1; thus, PKC-ζ overexpression appeared to alter the translocation of both GLUT4 and GLUT1.

In keeping with the possibility that PKC-ζ may participate in the regulation of glucose transport, a dominant-negative form of PKC-ζ inhibited basal and insulin-stimulated glucose transport in both fibroblasts and adipocytes. Here again, the inhibition of glucose transport could not be readily explained by changes in total GLUT1 and/or GLUT4 levels, and blunted responses of both transporters appeared to contribute to decreases in glucose transport. In addition, the intactness of glycogen synthesis responses in dominant-negative PKC-ζ transfecants suggested that initial insulin signaling sequences were intact in these cells. Nevertheless, further studies will be needed to determine whether the observed inhibitory effects on glucose transport were directly due to the dominant-negative action of the expressed mutant PKC-ζ.

In summary, insulin activated PKC-ζ, as well as PKC-α and PKC-β, in 3T3/L1 cells. Wherea, the stable expression of both wild-type and constitutively active forms of PKC-α, PKC-β1 and PKC-β2 failed to alter basal or insulin-stimulated glucose transport, the stable overexpression of PKC-ζ stimulated, and a dominant-negative form of PKC-ζ inhibited, basal and insulin-stimulated glucose transport in 3T3/L1 cells. Our findings therefore suggested that PKC-ζ may contribute to insulin-stimulated glucose transport in 3T3/L1 cells. Further studies will be required to test this hypothesis.

REFERENCES

1. Merrall, N. W., Wakejam, M. J., Plevin, R., and Gould, G. W. (1993) Biochim. Biophys. Acta 1177, 191–198
2. Blackshear, P. J., Haput, D. M., and Stumpo, D. J. (1991) J. Biol. Chem. 266, 10946–10952
3. Klip, A., Ramal, T., and Kolvitz, U. M. (1988) Endocrinology 123, 296–304
4. Morley, S. J., and Traugh, J. A. (1990) J. Biol. Chem. 269, 10611–10616
5. Gibbs, M., Calderhead, D. M., Holman, G. D., and Gould, G. W. (1993) Biochem. J. 275, 145–150
6. Kazma, I., Baltensperger, K., Klarlund, J., Porras, A., Santos, E., and Czech, M. P. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 4460–4464
7. Ishizuka, T., Cooper, D. R., and Farese, R. V. (1989) FEBS Lett. 257, 337–340
8. Yamada, K., Staendaert, M. L., Yu, B., Mischak, H., Cooper, D. R., and Farese, R. V. (1994) Arch. Biochem. Biophys. 312, 167–172
9. Farese, R. V., Staendaert, M. L., Francia, A. J., Ways, K., Arnold, T. P., Hernandez, H., and Cooper, D. R. (1992) Biochem. J. 288, 319–323
10. Roth, B. L., Mehegen, J. P., Jacobowitz, D. M., Robey, F., and Iadarola, M. J. (1989) J. Neurochem. 52, 215–221
11. Yamada, K., Avignon, A., Staendaert, M. L., Cooper, D. R., Spencer, B., and Farese, R. V. (1995) Biochem. J. 308, 177–180
12. Avignon, A., Staendaert, M. L., Yamada, K., Mischak, H., Spencer, B., and Farese, R. V. (1995) Biochem. J. 304, 141–147
13. Goodnight, J., Mischak, H., Kelch, W., and Mushinski, J. F. (1995) J. Biol. Chem. 270, 9991–10001
14. Housey, G. M., Johnson, M. D., Hsiao, W. L., O’Brian, C. A., Murphy, J. P., Kirschmeier, P., and Weinstein, I. B. (1988) Cell 52, 543–554
15. Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S., and Weinstein, I. B. (1988) DNA 7, 219–225
16. Chalfant, C. E., Mischak, H., Watson, J. E., Winkler, B. C., Goodnight, J., Farese, R. V., and Cooper, D. R. (1995) J. Biol. Chem. 270, 13326–13332
17. Diaz-Meco, M. T., Muniego, M. M., Sanchez, P., Lozano, J., and Moscat, J. (1996) Mol. Cell. Biol. 16, 105–114
18. Genet, E. M., Parker, P. J., and Cantrell D. A. (1995) J. Biol. Chem. 270, 9833–9839
19. Kaibuchi, K., Fukumoto, Y., Oku, N., Takai, Y., Araki, K., and Muramatsu, M. (1989) J. Biol. Chem. 264, 13492–13498
20. Kazianietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg, P. M. (1993) Mol. Pharmacol. 44, 296–307
21. Cazanave, S. M., and Parker, P. J. (1993) J. Biol. Chem. 268, 17559–17563
22. Days, D. K., Posecanay, K., deVente, J., Garria, T., Chen, J., Hooker, J., Qin, W., Cook, P., Fletcher, D., and Parker, P. (1994) Cell. Growth Diff. 5, 1195–1203
23. Nakamura, S., and Nishizuka, Y. (1994) J. Biochem. (Tokyo) 115, 1029–1034
24. Nakashima, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 288, 13–16
25. Limatola, C., Schaap, D., Mosdaera, W. H., and van Blitterswijk, J. (1994) Biochem. J. 304, 1001–1008
26. Kotani, K., Caruzzi, A. J., Sakaue, H., Hara, K., and Robinson, L. J. (1995) Biochem. Biophys. Res. Commun. 209, 343–348
27. Heydenreich, K. A., Toledo, S. P., Brunton, L. L., Watson, M. J., Daniel-Issakani, S., and Strulovici, B. (1990) J. Biol. Chem. 265, 15076–15082
28. Roach, P. J., and Goldman, M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 1710–1712
29. Ahmad, Z., Lee, P. T., DePauli-Roach, A., and Roach, P. J. (1984) J. Biol. Chem. 259, 8743–8747
30. Blackmore, P. F., Strickland, W. G., Boecino, S. B., and Exton, J. H. (1986) Biochem. J. 237, 255–242