Protein Kinase C-ζ as a Downstream Effector of Phosphatidylinositol 3-Kinase during Insulin Stimulation in Rat Adipocytes

POTENTIAL ROLE IN GLUCOSE TRANSPORT*

Mary L. Standaert, Lamar Galloway, Purushotham Karnam, Gautam Bandyopadhyay, Jorge Moscat‡, and Robert V. Farese‡§

From the J. A. Haley Veterans’ Hospital Research Service and Departments of Internal Medicine and Biochemistry/Molecular Biology, University of South Florida College of Medicine, Tampa, Florida 33612 and 3Centro de Biología Molecular “Severo Ochoa,” Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Insulin provoked rapid increases in enzyme activity of immunoprecipitable protein kinase C-ζ (PKC-ζ) in rat adipocytes. Concomitantly, insulin provoked increases in 32P labeling of PKC-ζ both in intact adipocytes and during in vitro assay of immunoprecipitated PKC-ζ; the latter probably reflected autophosphorylation, as it was inhibited by the PKC-ζ pseudosubstrate. Insulin-induced activation of immunoprecipitable PKC-ζ was inhibited by LY294002 and wortmannin; this suggested dependence upon phosphatidylinositol (PI) 3-kinase. Accordingly, activation of PI 3-kinase by a pYXXM-containing peptide in vitro resulted in a wortmannin-inhibitable increase in immunoprecipitable PKC-ζ enzyme activity. Also, PI, 3,4,5-(PO4)3, PI, 3,4,5,6-(PO4)3, and PI, 4,5,6-(PO4)3 directly stimulated enzyme activity and autophosphorylation in control PKC-ζ immunoprecipitates to levels observed in insulin-treated PKC-ζ immunoprecipitates. In studies of glucose transport, inhibition of immunoprecipitated PKC-ζ enzyme activity in vitro by both the PKC-ζ pseudosubstrate and RO 31-8220 correlated well with inhibition of insulin-stimulated glucose transport in intact adipocytes. Also, in adipocytes transiently expressing hemagglutinin antigen-tagged GLUT4, co-transfection of wild-type or constitutive PKC-ζ stimulated hemagglutinin antigen GLUT4 translocation, whereas dominant-negative PKC-ζ partially inhibited it. Our findings suggest that insulin activates PKC-ζ through PI 3-kinase, and PKC-ζ may act as a downstream effector of PI 3-kinase and contribute to the activation of GLUT4 translocation.

The atypical protein kinase C (PKC),1 PKC-ζ, is ubiquitously expressed, but little is known about its activation or actions. This ignorance partly derives from the fact that PKC-ζ is not activated by membrane-associated diacylglycerol (DAG) or phorbol esters, generally does not translocate appreciably from cytosol to membrane when activated, and is not depleted by prolonged phorbol ester treatment. Consequently, methods used to evaluate DAG-sensitive conventional (α, β, and γ) and novel (δ, ε, η, and θ) PKCs are not relevant to PKC-ζ and other DAG-insensitive, atypical PKCs. Although not activated by DAG, PKC-ζ is activated in vitro by phosphatidylerine and polyphosphoinositides, including D3-PO4 derivatives of phosphatidylinositol (PI) (1, 2). Because of its activation by polyphosphoinositides, PKC-ζ has been suspected to operate downstream of PI 3-kinase; however, direct experimental evidence for this suspicion is lacking, particularly in intact cells. Since insulin increases total polyphosphoinositide levels (3–6), probably largely through PI 3-kinase activation (7), we examined the possibility that insulin activates PKC-ζ by a PI 3-kinase-dependent mechanism. To this end, we assayed immunoprecipitable PKC-ζ (a) following treatment of intact adipocytes with insulin in the presence and absence of PI 3-kinase inhibitors; (b) following PI 3-kinase activation in vitro by a pYXXM-containing peptide; and (c) in response to polyphosphoinositides added directly to the assay of PKC-ζ in vitro. Also, since PI 3-kinase appears to be required for insulin-stimulated GLUT4 translocation and subsequent glucose transport, we questioned whether PKC-ζ, as an effector of PI 3-kinase, may play a role in this process. To this end, we used PKC-ζ inhibitors and examined the effects of transiently transfected wild-type, constitutive, and dominant-negative PKC-ζ in adipocytes co-transfected with hemagglutinin antigen (HA)-tagged GLUT4.

MATERIALS AND METHODS

As described (8), rat adipocytes were prepared from epididymal fat pads of 200–250-g male Sprague-Dawley rats. For acute incubations, the cells were suspended and incubated in glucose-free Krebs-Ringer phosphate (KRP) buffer containing 1% bovine serum albumin with or without insulin (Elanco), tetradecanoylphorbol-13-acetate (TPA; Sigma), wortmannin (Sigma), LY294002 (Biomol), RO 31-8220 (Alexis), and/or myristoylated PKC-ζ or PKA pseudosubstrate peptides (Quality Controlled Biochemicals) as indicated in the text. For overnight incubations, the cells were suspended in Dulbecco's modified Eagle’s medium (Life Technologies, Inc.) containing 1% bovine serum albumin, 5% gravine, and other indicated treatments; after overnight incubations, the cells were transferred to glucose-free KRP for acute treatments. Following acute treatments, glucose transport was assayed by measurement of [3H]2-deoxyglucose (2-DOG; 0.1 mM; NEN Life Science Products) uptake over 1 min as described (8), or PKC-ζ enzyme activity was assayed as described below.

In some experiments, adipocytes were co-transfected by electroporation (as described by Quon et al. (9, 10) in the presence of (a) pCIS2 eukaryotic expression vector containing cDNA encoding HA-tagged GLUT4 (kindly supplied by Drs. Michael Quon and Simeon Taylor) and (b) pCDNA3 eukaryotic expression vector alone (vector) or pCDNA3 containing cDNA encoding either (i) various forms of PKC-ζ (wild-type

* Funds for this research work were provided by the Department of Veterans Affairs Merit Review Program and National Institutes of Health Research Grant DK38079. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Research Service (VAR 151), J. A. Haley Veterans’ Hospital, 13000 Bruce Downs Blvd., Tampa, FL 33612. Tel.: 813-972-7622; Fax: 813-972-7623.

1 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PI, phosphatidylinositol; KRP, Krebs-Ringer phosphate; TPA, tetradecanoylphorbol-13-acetate; HA, hemagglutinin antigen; 2-DOG, [3H]2-deoxyglucose.
and dominant-negative forms of PKC-ζ were described previously (11), and the constitutive form of PKC-ζ was generated by mutating Ala\textsuperscript{119} in the pseudosubstrate region to Asp\textsuperscript{119} (i.e. GGC to GAC) using a Trans
tamer Site-Directed Mutagenesis Kit from CLONTECH; the sequence of this construct was confirmed by sequence analysis) or (i) a dominant-
negative mutant form of the δ85 subunit of PI-3 kinase (δ85δ; kindly supplied by Drs. Wataru Ogawa and Masato Kasuga). These cells were 
incubated overnight in Dulbecco’s modified Eagle’s medium containing 
25 mM Hepes, 200 nM (\(N^\bullet-N^\bullet\)-2-phenylisopropyl)-adenosine, and 5% 
bovine serum albumin to allow time for expression of the inserts. Cells 
were then washed and resuspended in glucose-free KRP medium and 
treated with or without 10 nM insulin for 30 min prior to the addition of 
2 mM KCN and assessment of the translocation of HA-tagged GLUT4 to 
the plasma membrane, as described (9, 10). This treatment was per-
formed using anti-HA mouse monoclonal antibody (Berkeley Antibody 
Co.) and \(^{125}\)I-labeled sheep anti-mouse IgG (second) antibody (Amer-
sham Corp.) to measure cell surface content of expressed GLUT4 con-
taining the exofacial HA epitope.

To measure PKC-ζ enzyme activity, as described previously (11), the 
reactions were rapidly stopped by adding ice-cold KRP medium, and cells were washed and sonicated in Buffer A (20 mM Tris/HC1 (pH 7.5), 5 mM 
\(\beta\)-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 \(\mu\)g/ml leupeptin, 20 \(\mu\)g/ml aprotinin, 1 mM 
\(\text{Na}_2\text{VO}_4\), 1 mM \(\text{Na}_3\text{P}_2\text{O}_7\), and 1 mM NaF). Homogenates were cen-
trifuged at 500 \(g\) for 10 min to remove nuclei, debris, and the fat cake. 

**FIG. 1.** Time- and dose-dependent effects of insulin on immunoprecipi-
table PKC-ζ in rat adipocytes. Cells were treated with vehicle controls: open 
circles, CON) or 10 nM insulin (filled circles, INS) for indicated times (A and B); 
with 10 nM insulin for 10 min (A, inset); or with increasing doses of insulin for 10 
min (B, inset). PKC-ζ was then immunoprecipitated from total cell lysates and 
assayed. Biphasic time courses from two 
fully separate experiments are shown in the main curves of A and B. A, inset 
depicts the time course of the assay in vitro 
in control and insulin-treated immuno-
precipitates; note that absolute values are 
given in this inset (i.e. cpm \(\times 10^3\)immu-
noprecipitates). All other values (mean \(\pm\) 
S.E. of 4–6 determinations) depict insulin 
effects in intact cells (in situ) and are ex-
pressed relative to the corresponding mean control value, set at 1.

and were subtracted from total cpm to determine PKC-ζ-specific cpm. 
Reaction rates were linear with respect to time (see Fig. 1, inset) and 
were markedly dependent upon addition of phosphatidylinerine but not 
Ca\textsuperscript{2+} or diolien. Insulin treatment did not influence either the amount 
of PKC-ζ recovered in immunoprecipitates or the blank values. Approxi-
imately 50% of total cellular PKC was recovered in the immunoprecipi-
tates, and this recovery was not improved by the addition of a 2-fold 
excess of antibody. As reported previously (11), PKC-ζ immunoprecip-
tates contained no detectable PKC-α, β, δ, or ε, which are known to be 
present in rat adipocytes (13). The dependence of enzyme activity on 
phosphatidylinerine suggested that protein kinase B and protein kinase 
N were absent from PKC-ζ immunoprecipitates, since immunoprecipi-
table PKB and PKN activities are independent of phosphatidylinerine.\textsuperscript{2} In all experiments, control and treated cells were derived from the same 
batch of adipocytes, and all samples were subsequently assayed in 
parallel; consequently, results of each treatment were expressed rela-
tive to the corresponding mean control value, arbitrarily set at 1. An 
example of absolute cpm typically observed in control and insulin-
stimulated PKC-ζ immunoprecipitates in an individual experiment is 
shown in the inset of Fig. 1.

In some assays of immunoprecipitated PKC-ζ, exogenous substrate 
was omitted, and \(^{32}\)P labeling of PKC-ζ itself was determined after its 
resolution by SDS-polyacrylamide gel electrophoresis, electolytic 
transfer to nitrocellulose membranes, and analysis in the Bio-Rad Mo-
lecular Analyst phosphorimeter.

PKC isoforms were immunoblotted as described (11), except that 
polyclonal antibodies used for PKC-β\textsubscript{2}, and PKC-β\textsubscript{2}, were obtained from 
Santa Cruz Biotechnology. Blots were quantified in a Bio-Rad Mole-
cular Analyst Chemiluminescence Imagery.

PI-3 kinase activity in adipocyte homogenates was measured as 
described previously (8).

**RESULTS**

Insulin provoked rapid increases in the enzyme activity re-
covered in PKC-ζ immunoprecipitates prepared from total adi-
ocyte lysates (Fig. 1). Increases were observed throughout a 
20-min treatment period with 10 nM insulin, but the increases 
appeared to be biphasic, with peaks at 0.5–1 and 10 min, and 
were maximal at insulin concentrations of 1–10 nM (see Fig. 1, 
inset). In general, increases in immunoprecipitable PKC-ζ en-
zyme activity at 10 min of 10 nM insulin treatment were ap-
proximately 2–3-fold, although they varied from 1.5-fold to 
to 10-fold. This variability appeared to primarily reflect differ-
ences in basal activity, but in addition, the timing of the insu-
in-induced stimulatory peaks may have varied slightly in 
individual experiments.

As shown in Fig. 2, insulin-induced increases in immunoprecipi-
table PKC-ζ were observed, not only in acutely incubated 
adipocytes but also in adipocytes that were incubated overnight 
prior to acute insulin treatment. As expected, the marked 
down-regulation of DAG-sensitive PKCs (α, β1, β2, δ, and ε) by 
overnight TPA (1 \(\mu\)M) treatment failed to influence basal or 
insulin-stimulated immunoprecipitable PKC-ζ enzyme activity 
and interestingly, at least in some experiments (e.g. see Fig. 2),

\textsuperscript{2} M. L. Standaert, L. Galloway, P. Karnam, G. Bandyopadhyay, J. 
Moscat, and R. V. Farese, unpublished observations.
2-DOG uptake. (In other experiments (not shown), insulin-stimulated 2-DOG uptake was partly diminished, perhaps by alterations in the activity of the insulin receptor or other signaling factors caused by persistent activation of residual DAG-sensitive PKCs that in some cases (and for uncertain reasons) better survived overnight TPA treatment (e.g. see more substantial PKC-ε retention in experiments reported in Ref. 13).) These findings, along with the fact that PKC-ε immunoprecipitates contained little or no detectable immunoreactive PKC-α, -β, -δ, or -ε (see Ref. 11), (a) provided further evidence for the specificity of the presently used PKC-ε enzyme assay; (b) showed that PKC-α, -β1, -β2, -δ, and -ε can be dissociated from glucose transport effects of insulin in the rat adipocyte (also see below); and (c) documented that PKC-ε is activated by acute insulin treatment in adipocytes that are either used directly or first placed into primary culture and incubated overnight with or without TPA (see below).

To determine whether PKC-ε may be activated by a PI 3-kinase-dependent mechanism, we used three approaches. First, we used two relatively specific, but structurally different, inhibitors of PI 3-kinase. In concentrations that inhibit PI 3-kinase and insulin effects on glucose transport, both LY294002 and wortmannin inhibited insulin effects on PKC-ε activation in intact adipocytes (Fig. 3); in contrast, these inhibitors did not inhibit PKC-ε enzyme activity when added directly to PKC-ε immunoprecipitates (not shown). Second, we used a peptide (DAD(pY)ENMDNP-NH₂) that, by virtue of its pYXXM motif, activates the SH2 domain of PI 3-kinase (see FIG. 3).

LY294002 and wortmannin inhibited insulin effects on PKC-ε activation in intact adipocytes (Fig. 3); in contrast, these inhibitors did not inhibit PKC-ε enzyme activity when added directly to PKC-ε immunoprecipitates (not shown). Second, we used a peptide (DAD(pY)ENMDNP-NH₂) that, by virtue of its pYXXM motif, activates the SH2 domain of PI 3-kinase (see FIG. 3).
in these homogenates was increased nearly 2-fold by PI 3-kinase activation (Fig. 4). Note that PI 3-kinase activity, containing peptide and was fully blocked by LY294002 (Fig. 4).

Third, we added polyphosphoinositides (PI-3,4-PO4)2, PI-3,4,5-(PO4)3, and PI-4,5- (PO4)2 on control and insulin-stimulated immunoprecipitates. These findings suggested that PI 3-kinase is not only required for insulin-induced activation of immunoprecipitable PKC-ζ in rat adipocytes but, through its lipid products, can account for this activation.

Certain evidence (8, 15, 16) suggests that a protein kinase distal to PI 3-kinase (8, 17) may be required for insulin effects on GLUT 4 translocation and glucose transport. Insulin effects on these processes are sensitive to PKC inhibitors but, in general, the required concentrations of these inhibitors (8, 15, 16) have exceeded those required to inhibit conventional and novel DAG-sensitive PKCs. Along these lines, it may be noted that bisindolemaleimides are potent inhibitors of conventional PKCs (8, 15, 16) but inhibit PKC-ζ only at relatively high concentrations (18, 19), and this was presently found to be the case for the bisindolemaleimide derivative RO 31-8220, which inhibited recombinant forms of PKC-α, -β1, -β2, -γ, -δ, -ε, -η, and -ζ with EC50 values of approximately 40, 20, 15, 15, 30, 100, 20, and 1000 nM, respectively (Fig. 6). Presently, we also found that the inhibition of enzyme activity in adipocyte PKC-ζ immunoprecipitates in vitro closely matched the inhibition of insulin-stimulated glucose transport observed in intact adipocytes in response to increasing doses of RO 31-8220 (Fig. 7); EC50 values for inhibition of both processes were approximately 4 μM. (The differences between EC50 values of RO 31-8220 for inhibiting soluble preparations of recombinant PKC-ζ versus PKC-ζ immobilized in immunoprecipitates (or, for that matter, contained in intact, lipid-laden adipocytes) may reflect differences in effective local concentrations of RO 31-8220 at catalytic enzyme sites under these different conditions.) In other studies, we found that RO 31-8220 did not inhibit insulin-induced activation of PI 3-kinase (8) or PI 3-kinase-dependent activation of PKB or glycogen synthase.2 Thus, RO 31-8220 does not inhibit basic insulin signaling mechanisms that involve PI 3-kinase-dependent activation of PKB or glycogen synthase.3

In addition to RO 31-8220, the cell-permeable (see Ref. 20), myristoylated PKC-ζ pseudosubstrate (myr-SIYRGRAR-RWRKLR) but not the myristoylated PKA pseudosubstrate (myr-GRTGRRNAI) inhibited insulin-stimulated glucose transport in a time- and concentration-dependent manner in intact adipocytes (Fig. 7). Full inhibition was achieved at 90-
PKC-ζ as a Downstream Effector of PI 3-Kinase

150 min of treatment with 100 μM PKC-ζ pseudosubstrate (Fig. 7B), and the EC₅₀ was 10–20 μM (note that similar treatment times and concentrations were reported in studies of PKC inhibition by a myristoylated PKC pseudosubstrate in HEK cells (see Ref. 20)). Of particular interest, enzyme activity of immunoprecipitated PKC-ζ in vitro was inhibited by concentrations of PKC-ζ pseudosubstrate that were nearly identical to those required for inhibition of glucose transport in intact cells (Fig. 7). Of further interest, in other studies, we have found that the PKC-ζ pseudosubstrate does not inhibit immunoprecipitated PKB, and PKB is clearly not the PKC-ζ pseudosubstrate-sensitive protein kinase that is required for glucose transport.

As another approach to test the possibility that PKC-ζ may play a role in insulin stimulation of glucose transport, we co-transfected HA-tagged GLUT4 and various forms of PKC-ζ into rat adipocytes. After overnight incubation to allow time for expression (documented by transfecting HA-tagged PKC-ζ along with HA-tagged GLUT4 in some experiments; see Fig. 8), we found that wild-type PKC-ζ and, to a greater extent, point-mutated constitutive PKC-ζ increased HA-tagged GLUT4 translocation (Fig. 9 and Table I). Stimulatory effects of insulin on HA-tagged GLUT4 translocation were still observed in cells expressing wild-type and constitutive PKC-ζ, although the percent increases, because of relatively greater increases in basal HA-tagged GLUT4 translocation, were diminished in these cells. In contrast to consistently observed stimulatory effects of wild-type and constitutive PKC-ζ, a kinase-inactive, dominant-negative form of PKC-ζ (see Ref. 11) failed to significantly alter basal HA-tagged GLUT4 translocation, but in about half of the experiments, it inhibited insulin-stimulated HA-tagged GLUT4 translocation (Fig. 9 and Table I) by 53 ± 4% (mean ± S.E.; n = 4; p < 0.005; paired t test); this inhibition was only slightly less than that observed in adipocytes transfected with dominant-negative Δp85, viz., 61 ± 10% (n = 4; p < 0.01), which was also clearly inhibitory in about half of the experiments. Results from an experiment in which all forms of PKC-ζ and Δp85 were used in parallel (i.e., in the same batch of adipocytes) are shown in Fig. 9; results from multiple experiments are summarized in Table I. Relative increases in HA-tagged GLUT4 translocation owing to expression of wild-type and point-mutated constitutive PKC-ζ were 67 ± 18% (n = 6; p < 0.05) and 86 ± 28% (n = 7; p < 0.05), respectively; this may be compared with insulin-stimulated increases of 107 ± 24% (n = 13; p < 0.001). (Note that alterations in the translocation of HA-tagged GLUT4 could not be explained by changes in its expression in cells co-transfected with various forms of PKC-ζ or Δp85; see examples in Fig. 8.) Of further interest, as with point-mutated PKC-ζ, increases (60 ± 14%; n = 5; p < 0.025) in HA-tagged GLUT4 translocation were also observed in adipocytes expressing a constitutive form of PKC-ζ in which AA 1–241 (i.e., the inhibitory regulatory domain) was deleted. Although it was not possible to examine directly in transiently...
PKC-ζ as a Downstream Effector of PI 3-Kinase

FIG. 9. Effects of transiently expressed wild-type (WT), constitutive (Constit), and dominant-negative (Dom-Neg) PKC-ζ on control and insulin-stimulated HA/GLUT4 translocation in rat adipocytes. Cells were suspended in Dulbecco’s modified Eagle’s medium and co-transfected with pCIS2 containing cDNA encoding HA-tagged GLUT4 and with pCDNA3 alone (vector) or pCDNA3 containing cDNA encoding wild-type, constitutive, or dominant-negative PKC-ζ or dominant-negative Δp85. After overnight incubation, the cells were equilibrated for 15 min in glucose-free KRP medium with or without 100 nM wortmannin, and then incubated for 30 min with or without 10 nM insulin, as indicated. KCN (2 mM) was then added, and samples were examined for cell count and cell surface content of HA-tagged GLUT4 (reflected by specific binding of 125I-labeled second antibody). Values in A and B are means of closely agreeing duplicates from representative experiments. Similar results were observed in other experiments, except that in A there was some variability in absolute values and relative effects of insulin. See Table I for absolute values of HA-tagged GLUT4 translocation observed in multiple experiments. See text for findings in multiple experiments that were normalized by expressing values of treated cells as percentages of values observed in the corresponding control, i.e. cpm in unstimulated (control) cells transfected with pCIS2/HA-tagged GLUT4 and pCDNA3 vector.

TABLE I
Effects of transient expression of wild-type, constitutive, or dominant-negative PKC-ζ on translocation of HA-tagged GLUT4 to the plasma membrane in control and insulin-stimulated rat adipocytes

| Group | Transfection type | Cell surface HA/GLUT4 125I-labeled anti-IgG antibody (cpm/10^6 cells) |
|-------|------------------|---------------------------------------------------------------|
|       |                  | Control | 10 nM insulin |
| I     | Vector           | 675 ± 47 (13) | 1267 ± 71 (13) |
|       | Wild-type PKC-ζ  | 998 ± 83 (7) | 1482 ± 92 (7) |
|       | Constitutive PKC-ζ | 1156 ± 86 (7) | 1551 ± 76 (7) |
|       | Dominant-negative PKC-ζ | 760 ± 135 (4) | 1530 ± 145 (4) |
| II    | Vector           | 842 ± 142 (4) | 1199 ± 147 (4) |

a Number in parentheses indicates number of experiments performed.

Table I presents data for absolute values of HA-tagged GLUT4 translocation observed in multiple experiments. See text for findings in multiple experiments that were normalized by expressing values of treated cells as percentages of values observed in the corresponding control, i.e. cpm in unstimulated (control) cells transfected with pCIS2/HA-tagged GLUT4 and pCDNA3 vector.

transfected cells, we presume (from observed stimulatory effects of transfected wild-type PKC-ζ on co-transfected HA-tagged GLUT4 translocation) that a significant fraction of expressed transfected wild-type PKC-ζ was enzymatically active, even without insulin addition.

Since PKC-ζ would be expected to operate downstream of PI 3-kinase in the activation of glucose transport, it was of interest to see whether wortmannin altered GLUT4 translocation. As shown in Fig. 9, 100 nM wortmannin blocked insulin effects on HA-tagged GLUT4 translocation but did not inhibit the stimulatory effects of constitutive PKC-ζ. Thus, the activated form of PKC-ζ appeared to operate independently or downstream of PI 3-kinase.

In addition to increases in enzyme activity, insulin provoked rapid increases in 32P labeling of immunoprecipitated 70-kDa PKC-ζ in cells that had been incubated in the presence of 32PO₄ for 2 h prior to insulin addition (Fig. 10) (ATP is labeled to constant specific activity during a 2-h period (21)). This observation provided confirmary evidence that the activation of PKC-ζ observed in vitro truly reflected an activation of PKC-ζ in intact cells. On the other hand, it may be noted that maximal increases in 32P labeling of PKC-ζ occurred at 5 min, and this was different from the timing of maximal increases in enzyme activity (compare Figs. 10 and 1).

In addition to increasing 32P labeling of PKC-ζ in intact adipocytes, insulin provoked increases in 32P labeling of PKC-ζ that occurred during the in vitro assay of immunoprecipitated PKC-ζ (Fig. 11); this suggested that PKC-ζ autophosphorylated in vitro in response to insulin treatment in intact cells, and this postulation was confirmed by the finding that the PKC-ζ pseudosubstrate markedly inhibited 32P labeling of PKC-ζ in vitro (Fig. 11). Of further note, PI-3,4-(PO₄)₂ and, to a lesser extent, PI-3,4,5-(PO₄)₃ increased 32P labeling of PKC-ζ in control but not insulin-treated PKC-ζ immunoprecipitates (Fig. 11). These findings suggested that both insulin and D3-PO₄ polyphosphoinositides enhanced both the enzyme activity and autophosphorylation of PKC-ζ.

DISCUSSION

The requirement for PI 3-kinase in insulin-induced activation of PKC-ζ in intact adipocytes and the direct activating effects of both pYXXM-stimulated PI 3-kinase and D3-PO₄ polyphosphoinositides on PKC-ζ enzyme activity in vitro suggested that PI 3-kinase is both necessary and sufficient for PKC-ζ activation. The precise mechanism for insulin-induced PKC-ζ activation is not entirely certain, but since added polyphosphoinositides stimulated control but not insulin-treated immunoprecipitates and since added polyphosphoinositides narrowed or obliterated the difference in enzyme activity between control and insulin-stimulated PKC-ζ immunoprecipitates, it follows that polyphosphoinositides may have largely accounted for increases in enzyme activity observed in insulin-stimulated PKC-ζ immunoprecipitates. In this scenario, insulin-induced increases in either D3-PO₄ polyphosphoinositides themselves and/or other presently uncertain stimulatory processes or factors that are induced in response to increases in these polyphosphoinositides (e.g. autophosphorylation-
PKC-ζ as a Downstream Effector of PI 3-Kinase

Cell lines were incubated for 2 h in low phosphate (0.12 mM) KRP buffer containing 10 mM HEPES, 2 mM glucose, 1% bovine serum albumin, and 100 μCi of [32P]P/mL and then treated with or without 10 nM insulin for the indicated times. PKC-ζ was immunoprecipitated from whole cell lysates, and precipitates were resolved by SDS-polyacrylamide gel electrophoresis and analyzed for immunoreactivity and [32P] labeling of 70-kDa PKC-ζ. Inset shows a representative autoradiogram and its corresponding immunoblot (note equal loading of PKC-ζ). The graph shows results (mean ± S.E.) of 3 determinations in which [32P] labeling of PKC-ζ was quantified with a Bio-Rad phosphorimager.

FIG. 10. Effects of insulin on [32P] labeling of immunoprecipitable PKC-ζ in rat adipocytes. Cells were incubated for 2 h in low phosphate (0.12 mM) KRP buffer containing 10 mM HEPES, 2 mM glucose, 1% bovine serum albumin, and 100 μCi of [32P]P/mL and then treated with or without 10 nM insulin for the indicated times. PKC-ζ was immunoprecipitated from whole cell lysates, and precipitates were resolved by SDS-polyacrylamide gel electrophoresis and analyzed for immunoreactivity and [32P] labeling of 70-kDa PKC-ζ. Inset shows a representative autoradiogram and its corresponding immunoblot (note equal loading of PKC-ζ). The graph shows results (mean ± S.E.) of 3 determinations in which [32P] labeling of PKC-ζ was quantified with a Bio-Rad phosphorimager.


glucose transport; this conclusion was also supported by the fact that inhibitory effects of RO 31-8220 on insulin-stimulated glucose transport more closely matched the inhibition of PKC-ζ and occurred at considerably higher concentrations than those required to inhibit PKC-α, -β1, -β2, -γ, -δ, -ε, and -η.

As alluded to above, PKB does not appear to be the PKC-ζ pseudosubstrate-sensitive protein kinase that is required for insulin-stimulated glucose transport. This does not imply that PKB is not required for insulin-stimulated glucose transport, but it seems clear that at least one PKC or a closely related protein kinase is required, perhaps along with PKB. In this regard, as discussed above, DAG-sensitive PKCs appear to be ruled out. It is therefore tempting to suggest that PKC-ζ and/or another atypical PKC is the PKC-ζ pseudosubstrate-sensitive kinase that is required for insulin stimulation of glucose transport. However, we cannot rule out the possibility that the PKC-ζ pseudosubstrate, as well as RO 31-8220, may bind to and thus inhibit catalytic sites of other relevant, but presently unknown, non-PKC protein kinases.

The activation of GLUT4 translocation by wild-type PKC-ζ, and even more so by constitutive PKC-ζ, in transiently transfected adipocytes provided further evidence that PKC-ζ may participate in the activation of GLUT4 translocation by insulin or other agonists. Indeed, two forms of constitutive PKC-ζ, one point-mutated in the pseudosubstrate region (see above) and one in which the regulatory domain was deleted, were nearly as effective as insulin in stimulating HA-tagged GLUT4 translocation. However, as with inhibitor studies, the finding that transient transfection of wild-type and constitutive PKC-ζ led to increases in HA-tagged GLUT4 translocation must be interpreted cautiously, as these increases reflect slowly developing responses (i.e. over 16–24 hours) that could be due to activation of a variety of factors and other non-PKC-ζ signaling pathways. Similarly, the partial inhibition of insulin-stimulated glucose transport by dominant-negative PKC-ζ suggested that PKC-ζ may be required for this stimulation, but this inhibition may also reflect nonspecific alterations in other signaling components in cells transfected with this interfering PKC-ζ mutant. Obviously, other experimental approaches will be needed to further evaluate the importance of PKC-ζ in insulin-stimulated glucose transport.
PKC-ζ as a Downstream Effector of PI 3-Kinase

cDNA encoding wild-type and dominant-negative PKC-ζ led to increases and decreases, respectively, of glucose transport (11). Of further interest, we have found that PKC-ζ is activated by insulin in L6 myotubes (23) and rat skeletal muscles; it is therefore tempting to suggest that PKC-ζ is activated by insulin and may participate in regulating glucose transport in a variety of insulin-sensitive cell types.

In summary, our findings suggest that insulin activates PKC-ζ in rat adipocytes largely through PI 3-kinase-dependent increases in polyphosphoinositides. Our findings also suggest that PKC-ζ may be required for and may participate in the translocation of GLUT4 and the activation of glucose transport in rat adipocytes. Further studies are needed to test these hypotheses.

REFERENCES

1. Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
2. Palmer, R. H., Dekker, L. V., Woscholski, R., Le Good, J. A., Gigg, R., and Parker, P. J. (1995) J. Biol. Chem. 270, 22412–22416
3. Farese, R. V., Larson, R. E., and Sahar, M. A. (1992) J. Biol. Chem. 257, 4042–4045
4. Farese, R. V., Barnes, D. E., Davis, J. S., Standaert, M. L., and Pollet, R. (1984) J. Biol. Chem. 259, 7094–7100
5. Farese, R. V., Davis, J. S., Barnes, D. E., Standaert, M. L., Bahischekn, J. S., Hock, R., Rosic, N. K., and Pollet, R. J. (1985) Biochem. J. 231, 269–278
6. Pennington, S. R., and Martin, B. R. (1985) J. Biol. Chem. 260, 11039–11045
7. Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411–1415
8. Standaert, M. L., Avignon, A., Yamada, K., Bandyopadhyay, G., and Farese, R. V. (1996) Biochem. J. 313, 1039–1045
9. Quon, M. J., Butte, A. J., Zarnowski, M., Sesti, G., Cushman, S. W., and Taylor, S. I. (1994) J. Biol. Chem. 269, 27920–27924
10. Quon, M. J., Guerre-Millo, M., Zarnowski, M. J., Butte, A. J., Em, M., Cushman, S. W., and Taylor, S. I. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5587–5591
11. Bandyopadhyay, B., Standaert, M. L., Zhao, L., Yu, B., Avignon, A. M., Galloway, L., Karmam, P., Moscat, J., and Farese, R. V. (1997) J. Biol. Chem. 272, 2551–2558
12. Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg, P. M. (1993) Mol. Pharmacol. 44, 296–307
13. Avignon, A., Standaert, M. L., Yamada, K., Mischak, H., Spencer, B., and Farese, R. V. (1995) Biochem. J. 308, 181–187
14. Rordorf-Nikolic, T., Van Horn, D. J., Chen, D., White, M. F., and Backer, J. M. (1995) J. Biol. Chem. 270, 15246–15251
15. Standaert, M. L., Buckley, D. J., Ishizuka, T., Hoffman, J. M., Cooper, D. R., Pollet, R. J., and Farese, R. V. (1993) J. Biol. Chem. 268, 13–16
16. Rordorf-Nikolic, T., Van Horn, D. J., Chen, D., White, M. F., and Backer, J. M. (1995) J. Biol. Chem. 270, 15246–15251
17. Jullien, D., Tanti, J. F., Heydrick, S. J., Gautier, N., Gremeaux, T., Van Obberghen, E., and Le Marchand-Brustel, Y. (1993) J. Biol. Chem. 268, 21224–21231
18. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., and Schachtete, C. (1993) J. Biol. Chem. 268, 9194–9197
19. Wilkinson, S. E., Parker, P. J., and Nixon, J. S. (1993) Biochem. J. 294, 335–337
20. Eichholz, T., de Bont, D. B., de Wied, J., Laskamp, R. M., and Ploegh, H. L. (1993) J. Biol. Chem. 268, 1982–1986
21. Arnold, T. P., Standaert, M. L., Hernandez, H., Watson, J., Mischak, H., Kazanietz, M. G., Zhao, L., Cooper, D. R., and Farese, R. V. (1993) Biochem. J. 295, 155–164
22. Kelly, K. L., and Ruderman, N. B. (1993) J. Biol. Chem. 268, 4391–4398
23. Bandyopadhyay, G., Standaert, M. L., Galloway, L., Moscat, J., and Farese, R. V. (1997) Endocrinology, in press

FIG. 11. Effects of insulin treatment in intact adipocytes, or direct addition of polyphosphoinositides on [32P] labeling of immunoprecipitable PKC-ζ in vitro. PKC-ζ was immunoprecipitated from control and insulin-stimulated (10 nM insulin) adipocytes as indicated and assayed with or without 10 µM PI-3,4,5-(PO4)3, 10 µM PI-3,4-(PO4)2, and/or 100 µM PKC-ζ pseudosubstrate (PS), as indicated. After incubation, PKC-ζ was resolved by SDS-polyacrylamide gel electrophoresis and [32P]-labeled 70-kDa PKC-ζ was imaged by autoradiography (B) and quantified by phosphorimager analysis (A). For comparative purposes, C depicts alterations in immunoprecipitated PKC-ζ enzyme activity in samples developed in parallel with samples in which [32P] labeling of PKC-ζ was measured. Values in A and C are mean ± S.E. of 4–5 determinations. Although not shown here, addition of polyphosphoinositides did not increase [32P] labeling of PKC-ζ in insulin-treated samples.

It may be noted that the present findings on insulin-induced activation of PKC-ζ and its potential role in the activation of GLUT4 translocation and glucose transport in rat adipocytes are in keeping with findings in 3T3/L1 fibroblasts and adipocytes, in which stable transfection with pCDNA3 containing cDNA encoding wild-type and dominant-negative PKC-ζ led to increases and decreases, respectively, of glucose transport (11). Of further interest, we have found that PKC-ζ is activated by insulin in L6 myotubes (23) and rat skeletal muscles; it is therefore tempting to suggest that PKC-ζ is activated by insulin and may participate in regulating glucose transport in a variety of insulin-sensitive cell types.

In summary, our findings suggest that insulin activates PKC-ζ in rat adipocytes largely through PI 3-kinase-dependent increases in polyphosphoinositides. Our findings also suggest that PKC-ζ may be required for and may participate in the translocation of GLUT4 and the activation of glucose transport in rat adipocytes. Further studies are needed to test these hypotheses.