Insulin Activates Protein Kinases C-ζ and C-λ by an Autophosphorylation-dependent Mechanism and Stimulates Their Translocation to GLUT4 Vesicles and Other Membrane Fractions in Rat Adipocytes*

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In rat adipocytes, insulin provoked rapid increases in (a) endogenous immunoprecipitable combined protein kinase C (PKC)/ζ activity in plasma membranes and microsomes and (b) immunoreactive PKC-ζ and PKC-λ in GLUT4 vesicles. Activity and autophosphorylation of immunoprecipitable epitope-tagged PKC-ζ and PKC-λ were also increased by insulin in situ and phosphatidylinositol 3,4,5-(PO4)3 (PIP3) in vitro. Because phosphoinositide-dependent kinase-1 (PDK-1) is required for phosphorylation of activation loops of PKC-ζ and protein kinase B, we compared their activation. Both RO 31-8220 and myristoylated PKC-ζ pseudosubstrate blocked insulin-induced activation and autophosphorylation of PKC-ζ/λ but did not inhibit PDK-1-dependent (a) protein kinase B phosphorylation/activation or (b) threonine 410 phosphorylation in the activation loop of PKC-ζ. Also, insulin in situ and PIP3 in vitro activated and stimulated autophosphorylation of a PKC-ζ mutant, in which threonine 410 is replaced by glutamate (but not by an inactivating alanine) and cannot be activated by PDK-1. Surprisingly, insulin activated a truncated PKC-ζ that lacks the regulatory (presumably PIP3-binding) domain; this may reflect PIP3 effects on PDK-1 or transphosphorylation by endogenous full-length PKC-ζ. Our findings suggest that insulin activates both PKC-ζ and PKC-λ in plasma membranes, microsomes, and GLUT4 vesicles by a mechanism requiring increases in PIP3, PDK-1-dependent phosphorylation of activation loop sites in PKC-ζ and λ, and subsequent autophosphorylation and/or transphosphorylation.

Insulin has been reported to activate atypical forms of protein kinase C (PKC), i.e. PKC-ζ and/or PKC-λ, in 3T3/L1 adipocytes (1, 2), rat adipocytes (3), L6 myotubes (4), and 32D cells (5). These increases in atypical PKC enzyme activity appear to be largely dependent upon activation of phosphatidylinositol (PI) 3-kinase (1–5) and subsequent increases in D3-PO4 polyphosphoinositides, i.e. PI 3,4,5-(PO4)3 and PI 3,4-(PO4)2 (6). Moreover, transfection studies suggest that PKC-ζ and/or PKC-λ is/are required for and may be sufficient for insulin stimulation of GLUT4 translocation and subsequent glucose transport (1–4).

At present, there is only limited information on the mechanism whereby D3-PO4 polyphosphoinositides activate atypical PKCs and little or no information on the subcellular compartments in which atypical PKCs are activated or, for that matter, whether one or both atypical PKCs are activated by insulin in specific cell types. With respect to the first point, recent findings (6, 7) suggest that PI 3,4,5-(PO4)3 and PI 3,4-(PO4)2 activate, or allow access for, 3-phosphoinositide-dependent kinase-1 (PDK-1), which phosphorylates threonine 410 in the activation loop of PKC-ζ, thereby initiating the activation of this atypical PKC. Indeed, in other studies, we have found that PDK-1 action is required for insulin-induced activation of PKC-ζ in rat adipocytes. However, it is uncertain whether this requirement reflects a permissive effect of PDK-1 or whether PDK-1 mediates acute activating effects of insulin. Also, it is not clear whether other mechanisms, e.g. autophosphorylation or transphosphorylation, are also required for full enzymic activation of PKC-ζ, presumably subsequent to PDK-1-dependent loop phosphorylation, during insulin treatment. With respect to the question of whether insulin activates PKC-ζ and/or PKC-λ, in most of the above-mentioned studies (1, 3, 4, 5), immunoprecipitates that were assayed for enzyme activity probably contained both PKC-ζ and PKC-λ, because the antisera that were used for immunoprecipitation recognize a C-terminal epitope that is common to both PKC-ζ and PKC-λ.

Presently, we examined: (a) the subcellular localization and isoform specificity of atypical PKCs that are activated by insulin in rat adipocytes and (b) requirements for loop phosphorylation by PDK-1 and subsequent autophosphorylation in the activation of PKC-ζ by insulin in rat adipocytes. Also, because insulin primarily regulates glucose transport by stimulating the translocation of GLUT4 vesicles from the microsomal fraction to the plasma membrane and because GLUT4 translocali

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§ The abbreviations used are: PKC, protein kinase C; PI, phosphatidylinositol; PDK-1, 3-phosphoinositide-dependent kinase-1; KRP, Krebs-Ringer phosphate; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin antigen; PKB, protein kinase B.

2 M. L. Standaert, G. Bandyopadhyay, L. Perez, D. Price, L. Galloway, A. Poklepovic, M. P. Sajan, V. Cenni, A. Sirri, J. Moscat, A. Toker, and R. V. Farese, unpublished observations.
tion appears to be dependent upon increases in PI 3-kinase activity in specific membrane fractions, in particular microsomes (8–10) and GLUT4 vesicles (11), we examined the question of whether insulin provokes changes in atypical PKCs in these and other membrane fractions in rat adipocytes.

**EXPERIMENTAL PROCEDURES**

**Rat Adipocyte and Subcellular Preparations**—As described (3), adipocytes were prepared by collagenase digestion of rat epididymal fat pads, suspended in glucose-free Krebs-Ringer phosphate (KRP) buffer containing 1% bovine serum albumin, and incubated at 37 °C for indicated times with or without 10 nM insulin (Eli Lilly Co., Indianapolis, IN) and/or PKC-ζ inhibitors, RO 31-8220 (Alexis, San Diego, CA) or cell-permeable myristoylated PKC-ζ pseudosubstrate (myr-SIYRRGARRWRKL, Quality Controlled Biochemicals, Hopkinton, MA). After incubation, cells were homogenized in Buffer A, which contained 0.25 M sucrose, 20 mM Tris/Cl (pH 7.5), 1.2 mM EGTA, 20 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Na3VO4, 1 mM Na2P2O7, and 1 mM NaF. The fat cake and nuclear fraction were removed after centrifugation for 10 min at 500 g. Resultant defatted, post-nuclear homogenates were centrifuged at 20,000 g for 20 min to obtain crude plasma membrane-enriched fractions, and resulting supernatants were centrifuged at 400,000 g for 70 min to obtain microsomal membranes. Alternatively, homogenates were subjected to discontinuous sucrose gradient centrifugation to obtain highly purified plasma membranes and microsomal membranes as described (12). GLUT4 vesicles were isolated from low density microsomal membranes by immunoprecipitation using mouse monoclonal anti-GLUT4 IF8 antibodies (Biogenics) (13).

**3T3/L1 Adipocyte Preparations**—3T3/L1 fibroblasts were grown to confluence, differentiated to adipocytes, and subsequently incubated in serum-free, glucose-free KRP medium for indicated times with or without 100 nM insulin as described (1).

**Assays for PKC-ζ and PKC-λ Enzyme Activity**—As described previously (1, 3) post-nuclear, defatted homogenates or subcellular fractions suspended in Buffer A were supplemented with 0.15 M NaCl, 1% Triton X-100, and 0.5% Nonidet, and PKC-ζ and PKC-λ were immunoprecipitated by overnight incubation at 4 °C with a rabbit polyclonal antisera to (Santa Cruz Biotechnologies, Santa Cruz, CA) that targets the C terminal of both PKC-ζ and PKC-λ (their C-terminal 26 amino acids are identical except for one residue). Immunoprecipitates were collected on protein AG-Sepharose beads, washed, and incubated for 5 min at 30 °C in 50 μl of buffer containing 5 mM MgCl2, 100 μM Na3VO4, 100 μM NaF, 100 μM Na2P2O7, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 50 μM Tris (pH 7.5), 4 μg of phosphatidylserine, 50 μM ATP, 3–5 μCi of [γ-32P]ATP (NEN Life Science Products), 40 μm serine analogue of PKC-ε pseudosubstrate as a selective substrate for atypical PKCs (Quality Controlled Biochemicals, Hopkinton, MA), and, as indicated, PI 3,4,5-(PO)3 or PI 3,4-(PO)2 (Matreya, Pleasant Gap, PA, or Alexis, San Diego, CA). After incubation, an aliquot of the reaction mixture was spotted on Phast gel filter paper, washed with 5% acetic acid, and counted for [32P] radioactivity as described (3). Alternatively, the autophosphorylation of PKC-ζ and λ was examined by addition of Laemmli buffer and subjecting the reaction mixture to SDS-polycrylamide gel electrophoresis (PAGE) as described (3).

In some experiments, rat adipocytes were transfected with: (a) pcDNA3 that contained cDNA encoding hemagglutinin antigen (HA)-tagged PKC-ζ, Myc-tagged PKC-λ, or HA-tagged Δ1–247, using plasmids and methods described previously (3, 20) or (b) pCMV5 containing cDNA encoding either FLAG-tagged PKC-ζ/T410A, a mutant in which the threonine 410 site was constitutively activated by replacing threonine 410 with glycine, or FLAG-tagged PKC-ζ/T410E, in which threonine 410 is replaced by alanine, causing this mutant to be resistant to PDK-1 activation and therefore essentially inactive (see Ref. 7). After overnight culture to allow time for expression (see Ref. 20 for expression data), cells were washed and equilibrated in glucose-free KRP medium and treated for indicated times with or without 10 nM insulin. After incubation, cells were homogenized and subjected to immunoprecipitation as described above, using a mouse monoclonal antibody that targets the HA epitope (Babco, Berkely, CA), or a rabbit polyclonal antibody to the Myc epitope (Upstate Biotechnologies, Inc., Lake Placid, NY), or a rabbit polyclonal antiserum that targets the FLAG epitope (Zymed Laboratories Inc., San Francisco, CA) of these epitope-tagged PKCs. These immunoprecipitates were then collected and assayed for enzymatic activity or autophosphorylation as described above.

**Assays for PKB Activation**—PKB was immunoprecipitated and assayed using reagents supplied in kit form by Upstate Biotechnologies, because of a much greater length of time needed for purification.

**RESULTS**

**Enzymic Activation of PKC-ζ and PKC-λ in Subcellular Fractions of Rat Adipocytes**—As seen in Fig. 2, insulin provoked rapid increases in the enzymic activity of immunoprecipitable combined PKC-ζ/λ (precipitated with anti-C-terminal antisera) in preparations of both plasma membrane-enriched membranes and microsomal membranes. As in total cell homogenates (3), increases in PKC-ζ/λ activity in both membrane fractions were distinctly biphasic with peaks at 1 and 10 min. Increases in the enzymic activity of immunoprecipitable combined PKC-ζ/λ were also observed in preparations of highly purified plasma membranes and microsomes (Fig. 3), although increases in the highly purified plasma membranes were less than those observed in cruder preparations (Figs. 2 and 3); this difference may reflect a loss of stimulated activity because of a much greater length of time needed for purification.

**Activation of PKC-ζ and PKC-λ by Insulin**

![Image](359x564 to 503x729)
activation of highly purified plasma membranes or removal of a highly active nonplasma membrane pool. (Note that in control rat adipocytes, cytosol contained approximately 80% of total cellular protein and 60% of total cellular PKC-ζ enzyme activity and that highly purified plasma membrane and microsomal fractions each contained approximately 10% of total cellular protein and 20% of total PKC-ζ enzyme activity). These findings suggested that PKC-ζ and PKC-λ were activated in both plasma membrane and microsomal membrane fractions of the rat adipocyte.

**Specific Activation of PKC-ζ and PKC-λ by Insulin**—To examine the activation of individual atypical PKC isoforms in the rat adipocyte, we transiently expressed epitope-tagged forms of PKC-ζ and PKC-λ and precipitated these expressed forms with epitope-targeted antibodies. As seen in Fig. 4, insulin provoked increases in the activity of both HA-PKC-ζ and Myc-PKC-λ. It may be noted that the enzymic activity of HA-PKC-ζ was considerably greater than that of Myc-PKC-λ, despite the fact that expression and immunoprecipitability of both epitope-tagged isoforms (as measured with the C-terminal-targeted antiserum that would be expected to react equally with both PKC-ζ and PKC-λ) were similar (data not shown). Although the reason for this difference in enzymic activity of epitope-tagged, immunoprecipitable PKC-ζ and PKC-λ is unknown, our findings nevertheless provide seemingly clear evidence that insulin activates both PKC-ζ and PKC-λ in rat adipocytes. In this regard, it may also be noted that insulin is known to activate PKC-λ in 3T3/L1 adipocytes (1, 2), which apparently contain PKC-λ but not PKC-ζ (2).

**Specific Activation of PKC-ζ and PKC-λ by D3-PO4 Polyphosphoinositides**—We have reported (3) that insulin-induced increases in immunoprecipitable combined PKC-ζ/λ enzyme activity are dependent on PI 3-kinase and can be largely or fully reproduced by direct addition of PI 3,4,5-(PO4)3 or PI 3,4-(PO4)2 to the *in vitro* assay of immunoprecipitated combined PKC-ζ/λ (also see Ref. 14). Presently, we examined the effects of PI 3,4,5-(PO4)3 on *in vitro* assays of separate forms of PKC-ζ and PKC-λ. For this purpose, we used (a) HA-tagged PKC-ζ and Myc-tagged PKC-λ that were transiently expressed in rat adipocytes and precipitated with anti-HA and anti-Myc antibodies and (b) endogenous PKC-λ that was recovered from 3T3/L1 adipocytes with the anti-C-terminal antiserum (note that, as stated above, PKC-ζ has been reported to be absent in 3T3/L1 adipocytes; see Ref 2). As seen in Fig. 5, the addition of PI 3,4,5-(PO4)3 to the *in vitro* assay provoked similar concentration-dependent increases in the activity of both PKC-ζ and PKC-λ that had been immunoprecipitated from control rat and 3T3/L1 adipocytes; moreover, these PI 3,4,5-(PO4)3-induced increases in enzyme activity *in vitro* (approximately 1.5–2-fold) were comparable with or only slightly less than those provoked by insulin treatment in intact adipocytes (Figs. 4 and 5). These findings suggested that increases in PI 3,4,5-(PO4)3 may be sufficient (i.e., most likely in conjunction with PDK-1, which, as reported (6, 7), was presently found to co-immunoprecipitate with HA-tagged PKC-ζ; data not shown; also see below) to account for insulin-induced increases in the activity of both PKC-ζ and PKC-λ. In keeping with the latter suggestion and as reported previously in studies in which PI 3,4,5-(PO4)3 was added to immunoprecipitates of combined PKC-ζ and PKC-λ (3), the addition of PI 3,4,5-(PO4)3 in most experiments failed to stimulate or only mildly enhanced the kinase activity of specific PKC-ζ and PKC-λ immunoprecipitates that were obtained from insulin-treated adipocytes (data not shown); these latter findings were in keeping with the postulate (see Ref. 3) that both atypical PKCs were already maximally stimulated in insulin-treated adipocytes by a mechanism functionally comparable to that provoked by direct addition of PI 3,4,5-(PO4)3 to the *in vitro* assay of control PKC-ζ and PKC-λ immunoprecipitates.

**Inhibitor Studies Suggest That Autophosphorylation Is Required for Activation of PKC-ζ/λ**—Because both PKB (Akt) and atypical PKCs are activated by insulin through PI 3-kinase and because both PKB (15, 16) and PKC-ζ (6, 7) are phosphorylated and activated by PDK-1 (note that we have found2 that PDK-1 is required for insulin-induced activation of HA-tagged PKC-ζ as determined by findings in rat adipocytes transiently transfected with a kinase-inactive form of PDK-1), which is directly activated by or whose access to internal activation loop sites is facilitated by PI 3,4,5-(PO4)3 (6, 7), we questioned (a) whether there were discernible differences in factors that are required for the activation of PKB and atypical PKCs or, more specifically, (b) whether mechanisms subsequent to PDK-1 activation and action were required for PKC-ζ/λ activation. We gained insight into these questions by using RO 31-8220 and the...
PKC-ζ/λ pseudosubstrate (SIYRRGARRWRKL), both of which directly inhibit PKC-ζ and PKC-λ and thus interfere with their autophosphorylation or transphosphorylation (see Ref 3 and below). As seen in Figs. 6 and 7, the presence of RO 31-8220 during incubation of intact adipocytes led to an inhibition of insulin-induced activation of immunoprecipitable combined PKC-ζ/λ; in contrast, insulin-induced enzymic activation of immunoprecipitable PKB was not inhibited by the presence of RO 31-8220 during insulin treatment of intact adipocytes (Fig. 6). Similarly, RO 31-8220 failed to block the wortmannin-sensitive phosphorylation of serine 473 in PKB that was provoked by insulin in intact adipocytes (Fig. 7; the continued activation of PKB is also in keeping with the fact that RO 31-8220 does not inhibit the activation of PI 3-kinase by insulin; see Ref. 17).

In addition to RO 31-8220, the presence of the cell-permeable myristoylated PKC-ζ/λ pseudosubstrate (pseudosubstrate sequences in PKCs ζ and λ both contain SIYRRGARRWRKL; see Ref. 18) during the incubation of intact adipocytes with insulin completely inhibited the enzymic activation of combined immunoprecipitable PKC-ζ/λ (Figs. 7 and 8) but did not inhibit the enzymic activation of immunoprecipitable PKB (Fig. 8) or the wortmannin-sensitive phosphorylation of serine 473 in PKB (Fig. 7). (Note that addition of the myristoylated PKC-ζ pseudosubstrate to the cell lysate just before immunoprecipitation, i.e., after incubation with insulin, did not interfere with observance of insulin-induced effects on the activity of PKC-ζ/λ immunoprecipitates; accordingly, inhibitory effects of the PKC-ζ pseudosubstrate on the activation of PKC-ζ/λ observed in intact cells could not be explained by carryover of the inhibitory pseudosubstrate from the cell lysate to the in vitro assay of the immunoprecipitate.) Moreover, neither RO 31-8220 nor the myristoylated PKC-ζ pseudosubstrate (nor wortmannin, for that matter) altered the level of phosphorylation of threonine 410 in the activation loop of PKC-ζ (Fig. 7), which is the initial target of PDK-1 in PKC-ζ (6, 7); thus, these inhibitors did not appear to interfere with the action of PDK-1 on either PKC-ζ or PKB. These findings indicated that insulin activates PKC-ζ and PKC-λ by a mechanism that is at least partly different from that which underlies PKB activation. Moreover, because insulin-induced activation of PKC-ζ and PKC-λ in intact cells was inhibited by concentrations of RO 31-8220 and the myristoylated PKC-ζ pseudosubstrate that were similar to those that directly inhibit these kinases in vitro (see Ref. 3), it seemed likely that autophosphorylation or transphosphorylation was required for the enzymic activation of PKC-ζ and PKC-λ that is observed in PKC-ζ/λ immunoprecipitates following insulin treatment of intact adipocytes.

Studies on Autophosphorylation of PKC-ζ and PKC-λ—In view of the above-described findings that suggested the importance of autophosphorylation in the activation of PKC-ζ and PKC-λ, it was of interest to find that: (a) PI 3,4,5-(PO₄)₃ and PI 3,4-(PO₄)₂ provoked increases in the autophosphorylation of immunoprecipitable combined PKC-ζ/λ (Fig. 9) that were comparable, in magnitude and dose dependence, to increases in enzymic activity of immunoprecipitable PKC-ζ and PKC-λ (Figs. 5 and 9) and (b) effects of PI 3,4,5-(PO₄)₃ and PI 3,4-(PO₄)₂ in vitro on PKC-ζ/λ autophosphorylation were comparable with or exceeded those induced by insulin treatment in intact adipocytes (Fig. 9). (Note that PI 4,5-(PO₄)₂ did not stimulate this autophosphorylation; data not shown). In addition, insulin treatment in intact cells and PI 3,4,5-(PO₄)₃ in vitro also provoked increases in autophosphorylation of both HA-PKC-ζ and MYC-PKC-λ, as measured in specific epitope-
targeted immunoprecipitates (Fig. 10). Also note that the addition of PKC-ζ pseudosubstrate to the in vitro assay markedly diminished \( {^{32}P} \) incorporation into HA-PKC-ζ and MYC-PKC-λ; thus, assuming that PDK-1 is not inhibited by the PKC-ζ pseudosubstrate (see above and below), it follows that the \( {^{32}P} \) incorporation observed in vitro assays is largely reflective of autophosphorylation or transphosphorylation of PKC-ζ and PKC-λ (this however, does not imply independence from PDK-1, because PDK-1 effects may be amplified during continued autophosphorylation, and the stability of phosphorylation sites may vary considerably).

**Insulin Activates FLAG-tagged PKC-ζT410E but Not PKC-ζT410A**—The above findings suggested that insulin-induced activation of PKC-ζ may involve increases in autophosphorylation that are distinct from and presumably follow the phosphorylation of threonine 410 that is dependent on PDK-1. This possibility was supported by our observation that insulin in intact cells and PI 3,4,5-(PO₄)₃ in vitro both activated and stimulated the autophosphorylation in vitro of FLAG-tagged PKC-ζT410E in transiently transfected rat adipocytes (Fig. 11). In contrast, FLAG-tagged PKC-ζT410A, the threonine 410 alanine mutant that cannot be activated by PDK-1, was virtually devoid of activity both basally and following insulin treatment (not shown). Because the T410E mutant, by virtue of its glutamate residue, is constitutively active at the 410 site and therefore cannot be further activated by PDK-1 at this site, it seems clear that insulin and PI 3,4,5-(PO₄)₃ must act through a mechanism that is distinct from, and most likely distal to, PDK-1-dependent threonine 410 phosphorylation. On the other hand, it is also clear that PDK-1-dependent threonine 410 phosphorylation is absolutely essential for activity and, therefore, activation of PKC-ζ by insulin.

**Effects of Insulin on Threonine 410 Phosphorylation in PKC-ζ**—Because threonine 410 phosphorylation was essential for insulin-induced activation of PKC-ζ, it was surprising to find that insulin had little, if any, effect on the level of phosphorylation of threonine 410 over a period of 0.5–10 min (Fig. 7). Although these findings suggested that insulin may not acutely stimulate the autophosphorylation in vitro of full-length HA-PKC-ζ (above), it was of interest to see if activation of PKC-ζ could be observed in the absence of its regulatory domain, which contains the inhibitory pseudosubstrate peptide sequence, and which, as in other PKCs, is thought to serve as the major binding site for activating lipids such as PI 3,4,5-(PO₄)₃. As seen in Table I, insulin activated transfected HA-Δ1–247-PKC-ζ, in which amino acids 1–247 had been deleted from the N terminus, to approximately the same extent as full-length transfected HA-PKC-ζ (see above).
This finding therefore suggested that the N-terminal regulatory domain is not required for insulin-induced activation of the catalytic domain PKC-\(\zeta\); however, endogenous full-length PKC-\(\zeta\) and \(\lambda\) were present in these transfected cells, and it is possible that insulin may have initially activated these full-length forms, which in turn may have activated HA-D\(_{1-247}\)-PKC-\(\zeta\) by transphosphorylation. Another possibility is that PI 3-kinase-dependent lipids may have directly activated PDK-1, which in turn may have activated HA-D\(_{1-247}\)-PKC-\(\zeta\).

Studies on the Translocation of PKC-\(\zeta\) and PKC-\(\lambda\) to GLUT4 Vesicles—As shown in Fig. 12, insulin provoked rapid increases in the contents of both immunoreactive PKC-\(\zeta\) and PKC-\(\lambda\) in microsome-associated GLUT4 vesicles, as measured with specific antibodies that recognize the N terminus of PKC-\(\zeta\) and an internal epitope of PKC-\(\lambda\). GLUT4 content on the other hand, diminished rapidly, presumably reflecting the translocation of GLUT4 vesicles from low density microsomes to the plasma membrane. It may be noted that the observed increases in immunoreactive PKC-\(\zeta\) and PKC-\(\lambda\) in GLUT4 vesicles were comparable with increases in immunoprecipitable enzymic activity observed in total microsomal fractions (Figs. 2, 3, and 12); accordingly, increases in immunoreactive PKC-\(\zeta\) and PKC-\(\lambda\) levels in GLUT4 vesicles may simply be reflective of the activation of total microsomal PKC-\(\zeta\)/\(\lambda\).
FIG. 11. Effects of insulin in intact rat adipocytes and PI 3,4,5-(PO_4)_3 in vitro on activity and autophosphorylation of a threonine 410 constitutive mutant (T410E) form of PKC-ζ (A) and effects of insulin in intact rat adipocytes on the phosphorylation of threonine 410 in PKC-ζ (B). In A, adipocytes were transiently transfected with 3 μg of pCMV5 containing cDNA encoding FLAG-tagged PKC-T410E mutant/0.8 ml of 50% adipocyte suspension. After overnight incubation to allow time for expression, cells were washed and incubated for 10 min with or without insulin as indicated. After incubation, FLAG-tagged PKC-T410E was immunoprecipitated with anti-FLAG antiserum (Zymed Laboratories Inc.) and then assayed with or without 10 μM PI 3,4,5-(PO_4)_3 (PIP_3) added in vitro as indicated (PIP_3 was added only to control immunoprecipitates). Bar graph shows the mean values ± S.E. of the number of determinations shown in each bar in parentheses. Asterisks indicate p < 0.05 (paired t test).

FIG. 12. Insulin provokes increases in PKC-ζ and PKC-λ levels in GLUT4 vesicles of rat adipocytes. Cells were treated for 0, 1, or 10 min with 10 nM insulin (INS), following which GLUT4 vesicles were isolated and analyzed for contents of immunoreactive PKC-ζ (anti-N-terminal antiserum), PKC-λ (anti-internal epitope), and GLUT4. Representative blots are shown in insets. Bar graphs indicate the means ± S.E. of the number of determinations shown in each bar in parentheses. Asterisks indicate p < 0.05 (paired t test).

TABLE I
Activation of Δ1–247-PKC-ζ by insulin in rat adipocytes

| HA-Δ1–247-PC (PO_4)_3 | insulin (nM) | cpm/immunoprecipitate | p |
|------------------------|-------------|------------------------|---|
| Control                | 0           | 1519 ± 258             | <0.005 |
| Insulin                | 10          | 3066 ± 124             | <0.005 |

DISCUSSION

The present findings provided clear evidence that insulin activated both atypical PKCs, PKC-ζ and PKC-λ, in rat adipocytes. This conclusion is perhaps not surprising given the fact that these PKCs are 72% homologous (18). Nevertheless, there is significant nonhomology, and it is therefore important to be certain that both PKCs, rather than only one or the other, are in fact activated by insulin in specific cell types.

The present findings also clearly showed that the PI 3-kinase-dependent lipids, viz. PI 3,4,5-(PO_4)_3 and PI 3,4-(PO_4)_2, can activate both PKC-ζ and PKC-λ, as recovered from control adipocytes. Moreover, the present findings further suggested that (a) these D3-PO_4 polyphosphoinositides may be sufficient to account for insulin-induced increases in both the autophosphorylation (including transphosphorylation) and enzymic activation of both PKC-ζ and PKC-λ, and (b) insulin-induced activation of these atypical PKCs can be dissociated from the activation of PKB by using RO 31-8220 and the PKC-ζ/λ pseudosubstrate, which serve to inhibit the autophosphorylation and activation of PKC-ζ and PKC-λ but not PKB. Accordingly, the present findings suggested that the activation of atypical PKCs and PKB are parallel events that branch off from PI 3-kinase and PDK-1 and thereafter function without dependence upon each other during the action of insulin. In support of the latter, we have also found that expression of a dominant-negative mutant (T308A,T473A) form of PKB (see Refs. 2 and 19) does not inhibit HA-PKC-ζ activation or PKC-ζ-dependent HA-GLUT4 translocation during insulin treatment of transiently transfected rat adipocytes.

Although our findings could be interpreted to suggest that simple increases in PI 3,4,5-(PO_4)_3 may be sufficient to account for insulin-induced increases in the autophosphorylation and enzymic activation of PKC-ζ and PKC-λ in the rat adipocyte, recent findings in other cell-types suggest that PI 3,4,5-(PO_4)_3 may initially activate PKD-1, or may provide access for PDK-1 to critical activation loop sites in PKC-ζ and PKC-λ (6, 7), thus facilitating or triggering their activation. Indeed, as alluded to above, we have recently confirmed in transient transfection experiments that PDK-1 is required for insulin-induced activation of PKC-ζ in rat adipocytes; we also recently found that phosphorylation of threonine 410 (the target of PDK-1) in PKC-ζ is essential for intrinsic PKC-ζ activity and subsequent activation by insulin. Moreover, because PDK-1 co-immunoprecipitates with PKC-ζ (6, 7) (this too has been confirmed in our immunoprecipitates), it is possible that PDK-1 may have been responsible wholly or partly for mediating the stimulatory effects of PI 3,4,5-(PO_4)_3 and PI 3,4-(PO_4)_2 on PKC-ζ and PKC-λ enzymic activity and autophosphorylation observed during in
vitro assays of our immunoprecipitates. On the other hand, we presently did not observe consistent significant changes in the level of phosphorylation of threonine 410 in PKC-ζ following insulin treatment with insulin for 0.5–10 min, or following treatment with RO 31-8220 or wortmannin for 25 min, or following treatment with the PKC-ζ phospho-substrate for 70 min. In addition, we found that both insulin treatment in intact adipocytes and PI 3,4,5-(PO_4)_3 in vitro activated a mutant form of PKC-ζ in which the threonine 410 site is constitutively activated by conversion to glutamate and cannot be further activated by PDK-1. It is therefore possible that PDK-1-dependent phosphorylation of threonine 410 is relatively stable and is not acutely regulated by insulin. However, in view of the potent effects of PDK-1 (6, 7) and the requirement for phosphorylation of threonine 410 for activity and activation of PKC-ζ, it is also possible that PDK-1 may phosphorylate a small but highly active pool of PKC-ζ that in turn triggers a subsequent autophosphorylation/transphosphorylation response that is amplified and leads to activation of a larger pool of PKC-ζ. Further studies on acute ^32P labeling of specific phosphorylation sites in intact cells are needed to answer the question of whether PDK-1 or its action upon the threonine 410 site is acutely regulated or whether PDK-1 functions more chronically and permissively but is nevertheless required for insulin-induced activation of PKC-ζ/λ.

Our finding that insulin activated transfected HA-Δ1–247-PKC-ζ to approximately the same extent as full-length transfected HA-PKC-ζ was surprising, because this truncated form of PKC-ζ, which lacks the N-terminal regulatory domain and its inhibitory pseudosubstrate sequence, is generally considered to function as a constitutively active PKC-ζ. However, we have reported that, despite an elevated base line, insulin is able to provoke further increases in HA-GLUT4 translocation in cells expressing large amounts of the Δ1–247 truncated form of PKC-ζ, as well as another “constitutive” form of PKC-ζ in which the pseudosubstrate site has been mutated (1, 3, 20); obviously, the present findings provide a clear explanation for previously reported findings in studies of GLUT4 translocation. Somewhat similar to our finding that HA-Δ1–247-PKC-ζ was activated by insulin, Le Good et al. (6) found that expression of PDK-1 stimulated the activity of a co-expressed N-terminal truncated form of PKC-ζ in a PI 3-kinase-dependent manner. These authors suggested that D3-PO_4 polyphosphoinositides activate both truncated and full-length PKC-ζ at least partly through activating effects on PDK-1 rather than working solely by interacting with the regulatory domain of full-length PKC-ζ and promoting access of the threonine 410 activation loop site in the catalytic domain of PKC-ζ to PDK-1. Our finding that insulin activates Δ1–241-PKC-ζ is in accord with the postulate of Le Good et al. (6); however, as discussed above, it is also possible that truncated PKC-ζ was activated via transphosphorylating effects of endogenous full-length PKC-ζ/λ. Furthermore, it may be noted that IGF-1 does not alter the activity of immunoprecipitable PDK-1 in 293 cells (21), and insulin did not appear to activate PDK-1 in CHO/IR cells (22); the latter findings suggest that PDK-1 is not co-valently modified after IGF-1 or insulin treatment in a manner that of itself confers an increase in enzymic activity but nevertheless leaves open the possibility that PDK-1 may acutely activated or its action acutely facilitated by the PI 3,4,5-(PO_4)_3 ligand. Clearly, further studies are needed to see whether PDK-1 is acutely activated, co-valently or noncovalently, by insulin.

As alluded to above, it is clear from our studies of PKB activation that RO 31-8220 and the PKC-ζ/λ pseudosubstrate did not inhibit the activation or subsequent action of PDK-1 on PKB during insulin action (this conclusion follows if it is assumed that PDK-1 is largely responsible for activating PKB). Consequently, inhibitory effects of RO 31-8220 and the PKC-ζ/λ pseudosubstrate on the activation of PKC-ζ/λ in intact cells raised the possibility that a mechanism distinct from and probably subsequent to PDK-1 activation and action was required for PKC-ζ/λ activation. In this regard, our findings seem most compatible with the possibility that the autophosphorylation of PKC-ζ and PKC-λ is the non- or post-PDK-1 mechanism that is required for full activation of PKC-ζ and PKC-λ. As a corollary, our findings also seem very compatible with the possibility that changes in autophosphorylation are largely responsible for the more stable increases in enzyme activity that are observed in PKC-ζ/λ immunoprecipitates following insulin treatment. Along these lines, it may be noted that threonine 410 phosphorylation, as mediated by PDK-1, may be very short-lived, as opposed to more stable changes owing to autophosphorylation. It is also possible that PDK-1 effects on PKC-ζ are relatively stable and not subject to acute regulation; in this scenario, insulin-induced increases in D3-PO_4 polyphosphoinositides would be needed to acutely activate PKC-ζ via an autophosphorylation or transphosphorylation mechanism. Further studies on the turnover of phosphate groups at PDK-1-dependent threonine 410 and autophosphorylation-dependent sites may be helpful in deciding between these possibilities.

It is of interest that we have found in other studies (20) that insulin effects on GLUT4 translocation in rat adipocytes are comparably inhibited by kinase-inactive forms of both PKC-ζ and PKC-λ; moreover, inhibitory effects of each of these kinase-inactive atypical PKCs on GLUT4 translocation can be reversed by the wild-type form of either atypical PKC, ζ or λ (20). Coupling this information with the present findings, it may be surmised that both atypical PKCs, ζ and λ, are activated and seem to function interchangeably in supporting the translocation of GLUT4 during the action of insulin. Accordingly, it will be important to compare the levels and activation of both atypical PKCs in various insulin-sensitive cell types.

Finally, it was of interest to find that both PKC-ζ and PKC-λ are not only activated by insulin but that both PKCs are present and apparently increased in amount in GLUT4 vesicles during insulin treatment. On the other hand, this enrichment of PKC-ζ and λ may not be greater than that occurring in the more general microsomal compartment, and, moreover, it is presently not clear that the PKC-ζ and PKC-λ that are present in these vesicles are in fact important in promoting the translocation of GLUT4 vesicles to the plasma membrane. Along these lines, we have presently documented that insulin activates atypical PKCs in plasma membranes, as well as in microsomes and GLUT4 vesicles. It remains for future studies to determine which membrane site(s) and which atypical PKC substrate(s) is (are) specifically required and rate-limiting for GLUT4 translocation.

In summary, our findings provide evidence that insulin activates both PKC-ζ and PKC-λ by a mechanism that is dependent upon PI 3-kinase activation, generation of D3-polyphosphoinositides, acute or continued activating effects of PDK-1 on activation loop phosphorylation sites, and subsequent auto-phosphorylation of PKC-ζ and PKC-λ. Further studies are needed to identify the specific phosphorylation sites that are responsible for enzymic activation of PKC-ζ and PKC-λ.

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