Dependence of Insulin-Stimulated Glucose Transporter 4 Translocation on 3-Phosphoinositide-Dependent Protein Kinase-1 and Its Target Threonine-410 in the Activation Loop of Protein Kinase C-ζ

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Previous studies have suggested that 1) atypical protein kinase C (PKC) isoforms are required for insulin stimulation of glucose transport, and 2) 3-phosphoinositide-dependent protein kinase-1 (PDK-1) is required for activation of atypical PKCs. Presently, we evaluated the role of PDK-1, both in the activation of PKC-ζ, and the translocation of epitope-tagged glucose transporter 4 (GLUT4) to the plasma membrane, during insulin action in transiently transfected rat adipocytes. Overexpression of wild-type PDK-1 provoked increases in the activity of cotransfected hemagglutinin (HA)-tagged PKC-ζ and concomitantly enhanced HA-tagged GLUT4 translocation. Expression of both kinase-inactive PDK-1 and an activation-resistant form of PKC-ζ that is mutated at Thr-410, the immediate target of PDK-1 in the activation loop of PKC-ζ, inhibited insulin-induced increases in both HA-PKC-ζ activity and HA-GLUT4 translocation to the same extent as kinase-inactive PKC-ζ. Moreover, the inhibitory effects of kinase-inactive PDK-1 were fully reversed by cotransfection of wild-type PDK-1 and partly reversed by wild-type PKC-ζ, but not by wild-type PKB. In contrast to the T410A PKC-ζ mutant, an analogous double mutant of PKB (T308A/S473A) that is resistant to PDK-1 activation had only a small effect on insulin-stimulated HA-GLUT4 translocation and did not inhibit HA-GLUT4 translocation induced by overexpression of wild-type PDK-1. Our findings suggest that both PDK-1 and its downstream target, Thr-410 in the activation loop of PKC-ζ, are required for insulin-stimulated glucose transport. (Molecular Endocrinology 13: 1766–1772, 1999)

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INTRODUCTION

Glucose transporter 4 (GLUT4)-dependent glucose transport, the rate-limiting step in insulin-stimulated glucose disposal, appears to be activated, at least in part, by a protein kinase that operates distal to phosphatidylinositol (PI) 3-kinase. In this regard, insulin activates protein kinase C (PKC)-ζ and -λ via PI 3-kinase (1–5), and these atypical PKCs appear to be required for insulin-stimulated GLUT4 translocation and glucose transport in rat adipocytes (1, 2), 3T3/L1 adipocytes (3, 4), and L6 myotubes (5). Similarly, insulin activates protein kinase B (PKB) via PI 3-kinase (6, 7), and, although only a relatively small fraction of insulin-stimulated GLUT4 translocation in rat adipocytes appears to require PKB (8), constitutively active PKB (8–10), like constitutively active PKC-ζ (1, 4, 5), pro-

vokes strong insulin-like effects on GLUT4 translocation and/or glucose transport in rat adipocytes and 3T3/L1 adipocytes.

In addition to the above described similarities, PKB (11, 12) and atypical PKCs (13, 14) may be activated by comparable or seemingly related mechanisms that involve concomitant increases in D3-PO_4 polyphosphoinositides and action of a 3-phosphoinositide-dependent protein kinase-1 (PDK-1). Accordingly, in conjunction with increases in D3-PO4 polyphosphoinositides, PDK-1 phosphorylates analogous sites in the activation loops of both PKB and PKC-ζ, *viz*., Thr-308 in PKB (11, 12) and Thr-410 in PKC-ζ (13, 14). Phosphorylation of these sites by PDK-1 is thought to be required for and, indeed, may facilitate other phosphorylations, including autophosphorylation, and subsequent activation of PKB and PKC-ζ. In the case of PKC-ζ, however, it has not been shown that PDK-1 is, in fact, required for 1) insulin-induced increases in PKC-ζ activity, or 2) insulin effects on biological processes. Presently, we examined 1) the role of PDK-1 in insulin-induced activation of PKC-ζ, and 2) the importance of PDK-1 and its targets, Thr-308 in PKB and Thr-410 in PKC-ζ, during insulin stimulation of epitope-tagged GLUT4 translocation in transiently transfected rat adipocytes.

RESULTS AND DISCUSSION

As seen in Figs. 1 and 2, overexpression of wild-type (WT) PDK-1 in transiently transfected rat adipocytes provoked increases in the translocation of hemagglutinin (HA)-tagged GLUT4 to the plasma membrane that were approximately 55% of the increases observed with insulin treatment. Insulin provoked further increases in HA-GLUT4 translocation in cells overexpressing WT-PDK-1, presumably via activation of PI 3-kinase, increased availability of D3-PO_4 polyphosphoinositides (see Refs. 3, 5, and 15), and further activation of PKC-ζ (see Ref 14. and below). In contrast to WT-PDK-1, expression of kinase-inactive (KI) PDK-1 (Lys-110 mutated to Asn; see Ref. 13) inhibited insulin-stimulated HA-GLUT4 translocation, to about the same extent as KI-PKC-ζ (Fig. 1), which is known to inhibit this process (1, 2). As seen in Fig. 2, the inhibitory effect of KI-PDK-1 on insulin-stimulated HA-GLUT4 translocation was reversed or prevented by cotransfection of WT-PDK-1: this verified that inhibitory effects of KI-PDK-1 were due to the specific mutation in its catalytic domain. Of further note, the inhibitory effect of KI-PDK-1 on insulin-stimulated HA-GLUT4 translocation was also partially reversed or prevented by coexpression of WT-PKC-ζ significantly, but not WT-PKB (Fig. 3): these, along with other findings (see below), suggested that the inhibitory effect of KI-PDK-1 was more dependent upon a failure of insulin to activate PKC-ζ, as compared with PKB.

In addition to KI-PDK-1, expression of a mutated (T410A) form of PKC-ζ that is resistant to activation by

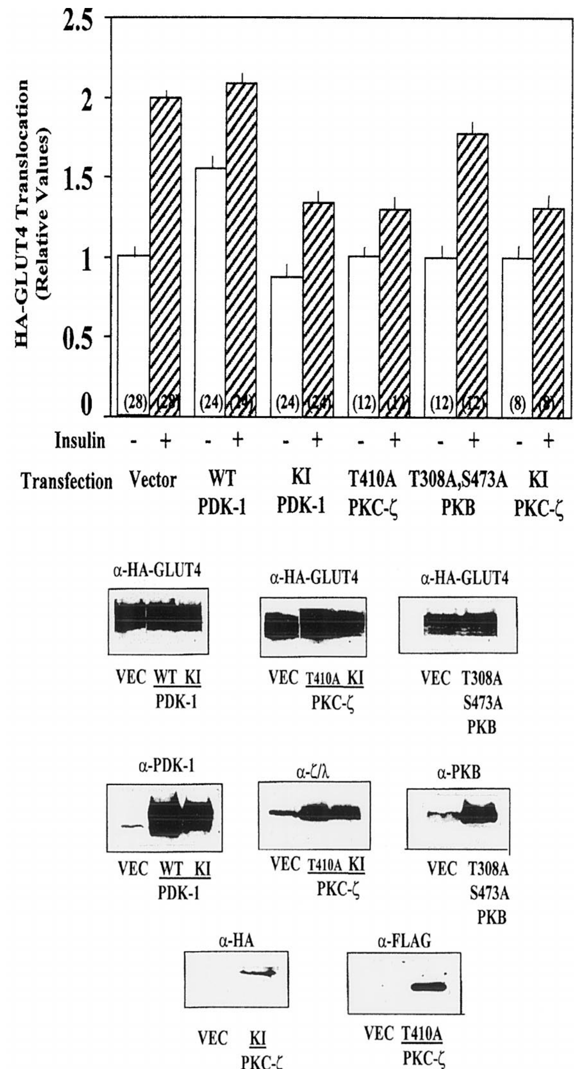

Fig. 1. Effects of WT-PDK-1, KI-PDK-1, KI-PKC-ζ, and Mutant Activation-Resistant Forms of PKC-ζ (T410A) and PKB (T308A,S473A) on Insulin-Stimulated Translocation of HA-GLUT4 to the Plasma Membrane of Rat Adipocytes

Adipocytes were prepared, transiently cotransfected with plasmids encoding HA-GLUT4 and indicated proteins, and subsequently incubated as described in *Materials and Methods*. Values for HA-GLUT4 translocation (normalized to allow pooling of results of multiple experiments) are mean ± SE of (n) determinations. Representative immunoblots shown here demonstrate large increases in expressed proteins; *viz*., WT-PDK-1, KI-PDK-1, and PKB●T308A●S473A, as blotted with anti-PDK-1 and anti-PKB antibodies; KI-PKC-ζ and PKC-ζ●T410A, as blotted with both anti-PKC-ζ and anti-epitope antibodies; and HA-GLUT4, as blotted with anti-HA antibodies. Note that, in multiple comparisons, HA-GLUT4 expression was not altered significantly by coexpression of these signaling proteins [HA-GLUT4 values (mean ± SE) of signaling protein transfectants, relative to corresponding vector-transfected controls set at 100%, were: KI-PDK-1, 99 ± 8% (n = 7); T410A-PKC-ζ, 107 ± 11% (n = 5); WT-PDK-1, 109 ± 7% (n = 6); KI-PKC-ζ, 100 ± 10% (n = 5) and T308A, S473A-PKB, 104 ± 10% (n = 8)]. Also note that the vectors did not alter control or insulin-stimulated HA-GLUT4 translocation. See Fig. 2 for actual experimental data in typical experiments.

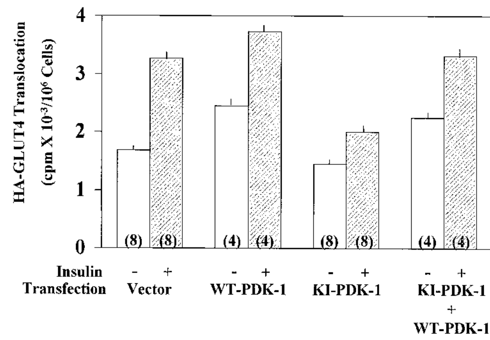

Fig. 2. WT-PDK-1 Reverses Inhibitory Effects of KI-PDK-1 on Insulin-Stimulated HA-GLUT4 Translocation

Experiments were conducted as in Fig. 1, except that 7 μg each of pCDNA3/WT-PDK-1 and/or pCDNA3/KI-PDK-1 were used, along with 3 μg pCIS2 encoding HA-GLUT4, and total DNA was kept constant by varying the amount of pCDNA3 vector used for electroporation (total, 14 μg/0.8 ml cell suspension). Values are mean ± SE of (n) determinations.

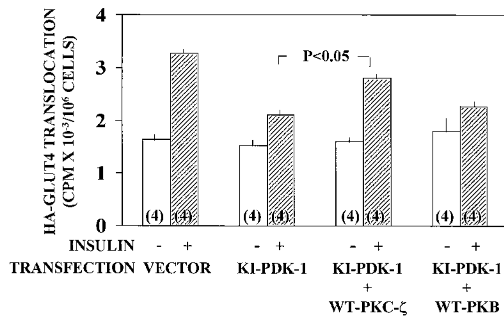

Fig. 3. Effects of WT-PKC-ζ and WT-PKB on Inhibitory Effects of KI-PDK-1 on Insulin-Stimulated GLUT4 Translocation

Experiments were conducted as in Fig. 2, except that 7 μg pCDNA3/WT-PKC-ζ or pCIS2/WT-PKB were co-transfected along with 7 μg pCDNA3 encoding KI-PDK-1 and 3 μg pCIS2 encoding HA-GLUT4. Values are mean ± SE of (n) determinations.

PDK-1 (13, 14) inhibited insulin-stimulated HA-GLUT4 translocation to the same extent as both PDK-1 and KI-PKC-ζ, *i.e.* by approximately 65% (Fig. 1). In comparison, expression of an analogous activation-resistant double-mutant (T308A,S473A) form of PKB, which functions as a dominant-negative for certain actions of insulin (but not glucose transport) in 3T3/L1 adipocytes (16), inhibited insulin-stimulated HA-GLUT4 translocation by only 20%; nevertheless, as discussed below, this modest inhibitory effect of double-mutant PKB is similar to that observed with KI-PKB in rat adipocytes (8). In comparing the effects of these activation-resistant mutant forms of PKC-ζ and PKB (see Figs. 1 and 4 and Ref 15), note that, relative to endogenous levels, increases in the level of total PKB in cells transfected with the double PKB mutant were, if anything, greater than the increases in total PKC-ζ observed in cells transfected with the T410A-PKC-ζ mutant: thus, it is clear that, relative to endogenous levels, there were marked increases in levels of the expressed PKB double mutant in transfected cells.

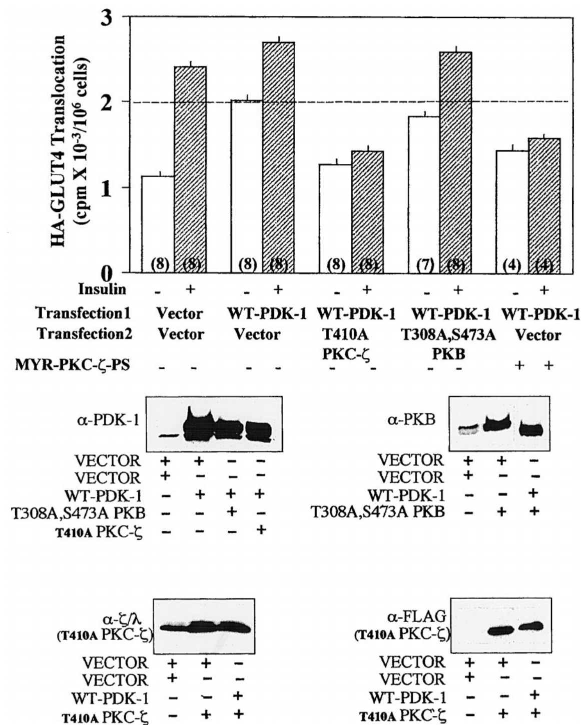

Fig. 4. Effects of Mutant Activation-Resistant Forms of PKC-ζ (T410A) and PKB (T308A,S473A) and the Cell-Permeable Myristoylated PKC-ζ Pseudosubstrate on HA-GLUT4 Translocation in Rat Adipocytes Overexpressing WT-PDK-1

Cells were transfected and incubated as described in Fig. 1. Where indicated, some cells were treated with 50 μM myristoylated PKC-ζ pseudosubstrate (MYR-PKC-ζ-PS) [which inhibits PKC-ζ and insulin-stimulated glucose transport (see Ref. 1)] for 60 min before insulin treatment. As shown in immunoblots: expression of WT-PDK-1 was markedly increased and was not significantly altered by coexpression of PKB●T308A●S473A or PKC-ζ●T410A; expression of PKB●T308A●S473A markedly increased total PKB immunoreactivity and was not influenced by coexpression of PDK-1; and expression of PKC-ζ●T410A increased total PKC-ζ immunoreactivity by approximately 2-fold and was not influenced by coexpression of PDK-1. Values are mean ± SE of (n) determinations. The *broken line* indicates the mean level of HA-GLUT4 translocation observed in cells overexpressing WT-PDK-1, and incubated in the absence of insulin.

Since expression of WT-PDK-1 stimulated HA-GLUT4 translocation even in the absence of insulin, we questioned whether this stimulatory effect of WT-PDK-1 alone was primarily due to activation of PKB or PKC-ζ. For this purpose, we used an activation-resistant T410A mutant form of PKC-ζ, which is only weakly active and cannot be activated by agonists (13), including, as we have recently confirmed, insulin (not shown). As seen in Fig. 4, the T410A mutant form of PKC-ζ markedly inhibited the stimulatory effects of WT-PDK-1 on HA-GLUT4 translocation. The analogous activation-resistant double-mutant (T308A,S473A) form of PKB, which serves as effective

dominant-negative signaling factor (see Ref. 16), on the other hand, had only a small insignificant effect on WT-PDK-1-stimulated HA-GLUT4 translocation (Fig. 4). In further support of the possibility that PKC-ζ serves as a downstream mediator during the action of overexpressed WT-PDK-1, the cell-permeable myristoylated PKC-ζ pseudosubstrate inhibited WT-PDK-1-stimulated HA-GLUT4 translocation (Fig. 4). Note that the myristoylated PKC-ζ pseudosubstrate, which fully inhibits the activity of PKC-ζ *in vitro* (1), as well as the effects of insulin on glucose transport and GLUT4 translocation (1, 2), also inhibits the activation of PKC-ζ by insulin in intact cells (our unpublished observations), apparently by virtue of a requirement for autophosphorylation of threonine-560 and other presently undefined sites after PDK-1-dependent phosphorylation of threonine-410 in the activation loop of PKC-ζ. In contrast, the myristoylated PKC-ζ pseudosubstrate does not inhibit PKB activation or action (our unpublished observations). From these findings, it may be surmised that 1) PKB activation is not dependent upon PKC-ζ, and that 2) inhibitory effects of the PKC-ζ pseudosubstrate on insulin- or PKD-1-stimulated glucose transport and/or GLUT4 translocation cannot be explained by inhibition of PKB.

The above-described findings suggested that PKC-ζ, and/or PKC-λ, since these atypical PKCs apparently function interchangeably in supporting insulin-stimulated GLUT4 translocation in the rat adipocyte (see Ref. 2), serves as a downstream effector(s) for PDK-1 during insulin stimulation; in further support of this possibility, we found that 1) transient expression of WT-PDK-1 markedly enhanced the activity of coexpressed HA-tagged PKC-ζ in control and insulin-treated adipocytes (Fig. 5A), and 2) expression of KI-PDK-1 inhibited the activation of HA-tagged PKC-ζ by insulin (Fig. 5, A and B). These findings provided clear evidence that PDK-1 is required for insulin-induced activation of PKC-ζ; they also provided an explanation for the increase in HA-GLUT4 translocation caused by overexpression of WT-PDK-1 in the absence of insulin (Figs. 1, 2, and 4), although, as is apparent, we did not observe a strict proportionality between PKC-ζ activation and HA-GLUT4 translocation when effects of PDK-1 alone were compared with those of insulin. The latter discrepancy could reflect a more generalized activation of PKC-ζ during simple PDK-1 overexpression, as opposed to more compartmentalized increases in D3-PO_4 polyphosphoinositides and subsequent PKC-ζ activation in specific pools, that probably occur during insulin treatment. The discrepancy may also reflect the participation of factors other than PI 3-kinase, PDK-1, PKC-ζ, and PKB during insulin-stimulated GLUT4 translocation.

The present experimental approach in which we simultaneously used mutant forms of PDK-1 and critical PDK-1-dependent phosphorylation/activation sites in the activation loops of PKC-ζ and PKB allowed us to not only evaluate the role of PDK-1, but also to directly compare the relative roles of two of its targets, PKC-ζ and PKB, in insulin-stimulated GLUT4 translocation. As is apparent, our findings provided further independent support for the hypothesis (1, 2, 4) that PKC-ζ and/or PKC-λ is/are required for insulin-stimulated GLUT4 translocation in the rat adipocyte. PKB, on the other hand, appeared to be required for only a small component of insulin-stimulated GLUT4 translocation, as suggested by relatively mild inhibitory effects of doubly mutated (T308A,S473A) PKB on insulin-stimulated HA-GLUT4 translocation. Nevertheless, the presently observed mild inhibitory effects of the activation-resistant PKB double mutant were similar in magnitude to inhibitory effects of KI-PKB observed previously (8), and it is therefore possible that a small component of the insulin effect on GLUT4 translocation in the rat adipocyte may require the activation of PKB. However, the situation in 3T3/L1 adipocytes may be different, as PKB was not found to be required, even partially, for insulin-stimulated glucose transport in these cells (16).

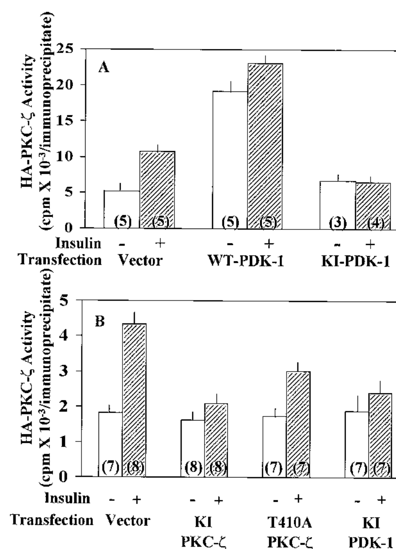

Fig. 5. Effects of WT and KI Forms of PDK-1 on Enzymic Activity of HA-Tagged PKC-ζ (A) and Effects of KI-PKC-ζ, Mutant T410A-PKC-ζ, and KI-PDK-1 on Enzymic Activity of HA-Tagged PKC-ζ (B)

Adipocytes were transiently cotransfected with plasmids encoding HA-PKC-ζ and indicated proteins, and then incubated and assayed as described in *Materials and Methods*. Although not shown here, the recovery of immunoprecipitable HA-PKC-ζ was not influenced by coexpression of WT-PDK-1, KI-PDK-1, KI-PKC-ζ, or PKC-ζ●T410A; also note that, as shown, basal HA-PKC-ζ enzyme activity was not influenced by expression of these cotransfected proteins. Values are mean ± SE of (n) determinations.

The strong activation of HA-PKC-ζ caused by simple overexpression of WT-PDK-1 was surprising, as it indicated that PDK-1 could act by mass action without concomitant increases in PI 3-kinase activity and the availability of D3-PO_4 polyphosphoinositides. However, overexpressed WT-PDK-1 may have amplified

the effects of resting, but nevertheless required levels of D3-PO_4 polyphosphoinositides (see Ref. 14) and superimposed insulin effects on HA-PKC-ζ activity and HA-GLUT4 translocation, albeit smaller in cells overexpressing WT-PDK-1, may reflect the activation of PI 3-kinase and further increases in D3-PO_4 polyphosphoinositides, followed by activation of HA-PKC-ζ and/or endogenous PKC-ζ, particularly in specific cellular locations that are important for GLUT4 translocation.

The above findings showed that expression of both the KI and the mutant (T410A) forms of PKC-ζ, as well as KI-PDK-1, inhibited insulin-stimulated HA-GLUT4 translocation. A plausible mechanism for these inhibitory effects was studied by examining the effects of these inhibitory mutant kinases on the activation of intact PKC-ζ. To this end, we cotransfected plasmids encoding these inhibitory mutant kinases, along with plasmid encoding HA-tagged PKC-ζ. As seen in Fig. 5B, insulin-stimulated HA-PKC activity was inhibited to the extent of 80%, 50%, and 80% by expression of KI-PKC-ζ, T410A-PKC-ζ, and KI-PDK-1, respectively. Importantly, basal HA-PKC activity was not affected by coexpression of these inhibitory proteins, thus militating against the possibility of nonspecific effects. Although the inhibitory effects of these mutant proteins on insulin-stimulated HA-PKC-ζ enzyme activity did not match up perfectly with the inhibitory effects on HA-GLUT4 translocation (*cf*. Figs. 1 and 5), it nevertheless seemed plausible, if not likely, that the inhibition of PKC-ζ was an important mechanistic factor to explain the observed inhibitory effects of KI-PKC-ζ, T410A-PKC-ζ, and KI-PDK-1 on insulin-stimulated HA-GLUT4 translocation.

In contrast to the inhibitory effects of KI-PKC-ζ and T410A-PKC-ζ on insulin-induced activation of both HA-tagged WT-PKC-ζ and HA-tagged GLUT4 translocation, the expression of these PKC mutants did not inhibit the activation of AU1-tagged PKB by insulin (Fig. 6): clearly, the inhibitory effects of these PKC-ζ mutants could not be explained by changes in PKB activity. On the other hand, as expected (13, 14), KI-PDK-1 inhibited insulin-induced activation of AU1-PKB (Fig. 6).

Finally, it should be emphasized that, although findings from the presently used transfection system allowed us to surmise that PKC-ζ participates in insulin-induced translocation of GLUT4, there are inherent caveats with this experimental approach, including 1) only a small fraction of adipocytes are successfully transfected during electroporation; 2) the expression of foreign mutated signaling proteins in transfected cells is very large relative to the level of endogenous wild-type proteins; 3) transfected epitope-tagged HA-GLUT4 may not be fully comparable to endogenous GLUT4; and 4) insulin effects on HA-GLUT4 in cultured adipocytes are modest in comparison to insulin effects on endogenous GLUT4 translocation and subsequent glucose transport in fresh adipocytes (this largely reflects an artefactual increase in basal activity during the overnight incubation of cultured adipocytes). On the other hand, our findings with the present transfection system are at least qualitatively similar to those observed in studies of adenoviral transfer of KI-PKC-λ in 3T3/L1 adipocytes, in which insulin effects on endogenous GLUT4 translocation and glucose transport were measured (4). Clearly, there is a need to utilize various experimental approaches to test the hypothesis that PI 3-kinase/PDK-1-dependent activation of atypical PKCs, ζ and/or λ, is required for insulin stimulation of glucose transport.

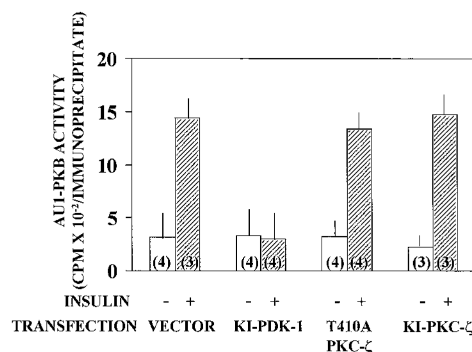

Fig. 6. Effects of KI-PDK-1, T410A-PKC-ζ, and KI-PKC-ζ on Enzymic Activity of PKB

Rat adipocytes were transiently cotransfected with plasmids encoding AU1-tagged PKB and indicated proteins, and then incubated and assayed as described in *Materials and Methods*. Values are mean ± SE of (n) determinations.

In summary, our findings show that PDK-1 and its immediate downstream target, *viz*. the Thr-410 phosphorylation site in the activation loop of PKC-ζ, are required for 1) insulin-induced activation of PKC-ζ, and 2) insulin stimulation of GLUT4 translocation in the rat adipocyte. Further studies are needed to determine whether insulin acutely regulates PDK-1 activity and/or subsequent Thr-410 phosphorylation.

MATERIALS AND METHODS

Adipocytes were prepared and transiently cotransfected as described previously (1, 2). In brief, to study epitope HA-tagged GLUT4 translocation, 0.8 ml 50% adipocyte suspension was electroporated in DMEM in the presence of 3 μg pCIS2 containing cDNA insert encoding HA-GLUT4 (all cells), along with 7 μg of the following plasmid/cDNA constructs: pCDNA3/WT-PDK-1; pCDNA3/KI-PDK-1; pCMV5/T410A-PKC-ζ (FLAG-tagged); pCIS2/T308A, S473A-PKB; pCIS2/WT-PKB; pCDNA3/KI-PKC-ζ (HA-tagged); pCDNA3/WT-PKC-ζ; or cDNA insert-free plasmids (vector groups). Constructs for WT-PDK-1, KI-PDK-1, and T410A-PKC-ζ were kindly supplied by Dr. Alex Toker and are described more fully in Ref. 13. The double-mutant form of PKB was made by site-directed mutagenesis of the wild-type construct (8) using the MORPH mutagenesis kit according to the manufacturer's instructions (5′ → 3′, Inc., Boulder, CO). Mutagenic oligonucleotides 5′-CCA CTA TGA AGG CAT TTT GCG GAA CGC CGG-3′ and 5′-TTC CCC CAG TTC GCC TAC TCG GCC AGT GGC ACA-3′ created point mutations T308A and S473A, as well as silent mutations that created a

new *Bgl*I site and disrupted an *Xmn*I site. Mutations were confirmed by direct sequencing. The pCDNA3/KI-PKC-ζ construct was described previously (1–3).

Cells were incubated overnight for 20–24 h to allow time for expression of cDNA inserts (see Refs. 1–3). As shown in immunoblots further described in the text, note that 1) HA-GLUT4 expression was not altered by coexpression of various proteins; 2) there were large increases in total cellular PDK-1, PKB, and PKC-ζ in transfected cells, as compared with cells transfected with vector alone (VEC); and 3) successfully transfected cells comprise only 5–10% of total cells, and increases in expressed proteins in transfected cells are therefore 10–20 times greater than that suggested by simple inspection of immunoblots of total cell lysates. Sources for antibodies used for immunoblotting were: PDK-1 and PKB (Upstate Biotechnology, Inc., Lake Placid, NY); PKC-ζ/λ (Santa Cruz Biotechnology, Inc.), HA (Covance); and FLAG (Zymed Laboratories, Inc., South San Francisco, CA)].

After overnight incubation with plasmids, cells were washed and incubated for 30 min in glucose-free Krebs Ringer phosphate (KRP) medium containing 1% BSA, with or without 10 nM insulin as indicated. Where indicated, some cells were treated with 50 μM myristoylated PKC-ζ pseudosubstrate (MYR-PKC-ζ-PS) [which inhibits PKC-ζ and insulin-stimulated glucose transport (see Ref. 1)] for 60 min before insulin treatment. After incubation, cell surface HA-GLUT4 was measured, using mouse monoclonal anti-HA antibodies (Covance, Berkeley, CA) and ^{125}I-labeled sheep antimouse IGG second antibody (Amersham Pharmacia Biotech), as described previously (1, 2).

To study HA-tagged PKC-ζ activation, cells (0.8 ml 50% suspension) were electroporated in the presence of 1 μg pCDNA3 containing cDNA encoding HA-tagged PKC-ζ (see Ref. 2), along with 7 μg pCDNA3 containing no insert (vector group) or cDNA insert encoding KI-PKC-ζ, T410A-PKC-ζ, KI-PDK-1, or WT-PDK-1. After incubation for 20–24 h to allow time for expression, cells were washed and incubated for 10 min in glucose-free KRP medium with or without 10 nM insulin as indicated. After incubation, cells were sonicated, and 400 μg cell lysate protein were subjected to immunoprecipitation with mouse monoclonal anti-HA antibodies (Covance), after which HA-PKC-ζ immunoprecipitates were collected (see Ref. 2), washed, and assayed for PKC-ζ enzyme activity as described previously (1, 2). Note that expression and recovery of immunoprecipitable HA-PKC-ζ was not altered by coexpression of other proteins.

To study AU1-tagged PKB activation, cells (0.8 ml 50% suspension) were electroporated in the presence of 3 μg pCDNA3 encoding AU1-tagged PKB (cDNA prepared as in Ref. 17 and subcloned into pCDNA3), along with 7 μg pCDNA3 encoding KI-PKC-ζ, T410A-PKC-ζ, or KI-PDK-1. After incubation for 20–24 h to allow time for expression, cells were washed and incubated for 3 min with or without 10 nM insulin. After incubation, cells were sonicated, and 200 μg cell lysate protein were immunoprecipitated with mouse monoclonal anti-AU1 antibody (Covance), after which AU1-PKB immunoprecipitates were collected and assayed for enzyme activity as described previously (18).

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