Insulin and PIP_3 Activate PKC-ζ by Mechanisms That Are Both Dependent and Independent of Phosphorylation of Activation Loop (T410) and Autophosphorylation (T560) Sites†

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ABSTRACT: Activation of protein kinase C-ζ (PKC-ζ) by insulin requires phosphatidylinositol (PI) 3-kinase-dependent increases in phosphatidylinositol-3,4,5-$(PO_4)_3$ (PIP_3) and phosphorylation of activation loop and autophosphorylation sites, but actual mechanisms are uncertain. Presently, we examined: (a) acute effects of insulin on threonine (T)-410 loop phosphorylation and (b) effects of (i) alanine (A) and glutamate (E) mutations at T410 loop and T560 autophosphorylation sites and (ii) N-terminal truncation on insulin-induced activation of PKC-ζ. Insulin acutely increased T410 loop phosphorylation, suggesting enhanced action of 3-phosphoinositide-dependent protein kinase-1 (PDK-1). Despite increasing in vitro autophosphorylation of wild-type PKC-ζ and T410E-PKC-ζ, insulin and PIP_3 did not stimulate autophosphorylation of T560A, T560E, T410A/T560E, T410E/T560A, or T410E/T560E mutant forms of PKC-ζ; thus, T560 appeared to be the sole autophosphorylation site. Activating effects of insulin and/or PIP_3 on enzyme activity were completely abolished in T410A-PKC-ζ, partially compromised in T560A-PKC-ζ, T410E/T560A-PKC-ζ, and T410A/T560E-PKC-ζ, and largely intact in T410E-PKC-ζ, T560E-PKC-ζ, and T410E/T560E-PKC-ζ. Activation of the T410E/T560E mutant suggested a phosphorylation-independent mechanism. As functional correlates, insulin effects on epitope-tagged GLUT4 translocation were compromised by expression of T410A-PKC-ζ, T560A-PKC-ζ, T410E/T560A, and T410A/T560E-PKC-ζ but not T410E-PKC-ζ, T560E-PKC-ζ, or T410E/T560E-PKC-ζ. Insulin, but not PIP_3, activated truncated, pseudosubstrate-lacking forms of PKC-ζ and PKC-λ by a wortmannin-sensitive mechanism, apparently involving PI 3-kinase/PDK-1-dependent phosphorylations but independent of PIP_3-dependent conformational activation. Our findings suggest that insulin, via PIP_3, provokes increases in PKC-ζ enzyme activity through (a) PDK-1-dependent T410 loop phosphorylation, (b) T560 autophosphorylation, and (c) phosphorylation-independent/conformational-dependent relief of pseudosubstrate autoinhibition.

Insulin is known to activate atypical protein kinase C (PKCs),[1] ζ and λ, in a variety of cell types through phosphatidylinositol (PI) 3-kinase-dependent increases in PI-3,4,5-$(PO_4)_3$ (PIP_3) (*1*−*5*), but the mechanisms that PIP_3 uses to activate atypical PKCs are presently unclear. Insulin-induced activation of PKC-ζ has been shown to be dependent upon phosphorylation of its activation loop site, threonine-410 (T410), by 3-phosphoinositide dependent protein kinase-1 (PDK-1) (*6*), which is either activated directly by PIP_3(*7*), or whose action on substrates, such as PKC-ζ and protein kinase B (PKB/AKT), may be facilitated by PIP_3 (*8*, *9*). However, whether PDK-1 activation and/or action is/are increased by insulin is presently uncertain, and, in this regard, in our initial studies, we did not obtain conclusive information as to whether insulin provokes increases in the phosphorylation of the target of PDK-1 in PKC-ζ, viz., T410 (*2*). On the other hand, we found that insulin and PIP_3 provoke increases in both the enzyme activity and the autophosphorylation of a T410E mutant form of PKC-ζ (*2*), indicating that PIP_3 can activate PKC-ζ independently of PDK-1-dependent phosphorylation of T410, presumably by a mechanism(s) that involves an increase in autophosphorylation, most likely at the T560 site (*10*, *11*), and/or through conformational (allosteric) alterations that presumably relieve autoinhibition by the pseudosubstrate sequence present in the N-terminal regulatory domain of PKC-ζ. Presently, we (a) revisited the question of whether PDK-1-dependent phosphorylation of T410 is increased by insulin and (b) used various T410 and T560 mutant and truncated forms of PKC-ζ to obtain further insight into the mechanisms that are used by PIP_3 to activate PKC-ζ during insulin action in rat adipocytes.

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[1] Abbreviations: PKC, protein kinase C; PKB/AKT, protein kinase B; PDK-1, 3-phosphoinositide-dependent protein kinase-1; T, threonine; A, alanine; E, glutamate; PI, phosphatidylinositol; PIP_3, phosphatidylinositol-3,4,5-$(PO_4)_3$; KRP, Krebs Ringer phosphate; DMEM, Dulbecco's minimal Eagle's medium; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WT, wild-type; V or VEC, vector; α, anti-; HA, hemagglutinin antigen; GLUT4, glucose transporter 4.

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EXPERIMENTAL PROCEDURES

Cell Preparations and Studies of PKC-ζ Activation. Adipocytes were prepared by collagenase digestion of rat epididymal fat pads as described (*1*, *2*). Cells were transfected as described below and subsequently incubated in glucose-free Krebs Ringer phosphate medium (KRP) containing 1% bovine serum albumin (BSA), with or without 10 nM insulin as indicated. After incubation, cells were sonicated and lysates were subjected to immunoprecipitation with antibodies that target either the C-termini of total cellular PKC-ζ and PKC-λ (antiserum from Santa Cruz Biotechnologies, Santa Cruz CA) or FLAG (antiserum from Zymed Laboratories Inc., San Francisco, CA) or HA (mouse monoclonal antibodies from Covance, Berkeley, CA) epitopes of various forms of transfected PKC-ζ, as described (*1*, *2*). Precipitates were then washed and assayed for PKC-ζ/λ-dependent substrate phosphorylation or autophosphorylation as described (*1*, *2*). The autophosphorylation assay was previously validated by showing that the in vitro phosphorylation of electrophoretically resolved 75-kDa PKC-ζ is inhibited by PKC inhibitors, viz., RO 31−8220 and the PKC-ζ pseudosubstrate (*1*, *2*).

Transfection of Adipocytes. As described (*1*, *2*, *6*, *12*), rat adipocytes, as 50% suspensions, were transiently transfected by electroporation in 0.8 mL of DMEM medium containing pCMV5 encoding FLAG-tagged forms of wild-type PKC-ζ or the following PKC-ζ mutants, T410A, T410E, T560A, T560E single mutants, or T410A/T560E, T410E/T560A, or T410E/560E double mutants, or HA-tagged truncated Δ1−247 or truncated/mutant Δ1−247/T410E/T560E forms of PKC-ζ with (in GLUT4 translocation studies) or without (in enzyme activation studies) pCIS encoding HA-tagged GLUT4 glucose transporter (*1*, *6*, *12*) or MYC-tagged GLUT4 glucose transporter (kindly supplied by Dr. Brent Reed), as indicated. The cells were then incubated overnight to allow time for expression, which was documented by Western analysis as described (*1*, *6*, *12*). The FLAG-tagged wild-type, T410A, and T410E forms of PKC-ζ were kindly provided by Dr. Alex Toker and have been characterized previously (*2*). The T560A and T560E PKC-ζ single and double mutants were prepared by site-directed mutagenesis of either wild-type or T410A or T410E mutant forms of PKC-ζ, using a Gene Editor kit provided by Promega and mutagenizing oligonucleotides, 5′-GAGCCCGTACAGCTG-GCGCCAGATGATGAG-3′, which substitutes an alanine GCG codon for a threonine ACC codon and introduces a unique *Nar*1 site, and 5′-GAGCCCGTACAGCTCGAGC-CAGATGATGAG-3′, which substitutes a leucine CTC codon for a leucine CTG codon, as well as a glutamate GAG codon for a threonine ACC codon, and introduces a unique *Xho*1 site. The truncated double EE mutant form of PKC-ζ, viz., HA-tagged Δ1−247/T410E/T560E- PKC-ζ, was prepared by site-directed mutagenesis of T410 and T560 sites of a pCDNA3 plasmid encoding this truncated HA-tagged Δ1−247 PKC-ζ (*1*, *2*, *12*) using the aforesaid primer for the T560E mutation and a 5′-CGGGGTTCCACAGAAT-TCGCTTGTTGTGTCA-3′ primer containing a unique GAATTC *Eco*R1 site for the T410E mutation. Mutations were confirmed by both restriction enzyme and sequence analyses.

Expression of Mutated and Truncated Forms of PKC-ζ and PKC-λ in L6 Myotubes. In a few experiments, where indicated, mutant FLAG-tagged T410E/T560E-PKC-ζ (see above) or truncated Δ1−247 HA-tagged PKC-ζ (in pCDNA3; *1*, *2*, *12*) was transfected into L6 myotubes using Lipofectamine as described (*3*). Myotubes were also infected with 10 MOI (multiplicity of infection or viral particles per cell) adenovirus encoding truncated Δ1−135 PKC-λ (kindly provided by Dr. Masato Kasuga; see ref *4*). After 48 h, plasmid-transfected and virus-infected myotubes were lysed, and epitope-tagged PKCs were immunoprecipitated and used for studies of effects of PIP_3 on PKC-ζ/λ activity.

Assay for HA-GLUT4 or MYC-GLUT4 Translocation. Mutation in the ATP-binding site of PKC-ζ (*1*, *12*) and T410A mutation of PKC-ζ (*6*) have been reported to inhibit the translocation of epitope-tagged (HA) GLUT4 to the plasma membrane in transiently cotransfected rat adipocytes. Presently, we used this cotransfection approach to develop functional correlates for other truncated or mutant forms of PKC-ζ, i.e., A and E mutations at T410 and/or T560. Cells were cotransfected with plasmids encoding HA-GLUT4 and wild-type and various truncated or mutant forms of PKC-ζ as described above. After overnight incubation to allow time for expression, the cells were washed and incubated in glucose-free KRP medium and treated for 30 min with or without 10 nM insulin. After incubation, cell-surface HA-GLUT4 or MYC-GLUT4 was measured, using mouse monoclonal anti-HA (Covance, CA) or anti-MYC (Upstate Biotechnologies Inc., Lake Placid, NY) primary antibody and ^{125}I-labeled sheep antimouse IGG second antibody (Amersham Pharmacia Biotech) as described (*1*, *6*, *12*).

Assessment of PDK-1-Dependent Phosphorylation of Threonine-410 in PKC-ζ. One milligram of lysate protein was immunoprecipitated with antibodies that target the C-terminus of PKC-ζ/λ (Santa Cruz Biotechnologies, CA), resolved by SDS−PAGE, transferred to nitrocellulose membranes, and blotted with rabbit anti-pT410 polyclonal antiserum, prepared by Dr. Alex Toker and previously validated as being specific for PDK-1-dependent phosphorylation of threonine-410 (*2*). With this amount of lysate/immunoprecipitate, immunoblot signals were sufficiently strong, and insulin effects were regularly observed (see below).

RESULTS

Expression of Threonine-560 Mutant Forms of PKC-ζ In Transiently Transfected Rat Adipocytes. Transfection of adipocytes with plasmids encoding wild-type and T560A, T560E, T410E/T560E, T410E/T560A, T410A/T560E, T410E, and T410A mutant forms of PKC-ζ led to comparable, substantial increases in total, i.e., endogenous plus expressed, immunoreactive PKC-ζ recovered in cell lysates (Figure 1). As reported and discussed previously (*1*, *2*, *6*, *12*), actual increases in transfected/expressed forms of PKC-ζ, relative to endogenous PKC-ζ, in successfully transfected adipocytes, were, in fact, considerably greater than the relative increases observed in total cell lysates.

Effects of Mutation of Threonine-560 On Activation and Activity of PKC-ζ. As compared to wild-type PKC-ζ, substitution of alanine for threonine-560 in PKC-ζ led to a partial loss in the ability of either insulin in intact cells or

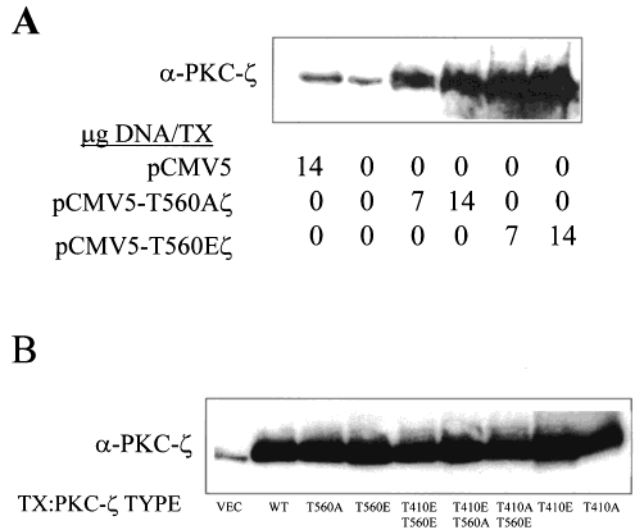

FIGURE 1: Expression of wild-type and mutant forms of PKC-ζ in transiently transfected rat adipocytes. As described in Experimental Procedures, threonine(T)-560 and threonine(T)-410 residues in PKC-ζ were replaced by alanine (A) or glutamate (E) residues, singly or in combination, by site-directed mutagenesis, and adipocytes were transfected (TX) by electroporation with 3−9 μg (per 0.8 mL of 50% adipocyte suspension) (panel B) or indicated amounts (panel A) of pCMV5 containing no cDNA insert (Vector or V) or cDNA inserts encoding FLAG-tagged wild-type (WT) or indicated mutant forms of PKC-ζ. After overnight incubation to allow time for expression, lysates were resolved by SDS−PAGE and blotted with anti-PKC-ζ antiserum (Santa Cruz) to allow comparison of endogenous and transfected PKC-ζ. Shown here are representative immunoblots.

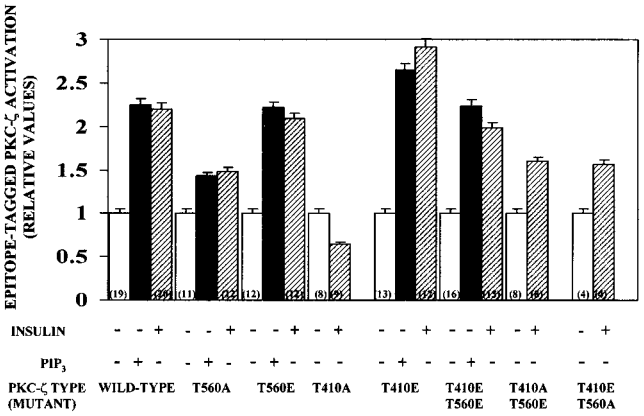

FIGURE 2: Effects of insulin treatment in intact adipocytes or PIP_3 treatment in vitro on enzyme activity of transfected, epitope-tagged, wild-type and mutant forms of PKC-ζ. Adipocytes were transfected with pCMV5 encoding FLAG-tagged wild-type or indicated mutants forms of PKC-ζ. After overnight incubation to allow time for expression, adipocytes were washed and incubated in glucose-free KRP medium for 10 min with or without 10 nM insulin, following which, cell lysates were precipitated with anti-FLAG antibodies, and precipitates were assayed for PKC-ζ enzyme activity, with or without addition of 10 μM PIP_3, as indicated. Shown here are mean values ± SE of (*n*) experiments that have been normalized to reflect effects of insulin or PIP_3, relative to the corresponding untreated control.

PIP_3 in vitro to activate the FLAG-tagged T560A-PKC-ζ mutant (Figure 2). As a functional correlate, expression of the T560A-PKC-ζ mutant in transiently transfected adipocytes was attended by decreases in insulin-induced translocation of HA-GLUT4 to the plasma membrane (Figure 3). Note that a large, most likely a saturating or near saturating, amount of mutant T560A PKC-ζ was used, and inhibitory effects (approx 55%) on HA-Glut4 translocation were most likely maximal or near maximal.

In contrast to substitution of an alanine residue, substitution of a phosphate-mimicking glutamate residue for threonine-560 in PKC-ζ had little or no effect on the ability of either insulin or PIP_3 to activate the FLAG-tagged T560E-PKC-ζ mutant (Figures 2 and 3). Also, expression of presumably saturating or near saturating amounts of this T560E mutant, if anything, mildly increased basal HA-GLUT4 translocation, and, moreover, did not significantly alter insulin effects on HA-GLUT4 translocation (Figure 4). These findings with A and E mutants collectively suggested that the phosphorylation of threonine-560 is required for full activation of PKC-ζ.

Effects of Mutation of Threonine-410 on Activation and Activity of PKC-ζ. As alluded to in a previous report (*6*), and, as compared to wild-type PKC-ζ, substitution of alanine for threonine-410 led to a complete loss in the ability of insulin to increase the activity of this FLAG-tagged T410A-PKC-ζ mutant (Figure 2). Comparable to findings reported previously (*6*), expression of this T410A mutant in transiently transfected adipocytes was attended by approximately a 65% inhibition of insulin-stimulated translocation of HA-GLUT4 to the plasma membrane (Figure 3). This level of inhibition of insulin-stimulated HA-GLUT4 translocation by the T410A mutant is comparable to that observed with kinase-inactive PKC-ζ and is probably maximal or near maximal (*1*, *12*). Note that expression of the T410A PKC-ζ mutant does not compromise the ability of insulin to activate PKB (*6*), and it is therefore clear that its inhibitory effects on HA-GLUT4 translocation cannot be attributed to a nonspecific inhibition of kinases that are activated through PI 3-kinase and PDK-1.

In contrast to the effects of substitution of an alanine residue, substitution of a phosphate-mimicking glutamate residue for threonine-410 had little or no effect on insulin-induced or PIP_3-induced activation of this FLAG-tagged T410E-PKC-ζ mutant (Figure 2; also see ref *2*) or on insulin-stimulated translocation of HA-GLUT4 to the plasma membrane (Figure 3). These findings with A and E mutants collectively provided further evidence that phosphorylation of threonine-410 is essential for the activation of PKC-ζ during insulin action.

Effects of Double Mutations of Threonine-410 and Threonine-560 on Activation and Activity of PKC-ζ. The above findings were compatible with our previous postulation that phosphorylations of both the threonine-410 loop site and the predicted threonine-560 autophosphorylation site are required for full activation of PKC-ζ. It was therefore of interest to find that (a) both insulin in intact cells and PIP_3 in vitro were capable of further activating a T410E/T560E-PKC-ζ double mutant (Figures 2 and 4), and (b) expression of this double mutant modestly increased basal translocation of HA-GLUT4 to plasma membrane and did not significantly alter insulin-stimulated HA-GLUT4 translocation (Figure 3). From these findings, it may be surmised that PIP_3 is capable of activating PKC-ζ, even without simultaneously increasing the phosphorylation of the threonine-410 loop site or the threonine-560 autophosphorylation site. Stated differently, PIP_3, in addition to contributing to the phosphorylation of threonine-410 and threonine-560, apparently acts through an ancillary, presumably conformational (see below) mechanism

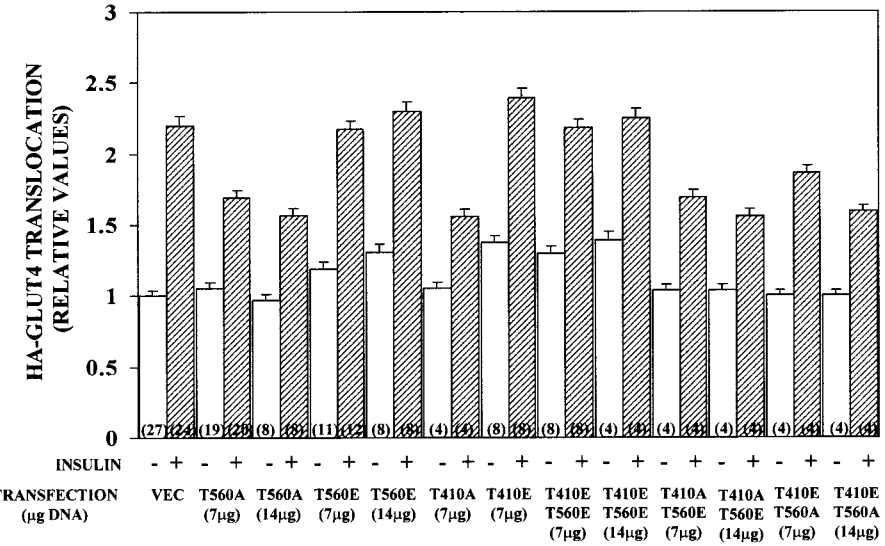

FIGURE 3: Effects of insulin on translocation of HA-tagged GLUT4 to the plasma membrane in rat adipocytes transiently cotransfected with pCMV5 encoding various mutant forms of PKC-ζ. Cells were cotransfected with indicated amounts (in parentheses) of pCMV5 alone (VEC) or pCMV5 containing cDNAs encoding indicated mutants, along with pCIS encoding HA-GLUT4. After overnight incubation to allow time for expression, adipocytes were washed and incubated in glucose-free KRP medium for 30 min with or without 10 nM insulin, following which, the cell surface level of HA-tagged GLUT4 was measured. Shown here are mean values ± SE of (*n*) experiments normalized to the mean control value.

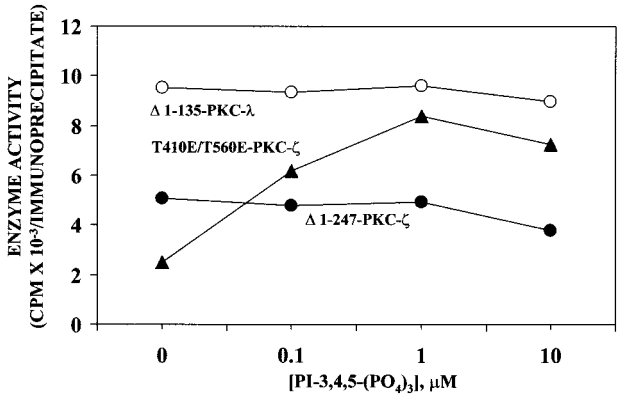

FIGURE 4: Effects of PIP_3 on enzyme activity of mutant and truncated forms of PKC-ζ and PKC-λ. FLAG-tagged T410E/T560E PKC-ζ double mutant and HA-tagged truncated forms of PKC-ζ and PKC-λ were prepared by immunoprecipitation of epitopes from lysates of plasmid-transfected or adenoviral-infected L6 myotubes as described in Experimental Procedures and then assayed in the presence of indicated amounts of PIP_3. Shown here are mean values of 2−3 determinations that have been normalized for the amount of PKC-ζ/λ recovered in the immunoprecipitates, as measured following resolution by SDS−PAGE and immunoblotting with antibodies (Santa Cruz) that target the C-termini of both PKC-ζ and PKC-λ.

that does not involve phosphorylation of threonine-410 and threonine-560.

In contrast to our finding of relatively strong activation of the T410E/T560E double mutant, insulin only modestly activated T410A/T560E-PKC-ζ and T410E/T560A-PKC-ζ double mutants (Figure 2). Moreover, expression of these T410A/T560E and T410E/T560A double mutants was attended by inhibition of stimulatory effects of insulin on HA-GLUT4 translocation (Figure 3). These findings with double mutants suggested that (a) phosphorylation of threonine-410 is required for full activation and activity of PKC-ζ and cannot be circumvented simply by phosphorylation of the threonine-560 autophosphorylation site; (b) in addition to threonine-410, phosphorylation of threonine-560 is required for full activation and activity of PKC-ζ; and (c) the defect in activation and activity of the T560A single mutant cannot be simply attributed to an inability to phosphorylate the threonine-410 residue of this mutant. On the other hand, it may be noted that the T410A/T560E double mutant was clearly more responsive to insulin than the T410A single mutant, suggesting that at least partial activation of PKC-ζ can theoretically be achieved in the absence of threonine-410 phosphorylation. It is, however, dubious that threonine-560 can be phosphorylated in the absence of threonine-410 phosphorylation during the activation of wild-type full-length PKC-ζ in intact cells.

Effects of N-Terminal Regulatory Domain Truncations on Activation of PKC-ζ and λ. The finding that the T410E/T560E double mutant can be activated suggested that PIP_3 activates PKC-ζ through a phosphorylation-independent mechanism, most likely conformational or allosteric in nature. We therefore questioned if effects of PIP_3 on PKC-ζ and PKC-λ enzyme activity may be dependent upon the presence of certain regions of their N-terminal regulatory domains. As shown in Figure 4, whereas PIP_3 provoked dose-dependent increases in the activity of the T410E/T560E PKC-ζ double mutant, there were no discernible effects of PIP_3 on the truncated Δ1−247 PKC-ζ, which lacks the entire N-terminal regulatory domain, or on the truncated Δ1−135 PKC-λ, which contains only that portion of the C1 region of the N-terminal regulatory domain that is distal to the pseudosubstrate sequence at amino acids 114−131. These findings suggested that PIP_3-induced activation of atypical PKCs, ζ and λ, is at least partly dependent upon the presence of the pseudosubstrate sequence in the N-terminus, and this apparent dependency is most readily explained by a relief of pseudosubstrate-dependent autoinhibition. With respect to this explanation, it may be noted that (a) in the absence of added PIP_3, truncated Δ1−247 PKC-ζ and Δ1−135 PKC-λ appeared to be intrinsically more active than the T410E/T560E PKC-ζ double mutant, which contains the N-terminal pseudosubstrate region (Figure 4); (b) exog-

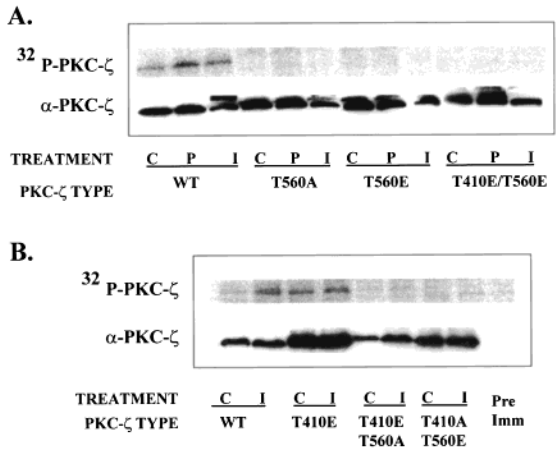

FIGURE 5: Autophosphorylation of wild-type and mutant forms of PKC-ζ. Adipocytes were transiently transfected with epitope-tagged wild type (WT) or mutant forms of PKC-ζ as indicated. After overnight incubation to allow time for expression, adipocytes were washed and incubated in glucose-free KRP medium for 10 min, without or with 10 nM insulin (I) as indicated. Resulting cell lysates were immunoprecipitated with anti-epitope antibodies, and immunoprecipitates were assayed for PKC-ζ autophosphorylating activity in the absence (C, control and I, insulin-treated) or presence (P) of 10 μM PIP_3. Shown here are autoradiograms of ^{32}P-labeled PKC-ζ (following resolution by SDS−PAGE and transfer to nitrocellulose membranes) that are representative of four determinations. Also shown here are PKC-ζ immunoblots, indicating levels of loading of various forms of PKC-ζ in these gels.

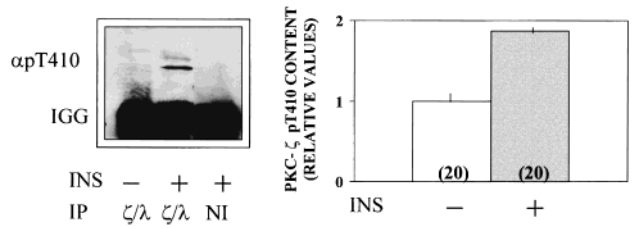

FIGURE 6: Effects of insulin on phosphorylation of threonine-410 in PKC-ζ. Adipocytes were incubated in glucose-free KRP medium for 10 min with or without 10 nM insulin, following which, cell lysates (1 mg of protein) were immunoprecipitated with anti-PKC-ζ/λ antiserum (Santa Cruz), subjected to SDS−PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phosphothreonine-410 antiserum as described in Experimental Procedures. Shown at left is a representative immunoblot of control and insulin-stimulated immunoprecipitates (IP) prepared with anti-PKC-ζ/λ antiserum (ζ/λ) or nonimmune (NI) serum. Bargrams reflect mean values ± SE of 20 comparisons between samples obtained from control and insulin-treated adipocytes.

enously added, free PKC-ζ/λ pseudosubstrate peptide completely inhibits both insulin- and PIP_3-induced increases in activity and autophosphorylation of full-length, wild-type PKC-ζ and PKC-λ (*1*, *2*); and (c) PI-4,5-$(PO_4)_2$ has little or no effect on PKC-ζ/λ activity (*1*). However, it is also possible that the truncated forms of PKC-ζ and PKC-λ may be unable to bind PIP_3, as PIP_3 binding sites are presently uncertain. As another possible explanation for the lack of effects of PIP_3 on enzyme activity, it may be reasonably argued that N-terminally truncated atypical PKCs are fully activated because they lack their pseudosubstrate autoinhibitory region. With respect to the latter possibility, however, it may be noted that (a) insulin (*2* and see below) and overexpression of PDK-1 (*10*) can potently activate truncated Δ1−247 PKC-ζ, apparently through a PI 3-kinase/phosphorylation-dependent mechanism, and (b) although expression of truncated Δ1−247 PKC-ζ substantially increases basal HA-GLUT4 translocation, insulin effects on HA-GLUT4 translocation are still readily apparent in rat adipocytes expressing this truncated PKC-ζ (*1*, *12*, *13*). It therefore seems clear that truncated Δ1−247 PKC-ζ is partially, but not fully, activated in the absence of PI 3-kinase activation.

Autophosphorylation of PKC-ζ Mutants. As alluded to above, it has been suggested that threonine-560 is a major autophosphorylation site in PKC-ζ. To test this hypothesis and to evaluate the possibility that there may be other autophosphorylation sites in PKC-ζ, we examined the autophosphorylation of mutant forms of PKC-ζ. As seen in Figure 5 panel A and as reported previously (*1*, *2*), both insulin treatment in intact cells and PIP_3 treatment in vitro provoked increases in the autophosphorylation of wild-type PKC-ζ. In contrast, there was no apparent autophosphorylation of T560A, T560E, or T410E/T560E mutant forms of PKC-ζ, even after long periods of exposure of autoradiograms in a Phosphorimager (Figure 5, panel A). The failure to see phosphorylation of the threonine-410 residue in T560 A and E mutants probably reflects an absence of PDK-1 in FLAG immunoprecipitates.

We have previously reported that both insulin in intact cells and PIP_3 in vitro stimulate the autophosphorylation, as well as the enzyme activity, of the T410E-PKC-ζ single mutant (*2*). As seen in Figure 5, panel B, despite being able to readily observe autophosphorylation of wild-type PKC-ζ and the T410E-PKC-ζ mutant, there was no significant phosphorylation of T410E/T560A or T410A/T560E mutants. Taken together, the inability to observe phosphorylation of EE, AE, and EA double mutants suggested that threonine-560 is the sole autophosphorylation site in PKC-ζ.

Assessment of PDK-1-Dependent Phosphorylation of Threonine-410 in PKC-ζ. As seen in Figure 6, insulin provoked increases in the level of phosphorylation of threonine-410 in PKC-ζ, as assessed by Western analysis, using an anti-pT410 antiserum. From these findings, it may be surmised that insulin acutely enhances either the activity or action of PDK-1 on PKC-ζ.

Dependence on PI 3-Kinase of Insulin-Induced Activation of Truncated Δ1−247 PKC-ζ and Double Mutant T410E/T560E PKC-ζ. The activation of truncated Δ1−247 PKC-ζ and double mutant T410E/T560E PKC-ζ by insulin appeared to largely reflect pseudosubstrate-independent/phosphorylation-dependent and phosphorylation-independent/pseudosubstrate-dependent, respectively, activating mechanisms. It was therefore of interest to find that activating effects of insulin on both of these truncated and double EE mutant forms of PKC-ζ were inhibited by wortmannin (Figure 7) and thus appeared to be similarly dependent upon PI 3-kinase activation.

Studies on Truncated Δ1−247/T410E/T560E-PKC-ζ. The above-described findings suggested that insulin activates PKC-ζ by PDK-1-dependent phosphorylation of the threonine-410 loop site, subsequent autophosphorylation at the threonine-560 site, and a phosphorylation-independent allosteric/conformational mechanism involving relief of pseudosubstrate-dependent autoinhibition. It was therefore of interest to find that, unlike simple truncated Δ1−247-PKC-ζ or T410E/T560E-PKC-ζ, insulin was unable to provoke further significant increases in the enzyme activity of a

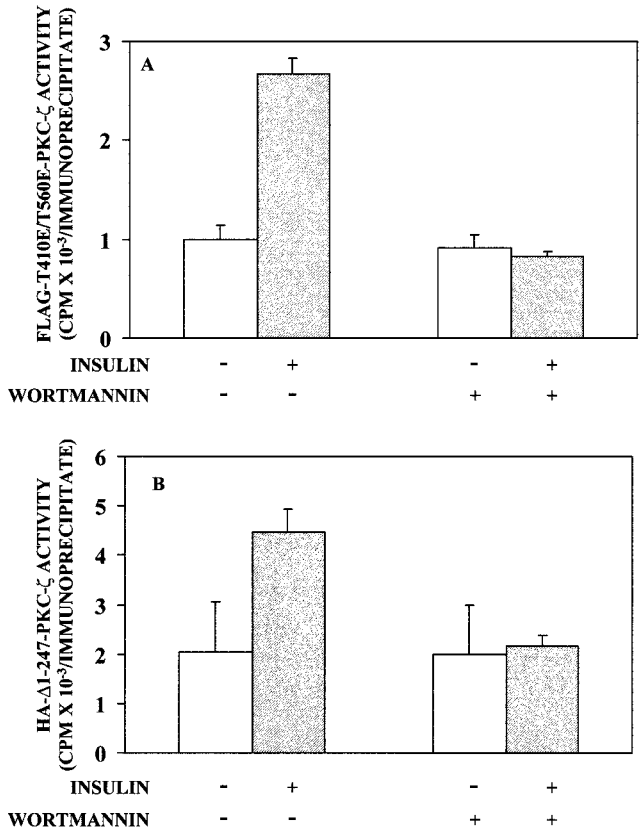

FIGURE 7: Effects of wortmannin on insulin-induced increases in enzyme activity of epitope-tagged truncated Δ1−247 PKC-ζ and double mutant T410E/T560E PKC-ζ. Adipocytes were transfected with plasmids encoding HA-tagged truncated Δ1−247 PKC or FLAG-tagged T410E/T560E PKC-ζ. After overnight incubation to allow time for expression, adipocytes were washed and incubated in glucose-free KRP medium, first for 15 min with or without 100 nM wortmannin, and then for 10 min, with or without 10 nM insulin as indicated. After incubation, epitope-tagged forms of PKC-ζ were immunoprecipitated and assayed for enzyme activity. Values are mean ± SE of (*n*) determinations.

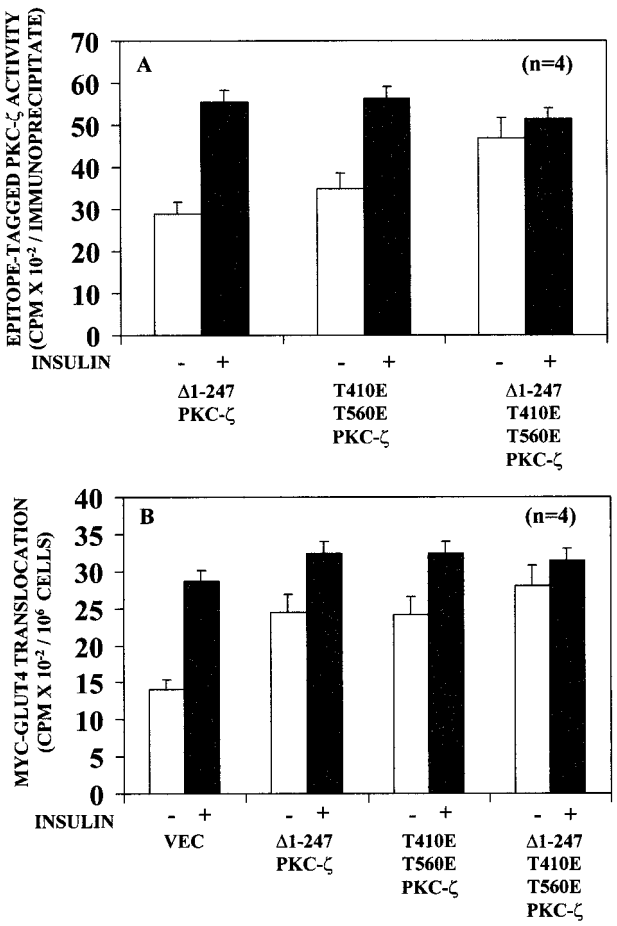

FIGURE 8: Effects of insulin on activity of HA-tagged truncated/double EE mutant Δ 1−247/T410E/T560E-PKC-ζ. Cells were transfected with HA-tagged truncated Δ 1−247 PKC-ζ, HA-tagged truncated/doubly mutated Δ1−247/T410E/ T560E-PKC-ζ, or FLAG-tagged T410E/T560E-PKC-ζ mutant, as indicated, with (for translocation studies) or without (for enzyme activation studies) pCDNA3 encoding MYC-GLUT4. After overnight incubation to allow time for expression (amounts of these three forms were similar, not shown, but note similar peak values), cells were incubated in glucose-free KRP medium for 10 min (for enzyme activation studies) or 30 min (for translocation studies) with or without 10 nM insulin, following which, HA- or FLAG-tagged PKC-ζ was immunoprecipitated and assayed for PKC-ζ enzyme activity (panel A), or translocation of MYC-GLUT4 to the plasma membrane (panel B) was measured as described in Experimental Procedures. Values are mean ± SE of (*n*) determinations.

PKC-ζ form that was both truncated to remove the N-terminal autoinhibitory pseudosubstrate sequence and mutated to contain activating glutamate residues at the 410 and 560 sites (Figure 8). Of further note, expression of this Δ1−247/T410E/T560E-PKC-ζ combined truncated double EE mutant led to nearly full insulin-like increases in HA-GLUT4 translocation that were slightly greater than those observed with expression of similar amounts of simple truncated Δ1−247-PKC-ζ or T410E/T560E-PKC-ζ (Figure 8). Thus, this Δ1−247/T410E/T560E-PKC-ζ combined truncated double EE mutant appeared to be more fully activated than the simple truncated or double EE mutant forms.

DISCUSSION

The present findings suggested that PIP_3 activates PKC-ζ by three distinct, but interrelated, mechanisms: (a) PDK-1-dependent increases in threonine-410 phosphorylation, (b) increased autophosphorylation at threonine-560, and (c) phosphorylation-independent activation, most likely involving a conformational/allosteric change and relief of pseudosubstrate-dependent autoinhibition. Both of the latter two mechanisms may readily be explained by postulating that PIP_3 binds to the N-terminal regulatory domain of PKC-ζ and thereby causes an unfolding of the molecule, which in turn allows the uninhibited catalytic unit in the C-terminal domain to further self-activate by auto(trans)phosphorylation and subsequently interact with and phosphorylate extrinsic substrates. In fact, this same conformational unfolding mechanism may serve to expose the threonine-410 activation loop site to PDK-1. However, it is also possible that PIP_3 may directly activate PDK-1 (*7*), which of itself may also lead to increased phosphorylation of threonine-410. On the other hand, such activation of PDK-1 by insulin was not observed in one reported study (*14*). In any event, from the present finding of increases in the level of threonine-410 phosphorylation, it seems clear that insulin acutely increases either the action or activity of PDK-1.

The acute activating effects of insulin on phosphorylation of threonine-410 in the activation loop of PKC-ζ and threonine-560, the autophosphorylation site, apparently differs from the mechanism recently suggested for activation

of convention and novel, diacylglycerol (DAG)-dependent PKCs (*15*). In the latter case, PDK-1-dependent loop and subsequent autophosphorylation are postulated to occur chronically, soon after synthesis of conventional and novel PKCs, independently and prior to agonist-induced activation, thus serving to prime these PKCs for subsequent acute conformational unfolding and activation by DAG generated during agonist action. This could explain how DAG-sensitive PKCs may be acutely activated in the absence of concurrent PI 3-kinase activation. In contrast, in the case of the atypical PKCs, it appears that agonist-induced, PI 3-kinase-dependent increases in PIP_3 act acutely by both phosphorylation and conformational mechanisms.

It was of interest to find that PIP_3 did not activate truncated forms of PKC-ζ and PKC-λ that lacked the pseudosubstrate-containing regions of their N-termini. In contrast, we have previously reported (*2*) and presently confirmed that insulin activates epitope-tagged, truncated Δ1−247 PKC-ζ in transiently transfected rat adipocytes, and Le Good et al. (*10*) have reported that overexpression of PDK-1 activates truncated PKC-ζ by a PI 3-kinase-dependent mechanism. Since we presently found that (a) truncated PKC-ζ is not directly activated by PIP_3 and (b) wortmannin blocks insulin-induced activation of truncated PKC-ζ, it may be speculated that insulin activates truncated PKC-ζ in the intact cell through a PIP_3-dependent activation of PDK-1 with subsequent phosphorylation of threonine-410 and autophosphorylation at threonine-560. However, another possibility is that truncated PKC-ζ may be activated through trans-phosphorylation at threonine-560 by endogenous full-length PKC-ζ that had been activated by insulin in situ. In either scenario, the present and previous findings collectively provide evidence that insulin, via PI 3-kinase, uses both phosphorylation-dependent and phosphorylation-independent mechanisms to activate atypical PKCs.

Our findings provided direct support for the notion that threonine-560 is the major, if not sole, autophosphorylation site in PKC-ζ. In addition, our findings suggested that phosphorylation of threonine-560 is required for full enzymatic activation and biological activity of PKC-ζ, as evidenced by the finding that substitution of alanine for threonine-560, like substitution of alanine for threonine-410 in PKC-ζ, was attended by substantial decreases in insulin-stimulated HA-GLUT4 translocation. Thus, it appears that mutations that interfere with phosphorylation at either the activation loop or autophosphorylation sites, like mutations involving the catalytic ATP-binding sites of PKC-ζ and PKC-λ (*1*, *12*), can serve in a dominant-negative fashion and inhibit PKC-ζ/λ-dependent processes.

Finally, it was of interest to find that substitution of phosphate-mimicking glutamate residues at threonine-410 and/or threonine-560 in PKC-ζ led to modest increases in basal, and continued stimulatory effects of insulin on HA-GLUT4 translocation. Similar partial activating effects of truncated PKC-ζ on basal HA-GLUT4 translocation have been observed previously (*1*, *12*, *13*). Presumably, these partial increases in basal HA-GLUT4 translocation reflected the fact that the presence of phosphate-mimicking glutamate residues at threonine-410 loop and/or threonine-560 autophosphorylation, like N-terminal truncation, would cause PKC-ζ to be partially constitutively active (*16*). However, the fact that insulin continued to provoke further substantial increases in GLUT4 translocation in cells expressing either glutamate-substituted or truncated mutants was in keeping with our findings that suggested that insulin, via PIP_3, activates PKC-ζ by at least three distinct mechanisms. Also in keeping with this notion is the finding that insulin did not activate a form of PKC-ζ that was both truncated to remove the autoinhibitory pseudosubstrate sequence and mutated to contain activating residues glutamate residues at 410 and 560 sites. Accordingly, phosphorylation or substitution of phosphate-mimicking glutamate residues at threonine-410 and/or threonine-560 residues would be expected to cause a partial activation of PKC-ζ (*16*), to which may be added PIP_3-induced activation that is phosphorylation-independent and is most likely due to conformational/allosteric changes that result in relief of pseudosubstrate-dependent autoinhibition. Further studies are needed to test the postulation that PKC-ζ is activated by both phosphorylation-dependent and conformational-dependent/phosphorylation-independent mechanisms, and full activating effects of insulin on PKC-ζ are required for full activating effects of insulin on GLUT4 translocation.

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