Effects of transiently expressed atypical (ζ, λ), conventional (α, β) and novel (δ, ε) protein kinase C isoforms on insulin-stimulated translocation of epitope-tagged GLUT4 glucose transporters in rat adipocytes: specific interchangeable effects of protein kinases C-ζ and C-λ

Gautam BANDYOPADHYAY*, Mary L. STANDAERT*, Ushio KIKKAWA†, Yoshitaka ONO†, Jorge MOSCAT‡ and Robert V. FARESE*[1]

*J. A. Haley Veterans' Hospital Research Service and Departments of Internal Medicine and Biochemistry/Molecular Biology, University of South Florida College of Medicine,Tampa, FL 33612, U.S.A., †Department of Biology, Faculty of Science, Kobe University and Biosignal Research Center, Kobe, Japan, and ‡Centro de Biologia Molecular 'Severo Ochoa', Universidad Autónoma, Canto Blanco, Madrid, Spain

Atypical protein kinase (PK)C isoforms, ζ and λ, have been reported to be activated by insulin via phosphoinositide 3-kinase, and have been suggested to be required for insulin-stimulated glucose transport. Here, we have examined the effects of transiently expressed wild-type (WT), constitutively active (Constit) and kinase-inactive (KI) forms of atypical PKCs, ζ and λ, on haemagglutinin antigen (HAA)-tagged glucose transporter 4 (GLUT4) translocation in rat adipocytes, and compared these effects with each other and with those of comparable forms of conventional (α, β) and novel (δ, ϵ) PKCs, which have also been proposed to be required for insulin-stimulated glucose transport. KI-PKC-ζ evoked consistent, sizeable (overall mean of 65%) inhibitory effects on insulin-stimulated, but not basal or guanosine-5′-[γ-thio]triphosphate-stimulated, HAA-GLUT4 translocation; moreover, inhibitory effects of KI-PKC-ζ were largely reversed by co-transfection of WT-PKC-ζ. Like KI-PKC-ζ, KI-PKC-λ inhibited insulin-stimulated HAA-GLUT4 translocation by approx. 40–60%, and the combination of KI-PKC-ζ and KI-PKC-λ caused nearly complete (85%) inhibition. Of particular interest is the fact that inhibitory effects of KI forms of PKC-ζ and PKC-λ were largely reversed by the opposite WT forms, i.e. PKC-λ and PKC-ζ respectively. In contrast with KI forms of atypical PKCs, KI forms of PKC-α, PKC-β2, PKC-δ and PKC-ϵ had little or no effect on insulin-stimulated HAA-GLUT4 translocation. Concerning the question of sufficiency, overexpression of WT-PKC-ζ enhanced insulin effects on HAA-GLUT4 translocation, whereas WT forms of PKC-α, PKC-β2, PKC-δ and PKC-ϵ did not affect GLUT4 translocation; furthermore, Constit PKC-ζ evoked increases in HAA-GLUT4 translocation approaching those of insulin, but Constit forms of PKC-α and PKC-β2 were without effect. Our findings suggest that, among PKCs, the atypical PKCs, ζ and λ, appear to be specifically, but interchangeably, required for insulin effects on HAA-GLUT4 translocation.

Key Words: glucose transport.

INTRODUCTION

Glucose transport, the rate-limiting step in insulin stimulation of glucose utilization, is regulated primarily by translocating glucose transporter 4 (GLUT4) from low-density microsomes to the plasma membrane. Phosphoinositide 3-kinase (PI-3K) is recognized to be required in this regulation, but downstream effectors of PI-3K are still uncertain. With respect to potential downstream effectors, it has recently been reported that: (a) insulin evokes rapid increases in the activity of immunoprecipitable protein kinase (PK)C-ζ in 3T3/L1 adipocytes [1], L6 myotubes [2], rat adipocytes [3] and 32D cells [4], largely via PI-3K-dependent increases in D3-PO_4 polyphosphoinositides [2–4]; (b) inhibitors of PKC-ζ concomitantly inhibit insulin effects on glucose transport in rat adipocytes [3] and L6 myotubes [2]; (c) stable expression of a kinase-inactive (KI) form of PKC-ζ inhibits insulin-stimulated GLUT4 translocation and glucose transport in transfected 3T3/L1 cells [1] and L6 myotubes [2]; and (d), in limited studies, transient expression of transfected KI-PKC-ζ in rat adipocytes appeared to inhibit insulin-stimulated translocation of epitope-tagged haemagglutinin antigen (HAA)-GLUT4 to the plasma membrane [3]. These findings suggested that the atypical diacylglycerol (DAG)-insensitive, polyphosphoinositide-sensitive PKC, PKC-ζ, and/or the closely related atypical PKC, PKC-λ (72% homology with PKC-ζ), might be important in insulin stimulation of GLUT4 translocation.

In addition to PKC-ζ and PKC-λ as downstream effectors of PI-3K, DAG-sensitive PKCs might be activated by insulin through PI-3K-dependent activation of phospholipase D (PLD) and subsequent generation of phosphatidic acid and DAG [5] or, for that matter, possibly more directly by D3-PO_4 polyphosphoinositides, as suggested by previous studies [6,7]. Moreover, DAG-dependent PKCs, most notably PKC-β2 [8], have been suggested to be important in the activation of glucose transport. Here, we compared the effects of various transiently transfected forms of PKC-ζ with those of PKC-β2 and other DAG-sensitive

Abbreviations used: Constit, constitutively active; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; 2-DOG, 2-deoxyglucose; GLUT4, glucose transporter 4; GTP[S], guanosine 5′-[γ-thio]triphosphate; HAA, haemagglutinin antigen; KI, kinase-inactive; KRP, Krebs–Ringer phosphate; PI-3K, phosphoinositide 3-kinase; PK(C/B/N), protein kinase C, B or N respectively; PLD, phospholipase D; WT, wild-type.

[1] To whom correspondence should be addressed (e-mail rfarese@com1.med.usf.edu).

PKCs on basal and insulin-stimulated HAA-GLUT4 translocation in rat adipocytes. We also compared the effects of transiently transfected forms of PKC-ζ and PKC-λ with each other on HAA-GLUT4 translocation, and we examined the question of whether these atypical PKC isoforms act interchangeably on GLUT4 translocation.

MATERIALS AND METHODS

Incubation and transfection conditions

Adipocytes were obtained by collagenase digestion of epididymal fat-pads of 200–250 g male Sprague Dawley rats, suspended in Dulbecco's modified Eagle's medium (DMEM), and transfected by electroporation, essentially as described in [3,9,10], but by using electroporator (Biorad, Richmond, VA, U.S.A) settings of Tanti et al. [11] with 3 μg of pCIS2 containing cDNA encoding HAA-GLUT4 (kindly provided by Drs. Michael Quon and Simeon Taylor, National Institutes of Health, Bethesda, MD, U.S.A.) in each electroporation cuvette (containing 0.8 ml of a 50 % cell suspension). The transfection also contained, as described below, indicated amounts of other plasmid/cDNA constructs (in most cases, 7 μg of DNA per cuvette and, in a few cases, 10.5–14 μg of DNA per cuvette; note that this is less than/comparable with amounts used in similar studies of PKB involvement in GLUT4 translocation; see [11,12]). In each experiment, the total amount of DNA used for transfection was kept constant by varying the amount of vector DNA relative to the amount of vector-plus-insert DNA used (as indicated below, DNA, in amounts used in these experiments, did not affect basal or insulin-stimulated HAA-GLUT4 translocation). After transfection, cells were incubated at 37 °C in DMEM containing 25 mM Hepes, 200 nM ($-N$)-N^6-(2-phenylisopropyl)adenosine and 5 % (w/v) BSA for 24 h to allow sufficient time for ample expression of HAA-tagged GLUT4 and other transfected proteins. Thereafter, the cells were washed and incubated at 37 °C in glucose-free Krebs–Ringer phosphate (KRP) medium containing 1 % (w/v) BSA, first with or without inhibitors (see text) to allow for sufficient uptake of inhibitor, and then for the times indicated with vehicle (control), insulin (Eli Lilly Co., Indianapolis, IN, U.S.A.) or PMA (Sigma, St. Louis, MO, U.S.A.), as described below. After incubation, cells were assayed (see below) for surface content of HAA-GLUT4 (i.e. translocation) or uptake of 2-[^{3}H]deoxyglucose (2-DOG).

Assays

Cell-surface HAA-GLUT4 was measured as described previously [3,9,10]. In brief, after incubation, 2mM KCN was added to prevent further inward or outward movement of GLUT4, and cells were incubated first with anti-HAA mouse monoclonal antibody (Berkeley Antibody Corp., Berkeley, CA, U.S.A.), and then with ^{125}I-labelled sheep anti-(mouse IgG) antibody (Amersham Corp, Arlington Heights, IL, U.S.A.), followed by extensive washing and counting of cell-surface radioactivity. Blank values, obtained by using cells transfected with pCIS2 vector lacking the HAA-GLUT4 insert [3,9,10], were subtracted from all values. Cells were counted in a haemocytometer. It is important to note that the expression of HAA-GLUT4 was not affected by co-expression of various forms of PKC and, in the amounts used in this study, transfected DNA did not exert toxic effects, as shown by the fact that basal and insulin-stimulated HAA-GLUT4 translocation were altered only by specific cDNA inserts, and not by simply varying the amount of DNA in the transfection (see below). In this respect, it should be noted that all samples within each experiment were transfected with the same amount of vector, and the only variable was the presence or absence of the cDNA insert.

2-[^{3}H]DOG uptake during a 1 min period was measured as described previously [3]. Western blot analyses were performed [3] using antibodies described below. Activity of immunoprecipitated PKC-ζ and PKC-λ [i.e. total, as precipitated with anti-(C-terminal PKC-ζ/λ antibodies)] was assayed as described previously [2,3].

Plasmids

In addition to the pCIS2/HAA-GLUT4 cDNA construct, we used the eukaryotic expression vector pCDNA3 containing cDNAs encoding HAA-tagged KI-PKC-ζ, HAA-tagged wild-type (WT) PKC-ζ, point-mutated (Ala^{119} in the pseudosubstrate sequence was mutated to Asp), constitutively active (Constit) PKC-ζ and a HAA-tagged, Constit PKC-ζ with the 247 amino acids deleted from its N $\times$ -terminus [Δ(1–247)]; these constructs have been described previously [1–3]. In a few experiments, we used a KI form of PKC-ζ, which was subcloned into pCIS2, and results (both expression and as affecting GLUT4 translocation) using this construct were similar to those in which the pCDNA3/KI-PKC-ζ construct was used. We also used pCDNA3/KI-PKC-α cDNA and pCDNA3/Constit PKC-α cDNA constructs; these cDNAs (kindly provided by Dr. Peter Parker, Imperial Cancer Research Fund Laboratories, London, U.K; see [13]) were subcloned into pCDNA3, and the orientation was checked by restriction-enzyme analysis and further verified by expression of increased amounts of full-length 80-kDa immunoreactive PKC in transfected adipocytes (see below). A Constit form of PKC-β2 was generated by site-directed mutagenesis (using the PCR method) of Ala^{25} $\rightarrow$ Asp (thus mimicking an anionic PO_4 group and relieving the inhibitory effects of the pseudosubstrate sequence) in a pCDNA3/WT-PKC-β2 cDNA construct [1,2]; this point-mutated construct (pCDNA3/Constit-PKC-β2) was checked by both restriction-enzyme and sequence analyses, in addition to expression of increased amounts of 80 kDa PKC-β2 in transfected adipocytes (see below). Constructs of pTB701 containing cDNAs encoding WT and/or KI (for the latter, each point-mutated by substituting methionine for lysine in the ATP-binding sites in its catalytic domain) forms of PKC-α, -β1, -β2, -δ, -ϵ and -ζ were generated; these KI-PKCs contained HAA-epitope tags at their N-termini [14]. WT and KI forms of PKC-λ (having N-terminal Myc tags) were also generated [15].

To generate a pCDNA3/KI-PKC-β2 cDNA construct, the pTB701/KI-PKC-β1 cDNA construct was treated with *Bam*HI and *Bst*EII, and the released fragment (1.4 kb), which contained the coding region of mutated KI-PKC-β1 from the initiation codon through base 1960 (i.e. distal to the point-mutation in the ATP-binding site, but proximal to the C-terminal PKC-β2-specific sequence), was gel-purified and ligated into pCDNA3/WT-PKC-β2 cDNA, which was gel-purified after removal of its corresponding 1.4 kb *Bam*HI/*Bst*EII fragment. This resulting pCDNA3/KI-PKC-β2 cDNA construct was checked by restriction-enzyme and sequence analyses, as well as by expression of the HAA-tag and increased amounts of immunoreactive 80 kDa PKC-β2 in transfected adipocytes (see below).

To generate a construct that contained cDNA encoding a form of PKC-β2 with the C-terminal 9 amino acids deleted {Δ(664–673); this construct was used in [8] as an effective dominant-negative inhibitor of insulin-stimulated glucose transport in L6 myotubes}, the pCDNA3/WT-PKC-β2 plasmid [1,2] was treated with *Hpa*I and *Xba*I to remove nucleotides that code for the 11 C-terminal amino acids of PKC-β2. The truncated remnant was gel-purified and ligated to an oligodeoxyribo-

nucleotide (5′-AACTCTTAATCTAGAGGG-3′), which contains codons for two C-terminal amino acids (Val and Ser), followed by a stop codon (TAA) and an *Xba*I site (TAG), thus yielding a pCDNA3/Δ(664–673) PKC-β2 construct. This construct was also checked by restriction-enzyme and sequence analyses, as well as by increased expression of immunoreactive PKC-β2 (see below).

Materials

Inhibitors used were the PI-3K inhibitor, wortmannin (Sigma), the general PKC inhibitor, RO 31-8220, and the specific inhibitor of PKC-α, PKC-β and PKC-γ, GO 6976 (Alexis Biochemicals, San Diego, CA, U.S.A.), and the myristylated PKC-ζ pseudosubstrate (Quality Controlled Biochemicals, Hopkington, MA, U.S.A.). Antibodies used for Western analyses included mouse monoclonal anti-HAA antibody (Berkeley Antibody Corp.), polyclonal antisera recognizing PKC-α, PKC-β1, PKC-β2, PKC-δ, the nearly identical C-termini of PKC-ζ and PKC-λ, the N-terminus of PKC-ζ and the N-terminus of PKC-λ (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), polyclonal anti-(PKC-ε serum) (Life Technologies, Gaithersburg, MD, U.S.A.), polyclonal anti-(Myc serum) (Upstate Biotechnologies Inc., Lake Placid, NY, U.S.A.) and mouse monoclonal anti-(PKC-λ) antibody (raised against an internal site: amino acids 397–558) (Transduction Laboratories, Lexington, KY, U.S.A.). Note that the antiserum raised against the C-terminal epitope of PKC-ζ also recognizes an almost identical sequence in the C-terminus of PKC-λ; in contrast, the antiserum that recognizes N-terminal and internal sites of PKC-ζ and PKC-λ respectively are specific and do not cross-react with opposite atypical PKCs.

RESULTS

Expression of PKCs in transiently transfected rat adipocytes

The expression of each PKC isoform was documented by surprisingly large increases in immunoreactivity of the PKC itself and, where possible, by expression of the HAA or Myc tag. For simplicity, representative increases in the expression of individual PKCs are depicted in relevant Figures that describe the GLUT4 translocation results of various groups in transfection experiments (see below). It should be noted that the increases in total PKC immunoreactivity reflected changes in only approx. 5% of the adipocytes [16]. For example, since total PKC-ζ immunoreactivity was approximately doubled in cells transfected with WT and KI forms of PKC-ζ, it may be surmised that PKC-ζ levels in these transfected cells increased by as much as 40-fold. As there were substantial relative increases in PKC levels (usually 2-fold as per immunoreactivity but, in some cases, e.g. KI-PKC-β2, much greater than this, probably depending upon the endogenous level of each PKC) with each of the currently used constructs, it is clear that transfected cells contained relatively large amounts of expressed PKC.

In addition to observing substantial increases in total immunoreactive PKC-ζ and PKC-λ levels (see below), in adipocytes transfected with WT-PKC-ζ and WT-PKC-λ we observed that there were increases in total PKC-ζ/λ enzyme activity that was precipitated by the C-terminally targeted antiserum. As shown in a representative experiment described in Table 1, transient expression of WT-PKC-ζ and WT-PKC-λ led to significant increases in immunoprecipitable PKC-ζ/λ enzyme activity, whereas transient expression of KI forms of PKC-ζ and PKC-λ had either no effect or diminished the activity of immunoprecipitable PKC-ζ/λ. As we will report in a separate study,

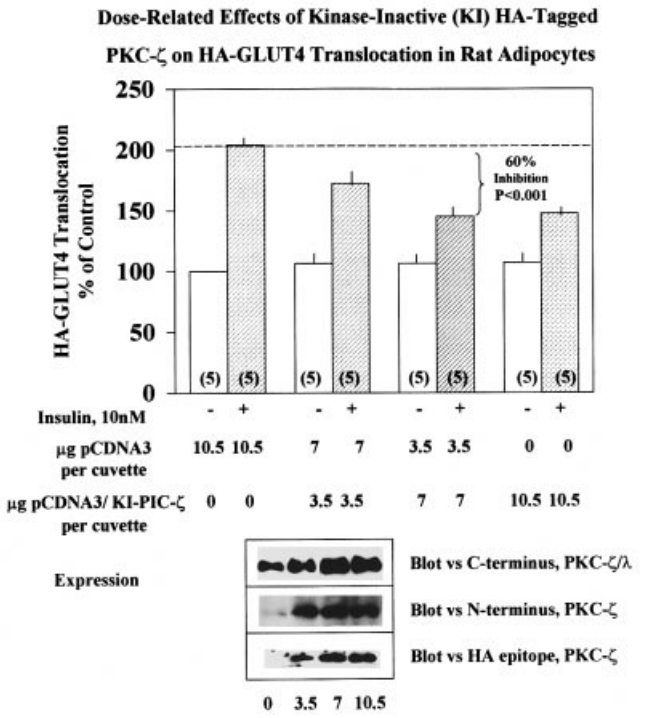

Figure 1 Effects of increasing amounts of KI-PKC-ζ on insulin-stimulated GLUT4 translocation in rat adipocytes

Cells were transfected with 3 μg/cuvette pCIS2/HAA-GLUT4 and indicated amounts of pCDNA vector or pCDNA2/HA-KI-PKC-ζ. After 24 h incubation, cells were equilibrated for 30 min in glucose-free KRP and then treated for 30 min with or without 10 nM insulin. Values are means ± S.E.M. for five determinations. Shown in the lower part of the Figure are immunoblots depicting levels of PKC-ζ in cell lysates (100 μg of protein), as assayed with three indicated antibodies.

Table 1 Alterations in enzymic activity of immunoprecipitable PKC-ζ/λ in adipocytes transfected with cDNAs encoding WT and KI forms of PKC-ζ and PKC-λ

Adipocytes were transfected with 7 μg/cuvette pCDNA3 containing no insert (vector), or cDNA insert encoding WT and KI forms of PKC-ζ or PKC-λ. After 24 h to allow time for expression, cells were equilibrated in glucose-free KRP medium and treated for 10 min with 10 nM insulin. After incubation, cell lysates (200 μg of protein) were subjected to immunoprecipitation with antiserum that recognizes the almost identical C-termini of both PKC-ζ and PKC-λ, and precipitates were assayed as described in the Materials and methods section. Values are means ± S.E.M. for four determinations. Significance (P values) was determined by the Student's t test. It should be noted that values in Groups B and D may be underestimated, since immunoprecipitation of PKC-ζ and PKC-λ is substantial, but not fully complete, with the C-terminally targeted antiserum (maximal, approx. 50–60% of the total, despite addition of higher concentrations of precipitating antibody; [2,3]); this incomplete immunoprecipitation probably also accounts for the lower levels of enzyme activity recovered in immunoprecipitates prepared from cells transfected with KI forms of PKC-ζ and PKC-λ (Groups C and D).

Group	Transfection	Immunoprecipitable PKC-ζ/λ enzyme activity [^{32}P incorporated (in c.p.m.) into substrate/immunoprecipitate]
A	Vector	1486 ± 143
B	Vector + WT-PKC-ζ	2598 ± 340 ($P < 0.05$, with respect to group A)
C	Vector + KI-PKC-ζ	926 ± 204
D	Vector + WT-PKC-λ	1936 ± 39 ($P < 0.05$, with respect to group A)
E	Vector + KI-PKC-λ	1273 ± 280

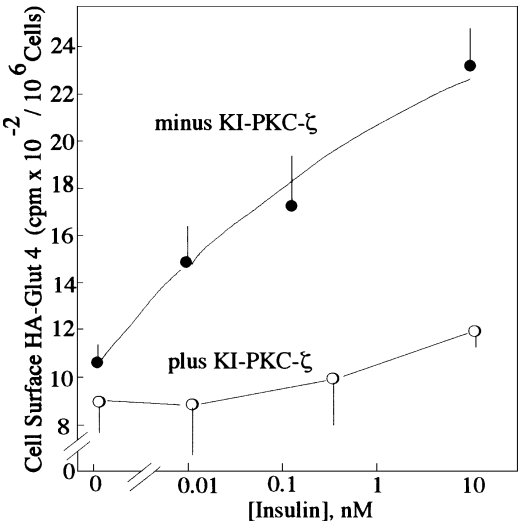

Figure 2 Effects of KI-PKC-ζ on dose-related effects of insulin on HAA-GLUT4 translocation in rat adipocytes

Cells were co-transfected with 3 μg/cuvette pCIS2 containing cDNA encoding HAA-GLUT4, and 7 μg/cuvette pCIS2 vector or pCIS2 containing cDNA encoding KI-PKC-ζ. After 24 h incubation, cells were suspended in glucose-free KRP medium and treated for 30 min with increasing amounts of insulin, as indicated. Values are means ± S.E.M. for six determinations.

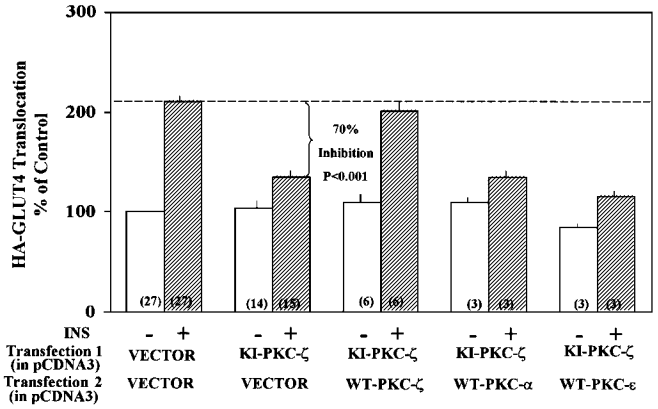

Figure 3 Co-transfected WT-PKC-ζ lowers the inhibitory affects of KI-PKC-ζ on insulin-stimulated HAA-GLUT4 translocation in rat adipocytes

Cells were transfected with 3 μg/cuvette pCIS2 containing cDNA encoding HAA-GLUT4, 7 μg/cuvette pCDNA3 containing cDNA encoding KI-PKC-ζ (where indicated), and 7 μg/cuvette pCDNA3 containing cDNA encoding WT-PKC-ζ, WT-PKC-α or WT-PKC-ε (where indicated). Total DNA used for transfection was kept constant at 17 μg per 0.8 ml cell suspension by adding supplemental vector, as required. After 24 h incubation, cells were equilibrated for 30 min in glucose-free KRP medium and then treated for 30 min with or without 10 nM insulin. Values are means ± S.E.M. for *n* determinations (as indicated in parentheses at the bases of the bars).

phospholipid-dependent enzyme activity was also recovered in precipitates prepared with anti-HAA and anti-Myc antibodies in lysates of adipocytes transfected with HAA-tagged WT-PKC-ζ and Myc-tagged WT-PKC-λ; moreover, insulin evoked increases in these activities, and it may be surmised that insulin activates both PKC-ζ and PKC-λ in the rat adipocyte.

Studies with transiently expressed forms of PKC-ζ

As alluded to above, our initial findings [3] had suggested that KI-PKC-ζ inhibits insulin-stimulated HAA-GLUT4 translocation. These findings were expanded in this study as follows: as portrayed in Figures 1–5, transient expression of KI-PKC-ζ inhibited insulin-stimulated, but not basal, HAA-GLUT4 translocation by approx. 60–70 % in these experimental groups. In the entire study, the mean % decrease in insulin-stimulated GLUT4 translocation due to expression of KI-PKC-ζ in 26 consecutive experiments (each with 3–4 individual determinations) was 65 ± 2 % (mean ± S.E.M, $n = 81$). As shown in Figure 1, both the expression of PKC-ζ and the inhibition of insulin-stimulated GLUT4 translocation appeared to approach maximal values when 7 μg of the pCDNA3/KI-PKC-ζ per electroporation cuvette was used, since neither expression nor inhibitory effects were increased significantly by using 10.5 μg of pCDNA3/KI-PKC-ζ per electroporation cuvette. Consequently, we used 7 μg of construct in most experiments. As shown in Figure 2, KI-PKC-ζ inhibited both overall responsiveness and sensitivity to insulin, i.e. the V_{max} was decreased and the half-maximally effective dose (ED_{50}) was increased. In contrast with inhibiting insulin effects, KI-PKC-ζ did not inhibit the effects of 500 μM guanosine 5′-[γ-thio]triphosphate {GTP[S]; introduced by electroporation [17]} on HAA-GLUT4 translocation. GTP[S]-induced increases were 66 % and 74 % in the absence or presence of co-transfected KI-PKC-ζ respectively. This finding correlated well with our finding that GTP[S], unlike insulin, does not activate or utilize PKC-ζ to activate glucose transport in the rat adipocyte [17], and demonstrated that KI-PKC-ζ specifically inhibits the insulin-dependent activation process, rather than the HAA-GLUT4 translocation process itself.

The inhibitory effect of KI-PKC-ζ on insulin-stimulated HAA-GLUT4 translocation appeared to be a specific consequence of the point mutation ($Lys^{281} \rightarrow Trp$) in the catalytic domain of KI-PKC-ζ [1], because this inhibitory effect of KI-PKC-ζ was largely reversed (or prevented) by co-transfection of an equal amount of WT-PKC-ζ, but not by co-transfection of equal amounts of WT-PKC-α or WT-PKC-ε (Figure 3). This finding of reversal (or prevention) by WT-PKC-ζ is particularly important as it ruled out the possibility that KI-PKC-ζ inhibited HAA-GLUT4 translocation non-specifically, e.g. by competing with other unrelated kinases (e.g. Akt/PKB) for activating polyphosphoinositides or substrates (in this case, the addition of supplementary WT-PKC-ζ would intensify, rather than reverse or prevent, inhibiting effects of KI-PKC-ζ). This finding of reversal of inhibition also provided clear evidence that the greater amounts of DNA used in some transfections (i.e. 17 μg of total plasmid/cDNA construct per 0.8 ml of cell suspension in each electroporation cuvette, as used in these rescue experiments, or in other experiments in which we wanted to ensure that maximal effects of various constructs had been achieved) were not toxic to the cells, as indicated by normal levels (both in relative and absolute terms) of both basal and insulin-stimulated GLUT4 translocation in cells co-transfected with both KI and WT forms of PKC-ζ (note that vector-treated cells received the same amount of total DNA). Finally, from the 90–95 % reversal rate, it may be surmised that co-transfection with plasmids containing cDNAs encoding WT-PKC-ζ and KI-PKC-ζ led to excellent levels of expression of both PKC isoforms, largely in the same cell population.

In addition to the pCDNA3 construct, we observed similar inhibitory effects of KI-PKC-ζ cDNA (with a $Lys^{281} \rightarrow Met$ mutation) when expressed in pTB701 (Figure 4). With respect to the question of whether PKC-ζ might actively contribute to insulin effects on GLUT4 translocation, we found that expression of WT-PKC-ζ from cDNAs in either pCDNA3 or pTB701

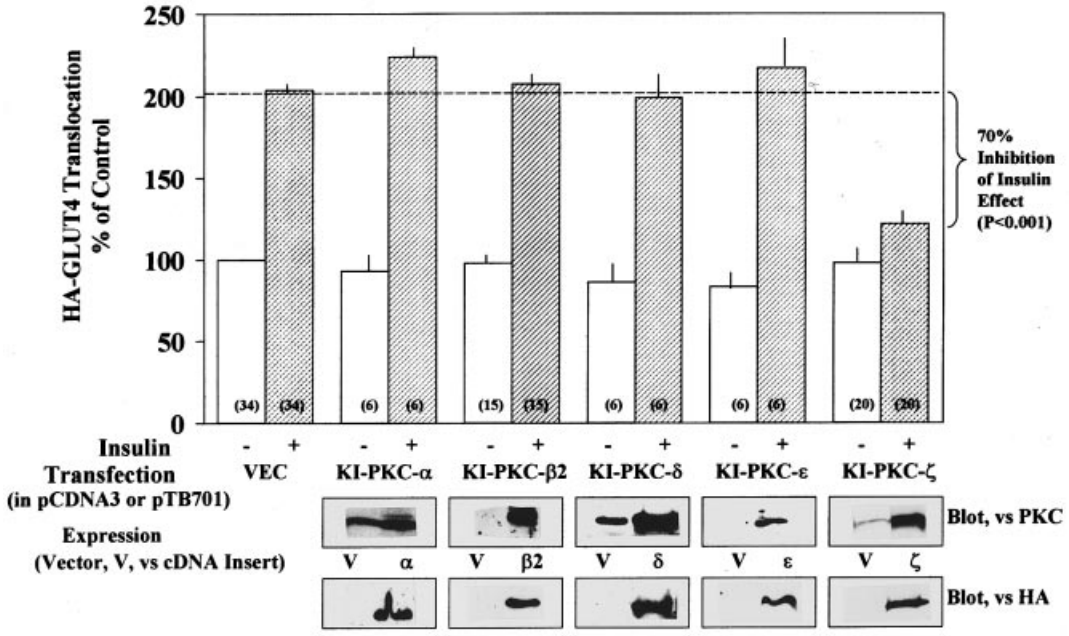

Figure 4 Effects of KI forms of PKC-α, PKC-β2, PKC-δ, PKC-ε and PKC-ζ on HAA-GLUT4 translocation in rat adipocytes

Cells were transfected with 3 μg/cuvette pCIS2 containing cDNA encoding GLUT4 and 7 μg/cuvette pCDNA3 or pTB701 containing cDNA encoding the indicated cDNA insert or no insert (VEC). (Results with these expression vectors were comparable, and were therefore pooled). After 24 h incubation, cells were suspended in glucose-free KRP medium and treated for 30 min with or without 10 nM insulin, as indicated. Values are means ± S.E.M. for *n* determinations (as indicated in parentheses at the bases of the bars). The blots show increases in expression of respective immunoreactive PKCs in cells transfected with PKC cDNAs, and expression of HAA tags migrating on SDS/PAGE at the level of the indicated PKC.

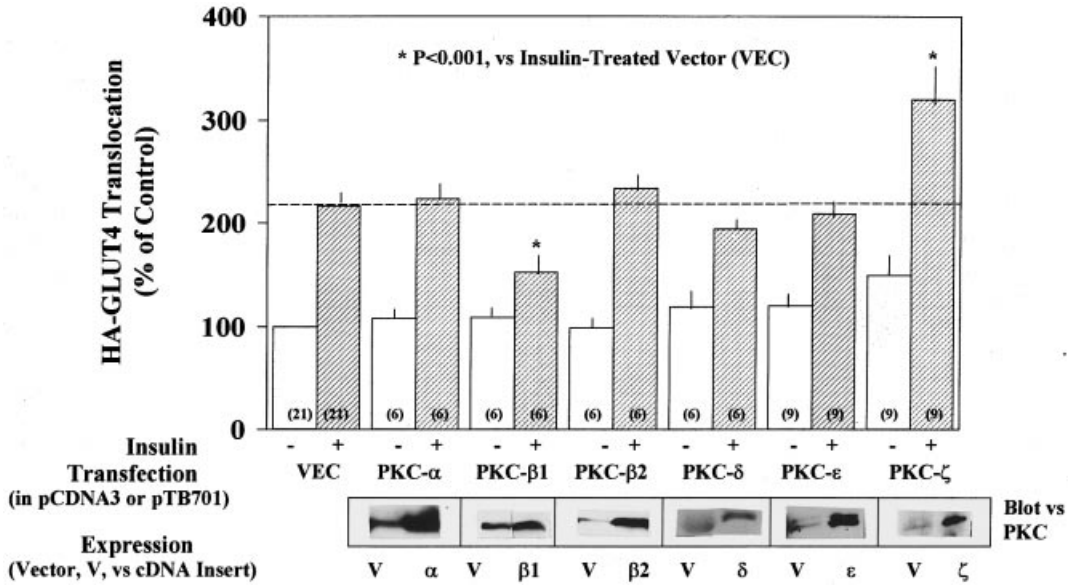

Figure 5 Effects of WT forms of PKC-α, PKC-β1, PKC-β2, PKC-δ, PKC-ε and PKC-ζ on HAA-GLUT4 translocation in rat adipocytes

As in Figure 4, cells were transfected with pCI32/GLUT4 (3 μg/cuvette) and either pCDNA3 or pTB701 (results were comparable and were therefore pooled) containing no insert (vector group) or cDNA inserts encoding WT forms of indicated PKCs (7 μg DNA/cuvette); after 24-hour incubation, cells were suspended in glucose-free KRP medium and incubated for 30 min with or without 10 nM insulin. Values are means ± S.E.M. for *n* determinations (as indicated in parentheses at the bases of the bars). The blots show increases in expression of transfected PKC cDNAs.

(results were comparable with both constructs, and these were therefore pooled) led to increases in insulin-stimulated HAA-GLUT4 translocation (Figure 5). As discussed below, this potentiating effect was observed with WT-PKC-ζ, but not with WT forms of other PKCs. With respect to the question of whether activation of PKC-ζ is sufficient to account for the stimulation of HAA-GLUT4 translocation during insulin action, expression of a point-mutated Constit form of PKC-ζ in

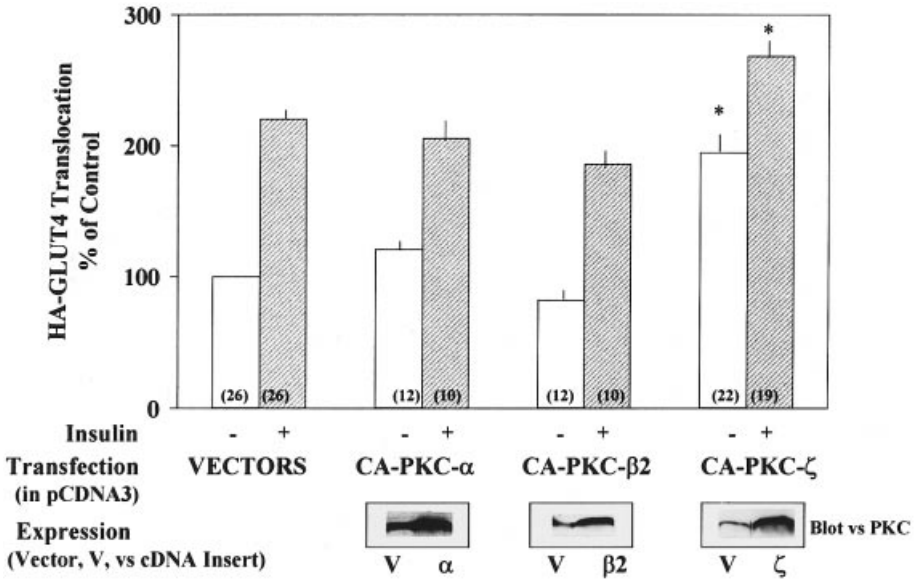

Figure 6 Effects of Constit forms of PKC-α, PKC-β2 and PKC-ζ on HAA-GLUT4 translocation in rat adipocytes

Experiments were identical with those of Figures 4 and 5, except that pCDNA3 containing no insert (vector group) or cDNA inserts encoding point-mutated Constit forms of indicated PKCs (7 μg DNA/cuvette) was used. Values are means $\pm$ S.E.M. for n determinations (as indicated in parentheses at the bases of the bars). Blots show increases in expression of transfected PKCs.

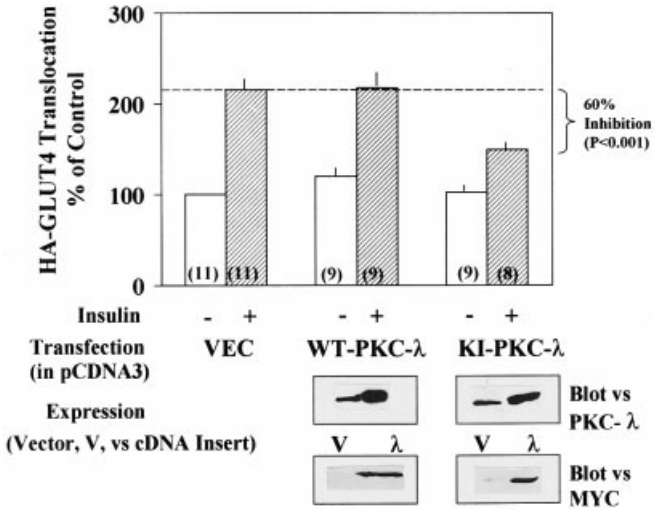

Figure 7 Effects of WT and KI forms of PKC-λ on HAA-GLUT4 translocation in rat adipocytes

Cells were transfected and incubated as in Figures 4–6, except that we used pCDNA3 containing no inserts (vector group) or cDNA insert encoding WT-PKC-λ or KI-PKC-λ (in each case, 7 μg DNA/cuvette). Values are means $\pm$ S.E.M. for n determinations (as indicated in parentheses at the bases of the bars). Blots show expression (measured with antibodies that target the specific internal site of PKC-λ or the Myc epitope) of transfected MYC-WT-PKC-λ and MYC-KI-PKC-λ.

pCDNA3 (Figure 6) stimulated basal activity and lowered, but did not fully obliterate, the difference between basal and insulin-stimulated HAA-GLUT4 translocation. Nevertheless, it is important to note that constitutive PKC-ζ increased the basal level of HAA-GLUT4 translocation to a level nearly comparable with that of insulin. It should also be noted that increasing the concentration of plasmid containing cDNA encoding constitutive PKC-ζ from 7 to 14 μg of DNA per electroporation cuvette (results not shown) did not significantly increase the expression of total PKC-ζ, or alter findings shown in Figure 6 (i.e. expression and activating effects of the constitutive PKC-ζ constructs were maximal at 7 μg of DNA per cuvette).

Studies with transiently expressed isoforms of PKC-α, -β, -δ and -ε

In contrast with WT-PKC-ζ, overexpression of WT forms of PKC-α, PKC-β2, PKC-δ and PKC-ϵ (in either pTB701 or pCDNA3) failed to alter basal or insulin-stimulated HAA-GLUT4 translocation (Figure 7). Interestingly, overexpression of WT-PKC-β1 expression inhibited HAA-GLUT4 translocation (Figure 7); the cause for this inhibitory effect of PKC-β1 is uncertain, but it should be noted that PKC-β1 and PKC-β2 have been reported to inhibit insulin receptor autophosphorylation in HEK293 cells [18].

As alluded to above, in contrast with Constit PKC-ζ, expression of constitutive forms (each in pCDNA3, and each mutated at comparable alanine sites in their pseudosubstrate sequence) of PKC-α and PKC-β2 had no effect on either basal or insulin-stimulated HAA-GLUT4 translocation (Figure 8). Perhaps more importantly, as shown in Figure 4, KI forms of PKC-α, PKC-β2, PKC-δ and PKC-ϵ, unlike KI-PKC-ζ, failed to inhibit insulin-stimulated HAA-GLUT4 translocation. Similarly, as with KI-PKC-β2, the C-terminal truncated form of PKC-β2, Δ(664–673) PKC-β2, which is presumably kinase-deficient by virtue of its loss of an important C-terminal sequence [8], had no effect on either basal or insulin-stimulated GLUT4 translocation (results not shown). In the case of KI-PKC-β2, the transfection concentration of pCDNA3/KI-PKC-β2 was also increased to 14 μg of DNA per electroporation cuvette, but results (not shown) were similar to those shown in Figure 4, i.e. basal and

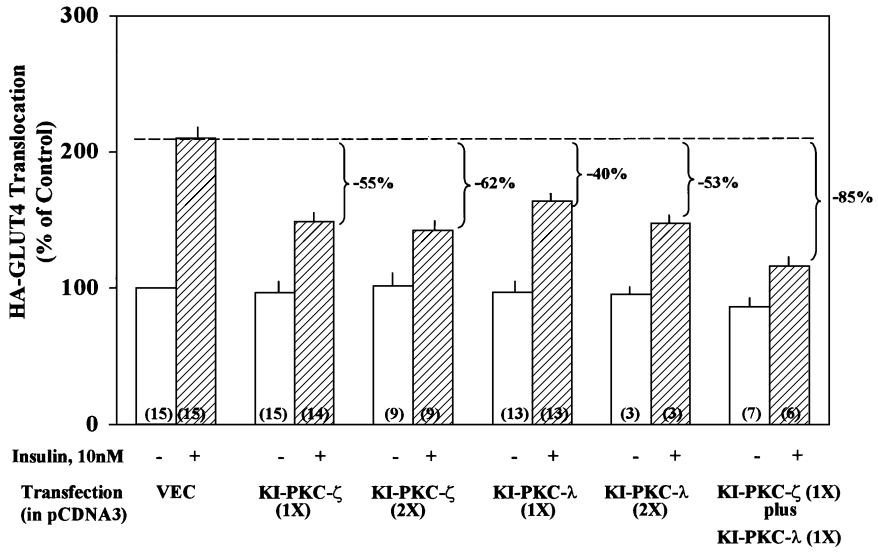

Figure 8 Effects of combined treatment with KI forms of PKC-λ and PKC-ζ on HAA-GLUT4 translocation in rat adipocytes

Cells were transfected and incubated as in Figures 4–7, except that we transfected pCDNA3 containing no insert (vector group) or cDNA inserts encoding KI forms of PKC-λ and/or PKC-ζ. 1×, 7 μg of DNA/cuvette; 2×, 14 μg of DNA/cuvette. Total DNA used for electroporation was kept constant at 17 μg DNA/cuvette (0.8 ml cell suspension) for all samples, including those in the vector group. Values are means ± S.E.M. for *n* determinations (as indicated in parentheses at the bases of the bars). Although not depicted, expression of PKCs was comparable with that observed in experiments described in Figures 1, 4 and 7.

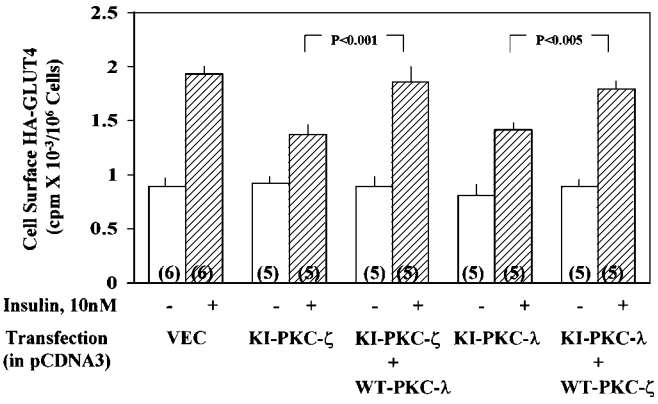

Figure 9 Interchangeable reversing effects of WT forms of PKC-ζ and PKC-λ on HAA-GLUT4 translocation in adipocytes inhibited by KI forms of PKC-λ and PKC-ζ

Cells were transfected with 3 μg/cuvette pCIS2 containing cDNA encoding HAA-GLUT4, 7 μg/cuvette pCDNA3 containing cDNA encoding either KI-PKC-ζ or KI-PKC-λ, and 7 μg/cuvette pCDNA3 containing no insert or, where indicated, insert cDNA encoding either WT-PKC-ζ or WT-PKC-λ. All cells were treated with the same amount of vector DNA. VEC, cells receiving 14 μg/cuvette pCDNA3 with no inserts. After 24 h incubation to allow time for expression, cells were equilibrated for 30 min in glucose-free KRP medium and then treated with or without 10 nM insulin for 30 min. Values are means ± S.E.M. for *n* determinations. Although not depicted, expression of PKCs was comparable with that observed in experiments described in Figures 1, 4, 5 and 7.

insulin-stimulated GLUT4 translocation were not altered. It should be noted that the failure of WT, Constit and KI forms of PKC-α, -β2, -δ and -ε to alter basal or insulin-stimulated HAA-GLUT4 translocation provided further evidence that alterations in GLUT4 translocation evoked by transfection of identical amounts of constructs encoding functionally comparable forms of PKC-ζ were not a consequence of noxious effects of transfected DNA, and could only be explained by the cDNA insert.

Studies with transiently expressed forms of PKC-λ

Since atypical PKCs ζ and λ (ι) are, in general, very similar structurally and, more specifically, highly homologous (72%) [19], it was of interest to see if expression of WT, Constit and KI forms of PKC-λ evoked changes in GLUT4 translocation that were similar to those observed with PKC-ζ variants. This interest was intensified by the fact that PKC-λ has very recently been reported to be required for insulin-stimulated GLUT4 translocation in 3T3/L1 adipocytes [20]. Although we did not observe changes in basal or insulin-stimulated HAA-GLUT4 translocation in cells transfected with WT-PKC-λ, the expression of transfected KI-PKC-λ (7 μg of construct/cuvette) inhibited insulin-stimulated HAA-GLUT4 translocation by approx. 60% and 40% in experiments depicted in Figures 7 and 8 respectively [note that expression of immunoreactive and enzymically active (as per C-terminally targeted antibodies) WT-PKC-λ was, in most cases, less than that seen with WT-PKC-ζ in transfected cells; nevertheless, increases in PKC-λ were very sizeable, i.e. up to 2-fold increases in immunoreactivity, as measured by antibodies that target an internal site of PKC-λ]. Interestingly, increasing the concentration of either pCDNA3/KI-PKC-λ or pCDNA3/KI-PKC-ζ from 7 to 14 μg per electroporation cuvette only slightly increased the level of inhibition of insulin-stimulated GLUT4 translocation (Figure 8), probably reflecting the fact that expression did not appear to increase appreciably with the higher concentration of transfected construct (results not shown). However, co-transfection of pCDNA/KI-PKC-ζ (7 μg/cuvette), along with pCDNA3/KI-PKC-λ (7 μg/cuvette), led to a more complete (85%) inhibition of insulin-stimulated GLUT4 translocation (Figure 8); this more complete inhibition might reflect the fact that total PKC-ζ plus PKC-λ levels (measured with the

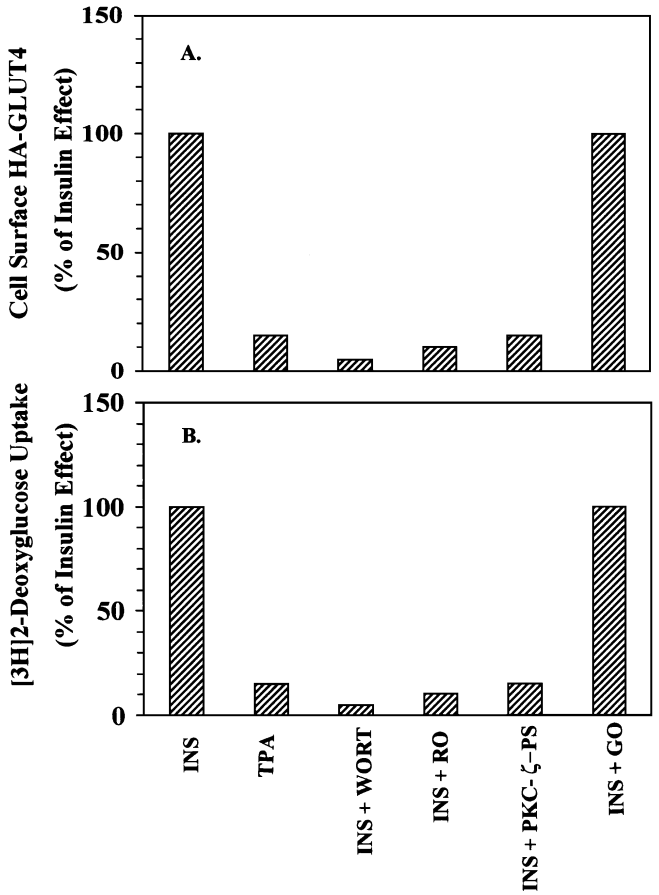

Figure 10 Comparative effects of PMA (TPA), wortmannin, RO 31-8220, myristylated PKC-ζ/λ pseudosubstrate and GO 6976 on HAA-GLUT4 translocation (A) and 2-DOG uptake (B) in the rat adipocyte

Adipocytes were suspended in DMEM and transfected with 3 μg/cuvette pCIS2 containing cDNA encoding HAA-GLUT4. The cells were incubated in DMEM as described in the Materials and methods section for 24 hours to allow time for expression of haemagglutinin antigen (HA)-tagged GLUT4, and subsequently washed and incubated in glucose-free KRP medium, first for 90 min with or without inhibitors to allow for sufficient uptake [3], and then for 30 min with vehicle (control), 10 nM insulin (INS) or 1 μM PMA (TPA), as indicated. After incubation, cells were assayed for surface content of HAA-GLUT4 or uptake of 2-DOG as described in the Materials and methods section. Note that, although not depicted, insulin effects on HAA-GLUT4 translocation and 2-DOG uptake were virtually the same, i.e. approx. 2–3 fold, in these experiments. Data (means for 3–4 determinations in representative experiments) are expressed as % of the insulin-induced increase over control, with the maximal increase set at 100%. WORT, wortmannin; RO, RO 31-8220; PS, myristylated PKC-ζ/λ pseudosubstrate; GO, GO 6976.

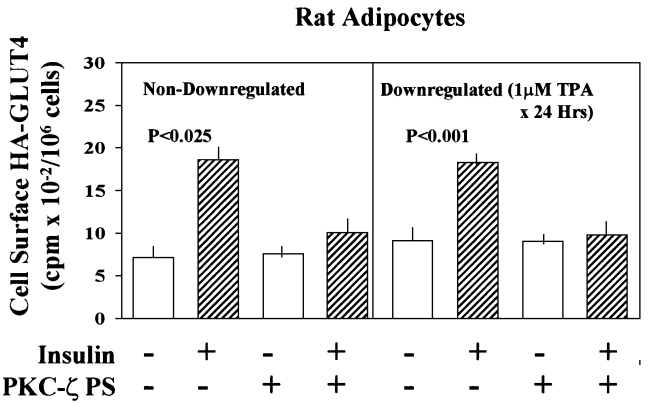

Figure 11 Effects of the cell-permeant myristylated (Myr) PKC-ζ/λ pseudosubstrate (PS) on insulin-stimulated GLUT4 translocation in rat adipocytes down-regulated or non-down-regulated by prolonged phorbol ester treatment

Cells were transfected with 3 μg/cuvette pCIS2 containing cDNA encoding HAA-GLUT4, incubated for 24 h in DMEM with (right) or without (left) 1 μM PMA (TPA), and then washed and incubated in glucose-free KRP medium, first with or without myristylated (Myr)-PKC-ζ/λ-PS for 90 min to allow for sufficient uptake [3], and then for 30 min with or without 10 nM insulin. Note that Myr-PKC-ζ/λ-PS might inhibit non-atypical PKCs (unpublished observations), but treatment with PMA overnight effectively depleted conventional and novel PKCs (PKC-α, -β1, -β2, -δ and -ϵ) by 80–100% [3].

antiserum that recognizes their similar C-termini) were greater (i.e. approx. 240% increases) in cells transfected with both PKCs than in cells transfected with either PKC alone (in each case, approx. 160–170% increases). Although we did not have a point-mutated constitutive form of PKC-λ, the N-terminal truncated, Δ(1–247) form of PKC-ζ is highly homologous (85%) to PKC-λ [19]. It was therefore interesting to discover that this truncated constitutive (as it lacks the inhibitory N-terminus) form of PKC-ζ evoked stimulatory effects on basal HAA-GLUT4 translocation that were virtually the same as those observed with the point-mutated constitutive form of PKC-ζ (results not shown).

In view of the seemingly additive effects of KI forms of PKC-ζ and PKC-λ, we questioned whether these atypical PKCs function independently or interchangeably during insulin stimulation of GLUT4 translocation. As seen in Figure 9, WT-PKC-ζ was found to reverse the inhibitory effects of KI-PKC-λ and, vice versa, WT-PKC-λ was found to reverse the inhibitory effects of KI-PKC-ζ. These findings suggested that these atypical PKCs could function interchangeably during the action of insulin.

Comparison of HAA-GLUT4 translocation with 2-DOG uptake

We compared the translocation of transiently expressed HAA-GLUT4 with overall 2-DOG uptake in a variety of experimental conditions that were relevant to the question of whether atypical PKCs, such as PKC-ζ and PKC-λ, or DAG-sensitive PKCs, such as PKC -α, -β, -δ and -ϵ, could be important for insulin stimulation of glucose transport. Accordingly, both of these processes were activated by virtually the same extent by insulin (2–3 fold; results not shown). Both processes were increased only slightly (approx. 15–20%) by acute phorbol ester treatment (Figure 10), and insulin effects on HAA-GLUT4 translocation were not affected by prolonged phorbol ester treatment (Figure 11), which depletes PKC-α, -β1, -β2, -δ and -ϵ by 80–100%, without depleting PKC-ζ/λ or inhibiting insulin-stimulated glucose transport [3]; furthermore, insulin effects on both processes were inhibited markedly by the PI-3K inhibitor, wortmannin (100 nM; Figure 10), and PKC-ζ/λ inhibitors [2,3], 20 μM RO 31-8220 (Figure 10) and 100 μM myristylated PKC-ζ/λ pseudosubstrate (Figures 10 and 11), but not by a selective inhibitor of PKC-α, -β and -γ, 100 μM GO 6976 [21] (Figure 10; note that although the PKC-ζ pseudosubstrate could inhibit most PKCs, the findings in down-regulated cells in Figure 11 indicate that this inhibitor alters GLUT4 translocation by inhibiting phorbol-ester-insensitive PKCs, such as PKC-ζ and PKC-λ). These findings suggested that atypical PKCs, such as PKC-ζ and -λ, but not conventional (α, β, γ) or novel (δ, ϵ, η) PKCs, might contribute to HAA-GLUT4 translocation and glucose transport. It should be noted that glucose transport and HAA-GLUT4 translocation were increased approx. 2–3-fold by insulin in

transfected cultured adipocytes; as described previously [17], these effects are less than those observed in fresh adipocytes, and this decrease primarily reflects a 2–3-fold increase in basal transport activity, coupled with either a small or an insignificant decrease in the maximal level of transport evoked by insulin.

DISCUSSION

These findings provide convincing evidence that transiently transfected KI-PKC-ζ inhibits insulin-stimulated translocation of HAA-GLUT4 in cultured rat adipocytes; the inhibition of insulin-stimulated GLUT4 translocation by KI-PKC-ζ was consistently observed in all experiments, regardless of the eukaryotic expression vector used or the initial source of variations in the amino acid used to replace lysine in the catalytic domain of the mutated KI-PKC-ζ cDNA insert. The inhibition achieved was reproducibly sizeable (overall mean ± S.E.M. of 65 ± 2 %) and, in some instances, almost complete (up to 90–95 %), approaching the inhibition observed with transfection of dominant-negative PI-3K [22] and PKC-ζ inhibitors. Moreover, we found that inhibitory effects of KI-PKC-ζ were prevented or reversed by co-transfection of cDNA encoding WT-PKC-ζ, and KI-PKC-ζ expression did not inhibit GTP[S]-stimulated HAA-GLUT4 translocation, which is independent of PKC-ζ [17]. We were therefore able to conclude that inhibitory effects of KI-PKC-ζ were (a) directly attributable to the mutation in the ATP-binding site in the catalytic domain of transfected KI-PKC-ζ; (b) at least relatively specific for the mechanism used by insulin to stimulate GLUT4 translocation; and (c) could not be explained by noxious or other non-specific effects of transfected DNA.

In contrast with KI-PKC-ζ and KI-PKC-λ (see below), expression of KI forms of PKC-α, PKC-β2, PKC-δ and PKC-ε failed to inhibit insulin-stimulated HAA-GLUT4 translocation. This specificity, among PKCs, of KI-PKC-ζ and KI-PKC-λ is in keeping with the notion that, whereas DAG-sensitive PKCs do not appear to be required for insulin effects on glucose transport, the atypical PKCs, i.e. PKC-ζ and PKC-λ, which are primarily activated by insulin-induced increases in D3-PO_4 polyphosphoinositides [2–4,20], appear to be required for insulin stimulation of GLUT4 translocation and glucose transport. As alluded to above, this notion was also supported by findings reported previously derived from inhibitor and PKC depletion studies in 3T3/L1 adipocytes [1], rat adipocytes [3] and L6 myotubes [2].

Although our findings in transfected cells suggested that enzymically active forms of PKC-ζ and/or PKC-λ is/are required for insulin stimulation of HAA-GLUT4 translocation, the following caveats should be noted. First, in the electroporated/cultured rat adipocyte, baseline glucose transport is elevated, the absolute level of the maximal insulin effect is either unchanged or decreased slightly, and the 2–5-fold (mean closer to 2–2.5-fold) increases in 2-DOG uptake observed with insulin treatment in these cells are diminished, relative to 4–10-fold (mean closer to 5-fold) increases seen in fresh cells [17]. Secondly, only about 5 % of adipocytes are transfected significantly by electroporation [16], and it may be argued that HAA-GLUT4 translocation might not reflect total GLUT4 translocation. Thirdly, in cells co-transfected with two plasmid/cDNA constructs, the frequency of uptake and expression of both proteins in the same cell population could be questioned. On the other hand, the present findings suggested that HAA-GLUT4 translocation in transfected cells accurately reflects the pattern of total overall glucose transport (i.e. 2-DOG uptake) in the entire population of cultured rat adipocytes in a variety of experimental conditions. In addition, it should be noted that the PKC-ζ pseudosubstrate inhibits 5–10-fold increases in 2-DOG uptake evoked by insulin in freshly incubated adipocytes [3]. Finally, the fact that HAA-GLUT4 translocation can be inhibited by 85 % in cells transfected with KI-PKC-ζ and KI-PKC-λ, coupled with the fact that inhibitory effects of KI-PKC-ζ were reversed to the extent of 90–95 % by co-transfection with WT-PKC-ζ, indicates that successful uptake and expression of co-transfected plasmid/cDNA constructs in the same cell population occur at high rates of frequency.

Our finding that expression of WT-PKC-ζ potentiated insulin effects on HAA-GLUT4 translocation, and that expression of constitutive PKC-ζ both stimulated basal HAA-GLUT4 translocation to a level comparable with that of insulin and narrowed the difference between basal and insulin-stimulated HAA-GLUT4 translocation, suggested that PKC-ζ might actively participate in stimulating GLUT4 translocation during the action of insulin. However, insulin continued to provoke a stimulatory effect on GLUT4 translocation in cells transfected with constitutive PKC-ζ. This suggested that another factor might be required for full effects of insulin on GLUT4 translocation; alternatively, exogenously added Constit PKC-ζ might be neither fully equivalent to nor entirely substitute for endogenous insulin-sensitive PKC-ζ and PKC-λ. In the latter scenario, insulin effects on endogenous PKC-ζ and PKC-λ [perhaps in specific pools that are activated by PI-3K-dependent increases in phosphatidylinositol 3,4,5-$(PO_4)_3$] might activate GLUT4 translocation above the level achieved with exogenously added constitutive PKC-ζ, which presumably acts indiscriminately at multiple sites by mass action. Further studies are needed to examine these possibilities.

It was of particular interest to find that KI-PKC-λ inhibited insulin-stimulated HAA-GLUT4 translocation almost as well as KI-PKC-ζ, and that inhibitory effects of KI-PKC-λ could be reversed by WT-PKC-ζ and, conversely, inhibitory effects of KI-PKC-ζ could be reversed by WT-PKC-λ. In this regard, it is important to note that: (a) both PKC-ζ and PKC-λ are present in rat adipocytes; (b) PKC-ζ and PKC-λ, as atypical PKCs, are highly homologous, particularly in key areas, i.e. in pseudosubstrate sequences, cysteine-rich zinc-finger-like regions in their regulatory domains, catalytic sites and C-termini [19]; (c) both atypical PKCs are activated by PI-3K-dependent D3-PO_4 polyphosphoinositides [3,20]; and (d), as discussed above, both PKC-ζ and PKC-λ [20] are activated by insulin. It therefore appears that both isoforms might be required for, and might function interchangeably in support of, insulin-stimulated GLUT4 translocation in the rat adipocyte. It is also possible that some cell types might primarily have and use only one atypical PKC isoform, e.g., PKC-λ in 3T3/L1 adipocytes [20]. Further studies are needed to evaluate these possibilities.

Transient transfection studies similar to those used in this study have suggested that insulin receptor substrate (IRS)-1 and/or IRS-2 [22], PI-3K [23], PKB [11,22], and both Rho and its downstream effector, PKN [17], might also be required for insulin stimulation of epitope-tagged GLUT4 translocation in rat adipocytes. Like PKC-ζ, PKB operates downstream of PI-3K [24–26], and, since PKB and PKC-ζ have been reported to interact physically [27], it could be argued that KI-PKC-ζ might inhibit PKB non-specifically (or vice versa), and therefore only PKB (or PKC-ζ/λ) is required for insulin-stimulated glucose transport. However, our finding that active forms of PKC-ζ activated basal and/or insulin-stimulated GLUT4 translocation, and restored insulin effects in cells treated with KI-PKC-ζ or KI-PKC-λ, militated strongly against the possibility that transfected KI-PKC-ζ and PKC-λ inhibited insulin-stimulated GLUT4 translocation via direct inhibitory effects on PKB. With respect to other possible relationships between PKC-ζ and PKB, it should also be noted that PKB (immunoprecipitated from insulin-treated adipocytes) is not inhibited (unpublished observations)

by the PKC-ζ pseudosubstrate (which, as shown here, strongly inhibits insulin-stimulated GLUT4 translocation and glucose transport), and PKB is clearly not the PKC-ζ pseudosubstrate-inhibitable protein kinase that is required for insulin-stimulated glucose transport. In addition, RO 31-8220 inhibits the activation of PKC-ζ, but not the activation of PKB, in intact rat adipocytes (unpublished observations); thus, except for shared requirements for PI-3K, subsequent activation mechanisms for PKC-ζ and PKB appear to be at least partly different and independent of each other. Finally, it is noteworthy that the mild (20%) inhibition of insulin-stimulated HAA-GLUT4 translocation observed with transfection of relatively large amounts (14 μg of construct/0.8 ml cell suspension) of plasmid containing cDNA encoding KI-PKB [12] was substantially less than that currently observed with lower concentrations of transfected plasmids containing cDNA encoding KI-PKC-ζ and/or KI-PKC-λ. Nevertheless, PKB, together with PKC-ζ, PKC-λ and possibly other kinases, might function as downstream effectors of PI-3K and contribute manifoldly to GLUT4 translocation during insulin action.

In summary, transient expression of KI-PKC-ζ evoked consistent, sizeable, specific, inhibitory effects on insulin-stimulated HAA-GLUT4 translocation in rat adipocytes. Moreover, the inhibitory effects of KI-PKC-ζ were reversed by WT-PKC-ζ, and were not mimicked by KI forms of PKC-α, PKC-β2, PKC-δ, and PKC-ϵ. Interestingly, KI-PKC-λ, like KI-PKC-ζ, inhibited insulin-stimulated GLUT4 translocation, and partial inhibitory effects of KI forms of both PKC-λ and PKC-ζ were partly additive and, most importantly, could be reversed by the opposite WT form, i.e. by PKC-ζ or PKC-λ respectively. It therefore appears that both atypical PKC isoforms, ζ and λ, might function interchangeably in supporting insulin-stimulated GLUT4 translocation in the rat adipocyte. Of further note, expression of WT-PKC-ζ potentiated insulin effects, and expression of constitutive PKC-ζ evoked insulin-like effects on HAA-GLUT4 translocation; in contrast, expression of WT and Constit forms of PKC-α, PKC-β2, PKC-δ and/or PKC-ϵ failed to elicit effects on GLUT4 translocation that were comparable with those of PKC-ζ. These findings suggest that, among PKCs, the atypical PKCs, PKC-ζ and PKC-λ, are together specifically required for, and might contribute interchangeably to, insulin stimulation of GLUT4 translocation and glucose transport in rat adipocytes. Further studies are needed to more fully define the roles of PKC-ζ, PKC-λ and other polyphosphoinositide-activated kinases, e.g. PKB and PKN, in insulin-stimulated glucose transport.

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