Repletion of Atypical Protein Kinase C following RNA Interference-mediated Depletion Restores Insulin-stimulated Glucose Transport*

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The role of atypical protein kinase C (aPKC) in insulin-stimulated glucose transport in myocytes and adipocytes is controversial. Whereas studies involving the use of adenovirally mediated expression of kinase-inactive aPKC in L6 myocytes and 3T3/L1 and human adipocytes, and data from knock-out of aPKC in adipocytes derived from mouse embryonic stem cells and subsequently derived adipocytes, suggest that aPKCs are required for insulin-stimulated glucose transport, recent findings in studies of aPKC knockdown by small interfering RNA (RNAi) in 3T3/L1 adipocytes are conflicting. Moreover, there are no reports of aPKC knockdown in myocytes, wherein insulin effects on glucose transport are particularly relevant for understanding whole body glucose disposal. Presently, we exploited the fact that L6 myotubes and 3T3/L1 adipocytes have substantially different (30% non-homology) major aPKCs, viz. PKC-ζ in L6 myotubes and PKC-λ in 3T3/L1 adipocytes, that nevertheless can function interchangeably for glucose transport. Accordingly, in L6 myotubes, RNAi-targeting PKC-ζ, but not PKC-λ, markedly depleted aPKC and concomitantly inhibited insulin-stimulated glucose transport; more importantly, these depleting/inhibitory effects were rescued by adenovirally mediated expression of PKC-λ. Conversely, in 3T3/L1 adipocytes, RNAi constructs targeting PKC-λ, but not PKC-ζ, markedly depleted aPKC and concomitantly inhibited insulin-stimulated glucose transport; here again, these depleting/inhibitory effects were rescued by adenovirally mediated expression of PKC-ζ. These findings in knockdown and, more convincingly, rescue studies, strongly support the hypothesis that aPKCs are required for insulin-stimulated glucose transport in myocytes and adipocytes.

Transport is the initial rate-limiting step for cellular uptake and utilization of glucose in skeletal muscle and adipocytes. Insulin is a major controller of glucose transport in adipocytes and skeletal muscle, and defects in insulin-stimulated glucose transport, particularly in skeletal muscle, contribute importantly to the development of insulin resistance in obesity and type 2 diabetes mellitus.

Insulin regulates glucose transport in adipocytes and skeletal muscle through the activation of insulin receptor substrate (IRS)-2-dependent phosphatidylinositol 3-kinase. Distal effectors of phosphatidylinositol 3-kinase that are postulated to mediate insulin-induced increases in glucose transport include protein kinase B (PKB/Akt) and atypical protein kinase C (aPKC) isoforms, ζ and λ. The initial evidence suggesting requirements for PKB (1–4) and aPKC (5–13) in insulin-stimulated glucose transport was based largely upon inhibitory effects ensuing from expression of kinase-inactive (dominant-negative) forms of these protein kinases in adipocytes and muscle cells. More recently, genetically manipulated adipocytes deficient in either PKB (14) or aPKC (15) have been used to further implicate these protein kinases in insulin-stimulated glucose transport. In these gene knock-out studies, insulin-stimulated glucose transport was markedly diminished in the absence of these protein kinases and restored by their expression. These methods, however, while highly suggestive, have inherent caveats, e.g. expression of inhibitory kinases may have untoward effects, and genetically manipulated and subsequently selected cells may have little relevance to native adipocytes and myocytes. As another experimental approach for evaluating the importance of aPKC and PKB during insulin-stimulated glucose transport, whole body knock-out of PKC-λ, the major aPKC in many mouse tissues, has not been feasible, as this aPKC is required for embryonic survival; in the case of PKBα or PKBβ, effects of maximally effective concentrations of insulin on glucose transport are not compromised in muscles of these knock-out mice (16, 17), perhaps reflecting the continued function of remaining untargeted PKB isoforms that may be sufficient or compensatorily increased.

A more recently developed approach for determining the importance of protein kinases during insulin-stimulated glucose transport is the use of small gene-silencing/interfering RNA (RNAi) to specifically diminish generation of mRNA that codes for the targeted protein kinase. Initial studies with RNAi have indeed suggested that PKBβ/Akt2 is required for insulin-stimulated glucose transport in 3T3/L1 adipocytes (18), whereas, in L6 myotubes, the impaired activation of PKBβ resulting from an RNAi-induced knockdown of IRS-2 does not inhibit insulin-stimulated glucose transport (19). This apparent dissociation of PKBβ from insulin-stimulated glucose transport in L6 myotubes may be explained by postulating that PKBα and/or PKBγ is the major functional PKB isoform, or can substitute for PKBβ during insulin-stimulated glucose transport in these cells. In the case of aPKC knockdown studies, in 3T3/L1 adipocytes, microinjection of RNAi that targets PKC-λ has been reported to inhibit insulin-stimulated GLUT4 translocation and insulin-stimulated glucose transport (20). On the other hand, the introduction of another RNAi-targeting PKC-λ by electroporation of detached and subsequently re-attached 3T3/L1 adipocytes has been reported to substantially diminish levels of PKC-λ while only minimally inhibiting insulin-stimulated glucose transport (21).
In view of the need to more critically test the requirement for aPKC during insulin-stimulated glucose transport in muscle cells, and, in view of the conflicting findings in 3T3/L1 adipocytes, we presently employed RNAi to knockdown aPKC in both L6 myotubes and 3T3/L1 adipocytes, two classical cultured cell types frequently used for studying insulin action. Moreover, we took advantage of the fact that the major aPKC in L6 myotubes is PKC-ζ (10), and the major aPKC in 3T3/L1 adipocytes is PKC-ε (9). Since these aPKCs have substantially different mRNA and amino acid compositions (30% non-homology), but nevertheless function interchangeably during insulin-stimulated glucose transport (8), this difference in aPKC composition in these two cell types allowed us to (a) examine the specificity of the presently used RNAi forms for the targeted endogenous aPKC and (b) most importantly rescue insulin-stimulated glucose transport in RNAi-treated cells by expression of an exogenous untargeted aPKC to substitute for the depleted endogenous aPKC.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Incubation Conditions—* As described (5, 13), 3T3/L1 adipocytes were cultured and fully differentiated prior to experimental usage on day 6. At this point, where indicated, the cells were incubated in serum-free Dulbecco’s modified Eagle’s medium for 96 h with 10 m.o.i. (multiplicity of infection) of adenovirus vector or adenovirus encoding wild type PKC-ζ and for 72 h (i.e. starting 24 h after viral infection) with Dharmafect reagent (DF-3, catalog number T-2000107-03, supplied by the Dharmacon Corp., Lafayette, CO) and the indicated RNAi (100 nM unless otherwise indicated). Fetal bovine serum (2%) (Sigma) was added 48 h after addition of RNAi. On the day of the experiment, the cells were incubated in serum-free Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin (BSA) for 3–4 h and finally incubated for 30 min in glucose-free Krebs-Ringer phosphate medium containing 1% BSA (Sigma), with or without 100 nM insulin or other agents prior to measurement of [3H]2-deoxyglucose uptake over 5 min, as described (5, 13).

As described (6, 10, 13), L6 myotubes were cultured and fully differentiated prior to experimental usage on day 5. At this point, where indicated, the myotubes were incubated in α-minimal essential medium for 96 h with adenovirus vector alone or adenovirus encoding wild type PKC-κ and/or Oligofectamine (Invitrogen, Chicago, IL) and the indicated RNAi (100 nM unless otherwise indicated), as previously used in studies of IRS-1/2 knockdown (19). On the day of the experiment, the culture medium was changed to α-minimal essential medium containing 1 mg/ml BSA, and after 3 h, the cells were finally incubated for 30 min in glucose-free Krebs-Ringer phosphate medium containing 1 mg/ml BSA, with or without 100 nM insulin or other agents prior to measurement of [3H]2-deoxyglucose uptake over 5 min, as described (6, 10, 13).

After incubation, the cells were washed three times with cold medium and subsequently released from the incubation plates with 0.1% sodium dodecyl sulfate solution. A small aliquot of the solubilized incubation mixture was then removed and placed into Laemmli buffer for Western analysis, and the remainder of the incubation mixture was used to measure [3H]2-deoxyglucose uptake. Note that, in all experiments, we routinely measured aPKC protein levels in the same incubation wells in which transport was measured.
RNAi Constructs—All RNAi constructs were obtained from Dharmacon. Construct A contained a single RNAi sequence targeting PKC-\(\zeta\)/H9261 mRNA (target sequence, 5'-UCCUCAAGAUGAGAGU-3'), as used by Ugi et al. (20) in 3T3/L1 adipocytes. Construct B consisted of a Smartset pool of four RNAi sequences targeting four sites in PKC-\(\zeta\)/H9261 mRNA, as used by Zhou et al. (21) in 3T3/L1 adipocytes. Construct C, used for PKC-\(\zeta\)/H9256 knockdown in L6 myotubes, consisted of a Smartpool mixture of four RNAi sequences targeting specific sequences in PKC-\(\zeta\)/H9256 mRNA (target sequences, accession number NM-022507): 5'-GAUGUGUAGACCUU-3', 5'-GGAAACAUGACAAUAUCAA-3', 5'-CGAUGCCGAUGGACA-CAAUU-3'; in more limited studies, where indicated, we also used the four individual RNAi sequences of this Smartset pool-targeting PKC-\(\zeta\).

Adenoviruses for Wild Type aPKCs—Adenoviruses that contain cDNAs to express wild type PKC-\(\zeta\)/H9256 and PKC-\(\zeta\)/H9261 were described previously (10, 13).

aPKC Assay—The enzyme activity of total aPKC was measured as described (5–8, 10, 13, 15). In brief, aPKCs were immunoprecipitated with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA) that recognizes a common epitope in the C termini of PKC-\(\zeta\).
and PKC-λ/ι, collected on Sepharose-AG beads, and incubated for 8 min at 30 °C in 100 μl buffer containing 50 mM Tris/HCl (pH 7.5), 100 μM Na3VO4, 100 μM NaF, 1 mM NaF, 100 μM phenylmethylsulfonyl fluoride, 4 μg phosphatidylserine (Sigma), 50 μM [γ-32P]ATP (PerkinElmer Life Sciences), 5 mM MgCl2 and, as substrate, 40 μM serine analogue of the PKC-ε pseudosubstrate (BioSource, Camarillo, CA). After incubation, 32P-labeled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter.

Western Analyses—As described (10, 13), proteins were resolved on SDS-PAGE and blotted with the following antibodies: (a) rabbit polyclonal anti-aPKC antiserum (obtained from Santa Cruz Biotechnologies); (b) mouse monoclonal anti-PKC-λ antibody (BD Transduction Laboratories, San Diego, CA); (c) rabbit polyclonal anti-PKC-ζ antiserum (kindly supplied by Dr. Todd Sacktor, New York); (d) goat polyclonal anti-PKBα/β antiserum (Santa Cruz Biotechnologies); (e) rabbit polyclonal anti-phospho-Ser-473-PKB antisera (Cell Signaling, Lake Placid, NY); (f) rabbit polyclonal anti-GLUT4 glucose transporter antiserum (Biogenesis, Kingston, NH); and (g) rabbit polyclonal anti-PKBα/β antisera (Santa Cruz Biotechnologies).

mRNA Analyses—Cells were scraped into TRIzol reagent (Invitrogen), and RNA was extracted and purified with the RNA-Easy mini-kit (Qiagen, Valencia, CA) and RNase-free DNase set (Qiagen) as per kit instructions, quantified by measuring A260/A280, further checked for purity by electrophoresis on 1.2% agarose gels, and mRNA quantified by quantitative real-time reverse transcriptase-PCR. PKC-ζ mRNA was measured with a SYBR Green kit (Applied Biosystems, Foster City, CA; catalog number 4309155) using: (a) PKC-ζ primers at nucleotides 1617 and 1797, GCCCTCCCTTCAGCCCCAGAGA (forward) and CAGG-GAATGGTCTAGCAGACGACAGCA (reverse); and (b) housekeeping gene, hypoxanthine phosphoribosyltransferase, and primers, TGAAAGAATCTCCACGAGCC (forward) and AAAGAACTTATAGCCTTTTTCCTT (reverse). PKC-λ mRNA was measured with TaqMan Universal PCR master mix kit (Applied Biosystems, catalog number 4304437) with PKC-λ primers and probe mix (as provided by Applied Biosystems, catalog number Mm00435769-m1) and internal control 18 S rRNA (Applied Biosystems, catalog number 4319413E).

Cellular Uptake of Fluorescently Labeled RNAi—Transfection efficiency was estimated by incubating 3T3/L1 adipocytes and L6 myotubes with a control fluorescently labeled RNAi (siGLO; Dharmacon, catalog number D00161-01; dual purpose siRNA-targeting cyclin B and carrying a fluorescent label) in the above-described transfection methods, followed by examination of paraformaldehyde-fixed cells for (a) fluorescent RNAi, which accumulates in a distinct perinuclear distribution, and (b) 4,6-diamidino-2-phenylindole dihydrochloride (Fluka-Biochime product supplied by Sigma)-stained nuclei. By comparing congruence of perinuclear yellow fluorescence and blue-stained nuclei in approximately 50 cells in n representative fields from multiple plates, we found that the percentage of cells successfully transfected, i.e. fluorescence-positive relative to total nuclei, was 71 ± 3 (mean ± S.E.; n = 6 fields) and 76 ± 4% (n = 8 fields) in 3T3/L1 adipocytes and L6 myotubes, respectively (also see Fig. 1). This estimate of transfection efficiency for RNAi was comparable in magnitude with the relative losses of targeted mRNA and protein in these cells, as described below.

RESULTS

aPKC Requirements in 3T3/L1 Adipocytes

Construct A, Knockdown Studies—Whereas RNAi-targeting PKC-ζ mRNA was without effect in 3T3/L1 adipocytes (Fig. 2A), Construct A, which contains a single RNAi sequence targeting PKC-λ mRNA (see 20), markedly inhibited insulin-stimulated glucose transport in 3T3/L1 adipocytes (Figs. 3, A and B, and 4B), while diminishing PKC-λ mRNA levels by 80–90% (Fig. 5B) and total aPKC levels (largely or exclusively PKC-λ, see Ref. 9 and Fig. 3, B and C) by approximately 60–90% (Figs. 3, A and B, and 4A). In confirmation of decreases in levels of total aPKC, Construct A RNAi-targeting PKC-λ also markedly diminished basal and insulin-stimulated aPKC activity (Fig. 6A). On the other hand, the levels and activation of PKBα/β, as determined by phosphorylation of Ser-473, were not affected by treatment with Construct A RNAi-targeting PKC-λ (Fig. 3, A and B). Thus, inhibitory effects of Construct A RNAi-targeting PKC-λ on insulin-stimulated glucose transport could not be explained by altered insulin signaling to PKB.

In contrast to inhibitory effects in 3T3/L1 adipocytes, Construct A RNAi-targeting PKC-λ did not diminish total aPKC levels or inhibit insulin-stimulated glucose transport in L6 myotubes (Fig. 2B), in keeping with the fact that the major aPKC is PKC-ζ and there are only trace amounts, if any, of PKC-λ in L6 myotubes (Ref. 10 and see Fig. 8B). Thus, assuming that the RNAi-targeting PKC-λ was effective in entering L6 myotubes, and assuming that initial insulin signaling requirements for activating glucose transport through aPKC and PKB are similar in 3T3/L1 adipocytes and L6 myotubes, it may be surmised that
Construct A RNAi, except for depletion of the targeted PKC-ζ, did not indiscriminately inhibit general signaling mechanisms required for insulin-stimulated glucose transport. In addition to signaling mechanisms, the RNAi-targeting PKC-ζ did not alter levels of GLUT4 glucose transporters in 3T3/L1 adipocytes (data not shown).

**Construct A, Rescue Studies**—To be certain that the above-described RNAi-mediated knockdown of PKC-ζ did not cause a nonspecific inhibition of glucose transport, and, moreover, inhibited insulin-stimulated glucose transport by specifically knocking down PKC-ζ, the major aPKC in 3T3/L1 adipocytes, we attempted to rescue insulin effects by expressing wild type PKC-ζ, which would not be targeted by Construct A RNAi. Accordingly, we found that expression of relatively modest amounts of this exogenous aPKC (see blot data in Figs. 2A and 3B) in fact substantially increased PKC-ζ mRNA levels (Fig. 5A), and, more importantly, fully “rescued” insulin-stimulated glucose transport, or stated differently, negated the ability of Construct A RNAi-targeting PKC-ζ to inhibit insulin-stimulated glucose transport in 3T3/L1 adipocytes (Fig. 3B). Moreover, as seen in Fig. 2A, adenovirally mediated expression of wild type PKC-ζ, despite increasing total aPKC levels, itself had little or no effect on basal or insulin-stimulated glucose transport in 3T3/L1 adipocytes. In addition, adenovirally mediated expression of PKC-ζ did not non-specifically diminish the ability of Construct A RNAi to decrease the level of either PKC-ζ protein, as determined with isoform-specific anti-PKC-ζ antibody (Fig. 3B) or PKC-ζ mRNA (Fig. 5B). Thus, the ability of wild type PKC-ζ to restore aPKC levels and rescue insulin-stimulated glucose transport in adipocytes in which endogenous PKC-ζ had been knocked down by Construct A RNAi cannot be ascribed to either a nonspecific stimulatory effect of wild type PKC-ζ on glucose transport or to an abrogation of the effectiveness of Construct A RNAi for knocking down PKC-ζ.

**Construct B, Knockdown Studies**—In addition to Construct A, we examined the effects of Construct B, a Smartpool that contains a set of four RNAi sequences that have been used to target PKC-ζ mRNA and diminish total aPKC levels in 3T3/L1 adipocytes (21). Findings in studies with Construct B were essentially the same as those seen with Construct A in 3T3/L1 adipocytes, in terms of: (a) diminishing total aPKC levels and insulin-stimulated glucose transport (Figs. 3C and 4C); (b) diminishing PKC-ζ mRNA levels (Fig. 5D); (c) diminishing basal and insulin-stimulated aPKC activity (Fig. 6B); (d) having no effect on levels or phosphorylation/activation of PKB (Fig. 3C); and (e) observing rescue
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**FIGURE 6. Effects of Construct A (A) or Construct B (B) RNAi-targeting PKC-ζ in 3T3/L1 adipocytes and Construct C RNAi-targeting PKC-ζ in L6 myotubes (C) on basal and insulin-stimulated total aPKC enzyme activity.** Cells were treated with scrambled RNAi (SCR) or indicated RNAi. Bar graphs depict mean ± S.E. values of n determinations of aPKC activity. Insets show representative blots reflective of changes in aPKC levels in lysates used for assays.

of insulin-stimulated glucose transport (Fig. 3C) in Construct B-treated 3T3/L1 adipocytes by expression of PKC-ζ, as evidenced by increases in both PKC-ζ mRNA (Fig. 5C) and protein (Fig. 3C), in the absence of changes in depressed levels of PKC-ζ mRNA (Fig. 5D) and protein (Fig. 3C).

**aPKC Requirements in L6 Myotubes**

**Construct C, Knockdown Studies**—In L6 myotubes, whereas Construct A RNAi-targeting PKC-ζ was without effect (Fig. 2B), Construct C RNAi-targeting PKC-ζ markedly inhibited insulin-stimulated glucose transport (Figs. 4A, 7, A and B, and 8, A and B), while diminishing total aPKC protein levels (largely PKC-ζ; Ref. 10 and see Fig. 8B)) by ∼60–90% (Figs. 4A, 7, A and B, and 8, A and B), and diminishing PKC-ζ mRNA levels by ∼80–90% (Fig. 5E). In confirmation of decreases in aPKC protein levels, this RNAi-targeting PKC-ζ also markedly inhibited basal and insulin-stimulated aPKC activation (Fig. 6C). In contrast to these inhibitory effects seen in L6 myotubes, as stated above, Construct C RNAi-targeting PKC-ζ was without effect on total aPKC levels and insulin-stimulated glucose transport in 3T3/L1 adipocytes (Fig. 2A), which have PKC-ζ as the major endogenous aPKC, and little or no PKC-ζ (Ref. 9 and Fig. 3, B and C). Thus, assuming that the RNAi-targeting PKC-ζ was effective in entering 3T3/L1 adipocytes, and, assuming similar initial insulin signaling requirements for activating glucose transport in 3T3/L1 adipocytes and L6 myotubes, it may be surmised that this RNAi-targeting PKC-ζ did not indiscriminately inhibit signaling mechanisms required for insulin-stimulated glucose transport. This conclusion is further supported by the finding that this RNAi-targeting PKC-ζ did not diminish PKB levels (Figs. 7, A and B, and 8, A and B) or inhibit the phosphorylation/activation of PKB (Fig. 7B). Also note that this RNAi-targeting PKC-ζ did not alter levels of GLUT4 glucose transporters (data not shown).

Although not shown, each of the four individual sequences in the Smartset pool-targeting PKC-ζ was effective, albeit to varying degrees, in diminishing aPKC levels and insulin-stimulated glucose transport in L6 myotubes. The fact that each of these RNAi sequences was effective suggests that the inhibition of glucose transport by the Smartset pool is due to the specific targeting of PKC-ζ, rather than to a nonspecific effect of a single component of the pool acting on a different mRNA.

**Construct C, Rescue Studies**—Analogous to findings in rescue experiments conducted in 3T3/L1 adipocytes, the expression of wild type PKC-ζ (which would not be targeted by RNAi-targeting PKC-ζ in Construct C (see above)) restored total aPKC protein levels (Fig. 8, A and B) and effectively rescued insulin-stimulated glucose transport in L6 myotubes treated with RNAi-targeting PKC-ζ (Fig. 8, A and B), while increasing PKC-ζ mRNA (Fig. 5F) and PKC-ζ protein (Fig. 8D) levels. In contrast to these rescue properties of wild type PKC-ζ in L6 myotubes
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in which PKC-ζ had been knocked down, the simple expression of wild type PKC-λ, despite increasing total aPKC levels, had little or no effect on either basal or insulin-stimulated glucose transport in L6 myotubes (Fig. 8A). In addition, adenosvirally mediated expression of wild type PKC-λ did not abrogate the ability of the RNAi in Construct C to diminish either PKC-ζ protein levels (Fig. 8B) or PKC-ζ mRNA levels (Fig. 5E) in L6 myotubes. Thus, the ability of wild type PKC-λ to restore aPKC levels and simultaneously rescue insulin-stimulated glucose transport in L6 myotubes in which endogenous PKC-ζ had been knocked down by RNAi cannot be ascribed to nonspecific stimulatory effects of wild type PKC-λ or to diminished ability of RNAi to specifically knock down PKC-ζ.

**DISCUSSION**

Presently, we were able to use RNAi in simple transfection experiments to substantially diminish mRNA and protein levels of the targeted major aPKC in L6 myotubes and 3T3/L1 adipocytes, i.e. PKC-ζ and PKC-λ, respectively. Moreover, the knockdown of these aPKCs by the respective RNAi constructs was accompanied by marked decreases in insulin-stimulated glucose transport in both cell types. Importantly, the specificity of these RNAi constructs was verified by finding that they were effective only in cells that contained the targeted aPKC; thus, RNAi-targeting PKC-ζ was effective in L6 myotubes but not in 3T3/L1 adipocytes. With respect to the issue of specificity, perhaps even more importantly, we were able to “rescue” insulin-stimulated glucose transport by using adenosvirally mediated expression of an exogenous aPKC that, unlike the major endogenous aPKC, was not targeted by the employed RNAi. This rescue of function by expression of an untargeted exogenous aPKC provided strong, if not compelling, evidence that the knockdown of the targeted aPKC, as such, was responsible for the observed inhibition of insulin-stimulated glucose transport in RNAi-treated cells.

Our findings of RNAi-mediated knockdown of PKC-λ and inhibition of insulin-stimulated glucose transport in 3T3/L1 adipocytes are similar to those reported by Ugi et al. (20). The similarity of our findings to those of Ugi et al. (20) most likely reflects the fact that we used the same RNAi construct to knock down PKC-λ in these adipocytes. On the other hand, we also used an RNAi Smartpool comparable to that used by Zhou et al. (21) for knocking down PKC-λ, but its inhibitory effects on insulin-stimulated glucose transport were different from those observed by Zhou et al. (21), although fully comparable with those obtained with the single RNAi sequence employed by Ugi et al. (20).

The reason for differences between the findings of Zhou et al. (21) versus those of both Ugi et al. (20) and those observed presently is uncertain. As the stated RNAi-mediated decreases in aPKC levels appeared to be comparable in all studies, the effectiveness of RNAi-induced knockdown of the targeted mRNA and its translated protein product does not appear to account for reported differences in alterations of insulin-stimulated glucose transport. On the other hand, there were considerable differences in methods used to introduce RNAi into adipocytes. In the experimental methods presently used and those of Ugi et al. (20), fully differentiated adipocytes were continuously maintained in their attached state, and RNAi was introduced by either microinjection or transfection. In contrast, in the method used by Zhou et al. (21), adipocytes were detached, electroporated in the presence of RNAi, and then reattached. In this latter method, it is thought that only successfully electroporated but relatively intact adipocytes are able to reattach for subsequent culturing. Presumably, this electroporation–reattachment method results in the selection of adipocytes that are functionally equivalent to those of native continuously attached adipocytes, but this is not entirely certain. In our attempts to use electroporation methods for adipocytes, we have encountered considerable cellular damage.

In addition to the question of adipocyte preparation/selection methods, other possible reasons for differences in results may be: (a) clonal differences in 3T3/L1 adipocytes, (b) different levels of actual aPKC depletion that were not apparent from immunoblot analyses, or (c) RNAi constructs may have had nonspecific effects unrelated to diminished levels of the targeted aPKC. With respect to the second possibility (i.e., b), we have recently found that a partial (approx 35–40%) loss of total aPKC in heterozygous mice in which PKC-λ has been knocked out specifically in skeletal muscle by Cre-LoxP methodology has little or no effect on insulin-stimulated glucose transport; on the other hand, in homozygous muscle-specific PKC-λ knock-out mice in which there is a greater loss (approximately 70–80%) of total aPKC, has a marked loss of insulin-stimulated glucose transport; thus, there appears to be a threshold level of aPKC knockdown that must be attained to observe a significant diminution of insulin-stimulated glucose transport. With respect to the third possibility (i.e., c), the specific efficacy of the RNAi to

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inhibit insulin-stimulated glucose transport only in cells containing the targeted endogenous aPKC, and the fact that expression of an untargeted exogenous aPKC effectively rescued insulin effects on glucose transport in cells in which the endogenous aPKC was knocked down, speaks strongly against the possibility of a nonspecific inhibitory effect of the RNAi forms used in the present studies.

To summarize, the present findings in which RNAi was used to deplete aPKC and simultaneously inhibit insulin-stimulated glucose transport are in keeping with previous studies in which adenovirally mediated expression of kinase-inactive aPKC was found to inhibit insulin effects on glucose transport in L6 myotubes and 3T3/L1 adipocytes (5, 6, 9, 10, 13). However, in present experiments, we are able to avoid the caveat of expression of inhibitory kinases, which conceivably may have untoward effects, and, instead, relied upon relatively acute aPKC depletion and repletion to diminish and rescue insulin-stimulated glucose transport in L6 myotubes and 3T3/L1 adipocytes. This novel experimental approach of combined RNAi-mediated knockdown and, perhaps even more importantly, rescue by adenovirally mediated expression, should prove to be a useful tool for studying glucose transport and certain other aPKC-dependent functions in these and other cell types that primarily express one aPKC, but nevertheless have functions that can be satisfied by either PKC-ζ or PKC-A.

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