Okadaic Acid Activates Atypical Protein Kinase C (ζ/λ) in Rat and 3T3/L1 Adipocytes

AN APPARENT REQUIREMENT FOR ACTIVATION OF GLUT4 TRANSLLOCATION AND GLUCOSE TRANSPORT*

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Okadaic acid, an inhibitor of protein phosphatases 1 and 2A, is known to provoke insulin-like effects on GLUT4 translocation and glucose transport, but the underlying mechanism is obscure. Presently, we found in both rat adipocytes and 3T3/L1 adipocytes that okadaic acid provoked partial insulin-like increases in glucose transport, which were inhibited by phosphatidylinositol (PI) 3-kinase inhibitors, wortmannin and LY294002, and inhibitors of atypical protein kinase C (PKC) isoforms, ζ and λ. Moreover, in both cell types, okadaic acid provoked increases in the activity of immunoprecipitable PKC-ζ/λ by a PI 3-kinase-dependent mechanism. In keeping with apparent PI 3-kinase dependence of stimulatory effects of okadaic acid on glucose transport and PKC-ζ/λ activity, okadaic acid provoked insulin-like increases in membrane PI 3-kinase activity in rat adipocytes; the mechanism for PI 3-kinase activation was uncertain, however, because it was not apparent in phosphotyrosine immunoprecipitates. Of further note, okadaic acid provoked partial insulin-like increases in the translocation of hemagglutinin antigen-tagged GLUT4 to the plasma membrane in transiently transfected rat adipocytes, and these stimulatory effects on hemagglutinin antigen-tagged GLUT4 translocation were inhibited by co-expression of kinase-inactive forms of PKC-ζ and PKC-λ but not by a double mutant (T308A, S473A), activation-resistant form of protein kinase B. Our findings suggest that, as with insulin, PI 3-kinase-dependent atypical PKCs, ζ and λ, are required for okadaic acid-induced increases in GLUT4 translocation and glucose transport in rat adipocytes and 3T3/L1 adipocytes.

Okadaic acid provokes insulin-like effects on the translocation of the GLUT4 glucose transporter and glucose transport by mechanisms that are presently obscure. In rat adipocytes, stimulatory effects of okadaic acid on a process that is largely dependent on glucose transport, viz. acute incorporation of labeled glucose into lipids, are inhibited by the inhibitor of phosphatidylinositol (PI) 3-kinase, wortmannin (1), and are associated with activation of protein kinase B (PKB) (2), which generally functions downstream of PI 3-kinase. These findings therefore raised the possibility that okadaic acid, like insulin, may activate PI 3-kinase and dependent processes, including PKB activation and glucose transport, in the rat adipocyte. However, effects of okadaic acid on PI 3-kinase and effects of PI 3-kinase inhibitors on okadaic acid-stimulated glucose transport in rat adipocytes have not been reported. In addition, in rat skeletal muscles (1) and human adipocytes (3), stimulatory effects of okadaic acid on glucose transport are wortmannin-insensitive and therefore appear to be largely independent of PI 3-kinase; moreover, in rat skeletal muscle (4), 3T3/L1 adipocytes (4), and human adipocytes (3), okadaic acid alone has no effect on phosphotyrosine-associated PI 3-kinase activity, and, in fact, inhibits insulin effects on this PI 3-kinase activity, apparently via enhanced serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1) (5). Accordingly, some findings suggest that okadaic acid can activate glucose transport independently of PI 3-kinase.

To add to the confusion as to whether okadaic acid activates PI 3-kinase and/or PI 3-kinase-regulated processes in rat adipocytes, there is uncertainty as to what protein kinase(s) serves as a distal activator of GLUT4 translocation and glucose transport during okadaic action. In this regard, although PKB is activated by okadaic acid in rat adipocytes and transient expression of a membrane-targeted constitutively active PKB results in the activation of GLUT4 translocation in the rat adipocyte (6), it is uncertain if PKB is required for effects of okadaic acid on GLUT4 translocation and glucose transport. Indeed, in rat adipocytes, only a relatively small fraction of insulin-stimulated GLUT4 translocation is inhibited by a kinase-inactive (KI) form of PKB (6). Also, in 3T3/L1 adipocytes, studies in which a double mutant (T308A,S473A), activation-resistant form of PKB was used as an effective dominant-negative inhibitor for insulin-sensitive PKB-mediated processes (e.g. protein synthesis) suggest that PKB is not required for insulin-stimulated GLUT4 translocation and glucose transport (7).

With respect to other potential mechanisms for activating glucose transport, PKC-ζ and PKC-λ are activated by insulin through a PI 3-kinase-dependent mechanism (8), and effects of insulin on GLUT4 translocation and glucose transport in rat adipocytes (8), 3T3/L1 adipocytes (9, 10), and L6 myotubes (11).

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1 The abbreviations used are: PI, phosphatidylinositol; PKB, protein kinase B; IRS-1, insulin receptor substrate-1; KI, kinase inactive; 2-Dog, 2-(H)deoxyglucose; PKC, protein kinase C; HA, hemagglutinin antigen; GTPγS, guanosine 5’-3-O-(thio)triphosphate; PDK-1, 3-phosphoinositide-dependent kinase-1.
appear to be dependent upon the activation of one or both of these atypical PKCs. We therefore examined the possibility that the effects of okadaic acid on glucose transport and GLUT4 translocation in rat adipocytes and 3T3/L1 adipocytes may be mediated through the activation of PKC-\(\zeta\) and/or PKC-\(\lambda\).

**EXPERIMENTAL PROCEDURES**

**Cell Preparations and Incubations**—As described (8), rat adipocytes were prepared by collagenase digestion of epididymal fat pads, equilibrated in glucose-free Krebs-Ringer phosphate medium containing 1% bovine serum albumin with or without PKC-\(\zeta\)/\(\lambda\) inhibitors (cell-permeable myristoylated PKC-\(\zeta\)/\(\lambda\) pseudosubstrate (myr-SIYRRGARRWRKL; Quality Controlled Biochemical, Hopkington, MA) or RO 31-8220 (Alexis, San Diego, CA)) or PI 3-kinase inhibitors (wortmannin (Sigma) or LY 294002 (Alexis)), and subsequently incubated, as described in the text, with or without okadaic acid or insulin.

Similarly, as described by Bandyopadhyay et al. (9), 3T3/L1 adipocytes were differentiated, equilibrated, and incubated in glucose-free Krebs-Ringer phosphate medium with the above described inhibitors and insulin or okadaic acid as described in the text.

**2-\([\text{H}]\)Deoxyglucose (2-DOG) Uptake**—Rat adipocytes were treated for 30 min with or without 10 nM insulin or 1 \(\mu\)M okadaic acid, 0.1 mM 2-DOG containing 0.1 \(\mu\)Ci [\(\text{H}\)]DOG (NEN Life Science Products) was added, and uptake of 2-[\(\text{H}\)]DOG over 1 min was measured as described (8). 3T3/L1 adipocytes were incubated in the same conditions, except that 100 nM insulin was used, and uptake was measured over 5 min (see Ref. 9).

**PKC-\(\zeta\)/\(\lambda\) Activation**—Activation of immunoprecipitable PKC-\(\zeta\)/\(\lambda\) was determined as described (8, 9). In brief, after incubation of intact cells with or without okadaic acid or insulin, cells were homogenized and cell lysates (defatted, post-nuclear homogenates in buffer containing 0.25 M sucrose, 20 mM Tris/HCl (pH 7.5), 1.2 mM EGTA, 20 mM \(\beta\)-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 \(\mu\)g/ml leupeptin, 20 \(\mu\)M aprotinin, 1 mM Na\(_3\)VO\(_4\), 1 mM Na\(_4\)P\(_2\)O\(_7\), 1 mM NaF, 0.15 M NaCl, 1% Triton X-100, and 0.5% Nonidet) were subjected to immunoprecipitation with a polyclonal antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA) that recognizes the C-terminal sequences of both PKC-\(\zeta\) and PKC-\(\lambda\) (these PKCs, except for one amino acid, have identical sequences in their C-terminal 26 amino acids). After overnight incubation at 0–4 °C, precipitates were collected on protein AG-Agarose beads, washed, and incubated for 8 min at 30 °C in 50 \(\mu\)l of buffer containing 50 mM Tris/HCl (pH 7.5), 5 mM MgCl\(_2\), 100 \(\mu\)M Na\(_3\)VO\(_4\), 100 \(\mu\)M aprotinin, 1 mM Na\(_4\)P\(_2\)O\(_7\), 100 \(\mu\)M phenylmethylsulfonyl fluoride, 3–5 \(\mu\)Ci \([\gamma\text{-}\text{32P}]\)ATP (NEN Life Science Products), 50 \(\mu\)M ATP, 4 \(\mu\)g of phosphatidylserine, and 40 \(\mu\)M 159Ser-PKC-\(\epsilon\) (amino acids 153–164)-NH\(_2\) substrate (Quality Controlled Biochemicals). After incubation, aliquots of the reaction mixtures were spotted on P81 filter paper, washed in 5% acetic acid, and counted for \(\text{32P}\).

**PI 3-Kinase Activation**—Membrane PI 3-kinase activity was measured in rat adipocytes as described (12). In brief, after treatment of intact cells with or without 500 nM insulin or 1 \(\mu\)M okadaic acid, cells were sonicated, post-nuclear defatted homogenates were centrifuged at 400,000 \(\times\) g for 45 min, and resultant membranes were assayed for PI 3-kinase activity. After assay, lipids were extracted and resolved by thin layer chromatography (12). \(\text{32P}\) radioactivity in PI-3-PO\(_4\) (which migrates just below PI-4-PO\(_4\) in this system) was quantitated in a Bio-Rad PhosphorImager. PI 3-kinase activity was also measured in phosphotyrosine immunoprecipitates (antiserum obtained from Santa Cruz Biotechnologies).

**Cell Transfections**—As described (6, 8, 13), rat adipocytes were tran-
siently co-transfected with hemagglutinin antigen (HA)-tagged GLUT4 with or without kinase-inactive (KI) forms of PKC-ζ and PKC-λ (described in Refs. 6, 8, and 13) or the activation-resistant double mutant (T308A,S473A) form of PKB. (The double mutant form of PKB was made by site-directed mutagenesis of the wild-type construct (6) using the MORPH mutagenesis kit according to the manufacturer's instructions (5 Prime — 3 Prime, Inc., Boulder, CO). Mutagenic oligonucleotides 5'-CCA CTA TGA AGT CAT TTT GCG GAA CGC CGG-3' and 5'-TTC CCC CAG TTC GGT CAT TAC TC GCC AGT GGC ACA-3' created point mutations T308A and S473A as well as silent mutations that created a new BglII site and disrupted an XmnI site. Mutations were confirmed by direct sequencing.) In brief, 0.8 ml of 50% cell suspension was electroporated in the presence of 3 μg of pCIS2 containing cDNA insert encoding HA-GLUT4, and 7 μg of pCDNA3 containing no insert (i.e. vector only) or cDNA insert encoding KI-PKC-ζ or KI-PKC-λ, or 7 or 14 μg pCIS2 containing no insert (i.e. vector only) or cDNA insert encoding T308A,S473A-PKB. After overnight incubation to allow time for expression of cDNA inserts, the cells were washed and equilibrated in glucose-free Krebs-Ringer phosphate medium, and treated for 30 min with or without 10 nM insulin or 1 μM okadaic acid. After incubation, 2 mM potassium cyanide was added to immobilize GLUT4, and cells were washed, counted, and examined for surface content of HA-GLUT4 using mouse monoclonal anti-HA primary antibody (Babco, Berkeley, CA) and 125I-labeled rabbit anti-mouse IgG second antibody (Amersham Pharmacia Biotech). Note that, in the absence of cDNA insert, the amounts of DNA used in these transfections have no effect on either basal or insulin-stimulated HA-GLUT4 translocation (8, 13); also, note that KI-PKC-ζ does not inhibit GTPγS-stimulated HA-GLUT4 translocation, which is independent of atypical PKCs (13). Also note that HA-GLUT4 expression (assessed by blotting the HA epitope) was not influenced by the presence of cDNA inserts. Thus, observed inhibitory effects of KI-PKC-ζ and KI-PKC-λ on HA-GLUT4 translocation specifically reflected changes in signaling factors used by insulin and okadaic acid, rather than the GLUT4 translocation process itself.

RESULTS

Studies of 2-DOG Uptake—In rat adipocytes, okadaic acid (1 μM) provoked increases in 2-DOG uptake that were approximately 30–50% of those provoked by maximally effective 10 nM insulin; moreover, PI 3-kinase inhibitors, wortmannin and LY294002, inhibited effects of okadaic acid, as well as insulin, on 2-DOG uptake (Fig. 1). Inhibitors of atypical PKCs, viz. RO 31–8220 and the cell-permeable myristoylated PKC-ζ/pseudosubstrate, also inhibited effects of okadaic acid and insulin on 2-DOG uptake (Fig. 1). Note that the doses of RO 31–8220 that were required to inhibit 2-DOG uptake (as stimulated by both okadaic acid and insulin) were similar to those previously found to inhibit atypical PKCs (8), but considerably greater than those required to inhibit conventional PKCs (14, 15).

Fig. 2. Effects of wortmannin, RO 31-8220 and cell-permeable myristoylated PKC-ζ/λ pseudosubstrate on okadaic and insulin-stimulated 2-DOG uptake in 3T3/L1 adipocytes. Experiments were conducted as in Fig. 1, except that 100 nM insulin was used and 2-DOG uptake was measured over 5 min. Values are mean ± S.E. of four determinations.
Cells were treated with 300 nM insulin or 1 μM okadaic acid for 15 min. After incubation, plates were scraped, cells were collected, and cell lysates were subjected to immunoprecipitation and PKC-ζ were scraped, cells were collected, and cell lysates were subjected to analysis (shown in parentheses). Values are mean ± S.E. of n determinations (shown in parentheses).

Okadaic acid provoked time-dependent increases in the activity of immunoprecipitable PKC-ζ/κ in rat adipocytes. Increases in immunoprecipitable PKC-ζ/κ enzyme activity varied between 50 and 80% at 15 min of treatment with 1 μM okadaic acid, and these increases were comparable to those observed with insulin treatment in rat adipocytes (Fig. 5). In contrast, okadaic acid had no effect on increases in membrane-associated PI 3-kinase activity, which was assayed. Values are mean ± S.E. of n determinations (shown in parentheses).

Okadaic Acid, PKC-ζ/κ, and Glucose Transport

Studies of PKC-ζ/κ Activation—Okadaic acid provoked increases in membrane-associated PI 3-kinase activity which were comparable to those observed with insulin treatment in rat adipocytes (Fig. 5). In contrast, okadaic acid had no effect on increases in membrane-associated PI 3-kinase activity, which was assayed. Values are mean ± S.E. of n determinations (shown in parentheses).

Transfection Studies—Okadaic acid provoked increases in the translocation of HA-GLUT4 to the plasma membrane that were approximately 40–60% of those induced by insulin in transiently transfected rat adipocytes (Figs. 6 and 7). Co-transfection of KI-PKC-ζ or KI-PKC-κ, or no insert (i.e. vector only). After overnight incubation to allow time for expression (see expression data in Ref. 13), cells were washed, suspended in glucose-free Krebs-Ringer phosphate medium, and treated for 30 min with 1 μM okadaic acid or 10 nM insulin. Cell surface HA-GLUT4 was then measured. See “Experimental Procedures” for other details. Values are mean ± S.E. of n determinations (shown in parentheses).

Okadaic acid provoked increases in HA-GLUT4 translocation during treatment of rat adipocytes with okadaic acid and insulin. Cells were co-transfected with pCIS2 containing cDNA encoding HA-GLUT4 and encoding KI-PKC-ζ or KI-PKC-κ, or no insert (i.e. vector only). After overnight incubation to allow time for expression (see expression data in Ref. 13), cells were washed, suspended in glucose-free Krebs-Ringer phosphate medium, and treated for 30 min with 1 μM okadaic acid or 10 nM insulin. Cell surface HA-GLUT4 was then measured. See “Experimental Procedures” for other details. Values are mean ± S.E. of n determinations (shown in parentheses).

Studies of PI 3-kinase Activation—Okadaic acid provoked increases in membrane-associated PI 3-kinase activity, which were comparable to those observed with insulin treatment in rat adipocytes (Fig. 5). In contrast, okadaic acid had no effect on increases in membrane-associated PI 3-kinase activity, which was assayed. Values are mean ± S.E. of n determinations (shown in parentheses).

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Note that PKC-ζ and PKC-λ apparently function interchangeably in supporting insulin-stimulated HA-GLUT4 translocation (see Ref. 13).

In contrast to KI-PKC-ζ, the double mutant T308A,S473A-PKB, which acts as a dominant-negative for PKB-mediated processes (7), had no appreciable effect on okadaic acid-stimulated increases in HA-GLUT4 translocation and caused only a mild inhibition (10–20%) of insulin-stimulated HA-GLUT4 translocation (Fig. 7).

DISCUSSION

We found that in both rat adipocytes and 3T3/L1 adipocytes, okadaic acid provoked increases in the activity of immunoprecipitable PKC-ζ/λ; moreover, based upon studies with wortmannin and LY294002, the activation of PKC-ζ/λ by okadaic acid appeared to be dependent upon PI 3-kinase. In keeping with this apparent dependence on PI 3-kinase, the activation of PKC-ζ/λ by okadaic acid was associated with apparent increases in membrane-associated PI 3-kinase activity in rat adipocytes. However, the mechanism whereby membrane-associated PI 3-kinase was activated by okadaic acid in rat adipocytes was unclear, because okadaic acid did not provoke increases in the activity of PI 3-kinase that was recovered in phosphotyrosine immunoprecipitates. Because the latter primarily reflects the activation of Src homology 2 domains in the p85 subunit of PI 3-kinase, the possibility remains that okadaic acid may alter the activity of the p110 catalytic subunit of PI 3-kinase independently of the p85 subunit but nevertheless inhibitable by wortmannin and LY294002.

Despite the evidence for PI 3-kinase activation during okadaic acid action, alternative explanations for PKC-ζ/λ activation should be also considered. One such alternative is that okadaic acid may have activated 3-phosphoinositide-dependent kinase-1 (PKD-1), which, in conjunction with D3-PO4 polyphosphoinositides, serves to activate both PKB (16, 17) and PKC-ζ (18, 19); in this case, basal PI 3-kinase activity may be required to support the activity of PKD-1, and this could explain the inhibition of PKC-ζ/λ activation by wortmannin and LY294002.

In keeping with the finding that okadaic acid-induced activation of PKC-ζ/λ appeared to depend upon PI 3-kinase or PKD-1, PKB, another protein kinase signaling factor that operates downstream of PI 3-kinase and PKD-1 (16, 17), has also been found to be activated by okadaic acid in rat adipocytes (2). Thus, several independent lines of evidence lend support to the postulation that okadaic acid activates or requires continued activity of PI 3-kinase and/or PKD-1. Obviously, activation of PI 3-kinase or PKD-1 may explain why effects of okadaic acid on glucose transport are inhibited by wortmannin and LY294002 in both rat adipocytes and 3T3/L1 adipocytes.

Whereas okadaic acid appeared to activate PI 3-kinase and PI 3-kinase-dependent processes, viz. PKB, PKC-ζ/λ, GLUT4 translocation, and glucose transport in rat and 3T3/L1 adipocytes, the situation in rat skeletal muscle (4) and human adipocytes (3) may be decidedly different. Indeed, the effects of okadaic acid on glucose transport in these tissues are wortmannin-insensitive, and therefore appear to be independent of PI 3-kinase. Further studies are needed to see if okadaic acid activates PKC-ζ/λ in human adipocytes and rat skeletal muscles.

Although okadaic acid activates PKB in rat adipocytes (2), and constitutively active PKB stimulates GLUT4 translocation (6) in rat adipocytes (1, 6) and 3T3/L1 (20) adipocytes, the present findings in transfection studies suggested that PKB was not required for okadaic acid-stimulated GLUT4 translocation in rat adipocytes. These findings are similar to those observed during insulin action in 3T3/L1 adipocytes (7), in which it was concluded that PKB is not required for insulin-induced activation of glucose transport. On the other hand, in rat adipocytes, a small fraction (approximately 20%) of insulin effects on HA-GLUT4 translocation is blocked by KI-PKB (6), as well as by an activation-resistant T308A,S473A-PKB double mutant,2 suggesting a partial requirement for PKB. Further studies are needed to see if there are different requirements for insulin- and okadaic acid-induced effects on GLUT4 translocation in different cell types.

In summary, okadaic acid activated PKC-ζ/λ in both rat adipocytes and 3T3/L1 adipocytes by a mechanism that appeared to be dependent upon PI 3-kinase or PKD-1. Moreover, the activation of PKC-ζ/λ was required for effects of okadaic acid on GLUT4 translocation and glucose transport.

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