Function and dysfunction of aPKC isoforms for glucose transport in insulin-sensitive and insulin-resistant states

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Farese, Robert V. Function and dysfunction of aPKC isoforms for glucose transport in insulin-sensitive and insulin-resistant states. Am J Physiol Endocrinol Metab 283: E1–E11, 2002; 10.1152/ajpendo.00045.2002.—Considerable evidence suggests that atypical protein kinase C isoforms (aPKCs), serving downstream of insulin receptor substrates and phosphatidylinositol (PI) 3-kinase, are required for insulin-stimulated glucose transport in skeletal muscle and adipocytes. More recent findings further suggest that aPKCs are activated and required for glucose transport responses while serving downstream of 1) proline-rich tyrosine kinase-2, extracellular signal-regulated kinase, and phospholipase D, as during the actions of high concentrations of carbohydrates (glucose, sorbitol) and agents that activate 5’-AMP-activated protein kinase (exercise, 5-amino-imidazole-4-carboxamide-1-β-D-ribose, dinitrophenol), and 2) Cbl-dependent PI 3-kinase, as during the action of insulin-sensitizing thiazolidinediones. It therefore seems reasonable to postulate that, regardless of the initial mechanism, aPKCs may serve as terminal molecular switches for activating glucose transport responses. This postulation is of critical importance, as it now appears that insulin-stimulated aPKC activation is compromised in various states of insulin resistance.

Maintenance of blood glucose levels within a relatively narrow physiological range is essential for normal health and well-being. This maintenance is accomplished through a finely regulated balance between glucose entry into the circulation from the gastrointestinal tract in the postprandial state and from the liver and kidney during the postabsorptive state and through clearance of glucose from the circulation, largely in skeletal muscle but also to some extent within the splanchnic circulation and, to a quantitatively lesser but nevertheless physiologically important extent, in adipose tissue. The initial rate-limiting step for glucose clearance in muscle and adipose tissues is the transport of glucose across the plasma membrane, which is effected by facilitated diffusion of glucose through a family of specific glucose transporters that are either constitutively present in the plasma membrane, in particular the GLUT1 transporter, or actively translocated to the plasma membrane in response to various stimuli, in particular the GLUT4 transporter. The most notable stimulus for transporter translocation and subsequent increases in glucose transport in skeletal muscle and adipose tissue is insulin, which also diminishes glucose production and release from the liver. Accordingly, deficiency of insulin...
in autoimmune type 1 diabetes mellitus and other disorders that primarily diminish insulin secretion from pancreatic islet β-cells leads to overt hyperglycemia, i.e., diabetes mellitus. In addition to insulin-deficient states, defective action of insulin in its target tissues leads to clinically important insulin-resistant states, including Syndrome X (or the dysmetabolic syndrome) and impaired glucose tolerance with associated obesity, which together serve not only as potential precursors to type 2 diabetes mellitus but also, in their own right, as clinically important cardiovascular risk factors. In addition to insulin, a number of agonists are capable of stimulating glucose transport in insulin-sensitive target tissues.

Because of the high prevalence of type 2 diabetes and insulin resistance syndromes in the general population, there is intense interest in identifying the signaling factors that are used by insulin and certain other stimuli to regulate glucose transporter translocation and subsequent glucose transport in muscle cells and adipocytes. Although there are undoubtedly a number of signaling factors that are required in this complex process, the present review will focus primarily on the atypical protein kinase C (aPKC) isoforms ζ, η, λ, and ι, because there is now strong and continually evolving evidence that these PKCs are not only required but may serve as actual molecular switches to turn on GLUT4 translocation/glucose transport responses during the actions of insulin and certain other relevant stimuli in skeletal muscles and adipocytes. Moreover, our interest in aPKCs has been further piqued by recent observations (see DEFECTS IN aPKC ACTIVATION IN SKELETAL MUSCLE IN TYPE 2 DIABETES MELLITUS IN HUMANS AND MONKEYS and DEFECTS IN aPKC ACTIVATION IN SKELETAL MUSCLE IN TYPE 2 DIABETES MELLITUS, OBESITY, AND OTHER INSULIN-RESISTANT STATES IN RODENTS) indicating that the activation of aPKCs by insulin in skeletal muscles is defective in type 2 diabetic humans, monkeys, and rodents, and this defect in aPKC activation seems to contribute importantly to the diminution in insulin-stimulated glucose disposal and muscle-dependent insulin resistance seen in these diabetic states. Additionally, thiazolidinediones (TZDs), which are important, clinically effective, insulin-sensitizing agents, have been found to improve this defect in aPKC activation, and this improvement seems to contribute importantly to TZD-induced increases in insulin sensitivity and glucose disposal in type 2 diabetes mellitus. Thus it seems likely that aPKCs are key elements in both the pathogenesis and treatment of type 2 diabetes mellitus and related insulin resistance syndromes.

INSULIN SIGNALING TO aPKCS

Consequent to the binding of insulin to its cell surface receptor and activation of the kinase activity of the β-subunit of the insulin receptor, a number of intracellular insulin receptor substrates (IRSs) are phosphorylated on tyrosine (Y) residues, and specific tyrosine-phosphorylated (pY) amino acid groups, in turn, activate downstream signaling cascades. One such cascade that is now generally accepted to be required for insulin-stimulated glucose transport, particularly in cells that contain GLUT4 glucose transporters, i.e., skeletal muscle cells and adipocytes, involves the activation of phosphatidylinositol (PI) 3-kinase by means of pYXXM motifs on IRS family members [and most likely other proteins, e.g., Cbl; see REQUIREMENTS FOR aPKCs DURING TZD ACTION] that interact with src homology (SH)2 domains of the p85 regulatory subunit of PI 3-kinase (see Fig. 1). The p110 catalytic subunit of PI 3-kinase is thereby activated, and this leads to increases in cellular membrane levels of PI-3,4,5-(PO4)3 (PIP3) and other D3-PO4 polyphosphoinositides, which then activate a number of downstream protein kinases, either directly or through enhanced activity or action of 3-phosphoinositide-dependent protein kinase-1 (PKD-1), which phosphorylates critical threonine residues in the activation loops of certain protein kinases. Two such PI 3-kinase/PKD-1-dependent protein kinases that have been postulated to be important for insulin-stimulated glucose transport are aPKC-ζ and -η (7, 8, 10, 27, 42) and protein kinase B (PKB or Akt) (20, 26, 45, 46). Of these two protein kinases, as already alluded to, there is mounting and strong, if not compelling, evidence that the aPKCs serve as terminal molecular switches that participate in turning on glucose transport responses not only during insulin action but also during the actions of a number of other important agonists, including TZDs (21, 22), carbohydrates [e.g., high glucose levels (5) and sorbitol (32)], and exercise (unpublished observations). As described in ACTIVATION OF aPKCS, the activation of aPKCs can be effected not only by means of PI 3-kinase/PKD-1-dependent increases in PIP3, as used by insulin and certain other growth factors in skeletal muscle cells and adipocytes but also by increases in certain other acidic phospholipids, including phospholipase D (PLD)-dependent increases in phosphatidic acid (PA) and bis-PA, as used by carbohydrates, glucose (5), and sorbitol (32), and exercise (unpublished observations).

With respect to the “typical” rather than “atypical” PKCs, it should be emphasized at the outset, that, in our experience, the conventional (α, β) and novel PKCs (ε, η, θ), which are activated largely by the neutral lipid diacylglycerol (DAG) rather than by acidic phospholipids PIP3 and PA, do not appear to be required for insulin-stimulated glucose transport (7). In fact, these conventional and novel PKCs, despite the fact that they are activated by insulin [perhaps largely by activation of de novo phospholipid synthesis independently of PI 3-kinase (28)], seem to serve primarily as negative feedback inhibitors of the insulin receptor and IRS family members and thus diminish the activation of PI 3-kinase, PKD-1, PKB, and aPKCs (see Ref. 28). These inhibitory effects of conventional and novel PKCs will be discussed in further detail.
ACTIVATION OF aPKCs

Like other PKCs, aPKCs exist in a folded state in which an autoinhibitory pseudosubstrate sequence in the NH2-terminal regulatory domain aligns with the substrate-binding site in the COOH-terminal catalytic domain and prevents the phosphorylation not only of extrinsic substrates, but also of specific threonine residues of the aPKCs themselves (i.e., auto- or transphosphorylation sites) that are needed for activation of aPKCs (9, 39, 40). The binding of acidic lipids such as PIP3 or PA to still undefined regions of the regulatory domains of the aPKCs apparently leads to molecular unfolding and increases in enzyme activity through at least three complementary mechanisms: 1) exposure of specific threonine residues (Thr410 in PKC-ζ and Thr411 in PKC-λ) in activation loops in the catalytic domains of aPKCs and subsequent phosphorylation by PDK-1, 2) enhanced autophosphorylation or transphosphorylation of other critical threonine residues within the catalytic domain of aPKCs, and 3) allosteric relief of pseudosubstrate-dependent autoinhibition (39). Thus there are mechanisms for aPKC activation that are dependent and independent of phosphorylation and, for that matter, of PDK-1.

With unfolding and activation, it is likely that aPKCs, like other PKCs, are more exposed to proteases and then converted first to short-lived, constitutively active M-type kinases that are no longer subject to pseudosubstrate-mediated autoinhibition and then to inactive moieties. Although relatively little is actually known about the degradation and turnover of aPKCs in the in vivo setting, uncontrolled proteolysis during homogenization and cell processing in vitro can spuriously increase activity in assays of immunoprecipitable aPKCs. Proteolytic clipping can also alter NH2-terminal and COOH-terminal epitopes required for immunoblotting and immunoprecipitation. These caveats must be kept in mind during studies of aPKCs.

In addition to PIP3 and related polyphosphoinositides, certain acidic phospholipids, such as PA and bis-PA, which are produced during PLD action on phosphatidylcholine (PC) and possibly other phospholipids, can activate aPKCs (29). Much less is known about this activation, except that PDK-1 does not appear to be required and PA provokes increases in autophosphorylation and a nonphosphorylation-dependent mobility shift on SDS-PAGE (29). Relative to PIP3, the Michaelis-Menten constant for PA activation is slightly higher, but PA provokes the same maximal level of activation of aPKCs as PIP3 (unpublished observations). Whereas PLD-derived PA can activate aPKCs, it remains to be determined whether PA derived from de novo synthesis or by action of DAG kinase has similar activating effects on aPKCs.

As alluded to in the foregoing, unlike conventional and novel PKCs, aPKCs are not activated by DAG. Consequently, the DAG analogs, phorbol esters, neither activate aPKCs directly nor cause their downregulation during prolonged treatment. On the other hand, phorbol esters, to variable degrees in different tissues, can activate PI 3-kinase or PLD, and this cross-talking may account for insulin-like effects of phorbol esters on aPKC activation and glucose transport.

REQUIREMENT FOR aPKC ACTIVATION DURING INSULIN-STIMULATED GLUCOSE TRANSPORT

Evidence from several independent experimental approaches strongly suggests that aPKCs are required...
for insulin-stimulated glucose transport. First, inhibitors of aPKCs, such as J) relatively high concentrations (as required for aPKC inhibition) of most, if not all, general PKC inhibitors and/or 2) the cell-permeable myristoylated aPKC pseudosubstrate peptide inhibit insulin-stimulated glucose transport in all tested cell types, including rat adipocytes (7, 22), mouse-derived 3T3-L1 adipocytes (7, 10, 43), rat-derived L6 myotubes (3, 7), rat skeletal muscle preparations (1, 7), and human preadipocyte-derived adipocytes (4). Although inhibitor studies have many caveats, note that these aPKC inhibitors do not inhibit the activation of PKB (40, 42), and, in this regard, there are no specific inhibitors of PKB presently available. Indeed, with respect to the latter issue, we have found (unpublished data) that the myosin light-chain kinase inhibitor ML-9, which has been suggested to serve as a relatively specific inhibitor of PKB, rather than of aPKCs, during insulin action, directly inhibits aPKCs in the same concentrations that inhibit insulin-stimulated glucose transport. Second, expression of kinase-inactive or activation-resistant aPKCs, by means of either plasmid or adenoviral gene transfer methods, inhibits GLUT4 translocation and glucose transport in each of the aforesaid cell types (3, 4, 7–10, 14, 27, 39, 40, 42, 43). Here again, it is important to note that the expression of kinase-inactive or activation-resistant aPKCs does not interfere with the activation of PKB. Third, we have recently found that, in mouse embryonic stem (ES) cells in which both alleles that encode the major aPKC in the mouse, i.e., PKC-λ, have been knocked out by recombination methods, insulin effects on glucose transport are severely compromised (relative to wild-type ES cells) but, remarkably, can be fully rescued by adenoviral-mediated expression of either PKC-λ or PKC-ζ (unpublished observations). However, to date, it has not been possible to examine aPKC requirements during insulin action in intact mice by standard gene knockout methods, because knockout of both PKC-λ alleles causes early embryonic lethality, and knockout of one PKC-λ allele does not measurably decrease aPKC levels in mouse muscles, possibly because of compensatory increases in the synthesis of PKC-λ through the remaining allele. Efforts to produce tissue-specific, conditional knockouts of PKC-λ are currently underway.

In addition to having found that aPKCs are required for insulin-stimulated glucose transport, the overexpression of wild-type aPKCs has either direct insulin-like or insulin-potentiating effects on glucose transport, and expression of constitutively active aPKCs produces full insulin-like effects on glucose transport during in vitro incubation of each of the aforesaid cell types (3, 4, 7–10, 14, 27, 39, 40, 42, 43). Of further note, adenoviral-mediated expression of wild-type PKC-ζ enhances insulin-stimulated glucose transport in rat skeletal muscle in vivo (16).

It appears that aPKC-ζ and -λ/ can function interchangeably to support insulin-stimulated GLUT4 translocation/glucose transport (3, 8, 14). This follows from the finding that inhibition of glucose transport caused by expression of kinase-inactive PKC-λ or kinase-inactive PKC-ζ can be rescued by coexpression of the wild-type forms of either PKC-ζ or PKC-λ (8). Similarly, the expression of either kinase-inactive aPKC inhibits insulin-stimulated glucose transport, regardless of which aPKC is present or dominant in the cell type under study (3, 14). Finally, in ES cells in which both PKC-λ alleles have been knocked out, insulin-stimulated glucose transport can be rescued by adenoviral-mediated expression of either PKC-ζ or PKC-λ (unpublished observations).

It should be noted that the mouse is somewhat unusual in that PKC-λ is the major aPKC expressed in its skeletal muscle and adipose tissues (3, 21, 22). In contrast, PKC-ζ is the major aPKC in skeletal muscles and adipose tissues of humans, monkeys, and rats (Refs. 3, 4, 21, 22, and unpublished observations). Also note that J) there is 98% amino acid homology between PKC-λ and its counterpart found in humans, PKC-ζ; 2) there is 70% amino acid homology between PKC-ζ and PKC-λ/ζ; 3) pseudosubstrate sequences are identical in aPKCs; 4) there are similar activation loop and autophosphorylation sites in all aPKCs; 5) both PKC-ζ and PKC-λ are comparably activated by PIP3 and PA; and 6) the COOH-terminus is nearly identical in all aPKCs, and most aPKC assays have used immunoprecipitates developed with anti-COOH-terminal antibodies and therefore reflect total aPKC activity. With these similarities, it is perhaps not surprising that aPKCs are activated by similar mechanisms and seem to activate the same substrates required for GLUT4 translocation/glucose transport. This does not necessarily imply, however, that aPKC-λ/ and -ζ have identical effects on all cellular processes.

The requirements for aPKCs during insulin action in adipocytes and muscle cells involve aPKCs operating downstream of PI 3-kinase and acting on factors that regulate the translocation of GLUT4 and, to a lesser extent, GLUT1 glucose transporters to the plasma membrane. However, in undifferentiated ES cells, which contain GLUT1 and GLUT8 but not GLUT4, glucose transporters, insulin [perhaps acting via the insulin-like growth factor (IGF)-I receptor] activates aPKCs and glucose transport by a mechanism largely independent of PI 3-kinase but sensitive to inhibitors of the extracellular signal-regulated kinase (ERK) pathway and PLD (unpublished observations). On the other hand, inhibitors of the ERK pathway either do not, or only modestly, inhibit insulin effects on aPKCs and glucose transport in differentiated myocytes and adipocytes. This difference may reflect that insulin primarily uses J) PI 3-kinase-dependent increases in PIP3 to activate aPKCs and glucose transport in myocytes and adipocytes and 2) the ERK pathway to activate PLD and aPKCs in ES cells. Moreover, in rat adipocytes (36) and L6 myotubes (32, 41), insulin-stimulated PLD is dependent on PI 3-kinase but is independent of ERK. As another perhaps surprising and confounding factor, PI 3-kinase, PDK-1, and aPKCs (in conjunction with Grb2/Sos/Ras/Raf/MEK1) operate upstream of ERK during insulin action not
only in rat adipocytes, as reported (33, 37), but also in 3T3-L1 adipocytes, L6 myotubes, and rat skeletal muscles (unpublished observations; also, for evidence of dependency of ERK on PI 3-kinase in L6 myotubes and rat skeletal muscles, see Refs. 15 and 47, respectively). Thus, even if ERK contributes in small part to aPKC activation during insulin action in these cells, inhibitors of PI 3-kinase would block this contribution. Finally, as two other considerations that may be pertinent to explain the activation or nonactivation of PLD and aPKCs following ERK activation, 1) factors operating upstream of Grb2 (e.g., proline-rich tyrosine kinase-2 (PYK2) in ES cells (see REQUIREMENT FOR APKCS DURING ACTIONS OF GLUCOSE, SORBITOL, AND OTHER CARBOHYDRATES) vs. Shc or IRS in adipocytes and muscle cells) may play a role in determining whether PLD and aPKCs are activated; and 2) PI 3-kinase/PDK-1/PIP3-dependent increases in aPKC activity may downregulate ERK/PLD/PA-dependent increases in aPKC activity and vice versa. Whatever the explanation(s), insulin apparently primarily utilizes PI 3-kinase-dependent increases in PIP3 to activate aPKCs in differentiatated adipocytes and muscle cells and ERK/PLD-dependent increases in PA to activate aPKCs in undifferentiated ES cells. Accordingly, it is tempting to suggest that the ERK/PLD/PA may be the more primitive mechanism, which is superseded by the PI 3-kinase/PDK-1/PIP3 mechanism as adipocytes and muscle cells differentiation during embryogenesis.

REQUIREMENT FOR APKCS DURING ACTIONS OF NONINSULIN GROWTH FACTORS

There is relatively little available information on signaling mechanisms used by growth factors other than insulin to stimulate glucose transport. In our experience, in 3T3-L1 adipocytes, like insulin, IGF-I, EGF, and fibroblast growth factor activate aPKCs and stimulate glucose transport largely by PI 3-kinase-dependent mechanisms (unpublished observations). However, we presently do not know what signaling pathway is used to activate the PI 3-kinase (i.e., IRS family members, Gab1, small or heterotrimeric G proteins, Ras, etc.) that is subsequently coupled to aPKC activation. In the case of platelet-derived growth factor, in 3T3-L1 adipocytes, both aPKCs and glucose transport are activated independently of both PI 3-kinase and ERK (unpublished observations) by a yet-to-be-defined mechanism.

It should be noted that simple activation of aPKCs does not necessarily imply that glucose transport will be concomitantly activated. In fact, we have found that aPKCs can be activated by certain growth factors in certain conditions by a poorly characterized mechanism that is independent of both PI 3-kinase and ERK and is not associated with increases in glucose transport. Thus it is likely that a certain degree of compartmentalization of aPKCs is required for their participation in the activation of glucose transporter translocation and glucose transport.

REQUIREMENTS FOR APKCS DURING EXERCISE AND AICAR ACTION

There is considerable interest in the mechanism of action of exercise or muscle contraction, which, like insulin, directly stimulates GLUT4 glucose transporter translocation and glucose transport in skeletal muscles and thereby increases total body glucose utilization.

Because overall glucose utilization is reasonably well maintained in diabetic, i.e., hyperglycemic, conditions, there has been a long-standing interest in the question of whether or not simple increases in blood glucose levels can activate glucose transporter translocation/glucose transport. Recently, we reported (6) that glucose-induced activation of the ERK pathway leads to activation of PLD, release of PA, and activation of aPKCs, which in turn is apparently required for increases in glucose transport and/or translocation of GLUT4 and GLUT1 transporters to the plasma membranes in rat adipocytes and rat skeletal muscles. This activation of ERK by relatively high concentrations of glucose, moreover, appears to be effected by interaction of glucose with cytochalasin B-inhibitable sites on the GLUT1 glucose transporter, which, apparently through specific residues in its cytoplasmic COOH terminus, interacts with and activates the Ca2+-dependent nonreceptor tyrosine kinase PYK2, which in turn activates Grb2 and the ERK pathway (5, 6). Presumably, it is this PYK2-dependent activation of the ERK pathway that glucose uses to activate PLD, aPKCs, and glucose transporter translocation (see Fig. 1).

In addition to glucose, other carbohydrates, including nonmetabolizable mono- and disaccharides and nontransported disaccharides, have been found to activate the ERK pathway in accordance with their ability to interact with glucose transporters (5). Like glucose, these carbohydrates stimulate GLUT4 translocation by a cytochalasin B-dependent mechanism that is dependent on specific residues in the GLUT1 COOH terminus. One such carbohydrate that has had considerable attention is sorbitol, which has been postulated to act “osmotically” to activate PYK2 and GLUT4 translocation/glucose transport independently of PI 3-kinase and PKB (12). In agreement, we (32) found that, like glucose, sorbitol activates not only PYK2, but also the ERK pathway, PLD, and aPKCs, and each of these activations is required for insulin effects on GLUT4 translocation and glucose transport in rat adipocytes, 3T3-L1 adipocytes, and L6 myotubes. Moreover, because these effects of sorbitol, like those of glucose, are cytochalasin B inhibitable and require specific residues in the COOH terminus of the GLUT1 glucose transporter (unpublished observations), we are intrigued by the idea that the GLUT1 glucose transporter may serve as the “osmoreceptor” for sorbitol and other carbohydrates that interact weakly with the glucose-binding site of the GLUT1 glucose transporter.
Exercise therefore represents an important therapeutic modality that may be useful for directly improving hyperglycemia in diabetes mellitus, as well as having other beneficial effects, e.g., improving caloric expenditure, plasma lipids, and insulin sensitivity.

The direct effects of exercise/muscle contraction on the glucose transport system are not entirely understood, but, differently from insulin action in skeletal muscle, PI 3-kinase and PKB are not required. Rather it is thought that there is at least partial dependence on increases in intracellular levels of 5′-AMP and subsequent activation of 5′-AMP-activated protein kinase (AMPK) (19, 31) and, possibly, dependence on Ca^{2+} (23). This involvement of AMPK has led investigators to use anoxia or mitochondrial uncoupling agents (23) to increase 5′-AMP levels or effective 5′-AMP analogs such as 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which is phosphorylated after cellular uptake, to activate AMPK and thus act as useful, albeit side (AICAR), which is phosphorylated after cellular uptake, to activate AMPK and thus act as useful, albeit moderate degrees of physical exercise rapidly increase ERK and aPKC activity in mouse and human skeletal muscles, and AICAR increases 1) PYK2 (which is Ca^{2+}-dependent), ERK, and aPKC activity in rat skeletal muscles and 2) PYK2, ERK, aPKC, and PLD activity in L6 myotubes (note that, at this point, PLD can be studied only in isolated cells in which PC is prelabeled). Moreover, glucose transport effects of AICAR can be blocked 1) in rat skeletal muscles by chemical inhibitors of the ERK pathway, aPKCs, and PLD and 2) in L6 myotubes by these same inhibitors and, more importantly, by adenoviral-mediated expression of kinase-inactive PKC-ζ and dominant-negative forms of ERK and upstream components of the ERK pathway (unpublished observations). Of further note, the mitochondrial uncoupling agent dinitrophenol, which increases 5′-AMP levels and thereby activates AMPK, has effects on these signaling factors and the glucose transport system in L6 myotubes that are essentially the same as those of AICAR. Taken together, our findings suggest that exercise and anoxia, at least partly, use AMPK and the ERK/PLD/aPKC pathway to activate GLUT4 translocation/glucose transport in skeletal muscle cells. Further studies are needed to test this hypothesis and determine how exercise, through AMPK and possibly another parallel-acting factor, activates the ERK/PLD/aPKC pathway.

REQUIREMENTS FOR aPKCS DURING TZD ACTION

TZDs, as clinically important insulin-sensitizers, have been found to increase levels of GLUT4 and/or GLUT1 glucose transporters in cultured cells and certain rodent models of obesity or diabetes, and it has generally been thought that these increases in glucose transporter levels largely accounted for effects of TZD-induced increases in glucose transport and glucose utilization in human type 2 diabetic subjects. However, it is not entirely clear that comparable increases in glucose transporters occur in muscles or adipocytes of TZD-treated human diabetic subjects. Furthermore, it does not necessarily follow that, of themselves, such increases in transporters would be sufficient to explain increases in glucose transport in adipocytes and skeletal muscle cells and subsequent increases in whole body glucose disposal rates, particularly in diabetic subjects in whom signaling defects are present. Indeed, in our studies of vastus lateralis muscles of TZD-treated type 2 diabetic human subjects (unpublished observations) and type 2 diabetic Goto Kakizaki (GK) rats (21, 22), we have not observed increases in glucose transporter levels following TZD treatment for periods of up to 1 mo. On the other hand, we have observed TZD-induced increases in insulin-stimulated aPKC activation in these same human (unpublished observations) and rat (22) skeletal muscles and also in vastus lateralis muscles of type 2 diabetic monkeys (unpublished observations). Moreover, it appeared that these TZD-induced increases in muscle aPKC activation contributed importantly to TZD-induced increases in glucose disposal rates observed during euglycemic-hyperinsulinemic clamp studies in diabetic humans and monkeys. Accordingly, TZDs may sensitize skeletal muscles to insulin by enhancing signaling mechanisms that lead to aPKC activation.

To our frustration, but perhaps not surprisingly, the signaling mechanisms underlying increases in muscle aPKC activation during TZD treatment in vivo have proved to be partly different in several species that we have examined. Thus, in vastus lateralis muscles of type 2 diabetic humans, TZDs provoked increases in IRS-1-dependent PI 3-kinase that could reasonably (but not necessarily solely) account for increases in aPKC activation (unpublished observations). In this case, TZDs seem to have caused a generalized improvement in insulin receptor signaling to IRS-1 and PI 3-kinase, but it remains unclear whether this improvement derives from direct effects of TZDs in muscle or by means of alterations in circulating factors that are released from adipose or other tissues, e.g., decreases in free fatty acids, tissue necrosis factor-α (TNF-α) and resistin or increase in adiponectin (Agrp30; see Fig. 1). In contrast, in vastus lateralis muscles of TZD-treated diabetic GK rats, there were no apparent changes in IRS-1-dependent PI 3-kinase activity (22), and we are therefore examining other possibilities, including increases in insulin-stimulated Chl-dependent PI 3-kinase (see below). In addition to these species differences, we have found that TZDs use partly different mechanisms to activate aPKCs in different cell types within species. Thus findings in one cell type cannot be simply extrapolated to other cell types.

Despite the aforementioned caveats, available cell lines have been useful for uncovering potential mechanisms for TZD-induced increases in aPKC activation. For example, during TZD treatment of cultured mouse-derived 3T3-L1 adipocytes (43), we have observed increases in levels of IRS-1 and IRS-2 and increases in activities of insulin-stimulated, but not basal, IRS-1/2-
dependent PI 3-kinase. Even more interestingly, we found that TZD provoked increases in a PI 3-kinase activity that were associated with and dependent on Cbl [which, like IRSs can be phosphorylated to yield pYXXM motifs (35)] both basally, i.e., without, and, more so, in response to subsequent insulin stimulation. Not unexpectedly, these TZD-induced increases in both IRS-1/2-dependent and Cbl-dependent PI 3-kinase activities were accompanied by increases in activities of aPKCs. Moreover, TZD-induced increases in Cbl-dependent PI 3-kinase and basal aPKC activities seemed to contribute importantly to the sizeable increases in basal GLUT4 and GLUT1 translocation and basal glucose transport that are seen in these cells after simple TZD treatment. Similarly, the increases in Cbl-dependent PI 3-kinase and insulin-stimulated IRS-1/2-dependent PI 3-kinase activities, along with subsequent increases in aPKC activities, provided a reasonable explanation for increases in insulin-stimulated glucose transport following TZD treatment. Of further note, the TZD-induced increases in Cbl-dependent PI 3-kinase activity were associated with, and probably due to, increases in tyrosine phosphorylation/activation of Cbl. Remarkably, unlike insulin-stimulated increases in IRS-1/2-dependent PI 3-kinase activity, TZD-induced increases in Cbl-dependent PI 3-kinase activity and subsequent PDK-1-dependent aPKC activity/phosphorylation were not accompanied by increases in PKB activity or phosphorylation, suggesting that aPKCs and PKB are not necessarily activated in unison or in tandem during at least certain forms of PI 3-kinase activation. Of further importance, this TZD-induced activation of Cbl-dependent PI-3-kinase and subsequent activation of aPKCs and stimulation of glucose transport were found to occur not only in 3T3-L1 adipocytes but also in cultured human adipocytes prepared from preadipocytes harvested during elective liposuction (unpublished observations). The activation of Cbl and Cbl-dependent PI 3-kinase and aPKCs in cultured 3T3-L1 adipocytes is most likely due to TZD-induced increases in the level of Cbl-associated protein [CAP; whose synthesis is increased via peroxisome proliferator-activated receptor-γ (PPARγ) activation], which facilitates tyrosine phosphorylation of Cbl by various tyrosine kinases, including the insulin receptor and nonreceptor tyrosine kinases of the Src family. This phosphorylation yields pYXXM motifs that interact with SH2 domains of the p85 subunit of PI 3-kinase, as has been found in other instances (35) and as has been confirmed to occur during rosiglitazone action in 3T3-L1 adipocytes (unpublished observations). Whether this activation of Cbl-dependent PI 3-kinase and aPKCs is restricted to cultured mouse and human adipocytes or whether it occurs in adipocytes and/or skeletal muscles during in vivo TZD treatment of humans or other species is under investigation in our laboratory. Interestingly, our preliminary studies suggest that there are increases in Cbl-dependent PI 3-kinase activity in human skeletal muscles following rosiglitazone and insulin treatment in vivo, but further work is needed to determine the importance of these increases in Cbl-dependent PI 3-kinase, vis-à-vis increases in IRS-1-dependent PI 3-kinase described above, for activating aPKCs and glucose transport in TZD-treated skeletal muscle.

To summarize, although there are many gaps in our present knowledge of how TZDs operate, as depicted in Fig. 1, it appears that these agents can provoke increases in basal and insulin-stimulated glucose transport by a variety of mechanisms, including 1) generalized increases in insulin receptor signaling, perhaps via alterations in factors released from adipocytes (fatty acids, TNF-α, resistin, and/or adiponectin) that in turn may act on various cell types, including adipocytes and muscle cells; 2) increases in IRS-1 and IRS-2 levels and IRS-1/2-dependent PI 3-kinase; and 3) increases in Cbl-dependent PI 3-kinase in certain cell types. From a clinical viewpoint, it is fortunate that each of these mechanisms fortuitously converges on aPKCs and thereby activates GLUT4/1 translocation and glucose transport.

**DEFECTS IN APKC ACTIVATION IN SKELETAL MUSCLE IN TYPE 2 DIABETES MELLITUS IN HUMANS AND MONKEYS**

In addition to nonobese type 2 diabetic GK rats (21, 22), we (unpublished observations) and others (24) have observed marked decreases in insulin-induced activation of aPKCs in biopsies of vastus lateralis skeletal muscles of type 2 diabetic humans obtained during hyperinsulinemic-euglycemic clamp studies. A similarly marked defect in aPKC activation was also seen during comparable clamp/muscle biopsy studies of obese type 2 diabetic monkeys (unpublished observations). These defects in skeletal muscle aPKC activation in diabetic humans and monkeys were accompanied by, and therefore seemed to be at least partly due to, decreases in IRS-1-dependent PI 3-kinase activation. Moreover, similar to findings in a previous study in diabetic humans (25), PKB activation was only mildly, if at all, decreased in these muscles of diabetic humans and monkeys, suggesting that PKB activation was maintained either by activation of PI 3-kinase activators other than IRS-1 or by better coupling of IRS-1-dependent PI 3-kinase to PKB. Whatever the reason for this dichotomy in aPKC and PKB activation, the defect in aPKC activation, rather than PKB activation, seemed to account for the associated decreases in insulin-stimulated glucose disposal observed during the hyperinsulinemic-euglycemic clamps in these diabetic humans and monkeys.

Of further interest, the defect in insulin-stimulated skeletal muscle aPKC activation in human diabetic subjects was attended by substantial (40%) decreases in muscle PKC-ζ levels. However, note that these decreases in aPKC levels could not account for the decreases in insulin-stimulated aPKC activity that we observed in these diabetic muscles. Indeed, insulin-stimulated aPKC activity was diminished by 80% in assays of diabetic muscle that contained amounts of...
immunoprecipitable aPKC equal to those of nondiabetic muscle. Thus it may be surmised that the defect in aPKC activation in skeletal muscles of human diabetic subjects may be further compounded by decreases in absolute PKC-ζ levels.

Oddly enough, in contrast to the situation in human diabetics, the defect in aPKC activation (as determined in assays of equal amounts of aPKCs) in skeletal muscles of obese diabetic monkeys was attended by moderate (35–40%) increases in PKC-ζ levels; accordingly, the defect in aPKC activation in monkeys is clearly not due to lower levels of aPKCs. In addition to defective activation of IRS-1-dependent PI 3-kinase as a cause for diminished activation of aPKCs in muscles of diabetic monkeys, there was a defect in aPKC activation in response to direct addition of PIP₃, the PI 3-kinase lipid product, to assays of immunoprecipitated aPKC in vitro. Similarly, from preliminary studies, we suspect that PIP₃-dependent activation of aPKCs is comparably compromised in skeletal muscles of human diabetics. The reason for this poor activation of aPKCs by PIP₃ in diabetic muscle is uncertain but may be due to diminished phosphorylation of the Thr⁴¹⁰/⁴¹¹ activation loop site by PDK-1 and/or other alterations that limit autophosphorylation or intrinsic activity of aPKCs.

Surprisingly, defects in aPKC activation and alterations in aPKC levels were seen at very early stages of development of the diabetic syndromes in both humans and monkeys. In humans, for example, defects in aPKC activation and PKC-ζ levels were nearly as severe in subjects who had impaired glucose tolerance (i.e., fasting blood glucose levels between 110 and 125 mg/dl) as those who had overt diabetes (fasting blood glucose levels >125 mg/dl), with only a modest relationship to the level of glycemia, i.e., hemoglobin A₁C levels. Furthermore, sizeable (albeit lesser compared with overt diabetics) defects in aPKC activation were apparent in obese insulin-resistant humans and monkeys who had essentially “normal” blood glucose levels, i.e., fasting blood glucose levels <110 mg/dl. Thus defects in aPKC activation may, in fact, precede the appearance of defects in glucose homeostasis in the pathogenesis of type 2 diabetes in both humans and monkeys.

The fact that impaired activation of IRS-1-dependent PI 3-kinase and aPKCs was observed in very early stages of development of obesity and type 2 diabetes, when blood glucose levels were only minimally, or not at all, elevated, suggested that factors other than hyperglycemia were responsible for impaired signaling through these factors. Obviously, a major challenge will be to determine the cause for the early defects in activation of these signaling factors. In this regard, in monkeys, because of very high prevalence rates of development of obesity and type 2 diabetes (>60%), and because in some instances longitudinal studies were possible, it seemed clear that these defects in IRS-1-dependent PI 3-kinase/aPKC signaling in obese prediabetic and diabetic monkeys were preceded by an earlier state in which the activation of these signaling factors was apparently normal, suggesting that this signaling defect was “acquired.” In keeping with the latter, calorie restriction and avoidance of obesity in these monkeys prevented the appearance of defects in IRS-1-dependent PI 3-kinase and aPKC activation, indicating the essential role of obesity in the development of these signaling defects. Such a clear-cut relationship between obesity and development of diabetes and associated defects in IRS-1/PI 3-kinase/aPKC signaling was, however, not evident in our studies of human subjects, and it remains for future studies to determine 1) the time of onset of abnormalities and 2) the effectiveness of caloric restriction in preventing or reversing abnormalities in aPKC activation and levels in obese diabetic humans.

DEFECTS IN aPKC ACTIVATION IN SKELETAL MUSCLE IN TYPE 2 DIABETES MELLITUS, OBESITY, AND OTHER INSULIN-RESISTANT STATES IN RODENTS

As alluded to, we have reported (21, 22) that there are defects in insulin-induced activation of IRS-1-dependent PI 3-kinase and aPKCs in adipocytes and skeletal muscles of type 2 diabetic GK rats. Interestingly, these GK diabetic rats are not obese, and the primary defect in these rats has been suggested to involve a defect in the coupling of glycolysis to insulin secretion in the pancreatic islet β-cells. Unlike the situation in obese type 2 diabetic humans and monkeys, the defect in insulin-induced activation of aPKCs in skeletal muscles of the GK diabetic rat was not attended by alterations in aPKCs levels and, even more importantly, appeared to be attributable largely to hyperglycemia, since this defect in a PKC activation could be largely reversed in association with improvement in blood glucose levels resulting from either 10 days of insulin therapy or 60 h of fasting (22). Increases in free fatty acids were also observed in these diabetic GK rats and may have contributed to decreases in insulin-induced activation of IRS-1-dependent PI 3-kinase and aPKCs, but the observed improvements in aPKC activation ensuing from the aforesaid treatments could not be explained by alterations in free fatty acid levels (22). Whatever the explanation for treatment-related improvements in aPKC activation, it seems likely that the defects in signaling to IRS-1-dependent PI 3-kinase and aPKCs seen in untreated GK diabetic rats may have been secondary to increases in plasma glucose and possibly free fatty acid levels caused by a partial defect in insulin secretion. In any event, it seems likely that the defect in skeletal muscle aPKC activation contributed importantly to the defect in peripheral glucose disposal observed in previous euglycemic-hyperinsulinemic clamp studies in diabetic GK rats (17). A similar situation of secondary reversible defects in aPKC activation and insulin resistance may exist in maturity onset forms of type 2 diabetes mellitus that are seen in younger human subjects (i.e., MODY) in which a variety of inherited defects within the β-cell lead to variable decreases in insulin secretion.
In addition to defects in diabetic GK rats, we (unpublished observations) have also observed defects in the activation of aPKCs in ob/ob diabetic mice and in streptozotocin-induced diabetes in rats. In both situations, it seems clear that these defects in aPKC activation are acquired. Thus, in ob/ob mice, defective activation of IRS-1-dependent PI 3-kinase, aPKCs, and PKB is most likely a result of leptin deficiency-dependent obesity and, perhaps, increases in plasma levels of free fatty acids and/or alterations in the release of other factors from adipocytes, including, leptin, TNF-α, resistin, and adiponectin. Also, in streptozotocin-induced diabetes, the defect in aPKC activation is probably due to increases in plasma levels of glucose and/or free fatty acids, but the mechanism for inhibition of aPKC activation remains uncertain, as IRS-1-dependent PI 3-kinase activation is not impaired.

Finally, we have found (unpublished observations) that the activation of aPKCs by insulin is compromised in both adipocytes and skeletal muscles of mice and rats that are placed on high-fat diets, even in the absence of increases in body weight or alteration of plasma glucose levels. In some cases, the decreases in aPKC activation were associated with, and probably due to, diminished activation of IRS-1-dependent PI 3-kinase. However, in other cases, IRS-1-dependent PI 3-kinase activation was not compromised, and the reasons for defects in aPKC activation are presently enigmatic.

CONVENTIONAL AND NOVEL aPKCS AS INHIBITORS OF INSULIN-INDUCED ACTIVATION OF aPKCS

As alluded to previously, unlike the aPKCs, which are activated by insulin in skeletal muscle and adipocytes largely through activation of IRS-1-dependent PI 3-kinase and generation of PIP3, the conventional (α and β) and, most likely, novel PKCs (δ, ε, and θ) as well (see Refs. 1, 2, 28, 36, 41, and 48) appear to be activated by insulin to a large extent by increases in DAG that are generated through the action of glycerol-3-phosphate acyltransferase and subsequent de novo phospholipid synthesis independently of PI 3-kinase (14, 27). Similarly, increases in serum glucose and free fatty acid levels, as occurring in states of diabetes and obesity, provide substrate and thereby, i.e., via the de novo pathway, increase tissue levels of DAG and activate conventional and novel PKCs (see Ref. 2). Increases in DAG-dependent PKCs, moreover, are known to inhibit insulin receptor function (11, 44) and subsequent activation of IRS-1-dependent PI 3-kinase (13). Further evidence for downregulating effects of conventional PKCs derives from studies of mice in which these PKCs have been knocked out by homologous recombination. Accordingly, knock-out of either PKC-α (28) or PKC-β (38) leads to decreases in insulin-stimulated glucose transport in both adipocytes and skeletal muscles. Moreover, in the case of knock-out of the PKC-α gene, there are increases in insulin-stimulated activities of IRS-1-dependent PI 3-kinase, PKB, and aPKCs (28), and it appears that this relatively abundant, conventional DAG-dependent PKC serves as a tonic physiological inhibitor of aPKC activation and subsequent glucose transport in muscle and adipose tissues.

Of further note, considerable evidence suggests that the novel, DAG-sensitive PKC-δ, which is abundant in skeletal muscle but is not present in other insulin-sensitive cell types such as adipocytes and hepatocytes, plays an important role in lipid-induced insulin resistance (18). Similarly, the novel, DAG-sensitive PKC-ε, which is more ubiquitous than PKC-δ, has been suggested to play an important role in lipid-induced insulin resistance (34), but information on this novel PKC is more limited. Finally, it may be noted that, like other PKCs, aPKCs may themselves serve as physiological feedback inhibitors of insulin effects on IRS-1, PI 3-kinase, PDK-1, PKB, and aPKCs (30).

CONCLUDING REMARKS

Perhaps the most surprising outcome of recent studies of aPKCs is that these PKCs have been found to be activated and, moreover, required for stimulation of glucose transport by multiple agents that initially signal through diverse pathways, including 1) insulin and other growth factors, which signal via IRS/PI 3-kinase; 2) glucose, sorbitol, and other carbohydrates, which signal via PYK2, the ERK pathway, and PLD; 3) TZDs, which signal via Cbl/PI 3-kinase as well as via IRS/PI 3-kinase; and 4) exercise, anoxia, and their chemical surrogates AICAR and dinitrophenol, which signal via AMPK to PYK2, the ERK pathway, and PLD. Collectively, these findings suggest that aPKCs serve as common terminal switches that have the capacity to activate the molecular machinery that leads to glucose transporter translocation and glucose transport. In keeping with this concept are recent findings indicating that, in conjunction with alterations in glucose transport and whole body glucose disposal, the aPKC-dependent switch is turned “off” in type 2 diabetes mellitus and, fortunately, can be turned “on” with exercise and insulin-sensitizing agents. Accordingly, aPKC activation may prove to be an important therapeutic modality for treating skeletal muscle insulin resistance.

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