MINIREVIEW

Insulin-Sensitive Phospholipid Signaling Systems and Glucose Transport: An Update[1] (44030)

ROBERT V. FARESE[2]

J. A. Haley Veterans' Hospital Research Service and Departments of Internal Medicine and Biochemistry/Molecular Biology, University of South Florida, Tampa, Florida 33612

During the past few years, remarkable progress has been made in elucidating the signaling systems that are used by insulin to regulate glucose metabolism and other metabolic functions. Most notably, tyrosine kinase functions of the insulin receptor have been more clearly linked to the activation of a network of interacting proteins, which in turn activate a number of downstream signaling systems. This review will focus largely upon a group of downstream, insulin-sensitive, phospholipid signaling systems that seem to play important roles in regulating glucose homeostasis. We will first review how these phospholipid signaling systems interdigitate both with each other, and with other nonphospholipid signaling systems. We will then examine how these phospholipid signaling systems may impact glucose transport, presumably a rate-limiting step in glucose metabolism in muscle, fat, and a number of other insulin target tissues.

Overview of Insulin-Sensitive Signaling Pathways

Insulin uses multiple cross-talking and, in some cases, seemingly redundant signaling systems (Fig. 1) to regulate many metabolic processes. Initially, insulin binds to the α subunit of the insulin receptor (IR) and activates an intracellular tyrosine kinase contained within the cytoplasmic domain of the IR β subunit, leading to tyrosine phosphorylation of the IR itself and a number of extra-receptor proteins, including IRS-1, Shc, pp60, p120, etc. Interactions ensue between specific phosphotyrosine-containing peptide sequences of these proteins and SH2 or other (e.g., newly recognized PID and PTB) domains in a second set of proteins (e.g., phosphatidylinositol 3-kinase [PI3K], GRB2, Shc, Syp, Nck, etc.). These interactions, in turn, trigger the direct activation of some of these proteins as kinases (PI3K) or phosphatases (Syp), as well as additional protein/protein interactions, for example, between SH3 domains of proteins, such as GRB2, and proline-rich or other amino acid sequences in downstream proteins, such as SOS. SOS, for instance, in turn increases GTP loading of ras, which successively activates a series of protein kinases (viz., raf-1, MEK, MAPK, and MAPKAP; this pathway may also be activated through other mechanisms).

In addition to the signaling pathways that utilize phosphotyrosine to activate SH2 and other newly recognized domains in downstream proteins, there appear to be other more poorly understood mechanisms whereby the insulin receptor activates other proteins, e.g., Gi or another pertussis toxin-sensitive protein that appears to be involved in the activation of a phospholipase C [PLC] and/or phospholipase D [PLD] that hydrolyzes glycosyl-phosphatidylinositol [GPI].

As a result of the above-described reactions, a number of signaling systems are activated by insulin, including, but not limited to, three systems that are particularly relevant to the present discussion: (i) the GRB2/SOS/ras/raf1/MEK/MAP kinase cascade; (ii)

[1] This manuscript is an update of a previously published minireview entitled, "Lipid-Derived Mediators in Insulin Action," Proc Soc Exp Biol Med **195**:312–324, 1990.

[2] To whom requests for reprints should be addressed at J. A. Haley Veterans' Hospital (VAR 151), 13000 Bruce Downs Boulevard, Tampa, FL 33612.

This material is based upon work supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, and National Institutes of Health Grant DK38079.

0037-9727/96/2131-0001$10.50/0

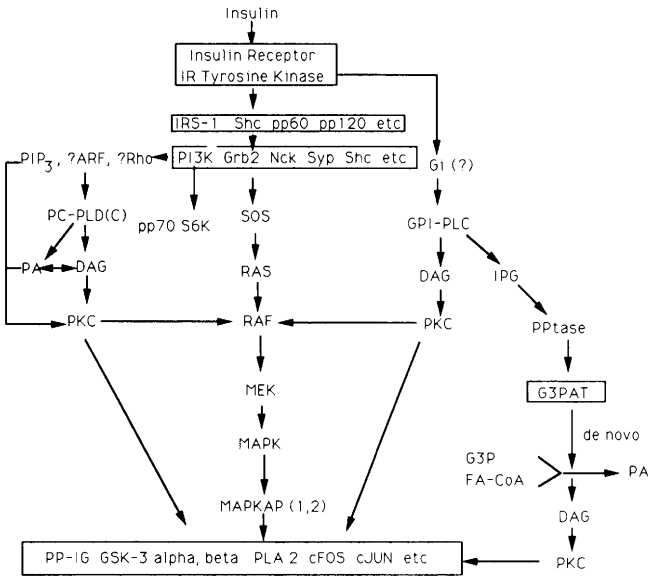

Figure 1. Insulin-sensitive signaling pathways. See text for abbreviations.

PI3K activation; and (iii) glycosyl-PI hydrolysis (Fig. 1). PI3K in turn activates pp70 S6 kinase, and, as suggested by more recent studies, also activates a phospholipase D (PLD) that hydrolyzes phosphatidylcholine (PC), causing increases in phosphatidic acid (PA) and diacylglycerol (DAG), and activation of protein kinase C (PKC). Glycosyl-PI hydrolysis also leads to DAG generation, along with the release of headgroups which appear to serve as "mediators" that, among other things, activate *de novo* PA synthesis through a protein phosphatase (PPtase) that activates glycerol-3-PO_4 acyltransferase (G3PAT). Increases in *de novo* PA synthesis also lead to increases in DAG and PKC activation, as does DAG derived from glycosyl-PI hydrolysis. These signaling systems activate other downstream protein kinases and phosphatases, and thus alter the phosphorylation state and activities of many proteins. In this review, we will focus upon several insulin-sensitive phospholipid signaling systems (viz., PI3K activation, PC hydrolysis, glycosyl-PI hydrolysis, and *de novo* PA synthesis), particularly as they relate to (i) the generation of lipid signaling substances (i.e., PA, DAG, PI-3-PO_4, and other D-3 phosphate derivatives of PI); (ii) the activation of protein kinase C (PKC); and (iii) the stimulation of glucose transport.

Insulin-Sensitive Phospholipid Signaling Pathways: General Aspects

Insulin provokes rapid changes in phospholipid metabolism in all target tissues examined, including BC3H-1 myocytes, L6 myotubes, rat skeletal muscle, rat adipocytes, rat hepatocytes, 3T3/L1 adipocytes, HIRcB rat fibroblasts, C6 glioma cells, and Xenopus oocytes. In most cell types, all or many of the above-mentioned effects (viz., PI3K activation, PC hydrolysis, glycosyl-PI hydrolysis, and *de novo* PA synthesis) have been documented to be activated by insulin, along with other changes in phospholipid metabolism (e.g., increased synthesis of PI and glycosyl-PI, as well as D-3 phosphate derivatives of PI, PIP and PIP_2). As alluded to above, signaling substances that are generated through the activation of these phospholipid pathways include: (i) inositol-phospho-glycan (IPG) mediators released from the headgroups of glycosyl-PI; (ii) DAG derived from the hydrolysis of both PC and glycosyl-PI, and from *de novo* PA synthesis; (iii) PA derived from PC-PLD activation and from *de novo* PA synthesis; and (iv) D-3 phosphate derivatives of PI PI-4′-PO_4 and PI-4′,5′-$(PO_4)_2$. Although DAG derived from each of the three sources cited above can activate PKC, there is undoubtedly some compartmentation—that is, PKC activation in the plasma membrane is largely due to PC and glycosyl-PI hydrolysis, and PKC activation in the endoplasmic reticulum is largely due to *de novo* PA synthesis (1, 2). In addition, it should be noted that PC hydrolysis accounts for most of the initial burst of DAG/PKC signaling that occurs in the plasma membrane in response to insulin (3, 4).

Insulin-sensitive phospholipid pathways appear to be separable but nevertheless integrated. A pertussis toxin-sensitive protein, most likely G_i, apparently couples the insulin receptor to a PLC or PLD that specifically hydrolyzes glycosyl-PI. In contrast, PC hydrolysis is not sensitive to pertussis toxin (3, 4) but, as discussed more fully below, seems to be activated by PI3K, perhaps through increases in PI-3-PO_4, PI-3,4-$(PO_4)_2$, and PI-3,4,5-$(PO_4)_3$, or by activation of small G proteins, such as rho, rac, and ARF. Like glycosyl-PI hydrolysis, the effect of insulin on *de novo* PA synthesis is pertussis toxin sensitive (3, 5) and is due to rapid activation of G3PAT (5), apparently through IPG headgroup mediators that are released from glycosyl-PI by a specific PLC or PLD (5, 6). Accordingly, the effect of insulin on G3PAT is blocked not only in intact cells by pertussis toxin (3, 5) but also in cell-free systems by antibodies that inhibit either (i) $G_{i\alpha}$ (5), (ii) glycosyl-PI–specific phospholipase (5), or (iii) IPG mediators (6). To reiterate, insulin-induced activation of the *de novo* PA synthesis pathway appears to be a secondary consequence of glycosyl-PI hydrolysis.

In addition to G3PAT (6), IPG mediators also appear to activate glycogen synthase and pyruvate dehydrogenase, and probably other enzymes, most likely through the activation of specific protein phosphatases (7, 8). Glycogen synthase and pyruvate dehydrogenase may also be activated by insulin through other mechanisms, e.g., MAP kinase, MAPKAP kinase, and/or other protein kinases, may activate a glycogen-associated phosphatase (PP-IG), and/or may inhibit

glycogen synthase kinase (GSK) α and β, ultimately resulting in glycogen synthase activation (9, 9a). In addition, there may be other, presently less defined, mechanisms for glycogen synthase activation by insulin.

As stated above, pertussis toxin does not inhibit PC hydrolysis but nevertheless inhibits the resynthesis of PC through the *de novo* pathway during insulin action (3–5). Since *de novo* PA synthesis is a consequence of glycosyl-PI hydrolysis, it may be surmised that the rapid replenishment of PC, from PA and DAG, during insulin action requires the concomitant activation of glycosyl-PI hydrolysis and, subsequently, *de novo* PA synthesis. Similarly, both PC hydrolysis and *de novo* PA synthesis provide substrate (viz., PA and DAG) to replenish both glycosyl-PI and PI (10), which are initially decreased during insulin action. The importance of PC hydrolysis for PI synthesis is further underscored by our recent finding (see below) that wortmannin, a PI3K inhibitor, blocks both PC hydrolysis and subsequent PI resynthesis during insulin action; on the other hand, wortmannin does not block insulin effects on glycosyl-PI hydrolysis and *de novo* PA synthesis.

To summarize, insulin-sensitive phospholipid pathways are integrated to provide for (i) rapid hydrolysis and resynthesis of both glycosyl-PI and PC, and (ii) the generation of phospholipid-derived signaling substances (viz., IPG mediators, PA, DAG, D-3 phosphate derivatives of PI, and PKC).

PC Hydrolysis

Insulin-induced decreases in PC levels in rat adipocytes (3, 11), BC3H-1 myocytes (3, 4), and rat hepatocytes (12–14) are surprisingly large (viz., 5%–10% of total membrane PC). The initial decreases in PC are maximal within seconds and are generally very short-lived, apparently because of the rapidity of *de novo* PA/DAG/PC synthesis (3, 15, 16). However, in pertussis toxin-treated BC3H-1 myocytes (3, 4) and in diabetic GK rat adipocytes (that have a genetic defect in glycosyl-PI hydrolysis and secondary *de novo* PA synthesis [6]), the decreases in PC are more exaggerated and prolonged.

In addition to rat adipocytes (3, 11), BC3H-1 myocytes (3, 4), and rat hepatocytes (12–14), insulin-induced increases in PC hydrolysis have been observed in C6 glioma cells (17), Xenopus oocytes (18), and rat skeletal muscle L6 cells (19). Moreover, in rat hepatocytes (14, 15), C6 glioma cells (17), BC3H-1 myocytes (4), rat adipocytes (20), and L6 cells (19), PC-PLD activation (as evidenced by choline release and/or phosphatidylethanol formation in cells incubated in the presence of ethanol) has been documented to occur very rapidly in response to insulin. Whether or not a PC-PLC is also activated by insulin is presently less certain.

As shown in other systems, PLC- and PLD-dependent PC hydrolysis can occur secondary to prior PKC activation and/or increases in Ca^{++}, but these mechanisms do not account for the initial effects of insulin on PC hydrolysis (3, 13). On the other hand, small G proteins such as rho (21) and ARF (22) have been found to play a role in the activation of PLD-dependent PC hydrolysis in other systems, and we have recently found (unpublished) that insulin rapidly translocates Rho and ARF from the cytosol to the membrane fraction in rat adipocytes and soleus muscles. It is therefore likely that Rho and ARF are important in insulin-induced activation of PLD. Moreover, since the PI3K inhibitor, wortmannin, blocks insulin-induced PC-PLD activation (22), it is possible that PI3K may act through rho/ARF to activate PC-PLD and DAG/PKC signaling in the plasma membrane. Alternatively, PC-PLD activation may occur secondarily to PI3K-mediated increases in D-3 phosphate derivatives of PI PIP and PIP_2 (see below).

PI3K Activation

As stated above, insulin increases the synthesis of PI, glycosyl-PI, and polyphosphoinositides PIP and PIP_2 (10, 23–25). Initially, it was assumed that the latter substances were simply 4′-PO_4 and 4′,5′-$(PO_4)_2$ derivatives of PI and that increases in their levels largely reflected increases in net PI synthesis resulting from PC hydrolysis and increases in *de novo* PA synthesis. Subsequently, it was found there were increases in D-3 phosphate derivatives of PI (viz., PI-3′-PO_4, PI-3′,4′-$[PO_4]_2$ and PI-3′,4′,5′-$[PO_4]_3$ [26]), and this was ultimately found to be due to the activation of the catalytic 110-kDa subunit of PI3K, apparently through interactions of the SH2 domains of the regulatory p85 subunit of PI3K and IRS1 and/or other phosphotyrosine-containing proteins. These D-3, and probably D-4 as well, phosphate derivatives of PI are of considerable interest, as they may function in several ways: (i) by directly activating certain enzymes such as PKC-ζ (27), other PKCs (28), and PC-PLD (29); and (ii) by perturbing membrane curvature and serving as budding and/or docking factors to regulate the trafficking of organelles such as GLUT 4 glucose transporter vesicles (30). In addition to increasing the synthesis of D-3 phosphate derivatives of PI, activated PI3K may interact with other proteins through SH3 or other domains, and PI3K may also serve as a serine/threonine protein kinase. With respect to interacting with other proteins, we have recently found (unpublished) that PI3K co-immunoprecipitates with a small G protein, Rho, which is rapidly translocated to the plasma membrane by insulin. Rho in turn, along with ARF and

possibly D-3-PO_4 derivatives of PI, activates PC-PLD (see above).

In contrast to PLD activation, PI3K is not required for insulin-induced activation of G3PAT and *de novo* PA synthesis (20). Nevertheless, PI3K and PC hydrolysis (as discussed above) appear to be required for PI synthesis, presumably to supply PA and DAG during the initial phases of insulin action.

To summarize, PI3K activation seems to play a pivotal role in insulin-induced activation of PLD and subsequent PC hydrolysis and DAG/PKC signaling. This pathway, as will be discussed below more fully, may play an important role in glucose transport during insulin action, at least in some cell types. On the other hand, in contrast to glucose transport, glycogen synthase activation is clearly not dependent upon PC hydrolysis and DAG/PKC signaling; in fact, the latter may inhibit glycogen synthase, perhaps by direct phosphorylation of this enzyme.

DAG Production

In BC3H-1 myocytes, insulin provokes rapid increases in DAG mass, as well as the labeling of DAG by glycerol, myristate, and arachidonate (3, 15, 16, 24, 25). Rapid increases in labeled and in most cases total DAG content have also been observed in the rat soleus (31–33, 33a), diaphragm (31), and gastrocnemius (33) muscles, rat liver (12, 13, 34), HIRc-B cells (35), Swiss 3T3 fibroblasts (36), CHO · IR cells (37), L6 myotubes (19), 3T3/L1 adipocytes (unpublished), and highly purified plasma membranes and microsomes of rat adipocytes (1, 20). Insulin-induced increases in DAG production, in most cell types tested, occur very rapidly, do not require extracellular glucose, and therefore cannot be attributed simply to glucose transport-dependent increases in DAG production through the *de novo* pathway.

As alluded to above, most of the initial large burst in DAG production that occurs during the first 0.5–1 min of insulin action is due to PLD-dependent PC hydrolysis in the plasma membrane (1, 3, 4, 20) and is clearly not due to glucose transport effects of insulin. On the other hand, at subsequent times, *de novo* PA synthesis in the endoplasmic reticulum becomes an important source of DAG (1–3), and, at least in some cell types, this effect can be amplified by insulin-induced increases in glucose transport, glycolysis, and fatty acyl-coenzyme A production, each providing substrate for the *de novo* pathway. Along these lines, in situations in which glucose transport is not rate-limiting, simple increases in extracellular glucose can increase DAG and consequent PKC activity. The latter mechanism appears to be important for certain pathological consequences of persistent hyperglycemia in the diabetic state.

General Aspects of PKC Activation

Before discussing insulin effects on PKC, it may be helpful to review some current thoughts about PKC structure and activation. Multiple PKCs have now been recognized (see Refs. 41 and 41a for reviews), and they may be grouped into four types: "conventional" or "classical" cPKCs (α, β_1, β_2, γ), "novel" or nPKCs (δ, ϵ, η, θ), "atypical" or aPKCs (ζ, λ), and membrane-anchored PKCs (PKC-μ and PKD). Within their N-terminal regulatory domain, cPKCs have two zinc fingers, one that contains a Ca^{++}-binding site in the C_2 region (C = conserved) and a second that contains DAG-binding or phorbol ester-binding site in the C_1 region: accordingly, the cPKCs can be activated by Ca^{++} and DAG or phorbol esters. nPKCs lack C_2/Ca^{++}-binding site, and consequently are not activated by Ca^{++} but nevertheless respond to DAG and phorbol esters through binding sites in their C_1 region. aPKCs have neither C_2/Ca^{++}-binding nor C_1/DAG-binding sites but, like other PKCs, require phospholipids (e.g., phosphatidylserine) for activation and moreover can be activated by *cis*-unsaturated fatty acids and phospholipids such as PI-3′,4′,5′-$(PO_4)_3$ and PA. Membrane-anchored PKCs lack the C_2/Ca^{++}-binding sites but have C_1/DAG-binding sites. Both cPKCs and nPKCs are activated by DAG which is exclusively or largely found in plasma membranes and other cellular membranes; this explains why these PKCs are translocated from the cytosol to the membrane fraction during DAG-induced PKC activation, and why translocation is generally accepted as an indicator of PKC activation. Simple increases in intracellular Ca^{++} can also result in the translocation of cPKCs but not nPKCs. In the case of insulin, increases in intracellular Ca^{++} concentrations are absent, minimal, or delayed. In addition, insulin translocates both cPKCs and nPKCs. It therefore seems likely that insulin-induced PKC translocations are largely due to DAG or other membrane-associated lipids.

In addition to DAG, PKC can be activated by certain other lipids and phospholipids (e.g., oleic acid, arachidonic acid, fatty acyl-CoA, phosphatidylserine, PA, lyso-PC, PI-4′,5′-$(PO_4)_2$, PI-3′-PO_4, PI-3′,4′-$(PO_4)_2$ and PI-3′,4′,5′-$(PO_4)_3$. In the case of atypical PKCs, such as PKC-ζ, increases in PA, fatty acids, and polyphosphoinositides may be responsible for their activation. Interestingly, insulin provokes increases in many of these PKC activators. Several PKCs (e.g., PKC-α and PKC-δ) have also been found to be activated by tyrosine phosphorylation, and certain serine/threonine phosphorylations of PKC are required for intrinsic PKC activity. In addition, the insulin receptor seems to directly bind and activate overexpressed PKC-α in CHO cells (38). Obviously,

insulin may use multiple mechanisms to activate specific PKCs in specific cellular domains.

Upon activation by DAG and possibly other lipids, there is an unfolding of PKC at the V_3 (V = variable) hinge region, and a dissociation of a V_1 (N-terminal) inhibitory pseudosubstrate peptide sequence from the substrate binding site in catalytic C-3 domain: this allows PKC to bind and phosphorylate specific serine/threonine residues (usually flanked by basic amino acids) in its substrates, which may vary for each PKC. The open hinge region also becomes more vulnerable to membrane-associated proteases, leading to cleavage and release of regulatory (30 kDa) and catalytic (50 kDa; "M-kinase" or PKM) fragments. As may also be surmised, prolonged activation may lead to PKC depletion, or "downregulation," unless there is a commensurate balance between degradation and new PKC synthesis. Interestingly, insulin acutely increases M-kinase in rat adipocytes (39), and prolonged continuous insulin action (e.g., as in hyperinsulinemic GK diabetic, aged/obese and genetically obese Zucker [fa/fa] rats) can lead to downregulation of certain PKC isoforms (submitted for publication). Of further interest, insulin increases mRNAs that encode PKC-α and PKC-β in rat adipocytes, skeletal muscle, and liver (40; unpublished observations), which probably contributes to the maintenance of PKC levels during insulin action.

Effects of Insulin on PKC

There is still a lingering controversy as to whether insulin activates PKC. This controversy partly stems from an initial assumption that only DAG derived from PI-4′,5′-$(PO_4)_2$ hydrolysis activates PKC. However, it is now abundantly clear that DAG derived from PC hydrolysis and the *de novo* PA synthesis can translocate and activate PKC (41, 41a). The controversy also derives from the failure of some initial studies (42, 43) to show that insulin provokes measurable *decreases* in cytosolic PKC enzyme activity, which was at first considered as essential evidence for the translocation of PKC from cytosol to membranes. However, similar "failures" to observe decreases in cytosolic PKC enzyme activity have been observed during the activation of PKC by other agonists in many other systems, and the fact that increases in membrane PKC activity were evident, even in the above-mentioned early studies of insulin action (42, 43) should not be lost sight of. Moreover, in subsequent studies in which PKC was partially purified by FPLC Mono Q column chromatography to remove more effectively noncovalent PKC modulators, typical PKC translocations patterns were observed in insulin-treated rat adipocytes (44), BC3H-1 myocytes (45), and various rat skeletal muscles (31–33): that is, increases in membrane, and decreases in cytosolic, PKC enzyme activity. Also, using PKC isoform-specific antisera, insulin-induced translocation of PKC-α PKC-β, PKC-ε, PKC-δ, and/or PKC-θ from the cytosol to the membrane has been observed in the above tissues (4, 31–33, 44–46), as well as in CHO-IR cells (37), A-10 vascular smooth muscle (VSM) cells (47), H4IIE hepatoma cells (48), 3T3/L1 cells (unpublished), HIRC-B cells (35), Xenopus oocytes (49), L6 skeletal muscle myotubes (19, 50), and rat hepatocytes (unpublished). Increases in PKC enzyme activity have also been observed in chick neurons, but this may not involve translocation and may rather be due to a covalent modification, of PKC (51). It should also be noted that insulin provokes rapid increases in DAG, PKC enzyme activity, and levels of immunoreactive PKC-α and PKC-β in purified plasma membranes and microsomes of rat adipocytes (1, 20, 46). Egan *et al.* (52) also observed similar increases in PKC enzyme activity of purified rat adipocyte plasma and microsomal membranes. These findings most likely reflect PC hydrolysis to the greatest extent, and glycosyl-PI hydrolysis to a lesser extent, in the plasma membrane and *de novo* PA synthesis in microsomes. On the other hand, it is possible that lipid vesicle trafficking between the plasma membrane and interior membranes may lead to significant mixing of lipid-derived signaling factors.

Another reason for doubting the importance of DAG/PKC signaling in insulin action derives from studies of PKC substrate phosphorylation in intact BC3H-1 myocytes, HIRc-B cells, and NIH3T3/HIR3.5 cells. In these studies (53), examination of trichloroacetic acid (TCA)-precipitable [^{32}P-labeled proteins (and other substances, e.g., lipids, nucleic acids) did not reveal significant increases in the phosphorylation of an endogenous PKC substrate (viz., MARCKS). It was therefore reasoned that insulin did not truly activate PKC in the intact cells. However, in BC3H-1 myocytes (1) and HIRc-B cells (35), when equal amounts of protein rather than equal amounts of [^{32}P]-label in TCA precipitates were examined, insulin-induced increases in the phosphorylation of immunoprecipitable MARCKS were observed. Thus, even by the criterion of MARCKS labeling, both in intact cells and in membrane preparations from these cells, it appears that insulin activates DAG/PKC signaling in BC3H-1 myocytes (1, 4), HIRc-B cells (35), rat adipocytes (1), rat soleus muscles (1), and probably in other cell types as well.

In addition to MARCKS, insulin-induced increases in [^{32}P]-labeling of other specific PKC substrates have been observed in a variety of intact cells. In rat diaphragm (54), insulin and phorbol esters stimulate [^{32}P]-labeling of identical tryptic peptides of a 15-kDa PKC substrate, and insulin effects are inhibited by PKC inhibitors and phorbol ester–induced PKC downregulation. Also, more recent studies in the

rat diaphragm (55) have shown that specific PKC-dependent sites in phospholemman are phosphorylated in response to insulin treatment. In 3T3/L1 cells (56), insulin and phorbol esters in intact cells, and PKC itself *in vitro,* have been reported to induce identical phosphorylations of phosphopeptides in tryptic fragments of eukaryotic protein synthesis initiation factors eIF-4F p25 and eIF-3 p120; moreover, insulin effects were lost after phorbol ester–induced PKC downregulation. In rat adipocyte membranes (57), insulin, phorbol esters, and PKC phosphorylated a 40-kDa protein identically, as shown in phosphopeptide mapping of tryptic digests. In CHO · IR cells (37), insulin and phorbol esters stimulated 40-kDa protein phosphorylation, and these effects were lost after PKC downregulation. In rat adipocytes (58), insulin and phorbol esters phosphorylate acetyl-CoA carboxylase identically, as shown by phosphopeptide mapping of tryptic digests. Finally, in human mammary epithelial cells (59), insulin, phorbol esters, and PKC identically phosphorylate p28 and p220 subunits of eIF-4E, as shown by phosphopeptide mapping of tryptic digests. Thus, certain insulin-stimulated protein phosphorylations in intact cells appear to be mediated through PKC. On the other hand, it is equally clear that MAP kinase and other kinases are responsible for the phosphorylation of many proteins during insulin action.

General Consequences of Insulin-Induced PKC Activation

Phorbol esters, as DAG analogs and thus PKC activators, have many insulin-like effects, including (i) increases in glucose transport and translocation of GLUT 1 and GLUT 4 from low density microsomes to the plasma membrane; (ii) activation of certain enzymes important in intracellular glucose metabolism (e.g., phosphofructokinase, enolase, and pyruvate dehydrogenase); (iii) activation of the rate-limiting enzyme in fatty acid synthesis, acetyl-CoA carboxylase; (iv) activation of the Na^+/H^+ antiport and Na^+/K^+ ATPase in the plasma membrane, and consequent regulation of ion fluxes and maintenance of intracellular ionic/acid milieu; (v) increases in amino acid transport; (vi) activation of eukaryotic initiation factors (eIFs), S6 ribosomal protein and thus protein synthesis; (vii) changes in gene expression, including (a) increases in mRNAs that encode c-*fos,* c-*myc,* P-33, IGF-I, c-Ki-*ras,* lutropin, β-actin, and ornithine decarboxylase, and (b) decreases in mRNAs that encode albumin, tyrosinase, and phosphoenolpyruvate carboxykinase (PEPCK); and (viii) activation of DNA synthesis and/or cellular differentiation in some cell types.

Unfortunately, the simple demonstration that phorbol esters mimic insulin does not necessarily indicate that insulin effects on the above-mentioned processes are mediated by PKC. Indeed, some insulin-like effects of phorbol esters are undoubtedly mediated through the activation of MAP kinase (*via* raf-1, see Fig. 1), rather than by PKC itself as the more terminal kinase. To confuse matters even more, MAP kinase may be activated by insulin by both PKC-dependent (50) or PKC-independent (60, 61) mechanisms, depending upon the cell type. In addition, PKC and MAP kinase may independently activate a number of common processes, most notably, alterations in gene expression. Obviously, there is considerable room for independent, parallel, additive, nonadditive, and redundant signaling through the PKC and MAP kinase pathways. Nevertheless, some effects of insulin appear to be largely or at least partly PKC dependent, as suggested by studies with PKC inhibitors or PKC depletion.

The Role of Lipid-Derived Signaling Substances in Glucose Transport

Phorbol esters and DAG increase glucose transport in many cell types, including BC3H-1 myocytes, rat adipocytes, mouse soleus muscles, rat soleus muscles, rat epitrochlearis muscles, cultured L6 muscle cells, 3T3/L1 cells, Swiss 3T3 cells, BALB/c 3T3 preadipose cells, cultured glial cells, chick embryo fibroblasts HeLa cells, brain endothelial cells, lymphocytes, polymorphonuclear leucocytes, rat heart, A-10 vascular smooth muscle (VSM) cells, HIRc-B cells, thyroid cells, and CHO · IR cells. The effects of phorbol esters on glucose transport, however, in some but not all cell types are less than those of insulin. For example, particularly in some cells that contain GLUT 4 glucose transporters (viz., rat adipocytes, rat or mouse soleus muscle, L6 myotubes, and 3T3/L1 adipocytes), insulin effects on 2-deoxyglucose uptake are generally about 3-fold greater than those of phorbol esters: however, it may also be noted that effects of exogenously added DAG or PLC (to generate DAG from PC in the plasma membrane) on glucose transport in some of these cell types are quantitatively more comparable to those of insulin. In contrast to GLUT 4–containing cells, in many cells that only contain GLUT 1 glucose transporters (viz., BC3H-1 myocytes, HIRc-B cells, 3T3/L1 fibroblasts, Swiss 3T3 cells, and CHO cells), phorbol ester effects on glucose transport are generally more comparable in magnitude to those of insulin and appear to be largely attributable in most cases to GLUT 1 translocation from internal stores to the plasma membrane.

The failure of phorbol esters to mimic fully insulin effects on glucose transport in GLUT 4–containing cells is not understood. Several possibilities must be considered. First, insulin may regulate glucose transport independently of PKC, and phorbol ester effects on this process may be fortuitous or may be mediated by a non-PKC factor (e.g., PI3K, PC-PLD, or PC-PLD

activation and PA/DAG production, etc.). Second, insulin and phorbol esters may activate PKC differently: some reports (62, 62a) suggest that there are different binding sites for DAG and phorbol esters on PKC, leading to different substrate preferences. Third, insulin, unlike phorbol esters, may strongly activate DAG-insensitive, atypical PKCs (e.g., PKC-ζ) *via* increases in PI-3,4-$(PO_4)_2$ and PI-3,4,5,-$(PO_4)_3$, or through other mechanisms. Fourth, insulin may use both PKC-dependent and PKC-independent mechanisms to either stimulate GLUT 4 translocation and/or activate GLUT 4 after its translocation, and dependence on these mechanisms may vary in different cell types. In support of a two-step mechanism, some groups have found that insulin effects on the translocation of GLUT 4 from low-density microsomes to the plasma membrane in rat adipocytes are equal to, or only slightly greater than, those of phorbol esters, despite the fact that overall glucose transport effects are much greater with insulin (63–66). In addition, similar equivalence of GLUT 4 translocation in response to insulin and phorbol esters was found in GLUT 4/*myc*-transfected CHO cells (67). On the other hand, other investigators have found insulin to have much greater effects than phorbol esters on GLUT 4 translocation but more comparable or equal effects on GLUT 1 translocation in rat adipocytes and 3T3/L1 adipocytes (68, 69, 69a).

As alluded to above, in addition to phorbol esters, exogenously added DAG activates glucose transport in BC3H-1 myocytes (70, 71) and rat adipocytes (66, 72, 73) and L6 cells (74). Also, exogenously added PLC, which increases DAG by hydrolyzing PC, activates glucose transport in BC3H-1 myocytes (71), rat adipocytes (72, 73), and rat skeletal muscle (31, 75). Electrical- or exercise-induced DAG and resultant PKC activation is associated with increases in glucose transport in rat skeletal muscle (76). A DAG kinase inhibitor, monoacylglycerol, and arachidonic acid, a non-DAG PKC activator, increase DAG/PKC signaling and glucose transport in Swiss 3T3 fibroblasts (77). Other non-DAG PKC activators, mezerein and SC-9, also increase glucose transport in BC3H-1 myocytes (71), rat adipocytes (72), and/or 3T3 fibroblasts (77a). Although not necessarily specific for PKC, the phosphatase inhibitor, okadaic acid, which is known to translocate and activate PKC, also activates glucose transport in rat adipocytes (78) and rat skeletal muscle (79). Finally, a long list of agonists that increase DAG and activate PKC by a variety of mechanisms have been found to increase glucose transport in their target tissues, including thyrotropin and carbachol in thyroid cells; carbachol in pancreatic acinar cells; phenylephrine in BC3H-1 myocytes; bombesin in Swiss 3T3 cells; EGF and IGF-1 in BC3H-1 myocytes; angiotensin II in VSM cells; and platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and serum in Swiss 3T3 cells. On the other hand, it should be recognized that many of these agonists also have effects on other signaling processes (e.g., PI3K and MAP kinase activation) that may contribute to the activation of glucose transport. In addition, increases in DAG and activation of PKC may in turn lead to other changes in phospholipid metabolism, for one, increases in PC-PLD activation and PA production; perhaps these lipid changes, rather than PKC *per se,* are important in stimulating glucose transport in some cases. On the other hand, it seems clear that a protein kinase that is downstream of PI3K is operative for insulin effects on glucose transport (20, 79).

In support of a role for PKC in glucose transport, phorbol ester–induced PKC downregulation (see above) concomitantly inhibits insulin–stimulated glucose transport in some cell types (i.e., rat adipocytes [40, 61, 80, 81], mouse soleus [82], rat soleus [unpublished], rat heart [83], rat A-10 VSM cells [47], and CHO · IR cells [37]. However, in other cell types, insulin effects are retained following such "downregulation" (i.e., in BC3H-1 myocytes [71], HIRc-B cells [35], 3T3/L1 cells [84], 3T3 fibroblasts [85], and L6 cells [86]). These differences in responses to phorbol ester–induced "downregulation" could reflect cell-specific differences in (i) regulatory mechanisms, (ii) failure to deplete all insulin-sensitive PKC isoforms sufficiently during this "downregulation," and/or (iii) untoward effects of prolonged phorbol ester treatment. With respect to these possibilities, we have recently found that PKC-ζ, which is not acutely activated or downregulated by phorbol esters, appears to play an important role in the activation of glucose transport in 3T3/L1 fibroblasts and adipocytes.

In addition to phorbol esters, antisense-DNA has been used to deplete PKC-α and PKC-β in rat adipocytes, and this too inhibits insulin-stimulated glucose transport (87). Further evidence for a requirement for PKC in glucose transport comes from the finding that the introduction of PKC into PKC-depleted rat adipocytes (by electroporation) apparently can restore insulin-stimulated glucose transport (88).

PKC inhibitors, including H7, staurosporine, sangivamycin, sphingosine, mellitin, polymixin B, R0 31-8220, acridine orange, bisindolemaleimide, chelerythrine, calphostin C, and, perhaps more specifically, the PKC pseudosubstrate (89), inhibit insulin- and TPA-stimulated glucose transport in rat adipocytes, rat soleus muscle preparations, L6 cells, 3T3/L1 cells, and/or other cell types. In some, but not all, cases, the inhibition of insulin effects on glucose transport requires higher inhibitor concentrations than those required for phorbol ester effects on glucose transport. This could be explained variously: (i) PKC isoforms have different sensitivities to inhibitors (cPKCs >

nPKCs > aPKCs, in order of sensitivity); (ii) there are separate binding sites for DAG and phorbol esters and differential inhibitor sensitivities (62, 62a); and/or (iii) some PKC inhibitors may inhibit other kinases. Although these PKC inhibitor studies may not unequivocally implicate PKC in insulin regulation of glucose transport, it seems clear that a protein kinase is involved, and this protein kinase(s) must be distal to PI3K, since the latter kinase, and insulin effects on PI3K, are not blocked by RO 31-8220 (20; also see Ref. 79).

To date, there is only fragmentary evidence to suggest which PKC isoforms may be required for the activation of glucose transport during insulin action. Insulin translocates virtually all DAG-sensitive PKC isoforms in certain cell types (e.g., α, β, ϵ, and δ in rat adipocytes [1, 20, 46], and PKC α, β, ϵ, δ, and θ in rat soleus muscles [90]). Also, although direct, fully convincing evidence is presently lacking, it is likely that insulin activates PKC-ζ through PI3K activation and production of polyphosphoinositides, a process which is known to occur in these and other cell types. Obviously, any or all of these isoforms could play a role in glucose transport in these cell types. However, in certain cultured cells there is more apparent selectivity in the translocation of PKC isoforms during insulin action (e.g., PKC-ϵ, but not PKC-α, in HIRcB cells [35]; PKC-β_1 and PKC-ϵ, but not PKC-α, in BC3H-1 myocytes [4, 91]; and PKC-α and PKC-β, but not PKC-ϵ, in 3T3/L1 cells [submitted for publication]). With respect to specific PKCs, studies with phorbol ester (40) and antisense-DNA (87) induced PKC downregulation have suggested that PKC-α and PKC-β may be required for insulin effects on glucose transport in rat adipocytes (87), but this evidence can only be considered suggestive at this time. Also, in recent studies (unpublished) we have found that transfection of 3T3/L1 cells with active and inactive forms of PKC-ζ leads to alterations in insulin-stimulated glucose transport. Thus, PKC-ζ seems to be important for glucose transport, and this would explain situations in which phorbol ester–induced PKC downregulation does not inhibit glucose transport. Clearly, a variety of molecular biology approaches will be needed to evaluate the question of involvement specific PKC isoforms in insulin-stimulated glucose transport in specific cell types.

As stated above, PKC-independent mechanisms and protein kinases other than PKC may be important in the activation of glucose transport. Along these lines, MAP kinase has received considerable attention, but its activation does not appear to be sufficient (92, 93) or necessary (94) to account for insulin-stimulated glucose transport. Some evidence also suggests that *ras* may contribute to the activation of glucose transport in 3T3/L1 adipocytes (95) and cardiomyocytes (96), however, this too is not clear, since in HIRc B cells (97) and CHO cells (98) insulin-stimulated *ras* activation can be dissociated from activation of PI3K and glucose transport.

As stated above, a growing body of evidence supports the possibility that PI3K plays a pivotal role during insulin-stimulated glucose transport (98–101). However, what happens downstream of PI3K is not well understood. PI3K itself has protein kinase, as well as PI kinase, activity, and, even though the role of PI3K as a protein kinase is unclear, it seems clear that a protein kinase downstream of PI3K functions in glucose transport (20, 79). A rapamycin-sensitive 70-kDa S6 kinase is known to be downstream of PI3K, but this S6 kinase does not appear to be involved in glucose transport (102). Another possibility is that GTP-binding proteins (e.g., rho, ARF, or rac [103]) or D-3 phosphate derivatives of PI might serve to couple PI3K activation to PLD-dependent PC hydrolysis, PA production, and DAG-dependent PKC activation (see above). Another possibility is PI3K-induced increases in PI-3′,4′-$(PO_4)_2$ and PI-3′,4′,5′-$(PO_4)_3$ may directly activate PKC-ζ or other PKC isoforms. The activation of PLD, moreover, would result in increases in PA, which along with PI-3,4-$(PO_4)_2$ PI-3,4,5-(PO_4), and DAG may alter membrane curvature and contribute to budding, vesiculation, and transport of GLUT 4 vesicles from the endoplasmic reticulum, and subsequent docking in the plasma membrane (30). PKC and G proteins may also participate directly in this trafficking, and the activation of glucose transport may occur through a multifactorial process. Obviously, more information will be needed to fit the pieces of the puzzle together, but it is important to keep in mind that the factors that are downstream of PI3K may vary in importance in regulating glucose transport in different cell types.

Concluding Remarks

To summarize, there is increasing evidence that insulin activates a number of phospholipid signaling systems (viz., PI3K, PLD-dependent PC hydrolysis, glycosyl-PI hydrolysis, *de novo* PA synthesis, and PKC) in most, if not all, target tissues. The major question is the definition of the role of each of these phospholipid signaling systems in specific actions of insulin. The similarity of effects of phorbol esters and insulin on various metabolic processes suggests that PKC could play an important role in insulin action. Moreover, a number of insulin effects on various metabolic processes, including glucose transport, in certain cell types seem to require PKC, though this conclusion can only be considered tentative at the present time. Similarly, a growing and convincing body of evidence suggests that PI3K is involved in effects of insulin on glucose transport. It is therefore of consider-

able interest that more recent findings suggest that PLD-dependent PC hydrolysis is downstream of PI3K activation in the action of insulin. If so, this could explain how PKC, along with other lipid signaling factors (e.g., DAG and acidic phospholipids, such as PA and D-3 phosphate derivatives of PI) could participate in the PI3K-dependent activation of glucose transport during insulin action. Needless to say, more definitive studies will be needed to evaluate the role of each of these lipid-derived signaling substances in insulin-stimulated glucose transport.

1. Arnold TP, Standaert ML, Hernandez H, Watson J, Mischak H, Kazanietz MG. Effects of insulin and phorbol esters on MARCKS (myristoylated alanine-rich C-kinase substrate) phosphorylation (and other parameters of protein kinase C activation) in rat adipocytes, rat soleus muscle and BC3H-1 myocytes. Biochem J **295**:155–164, 1993.
2. Farese RV, Standaert ML, Arnold TP, Yamada K, Musunuru K, Hernandez H, Mischak H, Cooper DR. Preferential activation of microsomal diacylglycerol/protein kinase C signaling during glucose treatment (*de novo* phospholipid synthesis) of rat adipocytes. J Clin Invest **93**:1894–1899, 1994.
3. Hoffman JM, Standaert ML, Nair GP, Farese RV. Differential effects of pertussis toxin on insulin-stimulated phosphatidylcholine hydrolysis and glycerolipid synthesis *de novo*. Studies in BC3H-1 myocytes and rat adipocytes. Biochemistry **30**: 3315–3322, 1991.
4. Standaert ML, Musunuru K, Yamada K, Cooper DR, Farese RV. Insulin-stimulated phosphatidylcholine hydrolysis, diacylglycerol/protein kinase C signalling and hexose transport in pertussis toxin-treated BC3H-1 myocytes. Cell Signal **6**:707–716, 1994.
5. Vila MC, Milligan G, Standaert ML, Farese RV. Insulin activates glycerol-3-phosphate acyltransferase (*de novo* phosphatidic acid synthesis) through a phospholipid-derived mediator. Apparent involvement of Gi-α and activation of a phospholipase C. Biochemistry **29**:8735–8740, 1990.
6. Farese RV, Standaert ML, Yamada K, Huang LC, Zhang C, Cooper DR, Wang Z, Yang Y, Suzuki S, Toyota T, Larner J. Insulin-induced activation of glycerol-3-phosphate acyltransferase by a *chiro*-inositol-containing insulin mediator is defective in adipocytes of insulin-resistant, type II diabetic, Goto-Kakizaki (GK) rats. Proc Natl Acad Sci USA **91**:11040–11044, 1994.
7. Larner J. Insulin-signaling mechanisms. Lessons from the old testament of glycogen metabolism and the new testament of molecular biology. Diabetes **37**:262–275, 1988.
8. Saltiel AR, Osterman DG, Darnell JC, Chan BL, Sorbara-Cazan LR. The role of glycosylphosphoinositides in signal transduction. Rec Prog Horm Res **45**:353–379, 1989.
9. Denton RM, Tavare JM. Does mitogen-activated protein kinase have a role in insulin action? The cases for and against. Eur J Biochem **227**:597–611, 1995.

9a. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378**:785–787, 1995.

10. Farese RV, Cooper DR, Konda TS, Nair GP, Standaert ML, Pollet RJ. Insulin provokes co-ordinated increases in the synthesis of phosphatidylinositol, phosphatidylinositol phosphates and the phosphatidylinositol-glycan in BC3H-1 myocytes. Biochem J **256**:185–188, 1988.
11. Macaulay SL, Larkins RG. Insulin stimulates turnover of phosphatidylcholine in rat adipocytes. Mol Cell Biochem **136**:23–28, 1994.
12. Baldini PM, Zannetti A, Donchenko V, Dini L, Luly P. Insulin effect on isolated rat hepatocytes: Diacylglycerol-phosphatidic acid interrelationship. Biochim Biophys Acta **1137**:208–214, 1992.
13. Donchenko V, Zannetti A, Baldini PM. Insulin-stimulated hydrolysis of phosphatidylcholine by phospholipase C and phospholipase D in cultured rat hepatocytes. Biochim Biophys Acta **1222**:492–500, 1994.
14. Petkova DH, Nikolova MN, Momchilova-Pankova AB, Koumanov, KS. Insulin effect on the phospholipid organization and some enzyme activities of rat liver membrane fractions. Comp Biochem Physiol **95B**:685–689, 1990.
15. Farese RV, Konda TS, Davis JS, Standaert ML, Pollet RJ, Cooper DR. Insulin rapidly increases diacylglycerol by activating *de novo* phosphatidic acid synthesis. Science **236**:586–589, 1987.
16. Farese RV, Cooper DR, Konda TS, Nair GP, Standaert ML, Davis JS, Pollet RJ. Mechanisms whereby insulin increases diacylglycerol in BC3H-1 myocytes. Biochem J **256**:175–184, 1988.
17. Wei JW, Yeh SR. Effects of insulin on glucose uptake in cultured cells from the central nervous system of rodent. Int J Biochem **23**:851–856, 1991.
18. Garcia de Herreros A, Dominguez I, Diaz-Meco MT, Graziani G, Cornett ME, Guddal PH, Johansen T, Moscat J. Requirement of phospholipase C-catalyzed hydrolysis of phosphatidylcholine for maturation of *Xenopus laevis* oocytes in response to insulin and *ras* p21. J Biol Chem **266**:6825–6829, 1991.
19. Standaert ML, Bandyopadhyay G, Zhou X, Galloway L, Farese RV. Insulin stimulates phospholipase D-dependent phosphatidylcholine hydrolysis, *de novo* phospholipid synthesis, and diacylglycerol/protein kinase C signalling in L6 myotubes. Endocrinology **137**:3014–3020, 1996.
20. Standaert ML, Avignon A, Yamada K, Bandyopadhyay G, Farese RV. The phosphatidylinositol 3-kinase inhibitor, wortmannin, inhibits insulin-induced activation of phosphatidylcholine hydrolysis and associated protein kinase C translocation in rat adipocytes. Biochem J **313**:1039–1046, 1996.
21. Bowman EP, Uhlinger DJ, Lambeth JD. Neutrophil phospholipase D is activated by a membrane-associated Rho family small molecular weight GTP-binding protein. J Biol Chem **268**:21509–21512, 1993.
22. Cockcroft S, Thomas GM, Fensome, Geny B, Cunningham E, Gout I, Hiles I, Totty NF, Truong O, Hsuan JJ. Phospholipase D: A downstream effector of ARF in granulocytes. Science **263**:523–526, 1994.
23. Farese RV, Larson RE, Sabir MA. Insulin acutely increases phospholipids in the phosphatidate-inositide cycle in rat adipose tissue. J Biol Chem **257**:4042–4045, 1982.
24. Farese RV, Barnes DE, Davis JS, Standaert ML, Pollet RJ. Effects of insulin and protein synthesis inhibitors on phospholipid metabolism, diacylglycerol levels and pyruvate dehydrogenase activity in BC3H-1 cultured myocytes. J Biol Chem **259**:7094–7100, 1984.
25. Farese RV, Davis JS, Barnes DE, Standaert ML, Babischkin JS, Hock R, Rosic NK, Pollet RJ. The *de novo* phospholipid effect of insulin is associated with increases in diacylglycerol, but not inositol phosphates or cytosolic Ca^{++}. Biochem J **231**:269–278, 1985.
26. Ruderman NB, Kapeller R, White MF, Cantley LC. Activation of phosphatidylinositol 3-kinase by insulin. Proc Natl Acad Sci USA **87**:1411–1415, 1990.
27. Nakanishi H, Brewer KA, Exton JH. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem **268**:13–16, 1993.
28. Singh SS, Chauhan A, Brockerhoff H, Chauhan VP. Activation of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. Biochem Biophys Res Commun **195**:104–112, 1993.
29. Pertile P, Liscovitch M, Chalifa V, Cantley LC. Phosphatidylinositol 4,5-bisphosphate synthesis is required for activation of

phospholipase D in U937 cells. J Biol Chem **270**:5130–5135, 1995.
30. Liscovitch M, Cantley LC. Signal transduction and membrane traffic: The PITP/phosphoinositide connection. Cell **81**:659–662, 1995.
31. Ishizuka T, Cooper DR, Hernandez H, Buckley D, Standaert M, Farese RV. Effects of insulin on diacylglycerol-protein kinase C signaling in rat diaphragm and soleus muscles and relationship to glucose transport. Diabetes **39**:181–190, 1990.
32. Hoffman JM, Ishizuka T, Farese RV. Interrelated effects of insulin and glucose on diacylglycerol-protein kinase-C signalling in rat adipocytes and solei muscle *in vitro* and *in vivo* in diabetic rats. Endocrinology **128**:2937–2948, 1991.
33. Yu B, Standaert M, Arnold T, Hernandez H, Watson J, Ways K, Cooper DR, Farese RV. Effects of insulin on diacylglycerol/protein kinase-C signalling and glucose transport in rat skeletal muscles *in vivo* and *in vitro*. Endocrinology **130**:3345–3355, 1992.
33a. Chen KS, Heydrick SJ, Brown ML, Friel JC, Ruderman NB. Insulin increases a biochemically distinct pool of diacylglycerol in the rat soleus muscle. Am J Physiol **266**:E479–E485, 1994.
34. Cooper DR, Hernandez H, Kuo JY, Farese RV. Insulin increases the synthesis of phospholipid and diacylglycerol and protein kinase C activity in rat hepatocytes. Arch Biochem Biophys **276**:486–494, 1990.
35. Zhao L, Standaert ML, Cooper DR, Avignon A, Farese RV. Effects of insulin on protein kinase C (PKC) in HIRC-B cells: specific activation of PKC-epsilon and its resistance to phorbol ester-induced downregulation. Endocrinology **135**:2504–2510, 1994.
36. Hesketh JE, Campbell GP, Reeds PJ. Rapid response of protein synthesis to insulin in 3T3 cells: Effects of protein kinase C depletion and differences from the response to serum repletion. Biosci Rep **6**:797–804, 1986.
37. Cherqui G, Reynet C, Caron M, Melin B, Wicek D, Clauser E, Capeau J, Picard J. Insulin receptor tyrosine residues 1162 and 1163 control insulin stimulation of myristoyl-diacylglycerol generation and subsequent activation of glucose transport. J Biol Chem **265**:21254–21261, 1990.
38. Liu F, Roth RA. Insulin-stimulated tyrosine phosphorylation of protein kinase Cα: Evidence for direct interaction of the insulin receptor and protein kinase C in cells. Biochem Biophys Res Commun **200**:1570–1577, 1994.
39. Ishizuka T, Cooper DR, Farese RV. Insulin provokes apparent increases in rat adipocyte M-kinase. Biochem Biophys Res Commun **169**:966–972, 1990.
40. Avignon A, Standaert ML, Yamada K, Mischak H, Spencer B, Farese RV. Insulin increases mRNA levels of protein kinase C-α and -β in rat adipocytes and protein kinase C-α, -β and -θ in rat skeletal muscle. Biochem J **308**:181–187, 1995.
41. Stabel S, Parker PJ. Protein kinase C. Pharmacol Ther **51**:71–95, 1991.
41a. Nishizuka Y. Membrane phospholipid degradation and protein kinase C for cell signalling. Neurosci Res **15**:3–5, 1992.
42. Walaas SI, Horn RS, Adler A, Albert KA, Walaas O. Insulin increases membrane protein kinase C activity in rat diaphragm. FEBS Lett **220**:311–318, 1987.
43. Cooper DR, Konda TS, Standaert ML, Davis JS, Pollet RJ, Farese RV. Insulin increases membrane and cytosolic protein kinase C activity in BC3H-1 myocytes. J Biol Chem **262**:3633–3639, 1987.
44. Ishizuka T, Cooper DR, Farese RV. Insulin stimulates the translocation of protein kinase C in rat adipocytes. FEBS Lett **257**:337–340, 1989.
45. Cooper DR, Ishizuka T, Watson JE, Standaert ML, Nair G, Farese RV. Protein kinase C activation patterns are determined by methodological variations. Studies of insulin action in BC3H-1 myocytes and rat adipose tissue. Biochim Biophys Acta **1054**:95–102, 1990.
46. Farese RV, Standaert ML, Francois AM, Ways K, Arnold TP, Hernandez H, Cooper DR. Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes. Biochem J **288**:319–323, 1992.
47. Cooper DR, Khalakdina A, Watson JE. Chronic effects of glucose on insulin signaling in A-10 vascular smooth muscle cells. Arch Biochem Biophys **302**:490–498, 1993.
48. Messina JL, Standaert ML, Ishizuka T, Weinstock RS, Farese RV. Role of Protein kinase C in insulin's regulation of c-*fos* transcription. J Biol Chem **267**:9223–9228, 1992.
49. Smith BL, Mochly-Rosen D. Inhibition of protein kinase C function by injection of intracellular receptors for the enzyme. Biochem Biophys Res Commun **188**:1235–1240, 1992.
50. Srinivasan M, Begum N. Stimulation of protein phosphatase-1 activity by phorbol esters. Evaluation of the regulatory role of protein kinase C in insulin action. J Biol Chem **269**:16662–16667, 1994.
51. Heidenreich KA, Toledo SP, Brunton LL, Watson MJ. Insulin stimulates the activity of a novel protein kinase C, PKC-ε, in cultured fetal chick neurons. J Biol Chem **265**:15076–15082, 1990.
52. Egan JJ, Saltis J, Wek SA, Simpson IA, Londos C. Insulin, oxytocin, and vasopressin stimulate protein kinase C activity in adipocyte plasma membranes. Proc Natl Acad Sci USA **87**:1052–1056, 1990.
53. Blackshear PJ, Haupt DM, Stumpo DJ. Insulin activation of protein kinase C: A reassessment. J Biol Chem **266**:10946–10952, 1991.
54. Walaas O, Horn RS, Walaas SI. Insulin and phorbol ester stimulate phosphorylation of a 15000 dalton membrane protein in rat diaphragm in a similar manner. Biochim Biophys Acta **1094**:92–102, 1991.
55. Walaas SI, Czernik AJ, Olstad OK, Sletten K, Walaas O. Protein kinase C and cyclic AMP-dependent protein kinase phosphorylate phospholemman, an insulin and adrenaline-regulated membrane phosphoprotein, at specific sites in the carboxy terminal domain. Biochem J **304**:635–640, 1994.
56. Morley SJ, Traugh JA. Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3, and ribosomal protein S6 by insulin and phorbol esters. J Biol Chem **265**:10611–10616, 1990.
57. Graves CB, McDonald JM. Insulin and phorbol ester stimulate phosphorylation of a 40-kDa protein in adipocyte plasma membranes. J Biol Chem **260**:11286–11292, 1985.
58. Haystead TA, Hardie DG. Insulin and phorbol ester stimulate phosphorylation of acetyl-CoA carboxylase at similar sites in isolated adipocytes. Lack of correspondence with sites phosphorylated on the purified enzyme by protein kinase C. Eur J Biochem **175**:339–345, 1988.
59. Donaldson RW, Hagedorn CH, Cohen S. Epidermal growth factor or okadaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. J Biol Chem **266**:3162–3166, 1991.
60. de Vries-Smits AM, Burgering BM, Leevers SJ, Marshall CJ, Bos JL. Involvement of $p21^{ras}$ in activation of extracellular signal-regulated kinase 2. Nature **357**:602–604, 1992.
61. Yang Y, Farese RV. Insulin activates myelin basic protein (p42 MAP) kinase by a protein kinase C-independent pathway in rat adipocytes. Dissociation from glucose transport. FEBS Lett **333**:287–290, 1993.
62. Robinson PJ. Differential stimulation of protein kinase C activity by phorbol ester or calcium/phosphatidylserine *in vitro* and in intact synaptosomes. J Biol Chem **267**:21637–21644, 1992.
62a. Slater SJ, Kelly MB, Taddeo FJ, Rubin E, Stubbs CD. Evidence for discrete diacylglycerol and phorbol ester activator sites on protein kinase C. Differences in effects of 1-alkanol inhibition, activation by phosphatidylethanolamine and calcium chelation. J Biol Chem **269**:17160–17165, 1994.
63. Mühlbacher C, Karnieli E, Schaff P, Obermaier B, Mushack J, Rattenhuber E, Haring HU. Phorbol esters imitate in rat fat-cells the full effect of insulin on glucose-carrier translocation, but not on 3-*O*-methylglucose-transport activity. Biochem J **249**:865–870, 1988.
64. Obermaier-Kusser B, Mühlbacher C, Mushack J, Seffer E, Ermel B, Machicao F, Schmidt F, Haring HU. Further evidence for a two-step model of glucose-transport regulation. Inositol phosphate-oligosaccharides regulate glucose-carrier activity. Biochem J **261**:699–705, 1989.

65. Vogt B, Mushack J, Seffer E, Haring HU. The phorbol ester TPA induces a translocation of the insulin sensitive glucose carrer (GLUT4) in fat cells. Biochem Biophys Res Commun **168:**1089–1094, 1990.
66. Farese RV, Standaert M, Yu B, Hernandez H, Cooper DR. 2-Hydroxypropyl-β-cyclodextrin enhances phorbol ester effects on glucose transport and/or protein kinase C-β translocation to the plasma membrane in rat adipocytes and soleus muscles. J Biol Chem **268:**19949–19955, 1993.
67. Kanai F, Nishioka Y, Hayashi H, Kamohara S, Ebina Y. Direct demonstration of insulin-induced GLUT4 translocation to the surface of intact cells by insertion of a c-*myc* epitope into an exofacial GLUT4 domain. J Biol Chem **268:**14523–14526, 1993.
68. Holman GD, Kozka IJ, Clark AE, Flower CJ, Saltis J, Habberfield AD, Simpson IA, Cushman SW. Cell surface labeling of glucose transport isoform GLUT4 by bis-mannose photolabel. Correlation with stimulation of glucose transport in rat adipose cells by insulin and phorbol ester. J Biol Chem **265:**18172–18179, 1990.
69. Saltis J, Habberfield AD, Egan JJ, Londos C, Simpson IA, Cushman SW. Role of protein kinase C in the regulation of glucose transport in the rat adipose cell. Translocation of glucose transport without stimulation of glucose transport activity. J Biol Chem **266:**261–267, 1991.
69a. Gibbs EM, Calderhead DM, Holman GD, Gould GW. Phorbol ester only partially mimics the effects of insulin on glucose transport and glucose-transporter distribution in 3T3-L1 adipocytes. Biochem J **275:**145–150, 1991.
70. Farese RV, Standaert ML, Barnes DE, Davis JS, Pollet RJ. Phorbol ester provokes insulin-like effects on glucose transport, amino acid uptake, and pyruvate dehydrogenase activity in BC3H-1 cultured myocytes. Endocrinology **116:**2650–2655, 1985.
71. Standaert ML, Farese RV, Cooper DR, Cooper RJ. Insulin-induced glycerolipid mediators and the stimulation of glucose transport in BC3H-1 myocytes. J Biol Chem **263:**8696–8705, 1988.
72. Christensen RL, Shade DL, Graves CB, McDonald JM. Evidence that protein kinase C is involved in regulating glucose transport in the adipocyte. Int J Biochem **19:**259–265, 1987.
73. Stralfors P. Insulin stimulation of glucose uptake can be mediated by diacylglycerol in adipocytes. Nature **335:**554–556, 1988.
74. Shillabeer G, Chamoun C, Hatch G, Lau DC. Exogenous triacylglycerol inhibits insulin-stimulated glucose transport in L6 muscle cells *in vitro*. Biochem Biophys Res Commun **207:**768–774, 1995.
75. Sowell MO, Boggs KP, Robinson KA, Dutton SL, Buse MG. Effects of insulin and phospholipase C in control and denervated rat skeletal muscle. Am J Physiol **260:**E247–E256, 1991.
76. Cleland PJF, Appleby GJ, Rattigan S, Clark MG. Exercise-induced translocation of protein kinase C and production of diacylglycerol and phosphatidic acid in rat skeletal muscle *in vivo*. J Biol Chem **264:**17704–17711, 1989.
77. Takuwa N, Takuwa Y, Rasmussen H. Stimulation of mitogenesis and glucose transport by 1-monooleoylglycerol in Swiss 3T3 fibroblasts. J Biol Chem **263:**9738–9745, 1988.
77a. Nishino H, Kitagawa K, Iwashima A, Ito M, Tanaka T, Hidaka H. N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide is one of a new class of activators for Ca^{++}-activated, phospholipid-dependent protein kinase. Biochim Biophys Acta **889:**236–239, 1986.
78. Haystead TA, Sim AT, Carling D, Honnor RC, Tsukitani Y, Cohen P, Hardie DG. Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature **337:**78–81, 1989.
79. Jullien D, Tanti JF, Heydrick SJ, Gautier N, Gremeaux T, Van Obberghen E, LeMarchand-Brustel Y. Differential effects of okadaic acid on insulin-stimulated glucose and amino acid uptake and phosphatidylinositol 3-kinase activity. J Biol Chem **268:**15246–15251, 1993.
80. Cherqui G, Caron M, Wicek D, Lascols O, Capeau J, Picard J. Decreased insulin responsiveness in fat cells rendered protein kinase C-deficient by a treatment with a phorbol ester. Endocrinology **120:**2192–2194, 1987.
81. Ishizuka T, Cooper DR, Arnold T, Hernandez H, Farese RV. Downregulation of protein kinase C and insulin-stimulated 2-deoxyglucose uptake in rat adipocytes by phorbol esters, glucose, and insulin. Diabetes **40:**1274–1281, 1991.
82. Tanti JF, Rochet N, Grémeaux T, Van Obberghen E, Le-Marchand-Brustel Y. Insulin-stimulated glucose transport in muscle. Evidence for a protein-kinase-C-dependent component which is unaltered in insulin-resistant mice. Biochem J **258:**141–146, 1989.
83. Van De Werve G, Zaninetti D, Lang U, Vallotton MB, Jeanrenaud B. Identification of a major defect in insulin-resistant tissues of genetically obese (*fa/fa*) rats. Impaired protein kinase C. Diabetes **36:**310–314, 1987.
84. Gibbs EM, Allard WJ, Lienhard GE. The glucose transporter in 3T3-L1 adipocytes is phosphorylated in response to phorbol ester but not in response to insulin. J Biol Chem **261:**16597–16603, 1986.
85. Kitagawa K, Nishino H, Iwashima A. Ca^{2+}-dependent stimulation of 3-*O*-methylglucose transport in mouse fibroblast Swiss 3T3 cells induced by phorbol-12,13-dibutyrate. Biochem Biophys Res Commun **128:**127–133, 1985.
86. Klip A, Ramlal T. Protein kinase C is not required for insulin stimulation of hexose uptake in muscle cells in culture. Biochem J **242:**131–136, 1987.
87. Farese RV, Standaert ML, Ishizuka T, Yu B, Hernandez H, Waldron C, Watson J, Farese JP, Cooper DR, Wickstrom E. Antisense DNA downregulates protein kinase C isozymes (β and α) and insulin-stimulated 2-deoxyglucose uptake in rat adipocytes. Antisense Res Dev **1:**35–42, 1991.
88. Cooper DR, Watson JE, Hernandez H, Yu B, Standaert ML, Ways DK, Arnold TT, Ishizuka T. Direct evidence for protein kinase C involvement in insulin-stimulated hexose uptake. Biochem Biophys Res Commun **188:**142–148, 1992.
89. Standaert ML, Sasse J, Cooper DR, Farese RV. Protein kinase C (19–31) pseudosubstrate inhibition of insulin action in rat adipocytes. FEBS Lett **282:**139–142, 1991.
90. Yamada K, Avignon A, Standaert ML, Cooper DR, Spencer B, Farese RV. Effects of insulin on the translocation of protein kinase C-θ and other protein kinase C isoforms in rat skeletal muscles. Biochem J **308:**177–180, 1995.
91. Standaert ML, Avignon A, Arnold T, Saba-Siddique SI, Cooper DR, Watson J, Zhou X, Galloway L, Farese RV. Insulin translocates PKC-ϵ and phorbol esters induce and persistently translocate PKC-β_2 in BC3H-1 myocytes. Cell Signal (in press), 1995.
92. Robinson LJ, Razzack ZF, Lawrence JC Jr., James DE. Mitogen-activated protein kinase activation is not sufficient for stimulation of glucose transport or glycogen synthase in 3T3-L1 adipocytes. J Biol Chem **268:**26422–26427, 1993.
93. Wiese RJ, Mastick CC, Lazar DF, Saltiel AR. Activation of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes. J Biol Chem **270:**3442–3446, 1995.
94. Lazar DF, Wiese RJ, Brady MJ, Mastick CC, Waters SB, Yamauchi K, Pessin JE, Cuatrecasas P, Saltiel AR. Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J Biol Chem **270:**20801–20807, 1995.
95. Kozma L, Baltensperger K, Klarlund J, Porras A, Santos E, Czech MP. The ras signaling pathway mimics insulin action on glucose transporter translocation. Proc Natl Acad Sci USA **90:**4460–4464, 1993.
96. Manchester J, Kong X, Lowry OH, Lawrence JC Jr. Ras signaling in the activation of glucose transport by insulin. Proc Natl Acad Sci USA **91:**4644–4648, 1994.
97. Reusch JE, Bhuripanyo P, Carel K, Leitner JW, Hsieh P, DePaolo D, Draznin B. Differential requirement for $p21^{ras}$ activation in the metabolic signaling by insulin. J Biol Chem **270:**2036–2040, 1995.
98. Sakaue H, Hara K, Noguchi T, Matozaki T, Kotani K, Ogawa W, Yonezawa K, Waterfield MD, Kasuga M. Ras-independent

and wortmannin-sensitive activation of glycogen synthase by insulin in Chinese hamster ovary cells. J Biol Chem **270:**11304–11309, 1995.

99. Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. J Biol Chem **269:**3568–3573, 1994.
100. Hara K, Yonezawa K, Sakaue H, Ando A, Kotani K, Kitamura T, Kitamura Y, Ueda H, Stephens L, Jackson TR. 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. Proc Natl Acad Sci USA **91:**7415–7419, 1994.
101. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis and glucose transporter translocation. Mol Cell Biol **14:**4902–4911, 1994.
102. Fingar DC, Hausdorff SF, Blenis J, Birnbaum MJ. Dissociation of pp70 ribosomal protein S6 kinase from insulin-stimulated glucose transport in 3T3-L1 adipocytes. J Biol Chem **268:**3005–3008, 1993.
103. Kotani K, Hara K, Kotani K, Yonezawa K, Kasuga M. Phosphoinositide 3-kinase as an upstream regulator of the small GTP-binding protein Rac in the insulin signaling of membrane ruffling. Biochem Biophys Res Commun **208:**985–990, 1995.