MINIREVIEW

Lipid-Derived Mediators in Insulin Action (43150C)

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From a pragmatic viewpoint, insulin is probably the most important hormone known to man. Diabetes mellitus, which is due to either a lack of, or peripheral resistance to, insulin, afflicts at least 5% of the general population, and diabetes mellitus is a major cause of blindness, end stage renal disease, leg amputations, and a variety of atherosclerotic cardiovascular disorders. In addition, in more subtle ways, insulin resistance may be an important contributor to states of obesity and hypertension.

It is becoming increasingly clear that type II diabetes mellitus, the most frequently occurring variety of diabetes, is largely due to resistance to peripheral effects of insulin. Therefore, a clear understanding of the mechanism of action of insulin is of paramount importance for understanding the pathogenesis of type II diabetes mellitus. Furthermore, as recent evidence suggests that resistance to insulin in this disorder is largely due to a postbinding defect(s), probably at, or somewhere between the insulin receptor and the glucose transporter, it is likely that disorders of intracellular signaling lie at the heart of clinical problems of insulin resistance.

For the past two decades, there have been important advances in our understanding of the structure and function of the insulin receptor. As expected, specific, molecular, genetic defects in the insulin receptor have been shown to result in peripheral insulin resistance. However, these genetic defects have been documented only in rare instances, and it is uncertain whether abnormalities of the insulin receptor will be uncovered in more common syndromes of insulin resistance, such as type II diabetes mellitus and obesity. The importance of tyrosine kinase activity of the insulin receptor, which is responsible for receptor autophosphorylation and phosphorylation of extrareceptor proteins, is still an unsettled issue. To be certain, some findings suggest that tyrosine kinase activity is essential for receptor-mediated activation of certain insulin-sensitive processes (1, 2). On the other hand, it appears that the insulin receptor can be activated (by antibodies) to provoke insulin-like responses in the absence of tyrosine kinase activation and autophosphorylation (3). In addition, it has been found that addition of insulin to plasma membrane preparations leads to the release of insulin mediators (see below) in the absence of added ATP, and, therefore, possibly in the absence of tyrosine kinase activation. Suffice it to say, more work will be required to elucidate the importance of tyrosine kinase activity of the insulin receptor as a signaling mechanism for specific actions of insulin.

During the past decade, there have been a number of breakthroughs which indicate that many hormones rapidly perturb phospholipid metabolism to produce intracellular signaling substances. Most notably, hydrolysis of phosphatidylinositol 4′,5′-bisphosphate (PIP_2) to yield (i) inositol 1′,4′,5′-trisphosphate, which mobilizes intracellular Ca^{2+} (4), and (ii) diacylglycerol, which activates protein kinase C (5), have been shown to occur in the actions of many hormones, particularly those which mobilize intracellular Ca^{2+} as a more conspicuous aspect of their mechanisms of action. However, it has also been realized that diacylglycerol, which is derived from sources other than PIP_2 hydrolysis, including both phosphatidylcholine hydrolysis (6) and *de novo* phosphatidic acid synthesis (7–9), can also activate protein kinase C. It therefore appears that there are multiple mechanisms to activate protein kinase C, and this activation can occur in the absence of Ca^{2+} mobilization.

In addition to hydrolysis of PIP_2 to yield inositol 1′,4′,5′-trisphosphate, there is recent evidence which suggests that some hormones hydrolyze a glycolipid, which has tentatively been identified as a phosphatidylinositol-glycan (PI-glycan) (10), and it appears that

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phospho-inositol-carbohydrate-containing head groups from these glycolipids are potent activators of various enzymes (10, 11), possibly through a phosphatase mechanism (12). The PI-glycan head groups (herein called "head group mediators" appear to be particularly prominent in the action of insulin, and it appears that insulin has the ability both to stimulate (see below) the phosphorylation of certain proteins through activation of protein kinase C (13, 19) and other serine/threonine kinases (20–24), and, on the other hand, to dephosphorylate another set of proteins by a phosphatase mechanism. On the whole, the phosphorylations provoked through tyrosine kinase, protein kinase C, and other kinases exceed the dephosphorylations which are thought to be provoked by head group mediators, such that the overall effect favors that of phosphorylation (25). Nevertheless, with respect to specific enzymatic functions, phosphorylation and dephosphorylation effects are site specific, and both mechanisms are probably equally important in the action of insulin.

Although insulin had been known to provoke increases in phospholipid turnover (including PI turnover) (26–28), it was not clear whether these increases reflected changes in primary synthesis and/or hydrolytic turnover and secondary resynthesis of phospholipids. As most investigators had focused upon hormones which increased hydrolytic turnover of PIP_2 and PI, it was at first thought that insulin also worked through this mechanism. However, it became apparent that insulin increased the mass of inositol-lipids (29–31), suggesting that the phospholipid effects of insulin were much different from those of hormones which primarily stimulate PIP_2 and PI hydrolysis. In fact, at a very early stage, it was realized that insulin either did not stimulate the hydrolysis of PIP_2 and PI in certain cells (31, 32), or provoked only small or transient increases in apparent PIP_2 and/or PI hydrolysis in other cells, such as rat adipocytes (33, 34). On the other hand, it was also clear that insulin increased the production of diacylglycerol (30, 31). Subsequently, it was demonstrated that insulin increases (i) *de novo* phosphatidic acid synthesis (35, 36) and (ii) the apparent hydrolysis of PI-glycan (10) and phosphatidylcholine (37). Each of these phospholipid effects of insulin generate diacylglycerol, and it is therefore not surprising that many investigators have documented that insulin increases the activity and/or translocation of protein kinase C. On the other hand, the only mechanism for increasing head group mediators appears to be hydrolysis of the PI-glycan and/or associated glycolipids. A major quest has been to understand how these phospholipid effects of insulin are interrelated, how they are stimulated, and the relationship between phospholipid-derived intracellular signaling substances and specific metabolic effects of insulin. In this review, a discussion of each of these phospholipid effects of insulin, both separately and integrated with each other, is presented. I also discuss the potential role of G proteins in transducing these phospholipid effects of insulin. Finally, an attempt to relate the dual effects of insulin on diacylglycerol-protein kinase C signaling and head group mediators signaling to specific metabolic effects of insulin is made.

De Novo Phospholipid Synthesis Effect of Insulin

The *de novo* phospholipid synthesis effect of insulin will be discussed first, not because it is more important than other phospholipid effects of insulin, but rather, because it is presently the most fully studied and best understood phospholipid effect of insulin. To begin with, increases in the synthesis and contents of inositol-phospholipids and other phospholipid during insulin action (29–32) provided the first clue that insulin has rapid effects on the *de novo* phospholipid synthesis pathway. As revealed in subsequent studies of [^{3}H] glycerol incorporation into lipids in BC3H-1 myocytes (35, 36), this effect of insulin was shown to specifically involve the initial step in phospholipid synthesis, namely, the production of phosphatidic acid from glycerol 3-phosphate and fatty acyl-CoA (35, 36, 38).

Increased incorporation of [^{3}H]glycerol into lipids during insulin action has now been documented in a number of cell types, including rat adipocytes (38), rat diaphragm and soleus muscles (18), rat hepatocytes (39), 3T3 cells (40), and EJ-H-*ras*-transformed cells (41). Clearly, this effect of insulin appears to be widespread and is activated within seconds (35, 36) of hormone stimulation. In addition, most of the newly synthesized phosphatidic acid is directly converted to diacylglycerol and subsequently to phosphatidylcholine, as well as triacylglycerol, which is stored. In most tissues, relatively little of the newly synthesized phosphatidic acid is directly converted to inositol-lipids, but, as discussed below, there are large increases in inositol-lipids, which appear to be derived from sources (e.g., phosphatidylcholine) other than newly synthesized phosphatidic acid. In any event, it would appear that the increase in *de novo* phospholipid synthesis contributes importantly to increases in diacylglycerol during insulin action, and studies in a variety of tissues (17–19) have provided convincing evidence that diacylglycerol derived from the *de novo* pathway is capable of activating protein kinase C. Along these lines, it should be realized that in tissues (e.g., adipocytes, hepatocytes, islet β cells, etc.) in which there is ready uptake of glucose and conversion to diacylglycerol, it is possible that glucose alone can stimulate diacylglycerol-protein kinase C signaling. On the other hand, in some tissues, glucose uptake is relatively slow, and/or may not be attended by increases in diacylglycerol and activation of protein kinase C. The latter appears to be the case in skeletal muscle (unpublished observations), which is

the major target organ for insulin-stimulated glucose utilization.

Insulin appears to activate *de novo* phosphatidic acid synthesis through an increase in the activity of glycerol-3-phosphate acyltransferase. In recent studies in BC3H-1 myocytes (42) and adipocytes (unpublished observations), we have found that insulin provokes a two-fold increase in the activity of the acyltransferase, apparently through a decrease in its K_m. Interestingly, the activation of this enzyme by insulin appears to require a pertussis toxin-sensitive G protein (Giα) and activation of a phospholipase C which hydrolyzes a PI-glycan or other glycolipid (42). Activation of glycerol-3-phosphate acyltransferase has been shown to occur in both intact and membrane preparations of BC3H-1 myocytes (42), rat adipocytes (unpublished observations), and rat skeletal muscle plasma membrane preparations (43). In cell-free systems, the enzyme is activated not only by insulin, but also by PI-specific phospholipase C (42). Moreover, the acyltransferase activation effects of insulin can be blocked, not only by pertussis toxin pretreatment, but also (in the cell-free systems) by antibodies to Giα and PI-glycan phospholipase C (42). The activation of the acyltransferase by insulin and PI-specific phospholipase C involves the release from membranes of a soluble factor having a molecular weight of <5000. Presumably, this stimulator of glycerol-3-phosphate acyltransferase is comparable to other "insulin mediators" (10, 12, 44), which are known to be released from membranes directly by insulin. As stated above, ATP is not required for these mediator-releasing effects of insulin on cell membranes, and it is therefore questionable whether receptor-mediated phosphorylation mechanisms are involved in the release of these head group mediators. Interestingly, it has recently been shown that insulin is capable of interacting with Giα by a mechanism which does not involve tyrosine phosphorylation (45): perhaps this interaction serves as the underlying mechanism for activation of the phospholipase C which hydrolyzes a PI-glycan or other glycolipid.

Hydrolysis of a PI-Glycan and/or Other Glycolipids

Although insulin increases PI turnover, as evidenced by studies of ^{32}P metabolism, as stated above, it soon became clear that insulin provoked no appreciable (31, 32, 38), or, at most, a small or transient activation (34, 35, 46) or a phospholipase C which hydrolyzes PIP_2 and/or PI. On the other hand, it has become apparent that insulin stimulates a phospholipase C, which hydrolyzes an apparent inositol-containing glycolipid (10, 11). The latter observation has been interpreted to suggest that insulin stimulates the hydrolysis of a PI-glycan (10), which is analogous to a lipid which serves as an anchor for a number of membrane-associated proteins (44). Accordingly, the addition of insulin and/or PI-specific phospholipase C to both intact tissues and membrane preparations has been shown to result in the release of biologically active insulin mediators, which apparently contain phosphate, inositol, glucosamine, and mannose (10, 11, 44, 47). It has therefore been suggested that a phospho-inositol-carbohydrate (glycan) moiety is released by action of an insulin-sensitive phospholipase C. Moreover, it has been found that activation of this phospholipase and hydrolysis of an inositol-glucosamine-containing glycolipid are inhibited by pertussis toxin pretreatment (48), and it would appear that a pertussis toxin-sensitive G protein serves to couple the insulin receptor to this phospholipase C. Obviously, this latter finding would fit well with our findings indicating that *de novo* phospholipid synthesis is activated by a PI-glycan phospholipase C, which, in turn, requires Giα (see above). As will be discussed further below, these head group mediators seem to explain a number of diverse effects of insulin, which have been postulated to be stimulated through a mechanism involving a phosphatase (12). In addition, hydrolysis of the PI-glycan itself yields diacylglycerol (49), or 1-ether analogues of diacylglycerol (11), in the plasma membrane. In some systems, only the ether analogues have been documented, whereas in other systems, it appears that a true diacylglycerol is produced. Only the true *sn*-1,2-diacylglycerols have the ability to activate protein kinase C, and, indeed, the 1-ether analogus of diacylglycerol may, in certain conditions, inhibit protein kinase C. Nevertheless, the findings on the acyltransferase (see above) suggest that activation of *de novo* phospholipid synthesis occurs secondarily to the release of head group mediators, and it would therefore appear that, regardless of whether or not diacylglycerol is generated directly from the PI-glycan, increases in diacylglycerol may occur through stimulation of the *de novo* phosphatidic acid synthesis pathway. Thus, the coupling of PI-glycan hydrolysis to *de novo* phosphatidic acid synthesis may provide a mechanism to amplify diacylglycerol-protein kinase C signaling, not only in the plasma membrane, but possibly in the endoplasmic reticulum as well, the site of most insulin-sensitive glycerol-3-phosphate acyltransferase.

It should be noted that there have been a number of differences in the chemical composition of substances which have been reported to be derived from PI-glycan-like lipids (10, 11, 47). This suggests that there are multiple molecular species of glycolipids which are hydrolyzed during insulin action. It would seem unlikely however, that there are multiple phospholipase C (or D) types that are responsible for effects of insulin on glycolipid hydrolysis. Whether or not all of the biologic functions which have been suggested to be stimulated by head group mediators are provoked by the same or different molecular forms of head group

mediators remains to be determined. Unfortunately, progress in this area has been slowed by the fact that it has been difficult to procure large amounts of putative headgroup mediators for structural analysis and correlation to biologic function.

Synthesis of Inositol-Phospholipids

Increases in inositol-phospholipid synthesis have been documented in BC3H-1 myocytes (30, 31) and rat adipocytes (29, 32, 38). Increases in the levels of PI and polyphosphoinositides, PIP and PIP_2, have been found, and this may reflect a decrease in hydrolytic turnover and/or an increase in synthesis. As alluded to above, insulin has not been found to increase hydrolysis of PIP_2 in certain cells (31) and in rat adipocytes, which are frequently used tissue for studies of insulin action, small and/or transient increases have been observed by several (33, 34, 46), but not all (32, 38), groups. Clearly, increased hydrolysis of PIP_2, PIP, and PI are not prominent phospholipid effects of insulin and do not seem likely to explain observed sustained increases in diacylglycerol production. Nevertheless, it appears that there are no apparent decreases in inositol-phosphate production, and observed increases in the levels of, or labeled precursor incorporation into, inositol-phospholipid (29–34, 48) seem to be explained by increased synthesis. Increased synthesis of inositol-phospholipids could conceivably occur through the pathway of *de novo* phosphatidic acid synthesis, or from hydrolysis of phospholipids, such as phosphatidylcholine, which may give rise to large amounts of diacylglycerol, phosphatidic acid, and ultimately PI and other inositol-phospholipid. In the BC3H-1 myocyte, it seems clear that insulin effects on *de novo* phosphatidic acid synthesis cannot fully explain increases in PI synthesis (35, 36), but, more recently, we have shown that hydrolysis of phosphatidylcholine can serve as a major source of PI (unpublished observations). Thus, since insulin stimulates phosphatidylcholine hydrolysis (see below), it seems likely that insulin-induced increases in inositol-lipid synthesis occur, at least partly, at the expense of preexisting phosphatidylcholine. On the other hand, in rat adipocytes, it appears that newly synthesized phosphatidic acid is also an important source of PI (38). In either event, PI may be metabolized to PIP, PIP_2, and the PI-glycan (50).

Effects of Insulin on Phosphatidylcholine Hydrolysis

Increases in labeled phosphocholine have been observed during insulin action in BC3H-1 myocytes (37), and it was suggested that this reflects hydrolysis of phosphatidylcholine by a specific phospholipase C. More recently, my laboratory found that phosphatidylcholine mass transiently decreased by approximately 10–20% within 30 sec of addition of insulin to either BC3H-1 myocytes or rat adipocytes (unpublished observations). Soon after this transient decrease, phosphatidylcholine mass rapidly returned to control or slightly elevated levels, and the latter resynthesis appears to reflect activation of the *de novo* pathway. Interestingly, pertussis toxin blocked the *de novo* pathway (42) (presumably by inhibition of PI-glycan hydrolysis [48] and consequent activation of glycerol-3-phosphate acyltransferase by PI-glycan-derived head group mediators, see above), but did not block the insulin effect on phosphatidylcholine hydrolysis (unpublished observations). Thus, with pertussis toxin, phosphatidylcholine levels rapidly decreased and remained at diminished levels because of apparent failure to resynthesize this lipid from the *de novo* pathway. These findings suggest that insulin effects on phosphatidylcholine hydrolysis are fully separable from insulin effects on PI-glycan hydrolysis and *de novo* phosphatidic acid synthesis. Thus, whereas Giα seems to be involved in PI-glycan hydrolysis and *de novo* phosphatidic acid synthesis, there is no present evidence to suggest involvement of this G protein in phosphatidylcholine hydrolysis. Nevertheless, further studies are required to investigate the role of G proteins in coupling the insulin receptor to phosphatidylcholine hydrolysis, as GTP-dependent proteins may stimulate phosphatidylcholine hydrolysis (51).

The effects of insulin on phosphatidylcholine hydrolysis have been found to be independent of protein kinase C activation (unpublished observations). Phorbol esters are, in fact, capable of stimulating phosphatidylcholine hydrolysis in BC3H-1 myocytes and rat adipocytes by a protein kinase C-dependent mechanism, but the effects of insulin on phosphatidylcholine hydrolysis in these cells seem to be mediated through a different mechanism, as they are insensitive to inhibitors of protein kinase C (unpublished observations).

Hydrolysis of phosphatidylcholine would be expected to contribute to increases in diacylglycerol and activation of protein kinase C in insulin action. Accordingly, in BC3H-1 myocytes, where it has been possible to inhibit PI-glycan hydrolysis and *de novo* phosphatidic synthesis by pertussis toxin pretreatment, initial increases in diacylglycerol production during insulin action appear to be intact, but increases after 1 min are diminished, although still evident. This may have bearing upon the fact that some responses to insulin, such as glucose transport in the some cell lines of BC3H-1 myocyte (48), are partially, but not fully, inhibited by pertussis toxin treatment. It is possible that those glucose transport effects of insulin which are resistant to pertussis toxin may reflect phosphatidylcholine hydrolysis as being a major source of diacylglycerol and subsequent protein kinase C activation.

Role of G Proteins in Phospholipid Effects of Insulin

Pertussis toxin has been shown to inhibit insulin-induced hydrolysis of an apparent PI-glycan in BC3H-

1 myocytes (48), and, more recently, my laboratory has found that insulin-induced activation of glycerol-3-phosphate acyltransferase and *de novo* phosphatidic acid synthesis are inhibited by pertussis toxin and/or antibodies to Giα (42). This suggests that a pertussis toxin-sensitive Giα is involved in both phospholipid effects of insulin (i.e., PI-glycan hydrolysis and *de novo* phosphatidic acid synthesis) in BC3H-1 myocytes. Furthermore, since *de novo* phosphatidic acid synthesis seems to be a consequence of PI-glycan hydrolysis (see above), it seems likely that the required G protein serves to couple the insulin receptor to the phospholipase C which hydrolyzes the PI-glycan and/or other glycolipids during insulin action: thus, inhibition of the *de novo* pathway by pertussis toxin probably occurs only as a secondary response. This secondary inhibition of the *de novo* pathway would further explain why pertussis toxin inhibits later (after 1 min) increases in (i) diacylglycerol content (unpublished observations) and (ii) increases in both myristoyl-labeled diacylglycerol and arachidonyl-diacylglycerol (48) during insulin action. (Myristoyl-diacylglycerol may derive from PI-glycan hydrolysis [49], phosphatidylcholine hydrolysis and the *de novo* pathway [36 and unpublished observations], whereas arachidonyl-diacylglycerol apparently derives from phosphatidylcholine hydrolysis and the *de novo* pathway, but not from PI-glycan hydrolysis [36 and unpublished observations]).

As stated above, insulin stimulates the hydrolysis of PI-glycan or other glycolipids in cell-free systems, and it has also been found that insulin inhibits the ADP-ribosylation of a Giα-protein *in vitro* in the absence of insulin receptor tyrosine kinase activation (45). These findings suggest that the insulin receptor may directly influence G proteins, which, in turn, may be important in coupling the receptor to phospholipases. However, it remains to be determined whether or not GTP, or its nonhydrolyzable analogues, is (are) capable of provoking insulin-like effects on PI-glycan hydrolysis and/or glycerol-3-phosphate acyltransferase activation.

As stated above, in contrast to PI-glycan hydrolysis and *de novo* phosphatidic acid synthesis, pertussis toxin does not inhibit insulin-stimulated phosphatidylcholine hydrolysis (unpublished observations). This suggests that the latter effect of insulin is not mediated by a pertussis toxin-sensitive G protein, and is fully separable from effects of insulin on PI-glycan hydrolysis and *de novo* phosphatidic acid synthesis. Thus, there are multiple mechanisms, both pertussis toxin sensitive and insensitive, whereby insulin may increase diacylglycerol production. Accordingly, we found that the early (30-sec) increase in diacylglycerol production in BC3H-1 myocytes was not blocked by pertussis toxin treatment, whereas later increases were partially inhibited (unpublished observations). This suggests that phosphatidylcholine hydrolysis contributes very importantly to the initial increase in diacylglycerol, but that latter increases are more dependent upon PI-glycan hydrolysis and the *de novo* phosphatidic acid synthesis pathway, the latter replenishing phosphatidylcholine during insulin action. This partial dependence of insulin-stimulated diacylglycerol production on pertussis toxin-sensitive G proteins (apparent) may have bearing upon the fact that, in some experimental conditions (48), insulin effects on glucose transport in BC3H-1 myocytes may be partially inhibited by pertussis toxin. On the other hand, in our laboratory, inhibitory effects of pertussis toxin on insulin-stimulated glucose transport have not been observed, and it appears that our lines of BC3H-1 myocytes have sufficient diacylglycerol production through phosphatidylcholine hydrolysis to satisfy the requirement for activation of protein kinase C and glucose transport, or, alternatively, it may be argued that glucose transport effects of insulin are not dependent upon diacylglycerol production and protein kinase C activation. (We have, in fact, found that insulin activates [translocates] protein kinase C in our BC3H-1 myocytes, regardless of pertussis toxin treatment.) In rat adipocytes, pertussis toxin has been reported to provoke no apparent changes (52), or decreases in sensitivity, or partial decreases in responsiveness of the glucose transport system (53) to insulin. Again, this could reflect different experimental conditions and different dependency of protein kinase C activation on various mechanisms for diacylglycerol production in these cell preparations, or, alternatively, there may be multiple mechanisms for activating glucose transport. Although the effects of pertussis toxin on glucose transport in rat adipocytes have been variable, certain effects of insulin, that have been attributed to head group mediators derived from the PI-glycan, have been reported to be inhibited by pertussis toxin, e.g., inhibition of stimulated lipolysis (54, 55), inhibition of adenylatecyclase activation (56), dense particle cAMP phosphodiesterase activation (56), and glycerol-3-PO_4 acyltransferase activation (42). These findings fit well with the idea that a pertussis toxin-sensitive Giα is required to couple the insulin receptor to the phospholipase C, which hydrolyzes the PI-glycan and thereby stimulates the release of head group mediators, that are responsible for activation or deactivation of the enzymes, mentioned above. This concept also fits well with the observation that insulin-induced increases in inositol-lipids (PI) are potentiated by pertussis toxin in rat adipocytes (57): the latter may be explained by the fact that phosphatidylcholine hydrolysis (as a source of PI, see above) is not inhibited by pertussis toxin, whereas pertussis toxin inhibits hydrolysis (degradation) of the PI-glycan, and, therefore, may diminish hydrolytic losses of PI as well (see above). Clearly, further work is required to evaluate the hypothesis that insulin activates two par-

allel phospholipid-signaling pathways by mechanisms involving G proteins.

Integration of Phospholipid Effects of Insulin

It is possible to integrate several of the above-described phospholipid effects of insulin into a "cycle," which serves to replenish phospholipids that are hydrolyzed during the action of insulin. In this postulated cycle (Fig. 1), insulin activates a pertussis toxin-sensitive Giα and, consequently, a specific phospholipase C, which hydrolyzes a PI-glycan. Head groups released from the PI-glycan apparently increase *de novo* phosphatidic acid synthesis through activation of glycerol-3-phosphate acyltransferase. *De novo* phosphatidic acid synthesis serves to replenish phosphatidylcholine, which is hydrolyzed by an insulin-sensitive, but pertussis toxin-insensitive phospholipase C or D, in parallel to hydrolysis of the PI-glycan. Phosphatidylcholine hydrolysis, in turn, seems to be a major source of diacylglycerol or phosphatidic acid for replenishing PI, which may be rapidly converted to the PI-glycan (50). As compared with the PIP_2 hydrolysis cycle (4), this cycle, which appears to be operative during insulin action, appears to be convoluted and unduly complex. On the other hand, it is becoming increasingly clear that PIP_2 hydrolysis rarely, if ever, occurs as an isolated phenomenon, and is frequently associated with hydrolysis of phosphatidylcholine and/or *de novo* phosphatidic acid synthesis, and/or PI-glycan hydrolysis. Thus, it is likely that multiple, seemingly divergent, phospholipid effects are integrated to produce signaling substances and regenerate phospholipid species during the action of many hormones. In other words, insulin's effects on phospholipid metabolism may be no more complex than those of other hormones.

It should be reemphasized that insulin may increase diacylglycerol production by at least three mechanisms (PI-glycan hydrolysis, *de novo* phosphatidic acid synthesis, phosphatidylcholine hydrolysis) and the diacylglycerol derived from each of these mechanisms may activate protein kinase C. On the other hand, certain of these effects on diacylglycerol may occur in specific subcellular compartments, and it seems likely that insulin increases diacylglycerol, both in the plasma membrane and the endoplasmic reticulum (presently there is no information concerning insulin-induced changes in nuclear diacylglycerol). In addition, there may be multiple molecular species of diacylglycerol that have different abilities to activate various protein kinase C isozymes. Indeed, considering the multiple subcellular compartments in which diacylglycerol and protein kinase C may be activated, the growing list of protein kinase C isozymes, and the diverse consequences of protein kinase C activation, diacylglycerol-protein kinase C signaling is of great potential importance for explaining a variety of insulin effects.

Activation of Protein Kinase C during Insulin Action

It would be a gross understatement to say that there has been considerable controversy concerning the question of whether insulin activates protein kinase C. As suggested above, it would be expected that increases in diacylglycerol during insulin action should activate protein kinase C. Nevertheless, initial studies of insulin action in adipocytes (58), hepatocytes (59), and BC3H-1 myocytes (60) failed to reveal effects of insulin on the apparent translocation of protein kinase C from the cytosol to the membrane fraction, a process which is generally considered to reflect membrane-associated diacylglycerol, causing recruitment and activation of the enzyme. On the other hand, increases in the activity of both membrane-bound and cytosolic protein kinase C were observed by several groups in BC3H-1 myocytes (13), rat diaphragm (14), and rat adipocytes (16). These

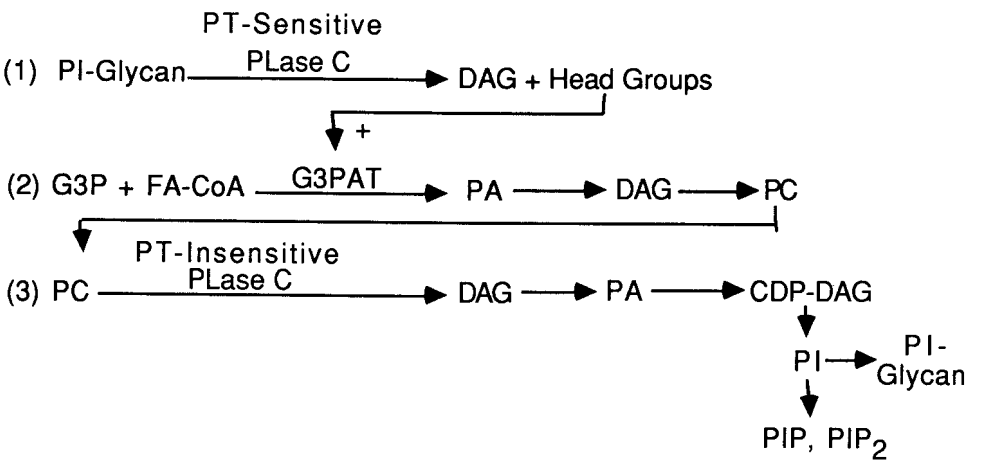

Figure 1. Integration of phospholipid effects of insulin. DAG, diacylglycerol; G3P, glycerol-3-PO_4; FA-COA, fatty acyl-coenzyme A; G3PAT, glycerol-3-PO_4 acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; CDP-DAG, cytidine diphosphate-diacylglycerol; PI, phosphatidylinositol; PIP and PIP_2, mono- and diphosphorylated derivatives of PI.

increases in enzyme activity were observed in extracts, which were either unpurified or purified by DEAE-Sephacel column chromatography. Unfortunately, this enzyme may not be sufficiently purified from "contaminating" diacylglycerol or other factors in the cytosol fraction (61), and an insulin-stimulated increase in diacylglycerol or other activating "cytosolic" factors may mask decreases in cytosolic enzyme content, that would otherwise be apparent during translocative activation of protein kinase C.

To add further confusion, many groups have compared the effects of insulin with those of phorbol esters, which invariably provoke simple translocation of protein kinase C from cytosol to plasma membrane. Unfortunately, the use of phorbol esters as a yardstick to determine whether or not insulin has activated protein kinase C has many caveats, including the following: phorbol esters primarily localize in the plasma membrane and only provoke activation patterns involving translocation to the plasma membrane; increases in the diacylglycerol which "contaminate" cytosolic fractions (61) (and thus may mask decreases in protein kinase C content) may occur during stimulation by hormones, but not by phorbol esters; and cytosolic protein kinase C activity measured in assays of histone phosphorylation may be masked by phorbol ester treatment, causing an exaggerated or overestimated loss of enzyme activity (62). Thus, it may have been unreasonable to expect that insulin effects on protein kinase C would necessarily be identical to those of phorbol esters.

In the case of insulin, in BC3H-1 myocytes (61, 63), rat adipocytes (17), rat soleus muscle (18), and in more recent studies (unpublished data) of H_4 hepatoma cells and vascular smooth muscle cells, we have been able to discern typical translocation patterns, providing that protein kinase C is assayed either by immunoblotting or enzymatically after Mono Q column chromatography purification. Apparently, Mono Q column chromatography removes diacylglycerol (and/or possibly other endogenous stimulators) more effectively than DEAE-Sephacel column chromatography (61), and it would appear that Mono Q column chromatography allows the protein kinase C assay to better reflect changes in enzyme content of the cytosol and/or membrane fraction, rather than activation of the enzyme by diacylglycerol and/or other endogenous factors. In agreement with our findings, another group has also found that insulin stimulates the translocation of protein kinase C to membranes in rat adipocytes (19).

In the case of rat skeletal muscle, studies of the effects of insulin on protein kinase C have yielded conflicting results. Two groups have observed insulin-induced increases membrane protein kinase C enzyme activity (14, 18), and apparent translocation has been observed in rat soleus using immunoblotting and enzyme activity measurement after Mono Q column chromatography to assess changes in protein kinase C (18). On the other hand, another group recently failed to observe increases in activity and/or translocation of protein kinase C in rat skeletal muscle (64). In the latter study, protein kinase C was assayed after DEAE-Sephacel column chromatography, and this method may not be optimal for showing decreases (reflective of translocation) in cytosolic protein kinase C. Obviously, further work will be required to resolve these controversies. Nevertheless, an increasing number of findings suggest that insulin activates protein kinase C.

Effects of Insulin Attributed to Head Group Mediators

A number of actions of insulin have been suggested to be due to release of head groups from a PI-glycan and/or other glycolipids during insulin action. These biologic functions include inhibition of adenylate cyclase (12, 65, 66, 67), activation of low K_m cAMP phosphodiesterase (10, 65, 67), inhibition of cAMP-dependent protein kinase A (66, 67), activation of glycogen synthase (12, 66), activation of glycerol-3-phosphate acyltransferase (42, 43), activation of acetyl-CoA carboxylase (12), inhibition of phospholipid methyltransferase (68), activation of pyruvate kinase (67), and activation of pyruvate dehydrogenase (65, 69). Many of these enzymes are responsible for intracellular glucose utilization and lipid synthesis. On the other hand, several studies have failed to show effects of head group mediators on glucose transport (69, 70), although it may be questioned whether these head group mediators are capable of entry into intact cells. (In adipocytes, it has been postulated that these head group mediators are taken up [69, 70].) Presumably, these head group mediators are the same insulin mediators which had previously been shown to be released from plasma membranes directly by insulin (see above). In most cases, the exact chemical composition of the mediator, which is responsible for altering the activities of these insulin-sensitive enzymes or transport systems, has not been determined. Thus, it is possible that there may be multiple mediators with specific biologic functions, or, alternatively, these mediators may be one and the same, or sufficiently alike chemically, to activate these enzymes through a common mechanism. Accordingly, it has been suggested that these mediators operate through activation of a phosphatase(s), which activates or inhibits enzymes through dephosphorylation (12). Again, the precise role of these mediators and their mechanism of activation remain to be fully established.

Importance of Diacylglycerol-Protein Kinase C Signaling during Insulin Action

Even accepting (see above) that insulin activates protein kinase C in its target tissues, the importance of this activation is presently uncertain. Clearly, activation

of protein kinase C by phorbol esters (71–73) and many other substances (74) has been found to result in stimulation of glucose transport. It would therefore seem logical to postulate that insulin-induced activation of protein kinase C results in the activation of glucose transport in its target tissue. However, several lines of evidence suggest the opposite conclusion. For example, although phorbol esters are potent activators of protein kinase C, the effects of phorbol esters on activation of glucose transport is considerably less than that provoked by insulin in some (72, 73), but not all (71), tissues. For example, in adipocytes, phorbol esters increase glucose transport only 10–30% as effectively as insulin, and it has been suggested by Haring's group that there may be a two-step activation of glucose transport, one dependent upon, and one independent of, protein kinase C (75). It has further been postulated that glucose transporter translocation may be the dependent step, since there appeared to be a relatively greater (or nearly full) effect of phorbol esters on this process than on overall glucose transport activity (75). More recently, Haring's group (76) observed a large, albeit not full (relative to insulin), effect of phorbol esters on specific translocation of the "Glut 4" glucose transporter. Similarly, Simpson's group (77) observed partial insulin-like effects of phorbol esters on specific Glut-4 glucose transporter translocation and, in addition, insulin appeared to have effects on glucose transport which were not readily explained by translocation of Glut-4 glucose transporters. These findings provide further evidence that insulin activates glucose transport by two processes, namely, translocation of the glucose transporter, which may be largely or partly protein kinase C dependent, and a second effect on activation of the translocated glucose transporter by a mechanism independent of protein kinase C.

The two-step hypothesis, while attractive, is largely based upon the assumption that phorbol ester-induced activation of protein kinase C would necessarily stimulate the glucose transport process with the same efficiency as diacylglycerol-induced activation of protein kinase C, which occurs during insulin action. Unfortunately, given the fact that phorbol ester-induced activation of protein kinase C has frequently been reported to differ from that of diacylglycerol-induced activation of the enzyme (78–86), it is presently uncertain whether this assumption is correct. Accordingly, it is uncertain if there are mechanisms for activating glucose transport during insulin action, other than those dependent upon diacylglycerol-protein kinase C signaling.

The suggestion that G proteins may directly activate glucose transport (87), independently of protein kinase C, is now clouded by the realization that G proteins are also important for activating phospholipid-signaling pathways during insulin action (see above). Thus, it is possible that the perturbations of G proteins may influence glucose transport through changes in phospholipid metabolism, diacylglycerol production, and protein kinase C activation, rather than through direct effects of G proteins on the glucose transporters. Indeed, some groups have reported that diacylglycerol alone can produce full insulin-like effects on glucose transport (88, 89), suggesting that it is not necessary to postulate the operation of other signaling factors. Along these lines, it has also been suggested that okadeic acid, which inhibits phosphatases and thereby potentiates the phosphorylation effects of serine/threonine protein kinases, can also provoke full insulin-like effects on glucose transport in rat adipocytes (90). These latter two findings are in keeping with (but do not prove) the postulate that diacylglycerol-protein kinase C signaling may play a dominant role during insulin-stimulated glucose transport. It is also possible that there may be redundancy, and one or more insulin-sensitive serine/threonine kinases, in addition to protein kinase C, may be capable of stimulating glucose transport.

There have been many other attempts to evaluate the role of diacylglycerol-protein kinase C signaling during insulin-stimulated glucose transport. Studies with a variety of inhibitors of protein kinase C (Table I), including sphingosine (91, 92), staurosporine (93), H-7 (93), sangivamycin (93), acridine orange (94), polymixin B (18, 95), and a tetradecapeptide pseudosubstrate, which binds to and thereby inhibits the substrate-binding site of protein kinase C (unpublished observations), have consistently shown that inhibition of protein kinase C concomitantly inhibits both insulin- and phorbol ester-stimulated glucose transport. As with all inhibitor studies, there may be nonspecific effects on signaling systems other than the diacylglycerol-protein kinase C system. On the other hand, in studies with H-7, sangivamycin, and staurosporine, we have found that these inhibitors did not inhibit insulin effects on (i) *de novo* phosphatidic acid synthesis (93) (presumably reflecting intactness of insulin receptor function, PI-glycan hydrolysis, and subsequent activation of glyc-

Table I. Agents Which Inhibit or Deplete Protein Kinase C and Inhibit Insulin-Stimulated Glucose Transport in Rat Adipocytes

Protein kinase C inhibitors	Protein kinase C depletors
Sphingosine	Phorbol esters
Staurosporine	Insulin
H-7	Glucose
Sangivamycin	Protein kinase C antisense
Acridine orange	
Polymixin B	
Protein kinase C pseudosubstrate	

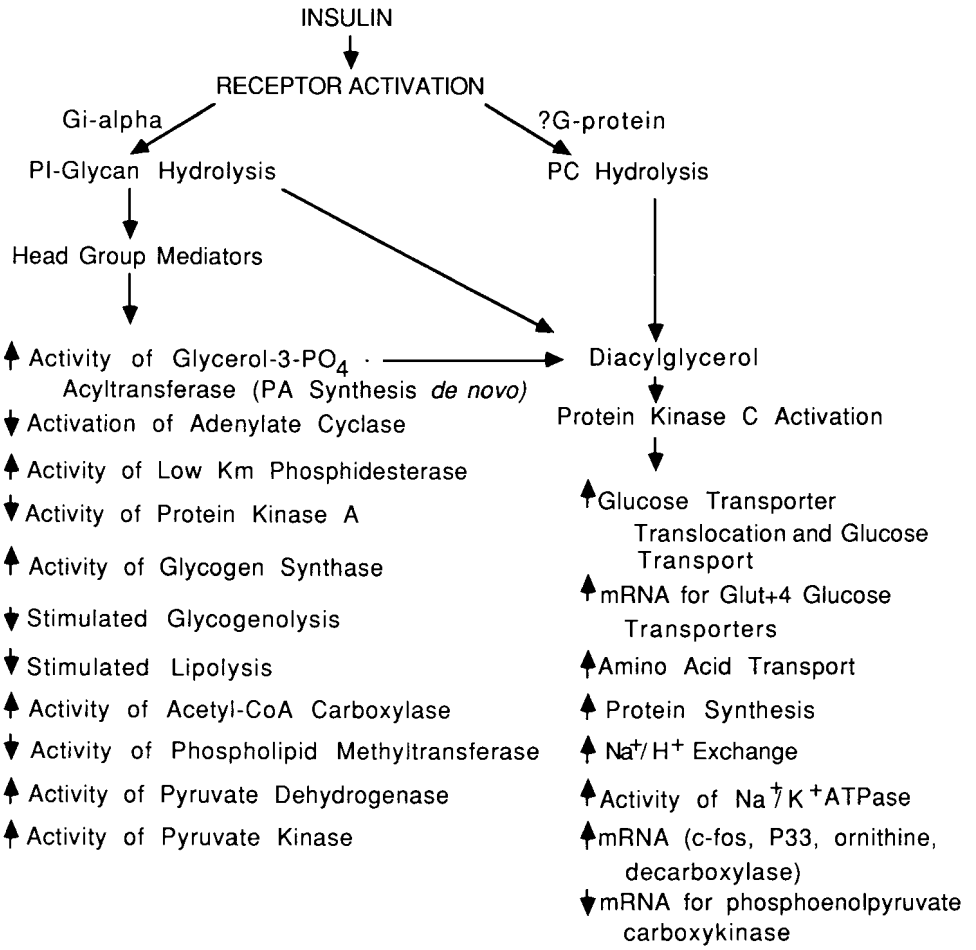

Figure 2. Dual phospholipid-signaling pathways and potential roles of lipid-derived mediators in insulin action. PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid.

erol-3-phosphate acyltransferase), or (ii) phosphatidylcholine hydrolysis. This suggests that these inhibitors are relatively specific and do not inhibit actions of insulin which precede protein kinase C activation.

In addition to protein kinase C inhibitors, depletion of protein kinase C by prolonged phorbol ester treatment has been found to down-regulate protein kinase C and result in decreases in insulin- and phorbol ester-stimulated glucose transport in some systems, such as rat adipocytes (96, 97), rat heart (98), and mouse soleus (95). More recently, we have found (summarized in Table I) that similar down-regulation of protein kinase C and insulin- and phorbol ester-stimulated glucose transport can be demonstrated in rat adipocytes after overnight exposure to (i) protein kinase C antisense DNA, which inhibits translation of mRNA which encodes protein kinase C (unpublished observations), or (ii) insulin and/or glucose, both of which, like phorbol esters, activate, translocate, and deplete protein kinase C in these cells (7, 17). Thus, multiple activators of protein kinase C in rat adipocytes seem to provoke a common effect, namely, down-regulation of both protein kinase C and insulin-stimulated glucose transport.

Although these findings suggest an important role for protein kinase C during insulin action in these tissues, the use of phorbol esters to "deplete" protein kinase C in several other tissues has yielded different results. In BC3H-1 myocytes (99), L-6 muscle cells (100), and Swiss 3T3 cells (101), for example, prolonged phorbol ester treatment results in large losses of Ca^{2+}/phospholipid-dependent histone phosphorylation and phorbol ester-stimulated glucose transport, but effects of insulin and exogenously added diacylglycerol (where tested) on glucose transport have not been impaired. Interestingly, the effects of insulin (and diacylglycerol) on glucose transport remain sensitive to inhibitors of protein kinase C, suggesting a continued involvement of residual protein kinase C (93, 99, 102). Moreover, in BC3H-1 myocytes (102), it has been found that phorbol ester treatment results in selective loss of protein kinase C-α, but retention, or preferential synthesis, of an apparent protein kinase C-β isoform. Thus, in these BC3H-1 myocytes, despite the loss of large amounts of histone phosphorylating activity and complete loss of protein kinase C-α, and despite the failure of phorbol esters to stimulate glucose transport, following prolonged phor-

bol ester treatment, it seems clear that there are still significant amounts of residual protein kinase C isoforms, which may continue to respond to insulin-induced diacylglycerol. Unfortunately, this experimental paradigm cannot be interpreted properly when hormone responsiveness and significant amounts of residual protein kinase C persist after prolonged phorbol ester treatment. Obviously, other experimental approaches will be required to resolve the issue of the importance of diacylglycerol-protein kinase C signaling during insulin-stimulated glucose transport in these cells.

In addition to glucose transport, activation of protein kinase C may be important for insulin effects on other biologic processes, including, membrane transport systems, enzyme activation or deactivation, and gene expression. Protein kinase C activators, namely, phorbol esters, have been found to provoke insulin-like effects on certain enzymes important for intracellular glucose utilization, including, 6-phosphofructo-1-kinase (103), 6-phosphofructo-2-kinase (103), enolase (104), and pyruvate dehydrogenase (71). Phorbol esters also have been reported to activate acetyl-CoA carboxylase (105), although this enzyme may also be activated by other mechanisms, perhaps involving head group mediators (see above). Na^+/H^+ exchange and cell alkalinization have been observed in the action of both phorbol esters and insulin (106, 107), and this may subsequently lead to enhanced Na^+/K^+-ATPase activity (107). Insulin has been reported to increase Ca^{2+} uptake in rat adipocytes (108), and phorbol esters may also increase Ca^{2+} uptake in some tissues. Activation of protein synthesis occurs in the action of both insulin and phorbol esters (40), and this may involve increases in amino acid uptake (71), S-6 kinase activation (21), and S-6 ribosomal protein phosphorylation (109, 110). Both insulin and phorbol esters have been shown to increase mRNA levels which encode c-*fos* (103), P33 (112), and ornithine decarboxylase (113). Interestingly, both insulin and phorbol esters diminish mRNA which encodes phosphoenolpyruvate carboxykinase (114). It is tempting to speculate that insulin effects on protein kinase C, and/or other serine/threonine kinases, may be important in these actions of insulin, but further work is required to determine whether specific phosphorylation sites on relevant enzymes are, in fact, phosphorylated through a protein kinase C-dependent and/or other kinase-dependent mechanism.

Conclusions

There seems to be little doubt that insulin provokes rapid changes in phospholipid metabolism in many of its target tissues. These changes include apparent hydrolysis of PI-glycan or other glycolipids, activation of *de novo* phosphatidic acid synthesis, and phosphatidylcholine hydrolysis. Diacylglycerol can derive from each of these three phospholipid effects of insulin, and more recent studies suggest that this diacylglycerol is functionally active and results in the activation of protein kinase C. A pertussis toxin-sensitive G protein appears to also be involved in PI-glycan (or other glycolipid) hydrolysis and *de novo* phosphatidic acid synthesis: this G protein (Giα) probably serves to couple the insulin receptor to a phospholipase C which hydrolyzes the PI-glycan, and the latter appears to release head group mediators which activate the *de novo* pathway. On the other hand, the activation of phosphatidylcholine hydrolysis appears to occur by a separate mechanism, which does not involve a pertussis toxin-sensitive G protein. It therefore appears that there are two separable mechanisms whereby insulin activates phospholipid metabolism, and while these parallel effects are separable mechanistically, they appear to be integrated to provide for coordinated hydrolysis and continued resynthesis of a PI-glycan (or other glycolipid) and phosphatidylcholine. Although the role of head group mediators and diacylglycerol-protein kinase C signaling remain to be further defined, preliminary findings suggest that many of the actions of insulin may be accounted for, wholly or in part, through these signaling pathways (Fig. 2). It remains for future studies to test the hypothesis that these signaling pathways are important, not only during insulin action physiologically, but also in clinical states of insulin resistance.

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