Some Thoughts on the Mechanism of Action of Insulin

JOHN H. EXTON

Proposed mechanisms by which insulin exerts its effects are discussed. Evidence for a role for the tyrosine kinase activity of the insulin receptor and of a phosphorylation/dephosphorylation cascade is presented. The possible roles of phospholipid breakdown, diacylglycerol, and protein kinase C are discussed. The hypothesis that insulin elicits the hydrolysis of a glycosyl phosphatidylinositol to form a mediator of certain of its actions is considered in detail. The evidence that a G protein is involved in insulin action is analyzed. Diabetes 40:521–26, 1991

Editor's note: Every now and then welcome humor grins out from under the mounds of paper found on editors' desks. It did so recently in correspondence from a renowned Southern humorist.

December 11, 1990

R. Paul Robertson, MD
Editor, Diabetes

Dear Paul,

By this hand, I do commit the enclosed work that thou didst ask this humble scribe to render forth upon this near Christmastide. Verily, I hath laboured mightily on this task, wearing thin many quills, draining many wells of ink, and pestering many purveyors of parchment. At times, it has been a veritable pestilence, forcing me to broach butts of marmsy or seek the counsel of soothsayers, wizards, and other learned gentlemen. I have heroically curbed the urge to jest and pun in a valiant effort to give the work an air of lofty scholarship melded with understanding of unfathomable depths. Attempts to keep miscreant lawyers and disgruntled scrivillians at bay have been made by combining exquisite judgment and impeccable balance with mellifluous phrasing.

Prithily, my liege, I yield unto thee this work of mere man that e'en the very gods would stumble to give birth to.

Your humble servant,
John H. Exton, MD, PhD

December 28, 1990

John H. Exton, MD, PhD

Dear Liegeman,

I am in receipt of your humbly proffered yet prodigious paradigm of potential phospholipid-provided perturbations in insulin action. It languished not even a fortnight on mine desk despite its untimely arrival near Winter Solstice, which greatly limits reading hours due to scant solar lumination in this bleak Northern clime. I deem your opus intriguing and thank thee for it. Despite thy consultations with various wizards and soothsayers, thy verbiage is not unduly homogenized. Howere', I understand not your use of "marmsy;" hereabouts we inbibe marble. Perhaps you could send me a draft of thine mysterious Southern concoction. More important, mislead not thyself by thinking mellifluous phrases will hold at bay the lawyers! To quote mine neighbouring Bard, "We must first kill all the lawyers and only then take our rest!"

Doubtless thou will feel pleasure in learning that thine words on parchment carry such persuasive force and clarity of thought that I will send them forthwith to ye olde publisher without burdening thou with inspection
MEMBRANE-ASSOCIATED SIGNALING SYSTEMS

There are several general mechanisms by which a signal from a surface receptor is passed to the interior of a cell. The most widespread involves guanine nucleotide-binding proteins (G proteins) that are activated by interaction of agonists with receptors and act on various effector proteins. A mechanism used by many growth factors involves activation of an intrinsic receptor-protein tyrosine kinase, and evidence is emerging that some signaling could involve receptor-associated protein tyrosine phosphatases. Another mechanism used by certain neurotransmitters involves regulation of an ion channel that is part of the receptor complex. Recently, a new system has been discovered that is used by atrial natriuretic and certain other peptides and involves guanylate cyclase as an intrinsic component of the receptor. There are also many agonists that interact with plasma membrane receptors but for which the signaling mechanisms are unknown.

In general, a receptor or receptor subtype is not directly coupled to two different types of signaling mechanism, e.g., to both tyrosine kinase and G proteins. However, a receptor may couple to more than one G protein, and a G protein may interact with more than one effector. Furthermore, some effectors, e.g., phosphoinositide phospholipase C, can be controlled by both G proteins and tyrosine phosphorylation. Although different isoforms are involved. Certain phospholipase isozymes and G proteins can also be phosphorylated by protein kinase C (PKC) or cAMP-dependent protein kinase, and certain cell proteins can be multiply phosphorylated by these and other kinases. These interactions provide many opportunities for cross talk between the different signaling systems.

PROTEIN PHOSPHORYLATION/DEPHOSPHORYLATION–SIGNALING SYSTEM FOR INSULIN

There is no dispute concerning the structure of the insulin receptor and of the location of its insulin-binding and tyrosine kinase domains (1–3). The tyrosine kinase activity of the β-subunit has been rigorously explored, and most studies indicate that this activity is essential for insulin action. Thus, deletions and truncations that result in loss of insulin-stimulated kinase activity or natural or induced mutations in the ATP-binding site cause loss of insulin effects on hexose transport and other processes (1–5). Likewise, inhibition of kinase activity by monoclonal antibodies or other inhibitors impairs insulin effects in several cell types (4–7). Although there is strong evidence that kinase activity is essential for insulin action, some studies with mutated or chimeric receptors indicate that certain other changes in the cytoplasmic tail of the β-subunit can interfere with signal transmission (2,3,5).

A major question is the identity of the immediate cellular targets of the insulin-stimulated tyrosine kinase. One substrate is the receptor itself (6–8). This raises the possibility that it is a conformational or other change induced in the receptor by autophosphorylation rather than the increased tyrosine kinase activity per se that is involved in generating or modulating some insulin effects. This possibility is supported by the finding that mutations of the autophosphorylation sites alter insulin responses (6,7). The postulated conformational change induced by autophosphorylation could either control access of certain substrates to the tyrosine kinase or permit the cytoplasmic tail to interact noncovalently with proteins involved in the signal transduction. If the latter change were induced by some monoclonal antibodies to the insulin receptor with little or no activation of the tyrosine kinase, this could explain why these antibodies stimulate glucose and amino acid transport (8,9).

The insulin receptor phosphorylates many substrates on...
tyrosine in vitro, and many substrates have been identified in intact cells, especially in the presence of phosphatase inhibitors. These cellular substrates include many proteins (e.g., 15,000, 60,000, 120,000, 185,000, or 240,000 M) of unknown function (4,5), phosphatidylinositol 3-kinase (10), and a 42,000-M, kinase that phosphorylates microtubule-associated protein 2 (MAP2) (11). There are also many proteins that are phosphorylated on serine and/or threonine in response to insulin in intact cells. Examples are the receptor itself, ATP citrate lyase, acetyl-CoA carboxylase, S6 ribosomal protein (5,12,13), an isozyme of cAMP phosphodiesterase (14). Raf-1 protein kinase (15,16), and some proteins of unknown function (5,13). The functional significance of these phosphorylations is unclear except in the case of cAMP phosphodiesterase, which is activated.

The simplest explanation of the above findings is that stimulation of the receptor tyrosine kinase and/or a resultant change in the receptor lead to the phosphorylation and activation of one or more protein serine/threonine kinases. In this regard, insulin treatment of several cell lines results in the phosphorylation and/or stable activation of various serine/threonine kinases assayed in vitro, e.g., MAP2 kinase, casein kinase II, protease-activated kinase II, two forms of S6 kinase, and Raf-1 kinase (4,5,11–13,15–18). However, none of these has been shown to be phosphorylated by the insulin-receptor tyrosine kinase in vitro, although MAP2 kinase is phosphorylated on tyrosine and threonine in intact cells, and dephosphorylation of either residue(s) results in inactivation (11,18). MAP2 kinase can phosphorylate and activate the 85,000-M, form of S6 kinase from Xenopus and may thus participate in a phosphorylation cascade initiated by insulin (8), but MAP2 kinase does not phosphorylate or activate the 70,000-M, form of S6 kinase that predominates in mammalian cells (17), indicating that another kinase is involved in its activation by insulin. Potential targets of phosphorylation are the insulin-sensitive forms of the glucose transporter, but there is no evidence that these proteins are phosphorylated in response to insulin. Obviously, a reason why no serine/threonine kinase has been found to be directly phosphorylated by the receptor tyrosine kinase is that its interaction with receptor may be noncovalent.

It is well known that insulin promotes the dephosphorylation of several proteins (5,13). In some cases, this is attributable to a decrease in cAMP, whereas in others, it is independent of cAMP. There is much indirect evidence that insulin stimulates protein phosphatase activity (5,13), and there have been reports that it activates a type 1 phosphatase in several tissues (19). Recently, evidence has been presented that an insulin-stimulated protein kinase activates type 1 phosphatase in skeletal muscle by phosphorylating its G subunit on a specific serine (19). This results in activation of glycogen synthase and inactivation of phosphorylase kinase and can thus explain the effects of insulin on glycogen metabolism. However, based on the identities of other proteins dephosphorylated in response to insulin, other phosphatases, e.g., type 2A, are probably involved. The mechanisms by which these phosphatases are stimulated are unknown.

Potential complications arising from the simultaneous activation of protein kinases and phosphatases could be obviated if these enzymes exhibited high substrate specificity or if they and/or their substrates were highly localized in the cell. Because insulin controls plasma membrane transport processes, cytosolic and mitochondrial enzyme activities, and nuclear transcriptional events, any mechanism of action must affect activities in all of these compartments. A phosphorylation/dephosphorylation mechanism operating in the cytosol could affect the phosphorylation state of proteins located in the cytosol and on the cytoplasmic surfaces of organelles. It could also result in the formation of molecules that enter organelles and alter the activity of proteins within them, or it could alter the translocation of such molecules into the organelles.

PHOSPHOLIPID-SIGNALING SYSTEM FOR INSULIN

In contrast to the mounting evidence that insulin initiates a phosphorylation/dephosphorylation cascade, data supporting a role for phospholipid or glycosphospholipid hydrolysis in insulin action remain fragmentary and controversial. Thus, some laboratories report that, in some cell types, insulin rapidly elevates diacylglycerol (DAG) measured chemically or isotopically, whereas other groups see no changes in cells that are major targets of the hormone (20–24). There have also been reports that insulin activates PKC (20,22,25,26), although this has been disputed (27,28), and the evidence that there is an associated translocation is inconsistent. Addition of active phorbol esters to many cells induces some effects similar to those caused by insulin but also some that are the opposite (13,20,27), and the patterns of protein phosphorylation are different (27,29,30). Furthermore, some insulin effects persist in cells depleted of PKC (27,29,30). Although, it is possible that an isozyme of PKC is involved in some actions of insulin, the data can also be explained if this kinase acted on some of the same substrates as the insulin-stimulated protein kinase(s).

Because insulin produces very different effects from Ca2+-mobilizing agonists, if insulin acts in part by generating DAG, this must be chemically different from that generated by these agonists or must be in a different cellular compartment. However, there have been no chemical analyses of the fatty acid composition and molecular species of the DAG in any cell type treated with insulin and no studies of its intracellular location.

Despite the inconsistency in the reported effects of insulin on DAG, which is surprising because of the large increases reported by one group (21,22), possible sources of this lipid may be considered. In many cells, insulin stimulates de novo synthesis of DAG, but this is usually glucose dependent. Other ways in which insulin could elevate DAG are by hydrolysis of phospholipids, glyco phospholipids, or triacylglycerol; inhibition of the synthesis of triacylglycerol, phosphatidylcholine, or phosphatidylethanolamine; increased phosphatidate phosphohydrolase activity; and reduced DAG kinase or DAG lipase activity. Of these possibilities, the only ones proposed are de novo synthesis and the hydrolysis of a phospholipid or glycosphospholipid. If insulin generates DAG through hydrolysis of a phospholipid, this should be identifiable by its characteristic fatty acid composition or molecular-species profile or its selective labeling by certain fatty acids. Labeling of the head group of the phospholipid, and isotopic or chemical measurement of the released head group should identify it more specifically.
MECHANISM OF INSULIN ACTION

Some early reports indicated that insulin stimulated the hydrolysis of inositol phospholipids in adipose tissue (e.g., 31). However, efforts to confirm these findings have been unsuccessful, and it is generally accepted that insulin does not increase inositol 1,4,5-trisphosphate (IP₃) or elevate cytosolic Ca²⁺ (23,32). On the other hand, there is evidence that insulin stimulates the synthesis of phosphoinositides and other phospholipids in adipose tissue and BC₃H₁ cells (21,23,33), due in part to increased de novo synthesis of phosphatidic acid (21,23,34). There is a single report that insulin stimulates the release of [³H]phosphocholine from BC₃H₁ cells previously labeled with [³H]choline (35), but the implication that phosphatidylcholine hydrolysis is stimulated needs further support.

GLYCOSYL PHOSPHATIDYLINOSITOL (GPI)-SIGNALING SYSTEM FOR INSULIN

The hypothesis that insulin signaling involves the release of an inositol phosphate glycan (IPG) from a GPI (20,36) arose out of earlier reports from the laboratories of Jarett (37) and Larner (38) of insulin-dependent second messengers that influenced the activities of several enzymes. Despite its initial attractiveness, most features of the system remain unclear and controversial.

In any cell signaling system, the chemical nature of the components must be defined before the system can be understood. This applies particularly to postulated second messenger molecules, which must be synthesized ultimately and shown to reproduce part or all of an agonist's action(s). It has been proposed that the insulin-sensitive GPI is similar in structure to the GPls that anchor many cell surface proteins, e.g., trypanosome-variant surface glycoprotein, 5'-nucleotidase, alkaline phosphatase, and acetylcholinesterase (20,36,39). The structure of the GPI anchor for the variant surface glycoprotein is defined, and there is extensive knowledge of the composition of the other anchors (39,40), but the structures of the insulin-sensitive GPI and its IPG product remain elusive.

Isotopic labeling studies of the insulin-sensitive GPI have provided some areas of agreement, but there are also some differences, perhaps arising from heterogeneity. Most reports indicate the presence of glucosamine and myo-inositol (20,36,39), although there is evidence for chiro-inositol in rat liver and H35 hepatoma cells (41). Most studies also report the incorporation of labeled myristic acid into a DAG moiety (20,36,39), whereas an alkylacylglycerol structure containing mainly palmitic acid has been proposed for the hepatoma cell GPI (42). The presence of phosphate and galactose has been reported (42) but not that of ethanolamine or mannose, which are present in all GPI anchors (39,40). However, it is not always demonstrated that the material being analyzed is insulin sensitive. The pitfalls in the area are illustrated by the finding that a material synthesized by liver microsomes and reported to be GPI is actually a mixture of lysophospholipids and does not contain hexosamine or ethanolamine (43).

With respect to the insulin-sensitive IPG, even less is certain. Surprisingly, no data on the chemical composition of the active material released from plasma membranes by insulin action have been published. Instead, investigators have mainly examined fractions produced by bacterial phosphoinositide phospholipases that mimic the actions of insulin on certain enzymes (20,36,39). Although the chromatographic and electrophoretic properties of these compounds have been stated to be identical to those of the material produced by insulin (20,36,44), published data supporting this crucial contention are very limited and not conclusive (45).

In most cases, the IPG fractions used for chemical and biological studies have undergone minimal or no purification after preparation by the action of bacterial phospholipases on GPI fractions isolated from cell or membrane lipid extracts by sequential thin-layer chromatography. In accordance with the GPI data, most IPG fractions can be labeled with glucosamine and either myo-inositol or chiro-inositol (20,36). The extent of labeling with phosphate or galactose is uncertain. Because of the absence of chemical or other assays, the concentrations of IPG and GPI are unknown in any tissue.

In accord with their function, the GPI anchors for cell surface proteins project from the surface of the cell (39,40). However, the orientation of the insulin-sensitive GPI is uncertain (20,36), and a surface projection seems a priori inappropriate for a molecule that is presumed to be cleaved to an intracellular messenger. Either it has the opposite membrane orientation or some mechanism exists for the cellular uptake of the IPG messenger. The idea that IPG can be taken up by cells is supported by reports that adding IPG preparations of undefined purity to intact cells modifies the activities of certain intracellular enzymes (20,46). However, hexose transport is not stimulated, and many insulin-sensitive enzymes are not affected (46). Furthermore, some enzymes that are altered are not generally recognized as insulin targets. Antibodies to the trypanosome-variant surface glycoprotein have been reported to block the action of insulin on pyruvate dehydrogenase but not hexose transport or glycogen synthase in BC₃H₁ cells (47), but the mechanism of the inhibition is uncertain.

Second messengers for hormones or neurotransmitters usually have primary cellular targets to which they bind with high affinity, e.g., the cyclic nucleotide protein kinases, PKC, calmodulin, and the IP₃ receptor. These proteins undergo specific changes and amplify the message by interacting with many proteins or by utilizing other mechanisms. The published effects of IPG indicate that it interacts with diverse enzymes in vitro and does not have a primary target (20,36,46). If these observations are correct, then an important amplification step is missing from this signaling system. It also means that all the target enzymes must have binding sites for IPG that are capable of influencing the diverse activities of the enzymes.

If insulin action is mediated in part through the hydrolysis of a unique GPI or phospholipid (20,36), there must be a mechanism by which the relevant phospholipase is activated. This could involve tyrosine phosphorylation or a G protein. There is much evidence that the γ₁-isozyme of phosphoinositide phospholipase C, is phosphorylated and activated by the tyrosine kinases of the epidermal growth factor and platelet-derived growth factor receptors, but efforts to show similar phosphorylation by the insulin receptor have been unsuccessful (48). A partial purification of a GPI-
specific phospholipase C from liver membranes has been achieved (49), but it is not known if this is insulin responsive or can be phosphorylated by the insulin receptor.

POSSIBLE ROLE FOR G PROTEINS

The idea that insulin action is partly mediated by a G protein has resulted in many studies. It has been found that high concentrations of guanine nucleotides (0.1–1 mM) inhibit insulin binding to plasma membranes from some but not all cells (50–52) and that insulin alters GTPyS binding to membranes from BC3H1 cells but not from liver or adipocytes (50,52). It has also been shown that pertussis toxin inhibits insulin binding and inhibits some but not all insulin effects in several cell types (52–55), but it is unclear that these effects are the direct result of ADP ribosylation of a G protein. Interaction between the insulin receptor and a G protein(s) is suggested by the observations that GTPyS inhibits insulin-induced tyrosine phosphorylation of several proteins in L6 muscle cells and adipocyte plasma membranes (50,51) and that insulin treatment of liver plasma membranes inhibits pertussis toxin-catalyzed ADP ribosylation of G (55), but again these effects may be indirect. An earlier report that insulin inhibits chola toxin-dependent ADP ribosylation of a 25,000-M, protein, termed Gtr, in liver membranes (56) has not been reproduced (57), and the 25,000-M, protein may have been the cholera toxin A subunit (57). There have also been studies showing that the insulin receptor from human placenta or rat liver can phosphorylate the α-subunits of Gtr, Gs, and G, (58–60), but there is no evidence that these proteins are phosphorylated in intact cells (55).

Insulin-stimulated tyrosine phosphorylation of a G protein would represent a novel means of cellular signal transduction. However, such a phosphorylation should alter the interaction of the G protein with known insulin-responsive effectors. Currently, few, if any, of the accepted enzyme targets of insulin are known to be regulated by G proteins.

SUMMARY

The major theme of this Perspective is to delineate experiments to confirm or refute key aspects of proposed mechanisms of insulin action. In an area where there is little general agreement, it seems that the tyrosine kinase activity of the receptor is essential for insulin action and that multiple signaling mechanisms are involved. Because it is not known at what steps the pathways diverge, the various proposed mechanisms are not mutually exclusive. Whatever particular mechanism(s) operates—e.g., a phosphorylation/dephosphorylation cascade initiated by the insulin-receptor tyrosine kinase: the hydrolysis of a specific glycosyl phospholipid or phospholipid to yield DAG, IPG, or other messengers; or a resultant or independent activation of a putative PKC isozyme—there are serious deficiencies in knowledge. Major uncertainties with the phosphorylation/dephosphorylation mechanism are its linkage to the insulin-receptor tyrosine kinase and the mechanisms involved in dephosphorylation. Problems with the proposed PKC mechanism are uncertainties about the extent to which it operates, if at all, and about the isozyme involved and its mechanism of activation. Difficulties with the GPI hypothesis relate to a serious lack of knowledge about its chemical structure and that of its IPG product, its location in the plasma membrane, the mechanism by which insulin promotes its cleavage, and the mechanism of action of IPG on its target enzymes. In addition, the possible involvement of G proteins lacks any direct support. Surprisingly, methods and approaches exist to explore many of these deficiencies, especially the rigorous chemical identification of postulated signaling components, but many investigators have been deterred by the slow progress in the area and the lack of unanimity on many points. I hope that the next few years will refute the jocular characterization of insulin as a hormone without a mechanism of action!

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