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*Topical Review*

# Role of Kinases in Insulin Stimulation of Glucose Transport

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## I. Introduction

Stimulation of glucose transport is one of the fundamental responses to insulin. This response occurs primarily in muscle and adipose tissue (Elbrink & Bihler, 1975), where glucose transport is a facilitated process mediated by a single membrane polypeptide of about 45 kDa (Shanahan et al., 1982; Klip et al., 1983; James et al., 1988). The signals that mediate insulin binding to stimulation of transport remain largely unknown. However, considerable progress in the last five years has suggested involvement of two possible routes in this response, namely the tyrosine-specific kinase activity of the insulin receptor, and the activation of the serine and threonine-specific protein kinase C (through generation of endogenous diacylglycerol). This review attempts to analyze critically the evidence for and against the roles of the insulin receptor kinase and of protein kinase C in the stimulation of glucose transport by insulin. Therefore, the potential role of these two kinases in other responses to the hormone are not discussed. Also, we do not discuss in great detail the effects of these kinases in tissues such as liver, where insulin does not stimulate glucose transport. For the sake of this review only, tissues classified as insulin responsive and unresponsive are those where the hormone stimulates or does not stimulate glucose transport.[1] In addition, other potential messengers of insulin action on glucose transport, such as $Ca^{2+}$, cytosolic pH, membrane potential and cyclic nucleotides, although having been the focus of much attention, are not the subject of this review. The reader is referred to outstanding reviews on the insulin receptor and insulin action (Czech, 1985).

**Key Words** insulin receptor kinase · protein kinase C · glucose transport stimulation · insulin resistance · phorbol esters

[1] In this review, insulin-responsive cells refers to cells that respond to the hormone with an elevation in the rate of glucose transport. Insulin unresponsive cells are those that, although displaying other responses to the hormone, do not increase glucose transport.

## II. The Insulin Receptor Kinase

The insulin receptor is an integral membrane glycoprotein consisting of two $\alpha$-subunits ($M_r$ ~135,000) and two $\beta$-subunits ($M_r$ ~95,000) linked by disulphide bridges to yield a symmetrical $\beta\alpha$–$\alpha\beta$ complex (Massague et al., 1980). The $\alpha$-subunit contains the insulin binding domain, is hydrophilic, glycosylated and is localized exclusively in the extracellular domain. The $\beta$ subunit is also glycosylated and consists of a short extracellular region linked to the $\alpha$-subunit through disulphide bonds, a hydrophobic transmembrane region and a longer cytoplasmic region containing an insulin tyrosine-specific kinase (Ebina et al., 1985; Ullrich et al., 1985). Insulin binding to its receptor is followed by rapid structural and chemical modifications within the receptor tetramer (Czech et al., 1981; Kasuga et al., 1982*a*,*b*). The purified insulin receptor rapidly autophosphorylates its $\beta$-subunit and other exogenous protein substrates in an insulin-sensitive, nucleotide (ATP)-specific, $Mn^{2+}$-dependent reaction (Kasuga et al., 1982*a*,*b*; Nemenoff et al., 1984; White et al., 1984; Czech, 1985).

The insulin receptor is synthesized as a single precursor polypeptide, and a cDNA sequence of this polypeptide has been cloned (Ebina et al., 1985; Ullrich et al., 1985), enabling transfection studies to elucidate the function of different regions of the receptor. It is believed that insulin-induced conformational changes of the receptor transfer a signal from the extracellular to the cytoplasmic receptor domains, leading to the activation of the $\beta$-subunit associated tyrosine kinase.

Insulin induces phosphorylation of several endogenous proteins on tyrosine residues, which differ from tissue to tissue: Polypeptides of 185 kDa in

intact Fao hepatoma cells (White et al., 1985) and of 110 kDa in rat liver; in rabbit brown adipose tissue, polypeptides of 110 kDa (Rees-Jones & Taylor, 1985; Sadoul et al., 1985) and in intact rat adipocytes a 46 kDa membrane polypeptide (Haring et al., 1987) and a 160 kDa cytosolic polypeptide (Madoff et al., 1988); in rat skeletal muscle a polypeptide of 15 kDa (Walaas et al., 1977) and in L6 muscle cells predominantly a 160–170 kDa polypeptide (Beguinot & Smith, 1987; Klip & Burdett, 1989). The correlation between the dose/response of insulin activation of the receptor kinase and the appearance of phosphoproteins suggests an involvement of these proteins in mediation of insulin action. However, apart from being phosphorylated, none of these phosphoproteins have been shown unambiguously to mediate insulin action, and hence are not discussed further in this review. In contrast, a 15 kDa polypeptide has been suggested to be involved in the stimulation of glucose transport by insulin (*see* section IIE). In addition to phosphorylation of endogenous proteins, the insulin receptor kinase also phosphorylates exogenously added substrates such as the synthetic copolymer $Glu_4:Tyr_1$ (Gherzi et al., 1986; Caro et al., 1987), casein (Zick et al., 1984), histone $H_2b$ and angiotensin II (Burant et al., 1986*a*) among others. A primary objective of this review is to evaluate the significance of insulin-stimulated receptor kinase activity in the stimulation of glucose transport.

A relationship between the insulin receptor kinase activity and stimulation of glucose transport was suggested by the time course of both events: In 3T3-L1 adipocytes, insulin-induced $\beta$-subunit phosphorylation was complete within 1 min ($t_{1/2}$ = 8 sec), while a 1-min latency period preceded insulin activation of hexose transport (Kohanski et al., 1986). Removal of insulin from the medium resulted in rapid loss of phosphorylation ($t_{1/2}$ = 2.5 min), while hexose transport decreased with a $t_{1/2}$ of 8 min. The close correlation between the time course of receptor phosphorylation and stimulation of glucose transport suggests that insulin receptor kinase activity may play a signalling role in stimulation of glucose transport. The following sections describe specific experiments designed to test a cause/effect relationship between activation of the receptor kinase and stimulation of glucose uptake.

## A. Site-Directed Mutagenesis of Receptor Sites Involved in Kinase Activity

### *1. Single Point Mutations*

Site-directed mutagenesis has been used to investigate the significance of the insulin receptor kinase in the activation of glucose transport. The human insulin receptor cDNA was mutated at the ATP binding site by replacement of lysine 1018 for alanine (A/K 1018) (Chou et al., 1987; McClain et al., 1987). Transfection of the cDNA's encoding the mutated receptors into rat 1 fibroblasts and chinese hamster ovary (CHO) cell lines resulted in loss of $\beta$-subunit phosphorylation (CHO cells) and of insulin receptor kinase activity. Importantly, this coincided with a decrease in insulin stimulation of hexose transport (both cell types) relative to cells transfected with normal human cDNA (Ebina et al., 1987; Chou et al., 1987; McClain et al., 1987). It should be noted, however, that CHO cells transfected with A/K 1018 exhibited a marked decrease also in basal hexose transport (Ebina et al., 1987; Chou et al., 1987). In fact, at maximal doses of insulin, the fold stimulation (insulin response/basal transport) observed in the transfected mutant was not significantly different from that in the parental or in the wild type transfected cells. This suggests that either the unoccupied insulin receptor in some way regulates basal transport, or that nonspecific cell damage occurred in the mutated cells resulting in the decrease in basal transport. In contrast, in rat 1 fibroblasts transfected with mutated receptor cDNA, basal transport was unaffected, and there was a marked decrease in insulin sensitivity (i.e., the response to a half maximal dose of insulin) of hexose transport relative to parental (rat endogenous) cells or to normal (overexpressed human) receptor transfectants. This suggests that the A/K 1018 receptor is unable to signal stimulation of glucose transport, and that expression of this mutated receptor may in fact inhibit the function of the normal (endogenous) rat insulin receptors (McClain et al., 1987). Responsiveness (maximal response) to insulin at high concentrations was not diminished in the mutant transfectant, suggesting that the glucose transport system was unaffected. Recent work by this group has suggested that expression of the A/K 1018 receptor inhibits insulin sensitivity, not at the level of the native receptor expression or activation but rather by preventing the phosphorylation of endogenous substrates of the insulin receptor (Maegawa et al., 1988).

Tyrosine residues 1162 and 1163 of the $\beta$-subunit of the receptor become phosphorylated upon addition of insulin. Replacement of these residues with phenylalanine by site-directed mutagenesis and expression of the mutated receptor in CHO cells resulted in a marked loss of insulin-dependent $\beta$-subunit autophosphorylation and of exogenous kinase activity of the purified receptors. Concomitantly, the transfected cells had a diminished stimulation by insulin of 2-deoxyglucose uptake (Ellis et

al., 1986). Debant et al. (1987) observed a similar inhibition of insulin-mediated tyrosine kinase activity and hexose transport in such CHO transfectants. However, the mitogenic effect of the hormone was not compromised in these transfected cells, suggesting that different pathways may be involved in the activation by insulin of hexose transport and mitogenesis.

### 2. *Truncated and Hybrid Receptors*

Ellis et al. (1986) showed that mutant-truncated insulin receptors lacking the last 112 amino acids possessed no kinase activity and had reduced ability to mediate stimulation by insulin of 2-deoxyglucose uptake. Conversely, truncated insulin receptors lacking the $\alpha$ subunit and most of the extracellular domain of the $\beta$-subunit possess permanently activated kinase activity (i.e., insulin-independent tyrosine kinase activity) (Ellis et al., 1987*a*). When transfected into CHO cells, the expressed membrane-bound receptors mediated a constitutively elevated, insulin-independent uptake of 2-deoxyglucose which was 135% of the maximal insulin response (Ellis et al., 1987*a*). Thus, these studies suggest that there is a correlation between the insulin receptor kinase activity and the hormonal stimulation of glucose transport.

A contrary conclusion was reached from studies with a hybrid receptor constructed from the extracellular insulin binding domain of the insulin receptor and the transmembrane and cytoplasmic protein kinase domains of chicken sarcoma virus UR2 transforming protein $p68^{gag\text{-}ros}$ (Ellis et al., 1987*b*). When expressed in CHO cells this receptor bound insulin with near wild type affinity, and underwent normal insulin-induced receptor autophosphorylation and phosphorylation of exogenous substrates. However, despite apparently normal kinase activity, this hybrid receptor had markedly reduced ability to mediate glucose transport. It is possible that the hybrid receptor lost regions other than those involved in kinase activity, which are essential for stimulation of glucose transport. This implies that the insulin receptor kinase may be necessary but not significant for stimulation of hexose transport.

## B. Effect of Antibodies to the Insulin Receptor

Studies with antibodies directed to the insulin receptor have also led to controversy regarding the role of the insulin receptor kinase activity in hormonal stimulation of glucose transport. A monoclonal antibody that specifically inhibits insulin receptor kinase activity was introduced by Morgan and Roth (1987) into CHO cells, isolated rat adipocytes and TA 1 mouse adipocytes by osmotic shock followed by cell recovery for several hours. This treatment resulted in obliteration of autophosphorylation of the receptor as well as in decreased ability of insulin to stimulate hexose transport when the cells were challenged acutely with the hormone (Morgan & Roth, 1987).

Studies with antiphosphotyrosine antibodies have also been used to test the role of the receptor kinase in insulin action. In a preliminary communication, Takayama et al. (1986) reported that antiphosphotyrosine antibodies inhibited both basal and insulin-stimulated tyrosine autophosphorylation of the $\beta$-subunit of purified insulin receptors from Fao cells. Inspite of being of hepatic origin, intact Fao cells respond to insulin with a modest increase in hexose uptake. Treatment of Fao cells with the mentioned antiphosphotyrosine antibodies decreased insulin-stimulated 2-deoxyglucose uptake. Other antiphosphotyrosine antibodies were recently reported to actually increase insulin receptor tyrosine kinase activity in a cell-free system (Balloti et al., 1988). These antibodies, when introduced into Fao hepatoma cells, enhanced both basal and insulin-stimulated glucose transport. Taken together, these findings suggests that insulin activation of the receptor kinase may be an important step in the enhancement of glucose transport.

In contrast, Forsayeth et al. (1987) produced monoclonal antibodies to the insulin receptor that failed to stimulate receptor autophosphorylation in both intact IM9 cells and purified human placental insulin receptors, yet stimulated glucose transport in human adipocytes. These antibodies competitively inhibited insulin binding to its receptor, indicating a direct interaction between the antibodies and the $\alpha$-subunit of the receptor. This observation suggested that insulin stimulation of glucose transport may not require the activation of the receptor kinase activity.

Further evidence suggesting that insulin receptor phosphorylation may not be a prerequisite for acute insulin action stems from investigations involving a polyclonal antiserum, B10, against the insulin receptor. B10 inhibited insulin binding to its receptor in human lymphoblastoid cells (Zick et al., 1984) and CHO cells (Gherzi et al., 1987). In rat adipocytes B10 mimicked hormonal stimulation of glucose transport, yet this antiserum did not stimulate and instead decreased phosphorylation of the receptor $\beta$-subunit in adipocyte plasma membranes (Simpson & Hedo, 1984). Furthermore, B10 stimulated lipogenesis in rat adipocytes, without affecting

the tyrosine kinase activity of partially purified insulin receptors from rat liver (Zick et al., 1984). Curiously, two groups have found that B10 does enhance phosphorylation of partially purified human placental insulin receptors as well as insulin receptors present in intact CHO cells (Gherzi et al., 1987), and in partially purified insulin receptors from BC3H-1 myocytes (Mojsilovic et al., 1986). The former study indicated that the antiserum activates the receptor kinase activity and stimulates hexose transport in CHO cells expressing wild type human insulin receptors (Gherzi et al., 1987). In contrast, although the antiserum induced phosphorylation of insulin receptor in BC3H-1 myocytes, in these cells it failed to provoke stimulation of glucose transport (Mojsilovic et al., 1986). Hence, the results of studies of insulin receptor kinase activity and insulin action involving the polyclonal antiserum B10 are inconsistent, since B10 increased or decreased $\beta$-subunit phosphorylation in different cell types. In most cases the two functions of insulin, receptor phosphorylation and stimulation of glucose uptake, were measured in different cell types, precluding the establishment of accurate correlations. Therefore, although the work done using B10 suggests that the insulin receptor kinase may not be an obligatory step in insulin action, the data are not conclusive and do not rule out a role of the insulin receptor tyrosine-specific kinase in signal transduction.

### C. Tyrosine Kinase Inhibitors and Stimulation of Glucose Transport

To test the physiological role of tyrosine kinase activity it would be desirable to have access to specific inhibitors of this function. In rat adipocytes, a polypeptide obtained from a tryptic digest of bovine serum albumin inhibited insulin-induced $\beta$-subunit autophosphorylation (Ueno et al., 1987). No phosphorylated form of the peptide was detected during phosphorylation of the receptor, and the peptide had no effect on dephosphorylation of the phosphorylated $\beta$-subunit. It was suggested that this peptide is a specific inhibitor of tyrosine-specific protein kinase. However, this fragment did not inhibit insulin-stimulated glucose oxidation, but in fact potentiated it (Ueno et al., 1987).

Tyrosine kinase activity is associated with cellular receptors for other growth factors in addition to insulin, such as the receptors to EGF (Ushiro & Cohen, 1980) and to IGF I (Rubin et al., 1983). It is possible then that the kinase activities of all these receptors play an important role in signal transduction. To test this possibility, specific inhibitors of these tyrosine kinases have been designed in recent years. Specific inhibition of the tyrosine-specific protein kinase of the EGF receptor was achieved *in vitro* by an isoflavone, genistein (Akiyama et al., 1987) and by synthetic 4-hydroxycinnamamide derivatives (Shiraishi et al., 1987). To our knowledge these compounds have not been tested on the insulin receptor kinase activity. We chose one of the cinnamamide derivatives, $\alpha$-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST 638), which inhibits EGF receptor tyrosine kinase activity with an $IC_{50}$ of 0.37 $\mu$M, to study its effect on the stimulation of 2-deoxyglucose transport by insulin in L6 muscle cells (Table 1). Pretreatment with ST 638 prior to addition of insulin, followed by addition of the hormone in the presence of ST 638, resulted in inhibition of hexose transport that was dose dependent with respect to the synthetic derivative. However, basal transport was inhibited in parallel (Table 1), even though membrane integrity remained intact in the concentration range studied (as determined by trypan blue exclusion and sensitivity of glucose uptake to inhibition by cytochalasin B). This suggests that ST 638 is not specific for inhibition of tyrosine kinases and precludes its use to test insulin stimulation of glucose transport.

**Table 1.** Concentration dependence of the inhibition by ST 368 of hexose uptake in L6 myotubes

| [ST638] ($\mu$M) | 2-Deoxyglucose uptake, pmol/min · mg protein (%) | |
|---|---|---|
| | Basal | Insulin |
| 0.0 | 8.56 ± 0.40 (100%) | 11.35 ± 0.04 (100%) |
| 1.0 | 7.27 ± 0.73 (85%) | 10.31 ± 0.45 (91%) |
| 5.0 | 3.94 ± 0.12 (46%) | 5.95 ± 0.24 (52%) |
| 10.0 | 2.44 ± 0.23 (29%) | 3.57 ± 0.53 (31%) |

Serum depleted monolayers of L6 myotubules were pretreated with varying concentrations of ST 638 for 15 min prior to a 30-min incubation with ST 638 (Basal) or ST 638 plus 100 nM insulin (Insulin). Cells were washed three times with HEPES-buffered saline solution and carrier mediated hexose transport of 10 $\mu$M 2-deoxy-D-[$^3$H]-glucose was measured for 10 min, always in the presence of either ST 638 or ST 638/insulin. Results are the mean ±SD of two independent experiments, each performed in quadruplicate.

### D. Insulin Receptor Kinase Activity and Insulin Resistance

#### *1. Cases where Insulin Resistance Is Associated with Insulin Receptor Kinase Dysfunction*

Insulin resistance in peripheral tissues, which occurs in type I and II diabetes as well as in several

**Table 2.** Insulin resistance associated with a *decrease* in insulin receptor autophosphorylation/kinase activity

| Resistance (type) | Species | Tissue | Insulin binding | Autophos-phorylation | Exogenous kinase | Reference |
|---|---|---|---|---|---|---|
| Type A | Human | Erythrocyte & fibroblast | *N* | *D* | *D* | Grigorescu, et al., 1984 |
| " | " | Mononuclear blood cells | *N* | *D* | *D* | Grunberger et al., 1984 |
| Lipoatrophy | " | Erythrocyte | *N* | $D_s$ | — | Grigorescu et al., 1987 |
| NIDDM | " | Muscle | *N* | *D* | — | Obermaier et al., 1987 |
| Leprechaunism | " | Fibroblast | *D* | *D* | — | Maassen et al., 1988 |
| Obesity & obese NIDDM, *vs.* lean C | " | Muscle | *N* | — | *D* | Arner et al., 1987 |
| Obesity & obese NIDDM, *vs.* lean C | " | " | *D* | $N_r$ | $D_r$ | Caro et al., 1987 |
| Obese NIDDM *vs.* obese C or lean C | " | Adipocyte | *D* | $D_r$ | $D_r$ | Freidenberg et al., 1987 |
| Obese NIDDM *vs.* obese C | " | " | *D* | — | $D_r$ | Sinha et al., 1987 |
| STZ | Rat | Muscle | *I* | $D_r$ | $D_r$ | Burant et al., 1986*a* |
| " | " | " | *N* | — | *D* | Block & Buse, 1987 |
| " | " | Liver | *I* | $D_r$ | $D_r$ | Kadowaki et al., 1984 |
| " | " | " | *I* | $D_r$ | $D_r$ | Gherzi et al., 1986 |
| STZ & BB | " | " | *I* | *D* | *D* | Okamoto et al., 1986 |
| In vitro hyperinsulinemia | " | Adipocyte | *D* | — | $D_r$ | Arsenis & Livingston, 1986 |
| Obesity | Mouse | Muscle | *D* | $D_r$ | $D_r$ | Le Marchand-Brustel et al., 1985 |
| " | " | Adipocyte | *D* | *D* but $N_r$ | *D* but $N_r$ | Tanti et al., 1986 |
| | Melanoma cell line | | *D* | *D* | | Haring et al., 1984*b* |

NIDDM = noninsulin-dependent diabetes mellitus; STZ = streptozotocin treated; *I* = increase; *D* = decrease; $D_s$ = decrease in sensitivity only; $D_r$ = decrease even when expressed per insulin receptor number; $D_n$ = decrease accounted for by the decrease in insulin receptor number; *N* = normal; $N_r$ = normal relative to receptor number; *C* = control (nondiabetic).

**Table 3.** Insulin resistance *not* associated with a decrease in insulin receptor autophosphorylation/kinase activity

| Resistance (type) | Species | Tissue | Insulin binding | Autophos-phorylation | Exogenous kinase | Reference |
|---|---|---|---|---|---|---|
| Acantosis nigricans | Human | Muscle, adipocyte | *I** | $N_r$ | $N_r$ | Misbin et al., 1988 |
| NIDDM | Rat | Liver | *N* | *N* | *N* | Kergoat et al., 1988 |
| STZ | " | " | *N* | *N* | — | Amatruda & Roncone, 1985 |
| Age related | " | Liver | *N* | *N* | *N* | Bryer-Ash et al., 1987 |
| Denerved skeletal-muscle | " | Muscle | *N* | *N* | *N* | Burant et al., 1986*b* |
| Chronic uremia | Rat | " | *N* | — | *N* | Cecchin et al., 1988 |
| " " | " | Liver | *I* | *I* | *I* | " " |
| " " | " | Adipocyte | *N* | *N* | *N* | Truglia et al., 1988 |
| STZ | " | " | *I* | $N_r$ | $N_r$ | " " |
| Fasted | " | " | *I* | $N_r$ | $N_r$ | " " |
| Glucocorticoid treated | " | " | *N* | *N* | *N* | " " |
| " " | " | Muscle | *N* | — | *N* | Block & Buse, 1987 |
| Obesity | " | " | — | *N* | *N* | Haring et al., 1984*a* |
| Obesity | Mouse (ob/ob) | " | *D* | — | $N_r$ | Vicario et al., 1987 |
| Diabetes | Mouse (db/db) | " | *N* | — | *N* | " " |

NIDDM = noninsulin-dependent diabetes mellitus; STZ = streptozotocin induced; *I* = increase; *I** = increase in vitro in the presence of an endogenous factor; *D* = decrease; *N* = normal.

nondiabetic syndromes (Tables 2 and 3) may be due to abnormal insulin binding, a defect in the receptor function at a post-binding site, and/or to post receptor defects. Several studies have identified post-binding defects at the level of the β-subunit tyrosine kinase activity in human diabetic states and animal models of diabetes (Table 2). For completeness, studies carried out in hepatic tissue are included

here, although glucose transport is insulin-independent in this tissue.

Some of the most striking evidence that insulin receptor kinase dysfunction coincides with insulin resistance comes from studies in patients with type A syndrome. These patients have severe insulin resistance, i.e., treatment requires insulin concentrations to be increased up to 1000-fold above the normal level to effectively control glucose homeostasis (Kahn et al., 1976). Despite the severity of insulin resistance in patients with type A syndrome, two studies described normal insulin binding in mononuclear blood cells (Grunberger et al., 1984) and erythrocytes and fibroblasts (Grigorescu et al., 1984) but interestingly identified a defect in the $\beta$-subunit tyrosine kinase activity. The authors proposed that this defect might be a possible cause of impaired insulin action. Dysfunction of the insulin receptor kinase in humans has also been observed in other states of severe insulin-resistance such as leprechaunism (Maassen et al., 1988) and lipoatrophy (Grigorescu et al., 1987).

A defect in the insulin receptor tyrosine kinase activity has also been described in tissues from patients with type II diabetes (NIDDM) (*see* Table 2). In human skeletal muscle from obese subjects with and without NIDDM, insulin resistance could be accounted for only in part by a decrease in receptor number. The receptor kinase activity of skeletal muscle was impaired in both obese diabetic patients and obese nondiabetic patients relative to lean controls (Caro et al., 1987). Thus obesity rather than diabetes correlated with impaired insulin receptor kinase activity in skeletal muscle. On the other hand, in a similar study, the kinase activity of the insulin receptor from skeletal muscle of insulin-resistant obese males as well as that of NIDDM (lean or obese) males, were 40% less than the receptor kinase activity of lean controls (Arner et al., 1987). This study, therefore, linked insulin resistance rather than obesity to impaired receptor kinase activity.

Similar studies have been carried out in human adipocytes. Insulin sensitivity and responsiveness of glucose transport are significantly reduced in adipocytes from morbidly obese NIDDM patients as compared to morbidly obese controls. In a comparison of these two groups of patients, the basal and insulin stimulated tyrosine protein kinase activities were significantly decreased in fat cells from NIDDM patients relative to those from obese nondiabetic patients (Sinha et al., 1987). Somewhat different results were obtained by Freidenberg et al. (1987) who compared adipocytes from three groups of individuals: lean nondiabetic, obese nondiabetic and obese NIDDM subjects. Basal state autophosphorylation and phosphorylation of exogenous substrates was comparable in all three groups. However, while insulin-stimulated kinase activity was comparable in control and obese individuals, subjects with NIDDM showed a 50% reduction in receptor kinase activity.

Animal models have also provided considerable evidence that insulin resistance is accompanied by tyrosine kinase dysfunction. In STZ diabetic rats, insulin binding capacity was enhanced in proportion to the severity of the diabetic state (Kobayashi & Olefsksy, 1979; Okamoto et al., 1986). STZ-induced diabetes elevated the number of insulin receptors 180% in liver cells (Kadowaki et al., 1984), 70% in rat skeletal muscle (Burant et al., 1986*a*) and 42% in rat adipocytes (Truglia et al., 1988). Paradoxically insulin-mediated glucose utilization was impaired despite increased insulin binding to its target tissues (Kasuga et al., 1978; Kobayashi & Olefsksy, 1979). Importantly, in this diabetic model, insulin resistance was associated with elevated insulin binding but reduced ability to phosphorylate the receptor $\beta$-subunit and other exogenously added substrates (Kadowaki et al., 1984; Burant et al., 1986*a*; Gherzi et al., 1986; Okamoto et al., 1986). STZ diabetes was associated with a 40% decrease in hepatic insulin receptor kinase activity (Kadowaki et al., 1984; Gherzi et al., 1986). Further, Okamoto et al. (1986) observed that insulin-stimulated autophosphorylation of the $\beta$-subunit decreased in proportion to the severity of the diabetes. Similarly, hepatic insulin receptors of BB rats, in which insulin-deficient diabetes occurs due to an autoimmune destruction of the islets cells, display reduced autophosphorylation and kinase activity (Okamoto et al., 1986). There are no studies to date on the insulin receptor kinase activity of insulin-responsive tissues from BB rats.

Reduced insulin receptor kinase activity also occurs in insulin-resistant obese mice (Le Marchand-Brustel et al., 1985). In this study skeletal muscle from 10-week-old, noninsulin-resistant, obese mice displayed a 20% reduction in receptor number with a corresponding decrease in receptor kinase activity compared to nonobese controls. However, skeletal muscle from 30-week-old obese insulin resistant mice showed a similar reduction in receptor number (20%), but the receptor kinase activity was reduced by 45%. Conversely, Debant et al. (1987) observed enhanced insulin receptor kinase activity in hyper-responsive adipocytes from young obese Zucker rats compared to lean control rats. These data imply that the underlying mechanism of insulin resistance/hypersensitivity in obesity correlates with decreased/increased insulin receptor kinase activity, respectively.

In vitro studies using isolated or cultured cells have also been used to investigate the insulin receptor kinase. Hyperinsulinemic insulin-resistant adipocytes show 60% loss in insulin sensitivity of glucose metabolism, accompanied by 37% loss in insulin binding due to a reduction in receptor number (Arsenis & Livingston, 1986). In these cells, insulin-induced phosphorylation of exogenous substrates was reduced by 40% when the activity was expressed per insulin binding. In another study, an insulin resistant variant of a Cloudman S91 melanoma cell line showed 30% decrease in insulin binding which was due to decreased receptor affinity rather than reduced receptor number (Haring et al., 1984*b*). Similar to the observations of Arsenis and Livingston (1986), insulin-induced phosphorylation of the receptor $\beta$-subunit was decreased 50% compared to the noninsulin resistant wild type.

Several insulin resistant states showed reduced insulin binding (Table 1); however, when the insulin receptor kinase activity of the diabetic and control states were normalized per insulin binding, a decrease in the kinase activity in the insulin-resistant cells was still evident. An exception is the case of brown adipose tissue of obese mice, where a direct correlation between the reduction in receptor number (60 to 70%) and the decrease in receptor kinase activity (60 to 70%) was observed (Tanti et al., 1986). The authors concluded, however, that even when the primary defect may not be at the level of phosphorylation, the decreased receptor number and the concomitant decreased kinase activity might explain insulin-resistance in this tissue.

It must be stressed that an association between receptor kinase dysfunction and insulin resistance is no proof of a causal relationship. Although it is possible that a defect in the receptor kinase would lead to resistance, it is also possible that the kinase defect is secondary to the disease. Additionally, it is also feasible that insulin resistance and insulin receptor kinase dysfunction are parallel manifestations of yet another phenomenon, rather than being related in series. In fact, not all states of insulin resistance are associated with a kinase defect (*see below*).

### *2. Cases where Insulin Resistance Is Not Associated with a Defect in Insulin Receptor Kinase Activity*

Table 2 listed results showing a clear association between decreased insulin receptor kinase activity and insulin resistance. However, as stated above, it is possible that decreased insulin receptor kinase activity in diabetes might be coincidental rather than causative. Indeed, several studies show insulin resistance not accompanied by a decrease in receptor kinase activity (Table 3). Most of the studies showing dissociation between receptor kinase activity and insulin resistance were done with animal models, and many of these conclude that abnormalities occur in signal transduction at a post receptor (post-binding and post-kinase) level.

Neonatal STZ administration induces NIDDM in rats (Kergoat et al., 1988). In this model of chronic insulin deficiency, liver cells showed normal receptor autophosphorylation and phosphorylation of exogenous substrates in both basal and insulin-stimulated states. In STZ-induced, nonketotic diabetic rats, insulin-dependent $\beta$-subunit autophosphorylation of lectin-purified, liver plasma membrane insulin receptors was normal compared to controls, when expressed either per $\mu$g protein or per unit of insulin binding activity (Amatruda & Roncone, 1985).

Elevated insulin binding was detected in partially purified hepatic insulin receptors from chronically uremic, insulin-resistant rats. Correspondingly, insulin-stimulated phosphorylation of the receptor $\beta$-subunit and the kinase activity towards exogenously added substrates was also elevated, suggesting that the receptor kinase functions normally in this diabetic model (Cecchin et al., 1988). Normal hepatic receptor kinase activity was also observed in age-related insulin resistant rats. Again, it should be noted that glucose transport in liver cells is not stimulated by insulin. Hence studies with this tissue suggesting noninvolvement of the receptor kinase in insulin resistance must be viewed as peripheral to the mechanism of stimulation of glucose transport.

However, normal receptor kinase function occurs in insulin-responsive tissues (i.e., adiopocyte, muscle[2]) of insulin-resistant rats (Table 3). In particular, Cecchin et al. (1988) found that insulin binding and receptor kinase activity were normal in skeletal muscles from chronically uremic, diabetic rats compared to controls. Skeletal muscle receptor kinase activity has also been measured in other insulin resistant models (Haring et al., 1984*a*; Burant et al., 1986*b*; Vicario et al., 1987). Denervation of skeletal muscle results in severe insulin resistance without loss in insulin binding (Burant et al., 1986*b*). In this model, insulin induced normal receptor autophosphorylation and phosphorylation of exogenous histone $H_2b$ in intact and 24-hour denervated rat hind limb muscles, although insulin-induced glycogen synthesis was markedly impaired after denervation.

[2] *See* footnote 1, p. 1.

Although measurements of glucose transport were not carried out in these studies, it is well documented that insulin stimulation of glucose transport is decreased in both uremic rat muscle and in denervated muscle.

Haring et al. (1984*a*) compared insulin binding glucose transport and receptor kinase activity in cardiac and skeletal muscle from normal and diabetic Zucker rats. Insulin stimulation of $\beta$-subunit autophosphorylation in purified receptor preparations was comparable in normal and diabetic rats. However, whereas insulin stimulated glucose transport in normal muscle, transport activity was decreased in diabetic muscles. Interestingly, a preliminary report suggests that, in vivo, receptor kinase dysfunction may be associated with some types of insulin resistance, i.e., STZ-induced diabetes, but not with others, i.e., hypercortisolemia (Block & Buse, 1987). It was suggested that insulin-resistance of muscle associated with hypercortisolemia may be due to a post receptor defect.

In a recent comprehensive study, Truglia et al. (1988) investigated the receptor kinase activity in four different animal models of insulin resistance. Adipocytes from STZ nonketotic diabetic, fasted, glucocorticoid-treated and chronically uremic rats showed reduced basal and maximally insulin-stimulated 2-deoxyglucose uptake compared to matched controls. Furthermore, insulin binding was normal in chronic uremia and glucocorticoid excess but was enhanced in STZ-diabetic and fasted rats relative to controls. Partially purified adipocyte receptors from these insulin-resistant rats showed normal insulin receptor kinase activity toward exogenous substrates when normalized to insulin binding. Similarly, insulin receptor autophosphorylation in intact adipocytes was unaltered in these diabetic states. In this study, stimulation of hexose transport by hydrogen peroxide, a post-binding insulin mimicker, was also reduced in adipocytes from these diabetic models without alteration of receptor kinase activity. This strongly suggests that in these models, insulin-resistance resides beyond insulin receptor phosphorylation.

Vicario et al. (1987) investigated insulin binding and receptor kinase activity in partially purified skeletal muscle membrane preparations in genetically insulin-resistant obese mice. Both ob/ob and db/db mice inherit their obesity through autosomal recessive genes. The ob/ob mouse is characterized by chronic hyperinsulinemia and transient phases of hyperglycemia, while the db/db mouse is characterized by chronic hyperglycemia and mild transient elevation in insulin levels. Insulin binding was decreased ~50% in hyperglycemic obese ob/ob mice. Correspondingly, receptor kinase activity was decreased when compared to normal lean littermates. However, when the receptor kinase activity was expressed per unit insulin binding, no significant difference was observed between the muscle kinase activities of obese and lean mice. Insulin binding and receptor kinase activity were normal in muscle from both obese and lean db/db mice despite the presence of hyperglycemia in the obese state.

The role of endogenous factors in the mechanism of insulin-resistance has recently received much attention (Misbin et al., 1988; Cooper et al., 1987*b*). Misbin et al. (1988) extracted a heat-stable, low molecular weight (~300 Da) factor, from serum of an insulin-resistant nondiabetic patient with acantosis nigricans, which inhibits stimulation of glucose transport in rat adipocytes by insulin, hydrogen peroxide and vanadate. In vitro incubation of rat soleus muscles with this factor resulted in inhibition of insulin-stimulated glucose transport. This factor enhanced insulin binding and receptor kinase activity; however, no difference in kinase activity was apparent when expressed per unit binding. It is of interest that low levels of this factor were detected in 9 out of 13 unselected patients with NIDDM. It was therefore suggested that insulin resistance in the original patient with acantosis nigricans could be due to this circulating factor which might uncouple insulin stimulation of glucose transport from receptor binding and phosphorylation.

### E. Target Phosphoproteins Associated with Glucose Transport

Although there is considerable evidence that activation of the insulin receptor kinase may be an important intermediary in insulin action, only one laboratory has provided evidence for the involvement of phosphorylated cytosolic proteins in insulin enhancement of glucose transport (Bernier et al., 1987; Frost et al., 1987). Phenylarsine oxide (PhAsO), a potent inhibitor of protein internalization (Wallace & Ho, 1972), inhibits insulin-stimulated but not basal hexose transport in 3T3-L1 adipocytes in culture (Frost & Lane, 1985). This agent does not impair insulin-induced $\beta$-subunit autophosphorylation, but in insulin-stimulated, PhAsO-treated cells, the arsenical induces accumulation of a tyrosine-specific phosphorylated protein, $M_r$ ~15 kDa (pp 15) (Bernier et al., 1987). Interestingly, in these cells PhAsO blocks insulin-stimulated serine specific phosphorylation of two endogenous phosphoproteins, pp 24 and pp 240 (Frost et al., 1987). The accumulation of the phosphotyrosyl-containing protein pp 15, and the accompanying decrease in the phosphoseryl proteins pp 240 and pp

24 in the presence of PhAsO, implies involvement of these proteins in the mediation of signal transduction. The following scenario is envisaged:

activated insulin receptor kinase → pp15 → pp240, pp24 → glucose transport.

Although the results obtained in 3T3-L1 adipocytes are encouraging, it was recently shown that in freshly isolated rat adipocytes PhAsO inhibits both basal and insulin-stimulated glucose transport (Douen & Jones, 1988; Douen et al., 1988). In fact, PhAsO inhibited binding of cytochalasin B (a specific ligand of the glucose transporter) to adipocyte plasma membranes. Therefore, in these cells inhibition of hexose transport is thought to result from a direct interaction of PhAsO with the glucose transporters (Douen & Jones, 1988; Douen et al., 1988) rather than by interruption of the signal from the insulin receptor. Interruption of the signal, though, cannot be ruled out in 3T3-L1 adipocytes. More work will be required to establish whether the endogenous pp 15, pp 24 and pp 240 proteins are indeed mediators of insulin stimulation of glucose transport. It will also be important to determine whether other actions of the hormone are affected by treatment with PhAsO.

## F. Conclusions

Section II summarized the role of insulin receptor kinase activity in insulin activation of glucose transport.

(i) A unified conclusion linking insulin resistance to defects in insulin receptor kinase cannot be reached. Thus, receptor kinase dysfunction is associated with leprechaunism, lipoatrophy, in vitro hyperinsulinemia and type A insulin resistance, and there have been no reports to the contrary in these conditions. On the other hand, defects in insulin receptor $\beta$-subunit phosphorylation/kinase activity have not been detected in insulin resistance accompanying acantosis nigricans, age, denervation of skeletal muscle, chronic uremia and hypercortisolemia, and no reports have challenged these results. In instances where the same type of insulin resistance has yielded conflicting results, the discrepancies may be attributed to differences in animal species and/or cell type. Thus, insulin receptor kinase function is normal in liver cells from NIDDM rats (induced by neonatal administration of STZ), but it is decreased in muscle and fat cells from humans with NIDDM. Similarly, STZ treatment results in normal kinase activity in adipocytes, but defective in skeletal muscle. Finally, most of the studies where insulin resistance was not associated with defective kinase activity were conducted in animal models, whereas a defect in insulin receptor kinase function was observed in several types of insulin resistance in humans. It is possible therefore, that the variations that exist in the literature regarding the role of the insulin receptor kinase function in insulin-resistance may be partly due to differences in species, tissues and origin of the resistance. A defect in the insulin receptor kinase function in insulin-resistant subjects implies the involvement of the kinase in signalling, but does not directly prove that the kinase is an essential mediator of signal. It is possible that the receptor may also have other functional defects.

(ii) Several studies utilizing antibodies to the insulin receptor, both monoclonal (Forsayeth et al., 1987) and polyclonal (Simpson & Hedo, 1984; Zick et al., 1984), have suggested that stimulation of glucose transport and of the kinase can be functionally dissociated. The earlier studies reported that B10 stimulated glucose transport and lipogenesis in rat adipocytes without stimulating receptor kinase activity (Simpson & Hedo, 1984; Zick et al., 1984). In contrast, the more recent studies showed that B10 enhanced receptor phosphorylation in CHO cells (Gherzi et al., 1987) and BC3H-1 myocytes (Mojsilovic et al., 1986). Furthermore, in CHO cells, B10-induced receptor autophosphorylation was accompanied by a rise in hexose uptake.

(iii) Probably the most convincing evidence that the receptor kinase activity plays a significant role in signal transduction comes from studies with cell lines expressing mutated insulin receptors. Human insulin receptors mutated at the ATP binding site or at the autophosphorylation sites Tyr 1162 and 1163 show dramatic loss in the ability of insulin to activate the kinase and to stimulate glucose transport (Ellis et al., 1986; Chou et al., 1987; Ebina et al., 1987; McClain et al., 1987; Debant et al., 1988). These finding were corroborated by additional studies with truncated mutant insulin receptors exhibiting either no kinase activity or augmented kinase activity and concomitant decreased or increased ability to mediate hexose uptake, respectively (Ellis et al., 1986; 1987*a*).

From the above it seems likely that activation of the receptor kinase may be an essential step in stimulation of hexose uptake. However, despite the shortcomings in some of the studies suggesting that receptor kinase activity is not essential in glucose transport activation, at present there is enough evidence suggesting that activation of the receptor kinase may be required albeit not sufficient for this function. A summary of the involvement of the in-

sulin receptor kinase in glucose transport stimulation is shown in Fig. 1.

## III. Protein Kinase C

Protein kinase C represents a ubiquitous family of kinases characterized by $Ca^{2+}$- and phospholipid dependence, as well as activation by diacylglycerol. In purified form, or in intact cells, the kinase is also activated by phorbol esters, which are structural analogues of diacylglycerol. The purified kinase phosphorylates polypeptides at serine and threonine residues, and indeed endogenous proteins phosphorylated at these residues are found in cells treated with phorbol esters. The majority of studies with phorbol esters have employed the co-mitogen and tumor promoter phorbol myristate acetate (PMA). The effects of PMA on carbohydrate and lipid metabolism are multiple, and a review of this topic is beyond our scope. This review focuses on the effect of phorbol esters on glucose transport, and on the potential role of protein kinase C in the stimulation of glucose transport by insulin.

### Is Protein Kinase C Involved in Glucose Transport Regulation?

#### A. Phorbol Esters Enhance Glucose Transport

In the past decade, numerous cell types have been shown to respond to phorbol esters with stimulation of hexose uptake. Table 4 summarizes most of the studies in which hexose transport was measured. In general, and with the exception of human epidermal cells and rat skeletal muscle, cells responded to the phorbol ester with a rise in glucose transport. It must be noted that the list contains mostly cells in culture. In fact, adult rat skeletal muscle was insensitive to phorbol esters, leading Sowell et al. (1986) to suggest that stimulation of glucose transport by phorbol ester is an exclusive property of cells in culture. However, it was recently demonstrated that in isolated rat adipocytes hexose uptake can be stimulated by phorbol esters (Muhlbacher et al., 1988; Stralfors, 1988).

##### *Specificity of Phorbol Esters in Stimulating Glucose Transport: Mediation through Protein Kinase C?*

In order to establish the mechanism of action of PMA on hexose transport, the effects of oleylacylglycerol and of phorbol dibutyrate were compared with those of PMA. Some studies (denoted APS under *Comments* in Table 4) demonstrated that stimulation of hexose transport was specific for phorbol esters that stimulate protein kinase C, whereas there was no response of hexose transport to phorbol derivatives that were protein kinase C inactive. A notable exception is a recent study by Stralfors (1988) who tested 1,2-diacylglycerols of diverse fatty acid composition in rat fat cells and demonstrated that those diacylglycerols that activate protein kinase C do not stimulate hexose transport and vice versa. Thus, a direct participation of protein kinase C in the stimulation of glucose transport by phorbol esters remains to be demonstrated.

In an attempt to further answer this question, Gibbs et al. (1986), Allard et al. (1987), and Joost et al. (1987) studied the level of phosphorylation of the glucose transporter immunoprecipitated from phorbol ester-treated human fibroblasts, 3T3-L1 adipocytes or rat adipocytes, respectively. All three studies reported phosphorylation of the glucose transporter, but no quantitative correlation between the degree of phosphorylation and the degree of stimulation of glucose transport was found. It must be mentioned, however, that the glucose transporter was immunoprecipitated in those studies by an antiserum that may recognize only one subtype of glucose transporters (James et al., 1988). Therefore, it remains to be confirmed whether phosphorylation of the transporter is linked to activation of transport. This ambiguity is further stressed by the phosphorylation of the glucose transporter in PMA-treated human erythrocytes, which does not result in stimulation of hexose transport (Witters et al., 1985). It also remains to be determined whether the phosphorylation of the transporter is caused by protein kinase C, and whether this occurs directly or through the mediation of a second kinase previously activated by protein kinase C. In this regard it should be mentioned that, *in vitro,* isolated glucose transporter from human erythrocytes is phosphorylated by purified protein kinase C (Witters et al., 1985).

##### *2. Phorbol Esters Can Stimulate Glucose Transport through Recruitment and Synthesis of New Transporters*

In most studies quoted in Table 4, micromolar concentrations of the phorbol ester and in general exposures of less than 1 hr sufficed to generate a consistent three-fold stimulation of hexose transport. Stimulation was observed in some cases as early as 4 sec after addition of PMA, suggesting that the response may not require *de novo* synthesis of

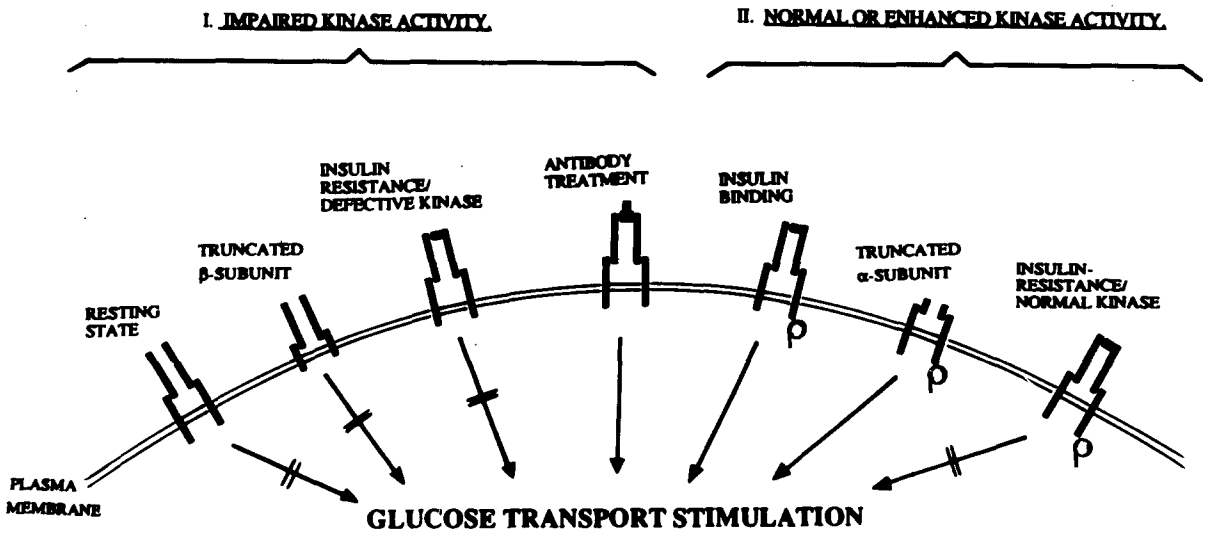


**Fig. 1.** Schematic summary of the effect of modifications in the insulin receptor kinase activity on hormonal stimulation of glucose transport. The solid bar structures represent the insulin receptor subunits. Absence of *P* signifies absence or decrease in autophosphorylation/tyrosine kinase activity of the insulin receptor. *P* signifies intact or enhanced autophosphorylation/kinase activity of the insulin receptor. Arrows indicate ability and crossed-arrows indicate inability to stimulate glucose transport above basal levels. For references on individual experiments, please refer to section II of the text

**Table 4.** Effect of phorbol esters on glucose transport

| Cell | P. ester | Conc. | Time | Glc. transport | Comments | Reference |
|---|---|---|---|---|---|---|
| Rat adipocyte | PMA | 1 nM | 4 sec | Incr. 3× | Translocation | Muhlbacher et al., 1988 |
| " " | DAG | 0.1 mM | 1 hr | Incr. 3–5× | No corr. PK-C | Stralfors, 1988 |
| Rat thymocyte | PMA | 0.16 μM | 0.3–1 hr | Incr. 3× | APS | Klip et al., 1984 |
| Mouse thymocyte | PMA | 0.16 μM | 0.3–1 hr | Incr. 3× | | Nordenberg et al., 1983 |
| Rat sk muscle | PMA/OAG | 0.8 μM | 0.5–1 hr | No Incr. | | Sowell et al., 1986 |
| Human neutrophil | PMA/OAG | 1 nM/33 μM | 1 hr | Incr. 3×, 6× | APS | McCall et al., 1985 |
| Human fibroblast | PMA | 0.3 μM | 0.3 hr | Incr. 0.5× | Transporter phosphorylated | Allard et al., 1987 |
| BALB/c3T3 preadipocyte | PMA | 0.16 μM | 4 hr | Incr. 2×/7× | glc-free/glc | O'Brien & Saladik, 1982 |
| CE fibroblast | PMA | 0.1 μM | 4 hr | Incr. 3–4× | Chx inhibitable, 2× Incr. in CB binding | Driedger & Blumberg, 1977 Yamada et al., 1986 |
| 3T3 fibroblast | MOG | 0.1 mM | 1 hr | Incr. 0.2× | Incr. DAG | Takuwa et al., 1988 |
| " " | PMA | 0.1 μM | 3 hr | | Incr. CB binding to PM | Kitagawa et al., 1985 |
| 3T3-L1 fibroblast | PDB | 1 μM | 0.5 hr | Incr. 1× | | Klip et al., 1988 |
| 3T3-L1 adipocyte | PDB | 1 μM | 0.5 hr | Incr. 1.3× | | " " " |
| " " " | PMA | 1 μM | 0.3–1 hr | Incr. 2× | Transporter phosphorylated | Gibbs et al., 1986 |
| HeLa cell | PMA | 1–40 nM | 0.5–1 hr | Incr. 2× | Decr $K_m$, = $V_{max}$ | Lee & Weinstein, 1979 |
| L6 muscle cell | PDB | 0.1 μM | 0.6 hr | Incr. 0.5× | APS | Klip & Ramlal, 1987*a* |
| BC3H-1 myocyte | PMA | 0.1 μM | 0.2 hr | Incr. 4× | APS | Farese et al., 1985 |
| " " | OAG | 0.25 mM | 0.2 hr | Incr. 0.4× | | " " |
| Human epidermal cells | PMA | 16 nM | 3 hr | Decr. 0.3× | | Chida & Kuroki, 1984 |

Incr.: increase; Decr.: decrease; PK-C: protein kinase C; CE: chick embryo; sk: skeletal; Chx: cycloheximide; CB: cytochalasin B; glc: glucose (in medium); PM: plasma membranes; PMA: phorbol myristate acetate (or TPA: 12-O-tetradecanoylphorbol-13-acetate); PDB: 4β-phorbol-12,13-dibutyrate; OAG: 1-O-octadecenyl,2-O-acetylglycerol; MOG: 1-Monooleylglycerol (diacylglycerol kinase inhibitor); DAG: 1,2-Diacylglycerol; APS: Active phorbol-specific (specific for phorbol esters that stimulate protein kinase C).

transport systems. Indeed, Kitagawa et al. (1985) demonstrated a cycloheximide-independent elevation in the number of glucose transporters in plasma membranes isolated from 3T3 fibroblasts treated with PMA for 3 hr. Hence, the increase did not result from net synthesis of transporters; instead, it appeared to be caused by translocation of pre-existing transporters to the plasma membrane, since it was accompanied by a decrease in glucose transporter number in a microsomal fraction. Unfortunately, there were no concomitant measurements of the stimulation of glucose transport so that a correlation between transport and transporter number cannot be established. The most detailed analysis of the mechanism of stimulation of glucose transport by phorbol esters to date is that of Muhlbacher et al. (1988) showing a decrease in the number of glucose transporters in intracellular membranes and a concomitant increase in plasma membranes isolated from rat adipocytes treated for only 4 sec with PMA. The threefold elevation in the number of glucose transporters in isolated membranes correlated with the threefold elevation in glucose transport in intact cells.

Longer exposure to phorbol esters may result in participation of protein synthesis mechanisms sustaining the stimulation of hexose transport. Indeed, Driedger and Blumberg (1977) showed that the stimulation of glucose transport observed in chick embryo fibroblasts after 4 hr of exposure to PMA was inhibited by the simultaneous addition of cycloheximide to inhibit protein synthesis, and Yamada et al. (1986) detected a doubling in the number of glucose transporters in total microsomes isolated from fibroblasts treated for several hours with PMA. This rise would presumably result from new synthesis of transporters. Recently, Flier et al. (1987) demonstrated that in rat 3T3 fibroblasts treated with PMA for 18 hr there was an induction of the glucose transporter mRNA. This increase correlated with a gain in glucose transporters detected by immunoreactivity with a specific antibody, and with an elevation in glucose transport.

The above results indicate that the early stimulation of glucose transport by PMA may be caused by translocation of glucose transporters from an intracellular pool to the cell membrane. Longer incubations with the phorbol ester may lead to synthesis of new transporters, presumably as a result of transcriptional regulation of the glucose transporter mRNA. Kinetically, both effects would be reflected in an increase in the $V_{max}$ of glucose transport. Indeed, this was observed in PMA-treated BC3H-1 myocytes (Standaert et al., 1988).

### *B. Exogenous Phospholipases Can Stimulate Glucose Transport*

Since stimulation of glucose transport occurs in response to exogenous phorbol esters, and since the latter are structural analogues of diacylglycerol, it is reasonable to expect that endogenously generated diacylglycerol would stimulate glucose transport. This possibility was tested by exposing intact cells to phospholipase C's known to augment the cell content of diacylglycerol. Exogenous bacterial phospholipase C with specificity for phosphoinositides stimulated glucose transport in rat adipocytes (Goldman & George, 1988) and in BC3H-1 myocytes (Standaert et al., 1988). However, an inositol phosphoglycan-specific phospholipase C, which also generates diacylglycerol (Saltiel, 1987), was ineffective in stimulating glucose transport in the latter cells (Standaert et al., 1988). In contrast, in rat adipocytes this phospholipase C stimulated glucose transport (Saltiel & Sorbara-Cazan, 1987). It remains to be determined whether the levels of diacylglycerol produced by these phospholipases correlates with their effects on glucose transport.

## C. Insulin and Phorbol Esters on Glucose Transport. Common Pathways of Action?

### *1. Similarities and Differences in the Stimulation of Glucose Transport by Insulin and Phorbol Esters*

It is widely accepted that, in adipocytes (Cushman & Wardzala, 1980; Suzuki & Kono, 1980) and in skeletal muscles (Wardazala & Jeanrenaud, 1981; Klip et al., 1987) insulin induces rapid recruitment of glucose transporters to the plasma membrane. This phenomenon is independent of new protein synthesis. The resulting enhancement in glucose transporters on the cell surface appears to account for a large part of the elevation in glucose transport. In contrast, the mechanism of stimulation of glucose transport by phorbol esters is less well defined.

When comparing the action of insulin and phorbol esters on glucose transport in rat adipocytes, several differences are noted. Whereas both agents stimulate glucose transport within minutes, the fold stimulation caused by the hormone excedes that caused by phorbol ester (Muhlbacher et al., 1988). Moreover, although both agents cause quantitatively similar translocation of glucose transporters to the cell surface in rat adipocytes, insulin further activates transport (Muhlbacher et al., 1988), sug-

gesting stimulation of the intrinsic activity of the transporters by the hormone. Furthermore, PMA but not insulin causes phosphorylation of glucose transporters in the intact cell (Gibbs et al., 1986; Joost et al., 1987). These observations suggest that phorbol esters are not "insulin like" in their action, although they clearly stimulate glucose transport.

### *2. Evidence of Common Pathways in the Stimulation of Glucose Transport by Insulin and Phorbol Esters*

If stimulation of glucose transport by phorbol esters and insulin share a common pathway then simultaneous addition of submaximal concentrations of both agents should produce additive stimulation, but maximal concentrations should not be additive. Conversely, if phorbol ester and hormone act through independent pathways, their stimulation of glucose transport should be additive even at maximal concentrations. This premise has the caveat that the number of glucose transporters may be the limiting factor, and hence maximal stimulation by one single agent may not be further increased by the second one, even if they act through different pathways.

In rat adipocytes, insulin causes a large (up to 30-fold) stimulation of glucose transport, whereas, as seen above, phorbol esters stimulate glucose transport only threefold. This complicates the study of the simultaneous effect of hormone and phorbol ester, since only small changes are expected to be caused by the latter on top of the large stimulation by the hormone. Accordingly, at maximal stimulatory concentrations of insulin, PMA did not cause any further stimulation of glucose transport (Cherqui et al., 1986). When different concentrations of insulin were tested in the presence of PMA, the phorbol ester had no effect at low insulin concentrations, but decreased stimulation of glucose transport by about 20% at hormone concentrations above 10 nM (Haring et al., 1986). Similarly, a decrease in the maximal insulin-stimulated hexose transport was caused by the simultaneous presence of phorbol dibutyrate (Kirsch et al., 1985). This was interpreted to result from antagonistic action of the phorbol ester at the receptor and post-receptor levels in these cells (*see* next section).

In cultured cell lines such as 3T3-L1 adipocytes, maximal stimulation of glucose transport by insulin exceeds only two- to threefold that by phorbol esters (Klip et al., 1988), whereas in 3T3-L1 fibroblasts (Klip et al., 1988) and L6 muscle cells (Klip & Ramlal, 1987*a*) the maximal stimulation by each agent is comparable, and in BC3H-1 myocytes phorbol esters are slightly more potent than insulin (Farese et al., 1985). Hence additive effects of phorbol ester and insulin can be assessed better in these cell types compared to rat adipocytes. In all of the above-mentioned cell cultures, maximal doses of insulin and phorbol ester (PMA or phorbol dibutyrate) did not produce additive stimulation of glucose transport. The stimulation by phorbol ester and insulin was similar to that caused by insulin alone. Submaximal doses of insulin and phorbol dibutyrate produced additive stimulation of glucose transport in L6 muscle cells (Klip & Ramlal, 1987*a*). These results are consistent with, but do not prove, common pathways of action of the hormone and the phorbol ester for stimulation of glucose transport.

### *3. Effects of Phorbol Esters on the Insulin Receptor*

Since only a fraction (about 10%) of the insulin receptors needs to be occupied for maximal stimulation of glucose transport, a decrease in insulin receptors is reflected in a decrease of sensitivity rather than of responsiveness. A decrease in sensitivity to insulin by phorbol esters has not been reported, but, as seen above, PMA caused a decrease in insulin responsiveness in rat adipocytes. This could be caused by post-binding effects, including modulation of kinase activity of the receptor. Indeed, PMA had no effect on insulin binding to rat adipocytes (Cherqui et al., 1986). However, contrasting reports by Kirsch et al. (1985) and Haring et al. (1986) show a PMA-induced 30% decrease in insulin binding in the same cells. As explained above, however, this change could not underlie the decrease in responsiveness observed in the same studies. Thus, the inhibitory effect of PMA on maximal stimulation of glucose transport by insulin cannot be explained by changes in insulin binding, although decreased binding was observed in some cases; post-binding action has been proposed to explain the stimulation of hexose transport by phorbol esters (*see* next section).

The effect of phorbol esters on the binding of insulin to insulin responsive cells[3] other than rat adipocytes has not been reported. However, insulin binding in the presence of phorbol esters has been measured in cells of hepatic origin. In Hep G2 cells, prolonged incubation with PMA actually raised insulin binding, presumably through inhibition of re-

[3] *See* footnote 1, p. 1.

ceptor recycling (Blake & Strader, 1986), and in H35 hepatoma cells, shorter exposure to PMA did not affect insulin binding (Takayama et al., 1984).

### *4. Phorbol Esters Can Regulate the Insulin Receptor Kinase*

As concluded in section II, participation of the insulin receptor kinase in stimulation of glucose transport is suggested by most studies, but it is not fully proven. Many studies on insulin receptor autophosphorylation and kinase activity have been performed in insulin unresponsive cells.[3] The same applies for studies on the effect of phorbol esters on receptor autophosphorylation and kinase activity. Hence it is difficult to relate these effects to the inhibition of insulin-stimulated glucose transport caused by PMA in adipocytes, or to the insulin-independent stimulation of glucose transport by phorbol esters in most cells. However, there are documented effects of phorbol esters on insulin receptor autophosphorylation and kinase activity, and these are summarized below. It is possible that in the future it may become possible to relate these effects to stimulation of glucose transport in responsive cells.

In Fao cells, of hepatic origin, PMA alone increased phosphorylation of the insulin receptor on serine residues (Takayama et al., 1984). Similar observations were made in H35 hepatoma cells treated with either diacylglycerol or PMA (Davies & Czech, 1985), and in Hep G2 cells treated with dioleylglycerol or PMA (Jacobs & Cuatrecasas, 1986). In the latter two cell types, PMA also caused a minor elevation in threonine phosphorylation. In intact cells, insulin causes serine phosphorylation of its receptor, in addition to the tyrosine autophosphorylation. It has been postulated that serine phosphorylation of the receptor could result from insulin-dependent activation of protein kinase C, with the receptor in turn serving as a substrate for this kinase. However, Jacobs et al. (1985) and Jacobs and Cuatrecasas (1986) found, by peptide map analysis, that three tryptic peptides of the receptor serine-phosphorylated by PMA are not phosphorylated in receptors isolated from insulin-treated IM9 or Hep G2 cells. IM9 cells are of lymphocytic origin, and although rich in insulin receptors they are also insulin unresponsive.[4] Moreover, at maximal doses, insulin and PMA or phorbol dibutyrate caused an almost additive augmentation in phosphoserine in insulin receptors from Hep G2 or IM9 cells (Jacobs et al., 1985). Thus, the insulin-dependent increase in receptor phosphoserine may be caused by a kinase other than protein kinase C.

PMA also inhibited the insulin-mediated stimulation of tyrosine phosphorylation by 50% in Fao cells (Takayama et al., 1988). The insulin receptor purified from PMA-treated Fao cells displayed decreased kinase activity towards exogenous substrates, caused by a decrease in $K_m$ for ATP. Interestingly, dephosphorylation of the receptor serine residues back to their resting level with alkaline phosphatase also resulted in reversal of the PMA-induced inhibition of insulin-dependent tyrosine phosphorylation (Takayama et al., 1988). This suggests that the serine phosphorylation caused by PMA can repress tyrosine autophosphorylation of the receptor.

Only a few studies have explored the effect of phorbol esters on insulin receptor phosphorylation in insulin responsive cells.[4] In rat adipocytes, PMA decreased insulin-dependent tyrosine autophosphorylation and tyrosine kinase activity towards synthetic substrates, by increasing fourfold the $K_m$ for ATP (Haring et al., 1986). Moreover, in intact adipocytes treated with PMA and insulin there was a decrease in the insulin-dependent phosphorylation of target proteins with $M_r$ of 40,000, 50,000 and 116,000 (Ermel et al., 1987). This suggests that the phorbol ester may alter the phosphorylation of putative intermediates in insulin signalling. Because neither the identity nor the function of these polypeptides are known, the functional consequence of the PMA-dependent inhibition of phosphorylation cannot be evaluated at present.

## D. Effect of Insulin on Phosphoinositide Metabolism

### *1. Insulin Elevates Diacylglycerol Levels through de Novo Synthesis*

Since phorbol esters mimic the ability of insulin to stimulate glucose transport, it can be hypothesized that insulin activates protein kinase C and thereby stimulates glucose transport. For insulin to stimulate protein kinase C the hormone should raise the levels of the endogenous activator of the kinase, diacylglycerol. Alternatively, one could envisage insulin stimulation of protein kinase C to occur through changes in the affinity of the kinase for its substrates or changes in its subcellular localization. This section reviews the evidence for and against elevated production of diacylglycerol by insulin. Following sections deal with the effect of insulin on protein kinase C activity and subcellular distribution.

A rise in diacylglycerol can occur by either increased *de novo* synthesis or increased phospholipid hydrolysis by phospholipase C. Because the lat-

[4] *See* footnote 1, p. 1.

ter reaction would concomitantly liberate polar head groups from phospholipids, considerable attention has been given to the production of inositol phosphates and choline phosphates in insulin-treated cells. These possibilities have been investigated in a variety of cells.

In IM9 unresponsive cells[5], insulin did not augment diacylglycerol levels (Jacobs et al., 1985). Similarly, in unresponsive[5] rat liver cells (Sakai & Wells, 1986) and in insulin-responsive[5] rat adipocytes (Pennington & Martin, 1985; Augert & Exton, 1988) insulin did not cause breakdown of inositol phospholipids at any concentration of the hormone. Instead, in rat adipocytes the hormone caused a fivefold increase in incorporation of inositol into inositol phospholipids through *de novo* synthesis of these phospholipids (Pennington & Martin, 1985; Augert & Exton, 1988). Unfortunately, the levels of diacylglycerol were not measured directly in those studies.

In contrast, in responsive[6] BC3H-1 myocytes, insulin raised the levels of diacylglycerol, and this was the direct consequence of an increase in *de novo* synthesis of diacylglycerol and increased levels of inositol phospholipids (Farese et al., 1984), specifically of phosphatidic acid, the direct precursor of diacyglycerol. Diacylglycerol was found to subsequently incorporate into phosphatidyl choline (Farese et al., 1987). It was also confirmed that there is no augmented hydrolytic activity of inositol phospholipids leading to the increase in diacylglycerol in these cells, based on the lack of change in the levels of inositol phosphates (Farese et al., 1985). However, recently Nair et al. (1988) showed insulin-induced hydrolysis of phosphatidylcholine, leading to a second generation of diacylglycerol. This secondary burst in diacylglycerol was suggested to be *caused* by activation of protein kinase C, insofar as it could be mimicked by phorbol ester. The following sequence of events was proposed: Insulin stimulates an unknown signal that leads to elevation in diacylglycerol and activation of protein kinase C, which in turn causes hydrolysis of phosphatidylcholine to liberate phosphocholine and diacylglycerol.

### *2. Insulin Stimulates Endogenous Phospholipase C Activity*

If insulin were increasing diacylglycerol levels through accelerated hydrolysis of phospholipids, then an insulin-dependent elevation in phospholipase C activity should be detected. Indeed, as reported by Koepfer and Wieland (1984), a phospholipase C activity is augmented in homogenates from insulin-treated rat adipocytes. Unfortunately, neither the subcellular localization of this enzyme nor its specificity were defined.

Recently, it was reported that insulin induces the liberation of polar head groups or inositol phosphoglycans from hepatocytes and BC3H-1 myocytes and transiently increases the levels of diacylglycerol (Saltiel & Cuatrecasas, 1986; Saltiel et al., 1986; 1987). It was postulated that the liberation of inositol phosphoglycans and elevation in diacylglycerol were due to hydrolysis of phosphatidylinositol glycans, through a specific phospholipase C. Indeed, a phospholipase C with specificity for phosphatidylinositol glycans has been isolated from rat liver (Fox et al., 1987). However, it is still unknown whether this enzyme is activated in response to insulin.

This elevation in diacylglycerol was detected by metabolic radioactive labeling of precursor phospholipids. However, the net amount of diacylglycerol produced was too small to represent a significant change over the steady-state levels of diacylglycerol in the membrane. Thus, this diacylglycerol could not produce significant activation of protein kinase C in insulin-treated cells.

In contrast, the liberated inositol phosphoglycans comply with the requirements to be insulin mediators (Saltiel, 1987). These polar head groups mimic several actions of insulin in cell free systems and in intact adipocytes including stimulation of glycogen synthase, inhibition of adenylate cyclase, stimulation of the low $K_m$ phosphodiesterase, etc., but conspicuously they fail to stimulate glucose transport (Kelly et al., 1987; Saltiel & Sorbara-Cazan, 1987).

## E. Effects of Insulin on Protein Kinase C

For protein kinase C to play a role in insulin signalling, its activity must change in insulin-treated cells relative to control cells. Studies on the effect of insulin on protein kinase C comprise three aspects: detection of protein kinase C by binding of phorbol esters, measurements of protein kinase C activity in cell extracts, and measurements of subcellular distribution of protein kinase C. In the latter two instances the activity of the enzyme is generally measured as $Ca^{2+}$- and phospholipid-dependent phosphorylation of histone type III-S.

### *1. Insulin Does Not Decrease Phorbol Ester Binding*

Phorbol esters interact with protein kinase C, and it has been suggested that binding of radiolabeled phorbol esters could potentially be used to estimate

[5] *See* footnote 1, p. 1.
[6] *See* footnote 1, p. 1.

the levels of protein kinase C. In isolated plasma membranes from insulin-treated rat adipocytes, there was a small *decrease* in phorbol dibutyrate binding, and a concomitant increase in the cytosol (Pershadsingh et al., 1987*a*). However, the changes were very small and not clearly statistically significant. Moreover, as reported by Skoglund et al. (1985), the dose-response curve of phorbol ester binding shows no correlation with the dose-response curve of the metabolic effects of the ester. Further, it appears that changes in phorbol dibutyrate binding can be detected in the absence of changes in protein kinase C activity in rat adipocytes (Draznin et al., 1988). These arguments preclude the reliable use of phorbol dibutyrate binding to assess participation of protein kinase C in hormone action.

### *2. Insulin Does Not Alter Protein Kinase C Subcellular Distribution but Can Regulate Protein Kinase C Activity*

It has been known for several years that phorbol esters induce rapid migration of protein kinase C from the cytosol to membranes, and this translocation has been correlated with biological activity. It was recently postulated that insertion into the membrane is the primary mechanism of protein kinase C activation (Bazzi & Nelsestuen, 1987).

In contrast to phorbol ester binding, the dose-response curve of translocation of protein kinase C does appear to correlate with the dose-response curve of phorbol ester metabolic actions (Skoglund et al., 1985). Since the insulin receptor is membrane bound, and since protein kinase C can migrate from cytosol to membranes, most studies analyzing the effect of insulin on protein kinase C have measured this activity in cytosolic and membrane fractions from insulin-treated and control cells. It must be noted, however, that in most studies the membrane fraction contains total cell membranes rather than purified plasma membranes.

In 3T3-L1 fibroblasts (Klip et al., 1988), 3T3-L1 adipocytes (Glynn et al, 1986; Klip et al., 1988), L6 muscle cells (Klip & Ramlal, 1987*a*) and rat adipocytes (Draznin et al., 1988) insulin did not increase protein kinase C activity in the membrane fraction, nor did it diminish this activity in the cytosol. In fact, a small but measurable *increase* in protein kinase C activity was detected in the soluble fraction. In insulin unresponsive hepatocytes,[7] there was no change in cytosolic activity of protein kinase C upon insulin treatment (Vaartjes et al., 1986). In contrast, all of these cells, i.e., 3T3-L1 fibroblasts, 3T3-L1 adipocytes, L6 muscle cells and hepatocytes, responded to phorbol esters with a decrease in cytosolic protein kinase C activity and an increase in microsomal protein kinase C activity. This confirms that insulin and phorbol esters have different effects on protein kinase C.

The above results indicate that insulin does not induce translocation of protein kinase C from the cysotol to membranes. However, an increase in protein kinase C activity in the microsomal fraction was reported for the insulin responsive[8] rat diaphragm (Walaas et al., 1987) and BC3H-1 myoctes (Cooper et al., 1987*a*). In the former tissue, insulin raised the activity in the membrane without changing it in the cytosolic fraction. Activity in this case was assessed by histone phosphorylation in vitro as well as by phosphorylation of an endogenous 87-kDa polypeptide.

In BC3H-1 myocytes insulin elevated protein kinase C activity in both the microsomal and cytosolic fractions (Cooper et al., 1987*a*). The cytosolic enzyme levels peaked after 60 sec of addition of the hormone, decreased and then increased a second time after 15 min. This effect was clearly different from that caused by PMA in the same cells. These authors have proposed that the reason for the lack of detection of activation of membrane-bound protein kinase C in other studies could be breakdown of the kinase. Indeed, it is known that, following phorbol ester-mediated migration of the kinase to membranes, there is a slow decrease in total enzyme activity due to its breakdown to a 50-kDa form which is still capable of kinase activity, albeit $Ca^{2+}$ independent (Girard et al., 1986). Thus, this activity would normally not be detected by traditional protein kinase C assays. This smaller form of the kinase is subsequently degraded. This sequence of events is the basis for the "downregulation" or depletion of protein kinase C (*see* next section).

Collateral information on the action of insulin on protein kinase C has emerged from studies on diabetic animal models. The rationale behind these studies is that, if insulin activates protein kinase C, then a decrease in this activity should accompany insulin deficiency or insulin resistance. In heart muscle from insulin-resistant obese fa/fa mice, phorbol ester failed to stimulate glucose transport. This correlated with augmented cytosolic and diminished microsomal protein kinase C activity, relative to control mice. Moreover, there was impaired translocation of protein kinase C activity in response to PMA in the obese mice (Van de Werve et al., 1987). This information suggests that phorbol

[7] *See* footnote 1, p. 1.

[8] *See* footnote 1, p. 1.

**Table 5.** Effect of prolonged incubation with phorbol ester on hexose transport and insulin action in 3T3-L1 adipocytes

| Pretreatment | Acute treatment (30 min) | | | |
|---|---|---|---|---|
| | (−) | Insulin | Phorbol dibutyrate | |
| | 2-deoxyglucose uptake, pmol/min · mg protein | | | Insulin/(−) |
| 36 hr (−) | 24 ± 2 | 47 ± 4 | 47 ± 5 | 2.0 |
| 36 hr + phorbol dibutyrate | 29 ± 2 | 43 ± 3 | 28 ± 1 | 1.5 |

Cells were incubated for 36 hr with 1 μM PDB. This treatment resulted in loss of over 75% of the protein kinase C activity. Cells were rinsed free of PDB and subjected to acute (30 min) exposure to: 1 μM insulin or 1 μM PDB. Hexose uptake (carrier-dependent) was next determined.

Pretreatment with PDB obliterated the subsequent response to acute exposure to PDB, consistent with "downregulation" of protein kinase C. However, the pretreatment did not prevent stimulation of glucose transport by insulin.

ester action is defective in heart muscle of these mice. However, this does not establish whether the defect in response to PMA is a cause or a consequence of the insulin-resistance. A different defect was detected in diabetic (STZ-induced) rat myocardium, where diacylglycerol levels were significantly elevated relative to controls (Okumura et al., 1988). However, these levels were partly corrected with progression of the diabetes, and furthermore insulin treatment did not decrease the levels of diacylglycerol back to normal. Hence, more work is needed to establish whether insulin resistance is generally accompanied by defects in PMA action or diacylglycerol levels, and which of these defects, insulin resistance or PMA insensitivity, precedes the other.

### *3. Insulin Action in Cells with "Downregulated" Protein Kinase C*

If insulin acted through activation of protein kinase C, then it would be expected that hormonal response would be blunted upon cellular depletion of protein kinase C. As indicated above, a protocol was developed by Rodriguez-Pena and Rosengurt (1984) to deplete or markedly decrease the level of protein kinase C through prolonged incubation with phorbol ester. We have observed that in 3T3-L1 fibroblasts or adipocytes, a 4-hr incubation with 1 μM PMA results in loss of over 75% of the total cellular activity of protein kinase C, and in L6 muscle cells >85% of the total activity disappears after 24 hr. PMA treatment results not only in loss of protein kinase C activity towards exogenous histone, but also in loss of immunologically detectable protein kinase C (Blackshear et al., 1985). This protocol has been used widely to test participation of protein kinase C on several pathways, including several actions of insulin. This discussion is restricted to the effect of protein kinase C depletion on stimulation of glucose transport.

In L6 muscle cells (Klip & Ramlal, 1987*a*), Swiss 3T3 fibroblasts (Kitagawa et al., 1986) and 3T3-L1 adipocytes (Klip & Ramlal, 1987*b*; also Table 5), decreasing protein kinase C activity of total cell extracts by over 80% resulted in an expected loss of stimulation of glucose transport by phorbol esters. In contrast, as seen in Table 5 for 3T3-L1 adipocytes the response to insulin remained largely unaffected. Lack of inhibition of insulin stimulation of glucose transport after protein kinase C depletion was also observed in L6 muscle cells (Klip & Ramlal, 1987*a*), 3T3-L1 fibroblasts (Klip & Ramlal, 1987*b*), Swiss fibroblasts (Kitagawa et al., 1986) and BC3H-1 myocytes (Standaert et al., 1988). This strongly suggests that stimulation of glucose transport by insulin is independent of protein kinase C activity. Of course, the untestable possibility remains that in protein kinase C-containing cells, stimulation of glucose transport may still occur through activation of this enzyme. Further, it is possible that the small amount of protein kinase C activity remaining after prolonged treatment with phorbol ester suffices to sustain in full response to the hormone. However, in this case one would still have to postulate a different mechanism of action for insulin and phorbol esters (since the latter failed to stimulate glucose transport in cells with substantially decreased protein kinase C). Interestingly, stimulation of glucose transport by an inositol phosphoglycan-specific phospholipase C was not decreased by protein kinase C depletion (Standaert et al., 1988), suggesting that glycerolipid metabolites can stimulate glucose transport in the absence of the kinase.

In contrast to results in the cell types described above, Cherqui et al. (1987) reported 50% inhibition of the response of glucose transport to insulin in rat adipocytes with decreased protein kinase C activity through pretreatment with phorbol ester. The fact that only 50% of insulin action could be prevented suggests that insulin stimulates glucose transport in these cells by two mechanisms, only one of which involves protein kinase C. Indeed, stimulation of glucose transport by insulin in rat adipocytes differs from that in other insulin-responsive cells in several aspects, i.e., (i) adipocytes show much larger stimulation of transport than most cells (*see* section IIIC1,2); (ii) recruitment of glucose transporters by insulin cannot fully account for stimulation of glucose transport in adipocytes (Simpson & Hedo, 1984), whereas it does in L6 muscle cells (Walker et al., 1989)[9]; (iii) extracellular $Ca^{2+}$ is required for 50% of the insulin response in adipocytes (Pershadsingh et al., 1987*b*), in contrast to independence of extracellular $Ca^{2+}$ for the stimulation of glucose transport by insulin in L6 muscle cells (Klip et al., 1984), heart myocytes (Cheung et al., 1987), Swiss 3T3 cells (Kitagawa et al., 1986) and 3T3-L1 adipocytes (Klip & Ramlal, 1987*c*).

The above results suggest that, whereas in rat adipocytes protein kinase C may participate at least in part in insulin action, response to the hormone in most other cells can occur independently of the presence of the kinase. However, with increasing knowledge of protein kinase C, two aspects must be considered when evaluating studies employing the protocol of depletion of this enzyme. These are the different isozymes of protein kinase C recently identified, and the reliability of histone phosphorylation as an unambiguous assay of protein kinase C. Kariya and Takai (1987), Yoshida et al. (1988) and Nishizuka (1988) have identified at least four types of protein kinase C, with different $Ca^{2+}$ and phospholipid dependence, whereas Cochet et al. (1986) reported that histone phosphorylation may disappear but vinculin phosphorylation may still occur after prolonged incubation with phorbol esters. These two aspects suggest that when protein kinase C was reported to be depleted, only one subgroup of protein kinase C might have been assayed, leaving open the possibility that other protein kinase C's might still be present and mediate insulin action. The most compelling argument against this, however, is that stimulation of glucose transport by phorbol ester was obliterated, while insulin action remained unaffected.

[9] Walker, P., Ramlal, T., Sarabia, V., Koivisto, U.-M., Pessin, J.E., Klip, A. 1989 (*submitted*).

### *4. Effectiveness of Protein Kinase C Inhibitors on Insulin Action*

Several compounds have been shown to inhibit protein kinase C activity, such as mellitin, glycyrrehetic acid, polymyxin B, gossypol, quercitin, sphingolipids, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and staurosporin. These agents inhibit protein kinase C in vitro, and in some cases inhibition has also been demonstrated in intact cells. However, most of these substances have not been tested on glucose transport.

In Swiss 3T3 mouse fibroblasts, pretreatment with glycyrrhetic acid impaired PMA stimulation of hexose transport (Kitagawa et al., 1984). In rat adipocytes mellitin inhibited both insulin- and PMA-stimulation of lipogenesis, at concentrations where basal levels were not affected (Cherqui et al., 1986). However, the stimulation by insulin required much higher doses of mellitin than the stimulation by PMA. The inhibitor was quoted to prevent stimulation of glucose metabolism by both insulin or PMA, presumably through the inhibition of protein kinase C, but unfortunately actual data were not shown. In the same cells, polymyxin B inhibited insulin-, PMA- and phospholipase C-stimulation of glucose transport, suggesting some commonality in the mechanism of action of these compounds (Christensen et al., 1987). However, polymyxin B inhibited more effectively the stimulation by phospholipase C and had the least effect on insulin action, allowing for the possibility that the three stimuli could act through different mechanisms. Gossypol also markedly inhibited the insulin-stimulation of glucose uptake, but curiously this inhibitor elevated basal uptake levels by ~90%, at concentrations where effective inhibition of the stimulated state occurred, and at higher concentrations it inhibited basal transport in parallel to inhibition of the stimulated state. Quercetin inhibited both basal and insulin-stimulated glucose transport. This suggests that gossypol and quercetin are not inhibitors of protein kinase C exclusively and therefore cannot be used reliably to study the role of the kinase in insulin action.

Sphingolipids (sphingosine) inhibit protein kinase C activity and phorbol ester binding in vitro (Hannun & Bell, 1987). This inhibitory action of sphingosine on protein kinase C is thought to be related to simple charge neutralization of the lipid, thereby preventing interaction with the kinase and/or its protein substrate (Bazzi & Nelsestuen, 1987). Indeed, in lymphocytes, sphingosine prevented PMA-induced downregulation of protein kinase C (Grove & Mastro, 1988). Robertson et al. (1987) showed that D-sphingosine inhibited both insulin- and PMA-stimulated hexose transport in rat adipocytes by ~60 and 54%, respectively, but did not

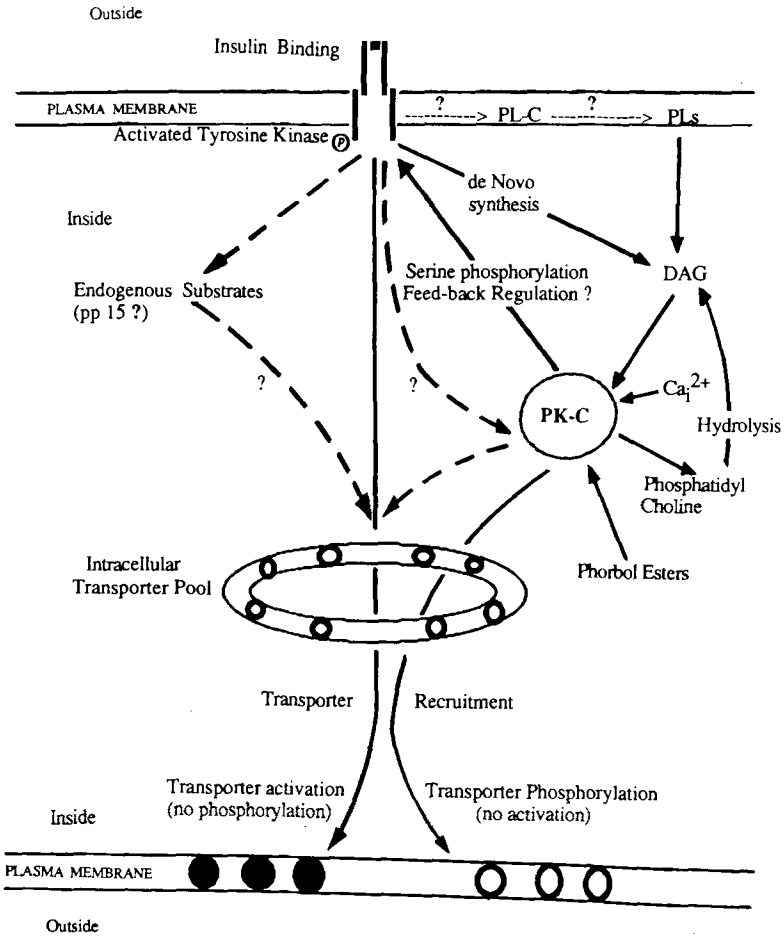


**Fig. 2.** Schematic representation of the multiple interactions between the insulin receptor, protein kinase C and glucose transporter translocation and activation. Solid arrows refer to demonstrated pathways. Broken arrows refer to pathways for which there is controversial evidence. Please refer to section III of the text for references on individual pathways.

prevent stimulation of transport in human fibroblasts. In contrast, in 3T3-L1 fibroblasts, stimulation of glucose transport by PMA or insulin was moderately inhibited by sphingosine and markedly by sphinganine (Nelson & Murray, 1986). The ability of sphingolipids to inhibit both insulin- and phorbol ester-stimulation of glucose uptake suggests again that the two agents may share a common pathway. However, the specificity of sphingosine to inhibit protein kinase C has recently been questioned (Winicov & Gershengorn, 1988; Jefferson & Schulman, 1988).

An inhibitor of protein kinase C denominated H-7 was synthesized by Hidaka et al. (1984). This compound is effective in some intact cells. However, in 3T3-L1 adipocytes, 200 $\mu$M H-7 did not completely abolish the stimulation of glucose uptake by phorbol ester. Under those conditions, the compound only moderately inhibited the effect of insulin[10]. Higher concentrations of H-7 resulted in cell deterioration and inhibition of basal glucose uptake. Recent work by Seifert and Schachtele (1988) demonstrated that the cellular effects of H-7 and polymyxin B exhibit cell specificity, stimulus dependence, and have no correlation with in vitro inhibition of protein kinase C. More specific inhibitors of the kinase are needed to establish the role of protein kinase C in insulin action by a pharmacological approach.

### *5. Possible Interactions between the Insulin Receptor and Protein Kinase C*

The above section A indicated that phorbol esters stimulate glucose transport and share the limiting step with insulin in this stimulation. It has been proposed that insulin stimulation of glucose transport may result from endogenous production of diacylglycerol, leading to activation of protein kinase C and subsequent stimulation of glucose transport (Cherqui et al., 1986; Farese & Cooper, 1988). Section B indicated that phorbol esters modulate the kinase activity of the insulin receptor and in some instances insulin binding. It has been proposed that protein kinase C may participate in insulin signalling by shutting off the receptor after it has been activated by the hormone, in a feedback mechanism

[10] Klip, A., Ramlal, T., and Koivista, U.-M. (*unpublished*).

(Haring et al., 1986). In addition, it is possible that protein kinase C could activate the insulin receptor into a signalling mode leading to stimulation of glucose transport. In this way the insulin-independent stimulation of transport by phorbol esters could be explained. The possible interrelation of these events is shown in Fig. 2.

### F. Conclusions

Section III summarized the action of phorbol esters on glucose transport, compared this action to the stimulation by insulin, and analyzed current evidence for and against participation of protein kinase C in insulin action. Although a definite conclusion of the summarized studies is precluded by the diversity in cell types and responses, it can be said globally that:

(i) Phorbol esters stimulate glucose transport, and this may be the consequence of activation/recruitment of protein kinase C. The latter is substantiated by the observation that phorbol esters lack response after downregulation of protein kinase C activity. Further, phosphorylation of the glucose transporter occurs in phorbol ester-treated cells. However, this phosphorylation does not correlate with the stimulation of transport. Moreover, the participation of the kinase is challenged by the lack of correlation between activation of protein kinase C and stimulation of transport by diacylglycerols of different acyl chain length.

(ii) Insulin and phorbol esters are not additive at maximal stimulatory concentrations, suggesting that they share at least a limiting component in their stimulation of glucose transport. However, insulin does not cause migration of protein kinase C from the cytosol to the cell membrane, although it does elevate the activity of this enzyme in the membrane. Insulin can still stimulate glucose transport in cells with substantially downregulated protein kinase C. Inhibitors of protein kinase C affect basal glucose transport, precluding their use to study participation of protein kinase C in the stimulation by insulin. Finally, insulin does not cause phosphorylation of the glucose transporter, although this awaits confirmation when more specific antisera are used to isolate the insulin-sensitive glucose transporter.

(iii) Insulin does not cause breakdown of inositol phospholipids leading to increases in either diacylglycerol or inositol phosphates. The insulin-dependent augmentation of inositol phosphoglycans is accompanied by a rise in diacylglycerol that is too small to represent a significant gain over the steady-state concentration of diacylglycerol in the membrane. This implies that activation of phospholipase C's by insulin may not participate in the stimulation of glucose transport. The hormone does, however, elevate diacylglycerol transiently by stimulating *de novo* synthesis of phospholipids. Exogenous diacylglycerol stimulates glucose transport, but it is not known if the insulin-induced transient increase in diacylglycerol is responsible for this stimulation. Also, it remains to be determined whether the stimulation of glucose transport by diacylglycerol is through activation of protein kinase C (*see above*).

(iv) It is possible that protein kinase C plays a regulatory role in insulin action, possibly as a feedback inhibitor. This action could be at the level of the receptor phosphorylation and possibly of receptor downregulation.

(v) Insulin also activates other protein kinases in the intact cell, as well as phosphatases. The exact role of these enzymes in insulin action will likely become apparent in the near future.

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## References

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S.-I., Itoh, N., Shibuya, M., Fukami, Y. 1987. *J. Biol. Chem.* **262:**5592–5595

Allard, W.J., Gibbs, E.M., Witters, L.A., Lienhard, G.E. 1987. *Biochim. Biophys. Acta* **929:**288–295

Amatruda, M., Roncone, A.M. 1985. *Biochem. Biophys. Res. Commun.* **129:**163–170

Arner, P., Pollare, T., Lithell, H., Livingston, J.N. 1987. *Diabetologia* **30:**437–440

Arsenis, G., Livingston, J.N. 1986. *J. Biol. Chem.* **261:**147–153

Augert, G., Exton, J.H. 1988. *J. Biol. Chem.* **263:**3600–3609

Ballotti, R., Scimeca, J.C., Kowalski-Chauvel, A., Obberghen, E. van 1988. *Diabetologia* **31:**467A

Bazzi, M.D., Nelsestuen, G.L. 1987. *Biochem. Biophys. Res. Commun.* **146:**203–207

Beguinot, F., Smith, R. J. 1987. *Diabetes* **36:**87A

Bernier, M., Laird, D.M., Lane, M.D. 1987. *Proc. Natl. Acad. Sci. USA* **84:**1844–1848

Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F., Quamo, S.N. 1985. *J. Biol. Chem.* **260:**13304–13315

Blake, A.D., Strader, C.D. 1986. *Biochem. J.* **236:**227–234

Block, N.E., Buse, M.G. 1987. *Clin. Res.* **35:**43A

Bryer-Ash, M., Olefsky, J.M., Freidenberg, G.R. 1987. *Clin. Res.* **35:**147A

Burant, C.F., Treutelaar, M.K., Buse, M.G. 1986*a*. *J. Clin. Invest.* **77:**260–270

Burant, C.F., Treutelaar, M.K., Buse, M.G. 1986*b*. *J. Biol. Chem.* **261:**8985–8993

Caro, J.F., Sinha, M.K., Raju, S.M., Ittoop, O., Pories, W.J., Flickinger, E.G., Meelheim, D., Dohm. G.L. 1987. *J. Clin. Invest.* **79:**1330–1337

Cecchin, F., Ittoop, O., Sinha, M.K., Caro, J.F. 1988. *Am. J. Physiol.* **254:**E394–E401
Cherqui, G., Caron, M., Wicek, D., Lascols, O., Capeau, J., Picard, J. 1986. *Endocrinology* **118:**1759–1769
Cherqui, G., Caron, M., Wicek, D., Lascols, O., Capeau, J., Picard, J. 1987. *Endocrinology* **120:**2192–2194
Cheung, J.Y., Constantine, J.M., Bonventre, J.V. 1987. *Am. J. Physiol.* **252:**C163–C172
Chida, K., Kuroki, T. 1984. *Cancer Res.* **44:**875–879
Chou, C.K., Dull, T.J., Russell, D.S., Gherzi, R., Lebwohl, D., Ullrich, A., Rosen, O.M. 1987. *J. Biol. Chem.* **262:**1842–1847
Christensen, R.L., Shade, D.L., Graves, C.B., McDonald, J.M. 1987. *Int. J. Biochem.* **19:**259–265
Cochet, C., Souvignet, C., Keramidas, M., Chambaz, E.M. 1986. *Biochem. Biophys. Res. Commun.* **134:**1031–1037
Cooper, D.R., Konda, R.S., Standaert, M.L., Davis, J.S., Pollet, R.J., Farese, R.V. 1987*a*. *J. Biol. Chem.* **262:**3633–3639
Cooper, G.J.S., Willis, A.C., Clark, A., Turner, R.C., Sim, R., Reid, K.B.M. 1987*b*. *Proc. Natl. Acad. Sci. USA* **84:**8628–8632
Cushman, S.W., Wardzala, L.J. 1980. *J. Biol. Chem.* **255:**4758–4762
Czech, M.P. (Editor) 1985. Molecular Basis of Insulin Action. Plenum, New York
Czech, M.P., Massague, J., Pilch, P.F. 1981. *Trends Biochem. Sci.* **6:**222–225
Davis, R.J., Czech, M.P. 1985. *In:* Cancer Cells 3: Growth Factors and Transformation. J. Feramisco, B. Ozanne and C. Stiles, editors. pp. 101–108. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Debant, A., Clauser, E., Ponzio, G., Filloux, C., Auzan, C., Contreres, J.-O., Rossi, B. 1988. *Proc. Natl. Acad. Sci. USA* **85:**8032–8036
Debant, A., Guerre-Millo, M., Le Marchand-Brustel, Y., Freychet, P., Laveu, M., Obberghen, E. van 1987. *Am. J. Physiol.* **252:**E273–E278
Douen, A.G., Jones, M.N. 1988. *Biochim. Biophys. Acta* **968:**109–118
Douen, A.G., Kacem, R., Jones, M.N. 1988. *Biochim. Biophys. Acta* **944:**444–450
Draznin, B., Leitner, J.W., Sussman, K.E., Sherman, N.A. 1988. *Biochem. Biophys. Res. Commun.* **156:**570–575
Driedger, P.E., Blumberg, P.M. 1977. *Cancer Res.* **37:**3257–3265.
Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C.S., Siddle, K., Pierce, S.B., Roth, R.A., Rutter, W.J. 1987. *Proc. Natl. Acad. Sci. USA* **84:**704–708
Ebina, Y., Ellis, L., Janagin, K., Edery, M., Graf, L., Clauser E., Ou, J.-H., Masiarz, F., Kan, Y.W., Goldfine, I.D., Roth, R.A., Rutter, W.J. 1985. *Cell* **40:**747–758
Elbrink, J., Bihler, H. 1975. *Science* **183:**1177–1184
Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A., Rutter, W.J. 1986. *Cell* **45:**721–732
Ellis, L., Morgan, D.O., Clauser, E., Roth, R.A., Rutter, W.J. 1987*a*. *Mol. Endocrinol.* **1:**15–24
Ellis, L., Morgan, D.O., Jong, S.-M., Wang, L.-H., Roth, R.A., Rutter, W.J. 1987*b*. *Proc. Natl. Acad. Sci. USA* **84:**5101–5105
Ermel, B., Obermaier, B., Schleicher, E., Haring, H.U. 1987. *Diabetologia* **30:**517A
Farese, R.V., Barnes, D.E., Davis, J.S., Standaert, M.L., Pollet, R.J. 1984. *J. Biol. Chem.* **259:**7094–7100
Farese, R.V., Cooper, D.R. 1989. *Diabetes Metab. (in press)*
Farese, R.V., Konda, T.S., Davis, J.S., Standaert, M.L., Potter, R.J., Cooper, D.R. 1987. *Science* **236:**586–589
Farese, R.V., Standaert, M.L., Barnes, D.E., Davis, J.S., Pollet, R.J., 1985. *Endocrinology* **116:**2650–2655
Flier, J.S., Mueckler, M.M., Usher, P., Lodish, H.F. 1987. *Science* **235:**1492–1495
Forsayeth, J.R., Caro, J.F., Sinha, M.K., Maddux, B.A., Goldfine, I.D. 1987. *Proc. Natl. Acad. Sci. USA* **84:**3448–3451
Fox, J., Soliz, N.M., Saltiel, A.R. 1987. *Proc. Natl. Acad. Sci. USA* **84:**2663–2667
Freidenberg, G.R., Henry, R.R., Klein, H.H., Reichart, D.R., Olefsky, J.M. 1987. *J. Clin. Invest.* **79:**240–250
Frost, S.C., Kohanski, R.A., Lane, M.D. 1987. *J. Biol. Chem.* **262:**9872–9876
Frost, S.C., Lane, M.D. 1985. *J. Biol. Chem.* **260:**2646–2652
Gherzi, R., Andraghetti, G., Ferrannini, E., Cordera, R. 1986. *Biochem. Biophys. Res. Commun.* **140:**850–856
Gherzi, R., Russell, D.S., Taylor, S.I., Rosen, O.M. 1987. *J. Biol. Chem.* **262:**16900–16905
Gibbs, E.M., Allard, J.W., Lienhard, G.E. 1986. *J. Biol. Chem.* **261:**16597–16603
Girard, P.R., Mazzei, G.J., Kuo, J.F. 1986. *J. Biol. Chem.* **261:**370–376
Glynn, B.P., Colliton, J.W., McDermott, J.M., Witters, L.A. 1986. *Biochem. Biophys. Res. Commun.* **135:**1119–1125
Goldman, J., George, A. 1988. *Diabetes* **37:**36A
Grigorescu, F., Flier, J.S., Kahn, C.R. 1984. *J. Biol. Chem.* **259:**15003–15006
Grigorescu, F., Jesuran, M., Jean, R., Mirouze, J. 1987. *Diabetes* **36:**2A
Grove, D.S., Mastro, A.M. 1988. *Biochem. Biophys. Res. Commun.* **151:**94–99
Grunberger, G., Zick, Y., Gorden, P. 1984. *Science* **223:**932–934
Hannun, Y.A., Bell, R.M. 1987. *Science* **235:**670–674
Haring, H.U., Kirsch, D., Hoelzl, J., Herberg, L., Eckel, J. 1984*a*. *Diabetologia* **27:**238A
Haring, H.U., Kirsch, K., Obermaier, B., Ermel, B., Machicao, F. 1986. *J. Biol. Chem.* **261:**3869–3875
Haring, H.U., White, M.F., Kahn, C.R., Kasuga, M., Lauris, V., Fleischman, R., Murray, M., Pawelek, J. 1984*b*. *J. Cell. Biol.* **99:**900–908
Haring, H.U., White, M.F., Machicao, F., Ermel, B., Schleicher, E., Obermaier, B. 1987. *Proc. Natl. Acad. Sci. USA* **84:**113–117
Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y. 1984. *Biochemistry* **23:**5036–5041
Jacobs, S., Cuatrecasas, P. 1986. *J. Biol. Chem.* **261:**934–939
Jacobs, S., May, S., Watson, S., Lapetina, E., Cuatrecasas, P. 1985. *In:* Cancer Cells **3:** Growth Factors and Transformation. J. Feramisco, B. Ozanne and C. Stiles, editors. pp. 139–143. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
James, D.E., Brown, R., Navarro, J., Pilch, P.F. 1988. *Nature (London)* **333:**183–185
Jefferson, A.B., Schulman, H. 1988. *J. Biol. Chem.* **263:**15241–15244
Joost, H.G., Weber, T.M., Cushman, S.W., Simpson, I.A. 1987. *J. Biol. Chem.* **262:**11261–11267
Kadowaki, T., Kasuga, M., Akanuma, Y., Ezaki, O., Takaku, F. 1984. *J. Biol. Chem.* **259:**14208–14216
Kahn, C.R., Flier, J.S., Bar, R.S., Archer, J.A., Gorden, P., Martin, M.M., Roth, J. 1976. *New Engl. J. Med.* **294:**739–745
Kariya, K., Takai, Y. 1987. *FEBS Lett.* **219:**119–124
Kasuga, M., Akanuma, Y., Iwamoto, Y., Kosaka, K. 1978. *Am. J. Physiol.* **235:**E175–E182
Kasuga, M., Karlsson, F.A., Kahn, C.R. 1982*a*. *Science* **215:**185–187

Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., Kahn, C.R. 1982*b*. *Nature (London)* **298:**4303–4306
Kelly, K.L., Mato, J.M., Merida, I., Jerett, L. 1987. *Proc. Natl. Acad. Sci. USA* **84:**6404–6407
Kergoat, M., Simon, J., Portha, B. 1988. *Biochem. Biophys. Res. Commun.* **152:**1015–1022
Kirsch, D., Obermaier, B., Haring, H.U. 1985. *Biochem. Biophys. Res. Commun.* **128:**824–832
Kitagawa, K., Nishino, H., Iwashima, A. 1984. *Cancer Lett.* **24:**157–163
Kitagawa, K., Nishino, H., Iwashima, A. 1985. *Biochem. Biophys. Res. Commun.* **128:**1303–1309
Kitagawa, K., Nishino, H., Iwashima, A. 1986. *Biochim. Biophys. Acta* **887:**100–104
Klip, A., Burdett, E. 1989. *Diabetes* **38(Suppl. 2):**16A
Klip, A., Ramlal, T. 1987*a*. *Biochem. J.* **24:**131–136
Klip, A., Ramlal, T. 1987*b*. *Diabetes* **36:**52A
Klip, A., Ramlal, T. 1987*c*. *J. Biol. Chem.* **262:**9141–9146
Klip, A., Ramlal, T., Koivisto, U.-M. 1988. *Endocrinology* **123:**296–304
Klip, A., Ramlal, T., Young, D.A., Holloszy, J.O. 1987. *FEBS Lett.* **224:**224–230
Klip, A., Rothstein, A., Mack, E. 1984. *Biochem. Biophys. Res. Commun.* **124:**14–22
Klip, A., Walker, D., Ransome, K.J., Schroer, D., Lienhard, G.E., 1983. *Arch. Biochem. Biophys.* **226:**198–205
Kobayashi, M., Olefsky, J.M. 1979. *Diabetes* **28:**87–95
Koepfer, B., Wieland, O.H. 1984. *Mol. Cell. Endocrinol.* **36:**123–129
Kohanski, R.A., Frost, S.C., Lane, M.D. 1986. *J. Biol. Chem.* **26:**12272–12281
Lee, L.S., Weinstein, I.B. 1979. *J. Cell. Physiol.* **99:**451–460
Le Marchand-Brustel, Y., Gremeaux, T., Ballotti, R., Obberghen, E. van 1985. *Nature (London)* **315:**676–679
Maassen, J.A., Klinkhamer, M.P., Zon, G.C.M. van der, Sips, H., Moller, W., Krans, H.M.J., Linkhout, D., Beemer, F.A. 1988. *Diabetologia* **31:**612–617
Madoff, D.H., Martensen, T.M., Lane, M.D. 1988. *Biochem. J.* **252:**7–15
Maegawa, H., Olefsky, J.M., Thies, S., Boyd, D., Ullrich, A., McClain, D.A. 1988. *J. Biol. Chem.* **263:**12629–12637
Massague, J., Pilch, P.F., Czech, M. P. 1980. *Proc. Natl. Acad. Sci. USA* **77:**7137–7141
McCall, C., Schmitt, J., Cousart, S., Flaherty, J., Bass, D., Wykle, R. 1985. *Biochem. Biophys. Res. Commun.* **126:**450–456
McClain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ullrich, A., Olefsky, J.M. 1987. *J. Biol. Chem.* **262:**14663–14671
Misbin, R.I., Green, A., Alvarez, I.M., Almira, E.C., Dohm, G.L., Caro, J.F. 1988. *Diabetes* **37:**1217–1225
Mojsilovic, L.P., Standaert, M.L., Rosic, N.K., Pollet, R.J. 1986. *Clin. Res.* **34:**224A
Morgan, D.O., Roth, R.A. 1987. *Proc. Natl. Acad. Sci. USA* **84:**41–45
Muhlbacher, C., Karnieli, E., Schaff, P., Obermaier, B., Muschack, J., Rattenhuber, E., Haring, H.U. 1988. *Biochem. J.* **249:**865–870
Nair, G.P., Standaert, M.L., Pollet, R.J., Cooper, D.R., Farese, R.V. 1988. *Biochem. Biophys. Res. Commun.* **154:**1345–1349
Nelson, D.H., Murray, D.K. 1986. *Biochem. Biophys. Res. Commun.* **138:**463–467
Nemenoff, R.A., Kwok, Y.C., Shulman, C.I., Blackshear, P.J., Osathanondh, R., Avruch, J. 1984. *J. Biol. Chem.* **259:**5058–5065
Nishizuka, Y. 1988. *Nature (London)* **334:**661–665
Nordenberg, J., Stenzel, K.H., Novogrodsky, A. 1983. *J. Cell. Physiol.* **117:**183–188
O'Brien, T.G., Saladik, D. 1982. *J. Cell. Physiol.* **112:**376–384
Obermaier, B., Su, Z., Muhlbacher, C., Haring, H. 1987. *Diabetalogia* **30:**563A
Okamoto, M., White, M.F., Maron R., Kahn, C.R. 1986. *Am. J. Physiol.* **251:**E542–E550
Okumura, K., Akiyama, N., Hidekasu, H., Kuoichi, O., Satake, T. 1988. *Diabetes* **37:**1168–1172
Pennington, S.R., Martin, B.R. 1985. *J. Biol. Chem.* **260:**11039–11045
Pershadsingh, H.A., Shade, D.L., Delfert, D.M., McDonald, J.M. 1987*b*. *Proc. Natl. Acad. Sci. USA* **84:**1025–1029
Pershadsingh, H.A., Shade, D.L., McDonald, J.M. 1987*a*. *Biochem. Biophys. Res. Commun.* **145:**1384–1389
Ramlal, T., Sarabia, V., Bilan, P.J., Klip, A. 1988. *Biochem. Biophys. Res. Commun.* **157:**1329–1335
Rees-Jones, R.W., Taylor, S.I. 1985. *J. Biol. Chem.* **260:**4461–4467
Robertson, D.G., Longo, N., Newby, F.D., DiGirolamo, M., Merrill, A.H., Lambeth, J.D. 1987. Endocrinol. Soc. 69th Annual Meeting, (Indianapolis) p. 36
Rodriquez-Pena, A., Rosengurt, E. 1984. *Biochem. Biophys. Res. Commun.* **120:**1053–1056
Rubin, J.B., Shia, M.A., Pilch, P.F. 1983. *Nature (London)* **305:**438–440
Sadoul, J.L., Peyron, J.F., Ballotti, R., Debant, A., Fehlmann, M., Obberghen, E. van 1985. *Biochem. J.* **227:**887–892
Sakai, M., Wells, W.W. 1986. *J. Biol. Chem.* **261:**10058–10062
Saltiel, A.R. 1987. *Endocrinology* **120:**967–972
Saltiel, A.R., Cuatrecasas, P. 1986. *Proc. Natl. Acad. Sci. USA* **83:**5793–5797
Saltiel, A.R., Fox, J.A., Sherline, P., Cuatrecasas, P. 1986. *Science* **233:**967–972
Saltiel, A.R., Sherline, P., Fox, J.A. 1987. *J. Biol. Chem.* **262:**1116–1121
Saltiel, A.R., Sorbara-Cazan, L.R. 1987. *Biochem. Biophys. Res. Commun.* **149:**1084–1092
Seifert, R., Schachtele, C. 1988. *Biochem. Biophys. Res. Commun.* **152:**585–592
Shanahan, M.F., Olsen, S.A., Weber, M.J., Lienhard, G.E., Gorga, J.C. 1982. *Biochem. Biophys. Res. Commun.* **107:**38–43
Shiraishi, T., Domoto, T., Imai, N., Shimada, Y., Watanabe, K. 1987. *Biochem. Biophys. Res. Commun.* **147:**322–328
Simpson, I.A., Hedo, J.A. 1984. *Science* **223:**1301–1304
Sinha, M.K., Pories, W.J., Flickinger, E.G., Meelheim, D., Caro, J.F. 1987. *Diabetes* **36:**620–625
Skoglund, G., Hansson, A., Ingelman-Sundberg, M. 1985. *Eur. J. Biochem.* **148:**407–412
Sowell, M.O., Treutelaar, M.K., Burant, C.F., Buse, M.G. 1986. *Clin. Res.* **34:**555A
Standaert, M.L., Farese, R.V., Cooper, D.R., Pollet, R.J. 1988. *J. Biol. Chem.* **263:**8696–8705
Stralfors, P. 1988. *Nature (London)* **335:**554–556
Suzuki, K., Kono, T. 1980. *Proc. Natl. Acad. Sci. USA* **77:**2542–2545
Takayama, S., Stegmann, E.W., Maron, R. Kahn, C.R. 1986. *Diabetes* **35:**8A
Takayama, S., White, M.F., Kahn, C.R. 1988. *J. Biol. Chem.* **263:**3440–3447
Takayama, S., White, M.F., Lauris, M., Kahn, C.R. 1984. *Proc. Natl. Acad. Sci. USA* **81:**7797–7801

Takuwa, N., Takuwa, Y., Rasmussen, H. 1988. *J. Biol. Chem.* **263:**9738–9745

Tanti, J.-F., Gremeaux, D., Obberghen, E. van, Le Marchand-Brustel, Y. 1986. *Diabetes* **35:**1243–1248

Truglia, J.A., Hayes, G.R., Lockwood, D.H. 1988. *Diabetes* **37:**147–153

Ueno, A., Arakaki, N., Takeda, Y., Fujio, H. 1987. *Biochem. Biophys. Res. Commun.* **144:**11–18

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfield, C., Rosen, O.M., Ramachandran, J. 1985. *Nature (London)* **313:**756–761

Ushiro, H., Cohen, S. 1980. *J. Biol. Chem.* **255:**8363–8365

Vaartjes, W.J., Haas, C.G.M. de, Bergh, S.G. van den 1986. *Biochem. Res. Commun.* **138:**1328–1333

Van de Werve, G., Zaninetti, D., Lang, U., Valloton, M.B., Jeanrenaud, B. 1987. *Diabetes* **36:**310–314

Vicario, P., Brady, E.J., Slater, E.E., Saperstein, R. 1987. *Life Sci.* **41:**1233–1241

Walaas, O., Walaas, E., Lystad, E., Alertsen, A.R., Horn, R.S., Fossum, S. 1977. *FEBS Lett.* **80:**417–421

Walaas, S.I., Horn, R.S., Adler, A., Albert, K.A., Walaas, O. 1987. *FEBS Lett.* **220:**311–318

Wallace, R.A., Ho, T. 1972. *J. Exp. Zool.* **181:**303–317

Wardzala, L.J., Jeanrenaud, B. 1981. *J. Biol. Chem.* **256:**7090–7093

White, M.F., Haring, H.U., Kasuga, M., Kahn, C.R. 1984. *J. Biol. Chem.* **259:**255–264

White, M.F., Maron, R., Kahn, C.R. 1985. *Nature (London)* **318:**183–186

Winicov, I., Gershengorn, M.C. 1988. *J. Biol. Chem.* **263:**12179–12182

Witters, L.A., Vater, C.A., Lienhard, G.E. 1985. *Nature (London)* **315:**777–778

Yamada, K., Tillotson, L.G., Isselbacher, K.J., 1986. *J. Cell. Physiol.* **127:**211–215

Yoshida, Y., Huang, F.L., Nakabayashi, H., Huang, K.-P. 1988. *J. Biol. Chem.* **263:**9868–9873

Zick, Y., Rees-Jones, R.W., Taylor, S.I., Gorden, P., Roth, J. 1984. *J. Biol. Chem.* **259:**4396–4400

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## *Note Added in Proof*

After submission of this review, several articles appeared that support participation of protein kinase C in insulin action in BC3H-1 myocytes (although not specifically addressing its role in the stimulation of glucose transport). Concisely, these indicate that (i) immunologically detected protein kinase C migrated from the cytosol to the membrane in insulin-treated cells (Duncan, M.A., Cooper, D.R., Standaert, M.L., Farese, R.V. 1989. *FEBS Lett.* **244:**174–176); (ii) prolonged exposure to phorbol ester-depleted protein kinase C isoform III but spared isoform II (Cooper, D.R., Watsion, J.E., Acevedo-Duncan, M., Pollet, R.J., Standaert, M.L., Farese, R.V. 1989. *Biochem. Biophys. Res. Commun.* **161:**327–334); and (iii) insulin and phorbol ester increased phosphorylation of three common endogenous proteins (Vila, M.C., Cooper, D.R., Davis, J.S., Standaert, M.L., Farese, R.V. 1989. *FEBS Lett.* **244:**177–180).