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Protein Kinase C: Mediator or Inhibitor of Insulin Action?

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Abstract The role of protein kinase C in insulin signal transduction is controversial It has been postulated that protein kinase C is activated by insulin and that the kinase is directly involved in insulin-mediated metabolic processes In opposition to this view is the hypothesis that protein kinase C is not activated by insulin and, more importantly, may be responsible for attenuation of the insulin signal The evidence for and against protein kinase C as a mediator of the insulin signal will be put in perspective followed by discussion of the possible role of the kinase in the pathogenesis of insulin resistance in type II diabetes © 1993 Wiley Liss Inc

Key words: signal transduction, metabolism, diabetes, PKC

Protein kinase C (PKC) was originally defined as a calcium, phospholipid-dependent enzyme which was found in virtually every cell studied [Nishizuka, 1984]. It is now known that PKC is actually a family of related kinases, with specific tissue distributions, regulation, and substrate specificity [Nishizuka, 1988; Parker et al., 1989]. PKC has found itself in the center of controversy with regard to the actions of insulin. On the one hand, PKC has been suggested to be directly activated by insulin and therefore involved in insulin signal transduction. On the other hand, several investigators have hypothesized that PKC may be responsible for attenuation of the insulin signal and, further, that the kinase may be involved in the pathogenesis of insulin resistance in type II diabetes. Prior to a discussion of the role of PKC in insulin action, it may be helpful to briefly review what is known about this unique kinase, the methods used to assess its activation, and, in particular, the pitfalls associated with each technique, as this may explain some of the controversy surrounding the interaction of insulin and PKC. For more extensive reviews of PKC metabolism the reader is referred to Nishizuka [1988] and Stabel and Parker [1991].

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CHARACTERIZATION AND MEASUREMENT OF PKC

PKC is composed of a regulatory domain and a serine/threonine specific kinase domain. The kinase domain of all of the isozymes studied to date is highly conserved; however, the regulatory domain exhibits considerable variability. Based on this regulatory domain variability the isozymes can be segregated into two groups. The mammalian PKC subspecies α, $\beta1$, $\beta2$, and γ possess very similar regulatory domains which differ considerably from the mammalian isozymes δ, ϵ, ζ, and η [Parker et al., 1989].

The first step in the activation of PKC is the formation of an enzyme + calcium + phospholipid complex. The second step is association of diacylglycerol (DAG) which promotes a conformational change and activation [Woodgett et al., 1987]. The isozymes exhibit similar affinities for phosphatidylserine and DAG; however, the calcium dependence is complex. Although PKC-α, -$\beta1$, -$\beta2$, and -γ do show a calcium dependence, it can be partially or completely attenuated with certain substrates or preparations. Further, PKC-δ and -ϵ are calcium independent. To the extent to which they have been characterized, the isozymes exhibit substrate specificity with respect to physiological substrates (EGF receptor and f1 protein) as well as nonphysiological substrates such as lysine-rich histone H1 [Stabel and Parker, 1991].

Assessment of the activation of PKC in vivo is done through variations of two basic procedures, the first of which has been termed the translocation assay. This assay is based on the observation that once activated by the production of a PKC-lipid-DAG complex at the plasma membrane, the kinase is stabilized and can be extracted in the presence of calcium chelators. Therefore, with appropriate stimulation, there is an increase in membrane-bound PKC and a decrease in the cytosolic activity, compared to nonstimulated controls. The membrane and cytosolic fractions are generated by ultracentrifugation of cell lysates and correspond to the pellet and supernatent, respectively. The membrane fraction is further treated with detergent to solubilize the associated PKC. The kinase in each fraction is then purified by column chromatography. The activity of PKC in both the cytosolic and membrane fractions is determined by the phosphorylation of lysine-rich histone in the presence and absence of phosphatidylserine, diolein, and calcium. A disadvantage to this method is that the extraction conditions can affect the partitioning of the kinase and subtle changes in the activity of the enzyme may not be detected [Phillips et al., 1989]. Further, histone protein is a suitable substrate for many kinases. The use of PKC specific peptide substrates increases the specificity of this assay [Casnellie, 1991]. Finally, the translocation assay as described does not differentiate between the activation of specific PKC isotypes. This information can be obtained by Western blot of the cytosol and membrane fractions with isozyme specific antibodies [Huang et al., 1991].

The second method to monitor PKC activation is to assess the phosphorylation state of specific substrates within intact cells. The best characterized intracellular PKC substrate is the myristoylated, alanine-rich C kinase substrate (MARCKS) protein originally described by Rozengurt et al. [1983] and subsequently cloned and sequenced by Blackshear's group [Stumpo et al., 1989]. This protein is rapidly and specifically phosphorylated by PKC both in vitro and in vivo and its distribution follows that of the kinase [Woodgett et al., 1987]. Phosphorylation can be monitored by gel electrophoresis and immunochemical or autoradiographic techniques [Blackshear et al., 1991]. This method does not require homogenization and fractionation of PKC as in the translocation assay. Further, this technique gives a measure of the actual activity, not amount of enzyme present in a particular pool, before cell disruption. This may be advantageous, as it has been demonstrated that translocation may not reflect enzyme activity, that certain isozymes may be differentially translocated, and that translocation in response to stimuli known to activate PKC can sometimes be difficult to demonstrate [Blackshear et al., 1991]. A potential disadvantage to this assay is that it does not differentiate between PKC isozymes, and the function of the MARCKS protein in the cell is not known.

PKC AS A MEDIATOR OF THE ACTIONS OF INSULIN

The ability of insulin to activate PKC has been a much debated topic. Several groups have demonstrated increases in both cytosolic and membrane-bound PKC activity in BC3H-1 myocytes [Cooper et al., 1987], rat adipocytes [Draznin et al., 1988; Pershadsingh et al., 1987], and rat hepatocytes [Cooper et al., 1990a]. However, an increase in both cytosolic and membrane-bound activity is not the typical response to hormones known to activate PKC [Parker et al., 1989]. Cooper et al. [1990b] propose that the increase in the cytosolic fraction is due to contaminating DAG that is not removed by DEAE-sephacel chromatography. Using a Mono-Q column to purify PKC after fractionation, these investigators observed an insulin-induced translocation pattern more in keeping with that of known activators of PKC. It should be noted that the activity of PKC in each of these studies was determined by the phosphorylation of histone protein. Farese and colleagues have utilized immunological techniques to localize PKC following insulin stimulation. These investigators have determined that insulin causes a transient translocation of immunoreactive PKC in BC3H-1 myocytes [Acevedo-Duncan et al., 1989], rat adipocytes [Ishizuka et al., 1989], and rat soleus muscle [Ishizuka et al., 1990]. These studies were limited in scope since the distribution of each of the PKC isozymes was not investigated individually.

In contrast to the observations above, other investigators have failed to observe, under similar conditions, an effect of insulin to translocate or increase PKC activity in rat adipocytes [Glynn et al., 1986], rat hepatocytes [Vaartjes et al., 1986], or BC3H-1 cells [Spach et al., 1986]. In addition, using the MARCKS protein as a marker of PKC activation, neither Blackshear's group

[Blackshear et al., 1985; Spach et al., 1986] nor Rozengurt et al. [1983] were able to demonstrate insulin-induced activation of PKC. To further substantiate this finding, Blackshear et al. [1991] developed five different fibroblast cell lines which overexpress normal human insulin receptors and contain MARCKS protein. In one cell line, insulin did stimulate MARCKS protein phosphorylation, but this represented only 14% of the response to PMA and was not time or dose dependent. In the other four cell lines the MARCKS protein was not phosphorylated following insulin stimulation.

As discussed above, DAG is necessary for the activation of PKC; therefore, for insulin to increase PKC activity it must stimulate the production of DAG. One source of DAG for PKC activation is derived from activation of phosphatidylinositol 4,5-bisphosphate–specific phospholipase C (PIP_2-PLC). In human liver Thakker et al. [1989] have shown that insulin does not stimulate PIP_2-PLC and therefore does not generate IP_3 or DAG. It has been speculated that DAG could also be generated by the insulin-stimulated breakdown of a glycosyl-phosphatidylinositol (GPI) [Saltiel et al., 1987]. The structural identity of the purported insulin-specific GPI and the inability of both insulin and GPI-specific PLC to hydrolyze this compound cast doubt on the possibility that this process would generate DAG [Thakker et al., 1990]. Finally, phosphatidylcholine hydrolysis has been suggested as a source of insulin-induced DAG [Farese et al., 1988]. This hypothesis requires further investigation as PKC and elevated calcium have been implicated as regulators of the hydrolysis of phosphatidylcholine [Shears, 1991].

Other observations which weigh in against insulin-stimulated PKC are derived from down-regulation experiments. In general, PKC can be almost completely removed from cells by prolonged incubation with high concentrations of PMA. This procedure, which apparently leads to proteolysis of the kinase, has been termed *down-regulation* [Stabel and Parker, 1991]. In seven different cell lines in which PKC was down-regulated, insulin responses such as S6 phosphorylation, ornithine decarboxylase induction, activation of Raf-1 kinase, and others were normal [reviewed in Blackshear et al., 1991]. However, when considering these experiments it should be noted that there appears to be variability in the extent of down-regulation of the various isozymes, and it has been suggested that some are resistent to PMA-mediated down-regulation [Stabel and Parker, 1991]. A final observation that goes against insulin-stimulated PKC is that PMA-induced activation of PKC often results in effects opposite to those of insulin [Denton, 1986; Saltiel et al., 1989].

A large body of evidence suggests that PKC is not directly activated by insulin and therefore calls into question the observations of insulin-induced PKC translocation. As discussed above, the translocation assay may reflect the amount of kinase present but not acurately reflect enzyme activity. Further, the assay is often difficult to do as exemplified by the necessity of a Mono-Q column to demonstrate an insulin effect on PKC [Cooper et al., 1990b]. PKC may be activated by insulin but this may represent a secondary or tertiary response to the hormone. Therefore, at the present time it appears that PKC is not a direct mediator of the effects of insulin.

PKC AS AN INHIBITOR OF INSULIN ACTION

The tyrosine kinase activity of the insulin receptor is crucial to its ability to transmit the insulin signal [Goldfine, 1987]. Bollag et al. [1986] originally demonstrated that the solubilized insulin receptor is a substrate for PKC and that phosphorylation results in a reduction in receptor tyrosine kinase activity. Subsequently, this observation was confirmed with isolated receptor from several tissues [Haring, 1991]. In whole cell preparations activation of PKC has also been shown to result in phosphorylation and inactivation of the insulin receptor kinase [Jacobs and Cuatrecasas, 1986; Takayama et al., 1988; Caro et al., 1992]. Inhibition of PKC with staurosporine blocks phorbol ester-induced phosphorylation of the insulin receptor in HepG2 cells [Duronio and Jacobs, 1990] and phorbol ester mediated down-regulation of PKC appears to potentiate receptor autophosphorylation [Pillay et al., 1990]. The PKC inhibitors staurosporine, H7, and polymyxin B also prevent the glucose-induced reduction in insulin receptor tyrosine kinase in fat cells [Muller et al., 1991].

Koshio et al. [1989] and Lewis et al. [1990] identified threonine 1336, located eight amino acids from the carboxy terminus of the β-subunit of the human insulin receptor, as the amino acid phosphorylated by PKC. Of interest are the recent observations of Anderson and Olefsky [1991] in transfected cells possessing truncated insulin receptors which lack threonine 1336. Exposure of these cells to PMA resulted in a reduction in the total phosphorylation state of the insulin receptor with a concomitant reduc-

tion in autophosphorylation, tyrosine kinase activity, and glucose incorporation into glycogen. These effects appear to be PKC dependent as staurosporine blocked the PMA effect on glucose incorporation into glycogen. Anderson and Olefsky suggest that PKC-mediated phosphorylation of the insulin receptor is not functionally significant, but that PKC-mediated dephosphorylation, perhaps through activation of a phosphatase, is more significant in intact cells. Although further work is necessary to completely understand these intriguing observations, they do support the hypothesis that PKC is involved in the desensitization of the insulin receptor.

PKC IN NIDDM

We and others have demonstrated a decrease in the tyrosine kinase activity of insulin receptor isolated from the liver [Caro et al., 1986], fat [Freidenberg et al., 1987; Sinha et al., 1987], and muscle [Arner et al., 1987; Caro et al., 1987; Obermaier-Kusser et al., 1989] of patients with NIDDM. In light of the fact that PKC can inhibit the insulin receptor tyrosine kinase in the experimental systems discussed above, it is tempting to speculate that PKC may be responsible for the decreased tyrosine kinase activity of the receptors isolated from NIDDM patients. In support of this hypothesis, we have demonstrated that the activity of PIP_2-PLC is increased in diabetic liver [Thakker et al., 1989]. An increase in the activity of PIP_2-PLC could result in increased release of IP_3 and DAG. The increase in DAG would result in activation of PKC which would phosphorylate the insulin receptor and decrease its tyrosine kinase activity. The mechanism of increased PIP_2-PLC is not known; however, possibilities include alterations in the phospholipid composition of the membrane or hyperglucagonemia. Glucagon is believed to activate PIP_2-PLC [Houslay, 1991], and hyperglucagonemia is present in patients with NIDDM.

Work in several different experimental models of NIDDM also implicates PKC in insulin resistance. A twofold increase in membrane PKC activity, concomitant with a 45% decrease in insulin receptor autophosphorylation, was found in the liver of rats made diabetic by starvation [Karasik et al., 1990]. In insulin-resistant, obese Zucker rats the levels of DAG were increased 82% in liver, 136% in calf muscle, 72% in soleus muscle, and 40% in plantaris muscle [Turinsky et al., 1990]. Interestingly ceramide levels, a precursor of the PKC inhibitor sphingosine, were also increased. Turinsky et al. also observed a sustained increase of 23–56% in DAG content of denervated, insulin-resistant soleus muscle. Heydrick et al. [1991] observed a 40% increase in DAG content which correlated with a two- to threefold increase in membrane PKC activity in denervated, insulin-resistant skeletal muscle. In a separate line of study it has been observed that elevated glucose stimulates de novo synthesis of DAG in a variety of tissues [Wolf et al., 1991]. Further, direct conversion of ^{14}C-glucose to ^{14}C-DAG has been demonstrated in retinal endothelial cells in culture [Lee et al., 1989]. These findings, as well as the observations in the animal models of NIDDM, provide impetus for the study of PKC in tissue from NIDDM patients.

FUTURE DIRECTIONS

At present the bulk of the evidence suggests that PKC modulates or inhibits insulin action. However, insulin-mediated activation of PKC, as a secondary or tertiary response, cannot be completely ruled out. One hypothesis that could encompass both observations is the following. A well-studied effect of the second messengers of pulsatile hormones is feedback inhibition of the activating signal. Since patients with NIDDM are hyperinsulinemic and lack coordinated pulsatile insulin secretion under basal conditions, it is conceivable that if PKC is the second messenger for insulin, the excess insulin signal could drive the kinase to feedback inhibit the β-subunit. One possible way to test this hypothesis would be by monitoring the response to insulin of NIDDM liver slices or cells in culture. Should the absence of high insulin or the reestablishment of a pulsatile pattern in culture restore insulin sensitivity and normal PKC activity, it would resolve some of the controversy.

On the ability of PKC to alter the insulin signal, although PKC phosphorylation of the β-subunit in vitro alters the tyrosine kinase activity, several questions remain to be answered. It is not known if all PKC isozymes can phosphorylate the insulin receptor β-subunit or if only certain isozymes are involved. How phosphorylation of threonine 1336 results in inhibition of the tyrosine kinase activity of the receptor is not completely understood. The possibility of other minor PKC phosphorylation sites deserves further attention. And it remains to be determined whether PKC-mediated activation of insulin receptor-specific phosphatases [Anderson and Olefsky, 1991] is more important than direct PKC phosphorylation of the receptor in vivo.

There is ample evidence to suggest that PKC may be responsible for insulin receptor desensitization in NIDDM. For this reason a comprehensive investigation of PKC in NIDDM tissue should be conducted. Experiments should include quantification of the phosphoserine/phosphothreonine content of the insulin receptor isolated from diabetic tissue as well as determination of membrane-associated PKC activity and the specific isozymes involved. It would also be important to determine the mechanism through which PKC activity was elevated. Possibilities include elevation of PKC activators such as DAG or calcium, or reduction in endogenous inhibitors of PKC such as sphingosine. On the point of PKC inhibitors, a recent paper suggests that sphingosine can inhibit the insulin receptor independently of its effects on PKC [Arnold and Newton, 1991]. This observation deserves further investigation. The role in PKC activation of increased PIP_2-PLC activity, observed in NIDDM liver, must be determined and if this finding is specific to the liver or occurs in other insulin sensitive tissues as well. Finally, at present it is unclear whether inappropriate PKC activation would occur as a primary cause of diabetes or is a secondary response to some other aberration such as hyperglucagonemia or elevated glucose levels. Answers to these and related questions will increase our understanding of the regulation of insulin signalling and the role of PKC in the pathology of NIDDM.

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