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Evidence for a role for protein kinase C in the stimulation of protein synthesis by insulin in swiss 3T3 fibroblasts

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We have examined the role of protein kinase C (PKC) in the stimulation of protein synthesis by insulin using two complementary approaches. In the first, fibroblasts were pretreated with phorbol esters to down-regulate PKC. In these cells, the effects of insulin and of phorbol esters on protein synthesis were completely abolished, although serum still elicited an effect approaching that seen in control cells. Secondly, we used newly developed inhibitors of PKC which, again, blocked the effects of insulin and phorbol esters without greatly reducing the response to serum. Thus PKC apparently plays an important role in the stimulation of translation by insulin.

Insulin; Protein synthesis; Protein kinase C

1. INTRODUCTION

Insulin acutely stimulates protein synthesis at the level of mRNA translation in a wide variety of mammalian cell types [1–3], this effect being exerted primarily on the process of peptide-chain initiation. However, the signal transduction mechanism(s) involved in this effect of insulin, and the translational components which are the targets for regulation by this hormone, remain poorly understood. Recently we have demonstrated that in Swiss 3T3 cells insulin rapidly activates an initiation factor termed eIF-2B [4], which plays a key role in regulating peptide-chain initiation by recycling the factor, eIF-2, which binds the initiator Met-tRNAi to the ribosome (reviewed in [5]). In this paper we wish to address another aspect of the control of translation by insulin, viz. the role of protein kinase C (PKC) in the stimulation of protein synthesis by insulin.

Several other research groups have studied the importance of PKC in the intracellular actions of insulin. Work from Farese and colleagues supports such a role for PKC (see, e.g. [6–8]), although other workers have questioned this [9], as discussed in detail in [10]. In the case of the stimulation of protein synthesis by insulin, two groups of workers have provided evidence for a role for PKC (reviewed in [11]), based largely on the effects of down-regulation of PKC by long-term phorbol ester treatment, on the ability of insulin to stimulate either overall protein synthesis or the phosphorylation of individual initiation factors [12–14].

We have extended this work by examining the effects of a series of novel protein kinase C inhibitors (Ro-31-7549, Ro-31-8220, and Ro-31-8425) on the activation of protein synthesis by insulin and other stimuli in Swiss 3T3 cells. These and related compounds have been shown to block effects believed to be mediated by PKC [15–17]. Our data support the involvement of PKC in the stimulation of protein synthesis by insulin.

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Abbreviations: eIF-, eukaryotic initiation factor; PKC, protein kinase C; PDBu, phorbol dibutyrate.

2. MATERIALS AND METHODS

2.1. *Materials*

All materials were obtained as described in [4] unless otherwise indicated. The PKC inhibitors, Ro-31-7549, Ro-31-8220 and Ro-31-8425, were a generous gift from Dr J. Nixon, Roche Products Ltd., Welwyn Garden City, Herts, UK. The phorbol ester, PDBu, was purchased from Sigma Chemical Co. (Poole, Dorset, UK). Insulin receptors, which were partially purified from CHO.T cell extracts by wheat-germ lectin–Sepharose chromatography and concentrated as described previously [18], were a generous gift from Mr S. Young, Department of Biochemistry, University of Bristol. Anti-insulin receptor antibody, 83–7, was a kind gift from Professor K. Siddle (University of Cambridge).

2.2. *Cell culture and protein synthesis assays*

Swiss 3T3 fibroblasts were grown and maintained as described in [4]. Protein synthesis assays were carried out as described in [4]. The PKC inhibitors were added at the same time as insulin. For down-regulation studies the phorbol ester, PDBu, was added at a concentration of 2 μM, 24 h before protein synthesis assays were carried out. The PDBu stock solution was dissolved in acetone whilst the PKC inhibitors were dissolved in 100% DMSO. At the final concentrations used in the protein synthesis assays none of these vehicles had an effect on control or insulin-stimulated rates of protein synthesis (data not shown). Controls contained appropriate amounts of these compounds.

2.3. *Assays of insulin receptor, tyrosine kinase activity*

The inhibitor studies on the insulin receptor kinase were performed using the immune-capture method of Zhang et al. [19] and utilized the synthetic peptide, RRDIFETDYFRK (synthesized by Dr. G. Bloomberg, University of Bristol) which is a substrate for the insulin receptor kinase [20].

3. RESULTS

3.1. *Phorbol esters, insulin and protein synthesis*

As we have shown previously [4], the phorbol ester, PDBu, rapidly (within 5 min) increases the rate of protein synthesis in Swiss 3T3 cells (in those experiments to 121 ± 7% of control rates seen in serum-depleted cells). Dose–response data for the activation of protein synthesis by PDBu shows maximal stimulation with about 2 μM and a half-maximal response at about 100 nM (data not shown).

3.2. *The effect of down-regulation of PKC on the stimulation of protein synthesis by insulin*

Total cellular levels of PKC can be down-regulated by prolonged exposure of cells to phorbol esters [21]. Treatment of Swiss 3T3 fibroblasts with 2 μM PDBu for 24 h led to the complete abolition of the stimulation of protein synthesis when the cells were re-challenged with these agents, consistent with the idea that stimulation of protein synthesis by phorbol esters is mediated by PKC. However, this pre-treatment had no effect on the control level of protein synthesis. In these phorbol ester-pretreated cells the effect of insulin on protein synthesis

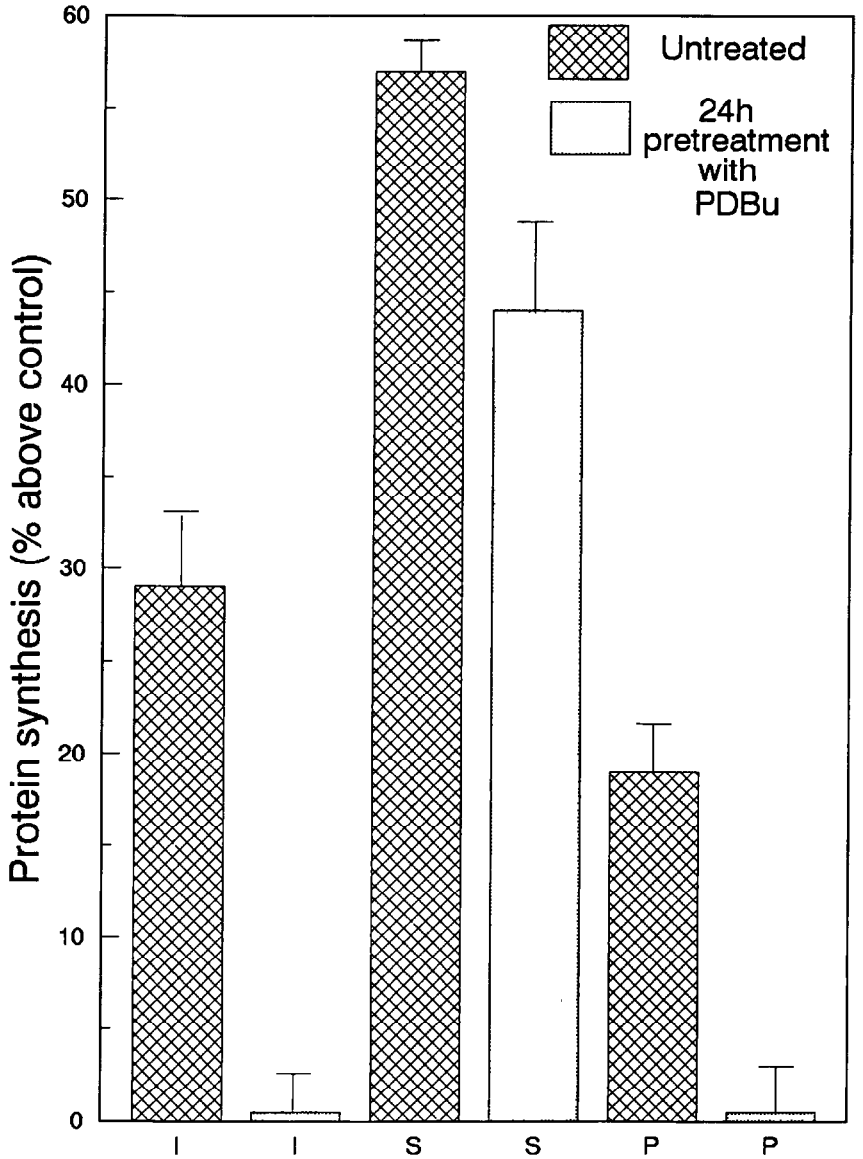

Fig. 1. Effect of pretreatment of Swiss 3T3 cells with phorbol esters on protein synthesis and its stimulation by insulin and serum. The effects of insulin (I, 10 nM), serum (S, 10%), or PDBu (P, 2 μM) were studied in cells treated with PDBu (2 μM)) or carrier only (ethanol) for 24 h prior to measurement of protein synthesis as described in section 2. The results are given as means ± S.D. from five independent experiments.

was completely abolished whereas the effect of 10% serum was only inhibited by about 15% (Fig. 1).

The observation that phorbol ester pretreatment had no effect on control levels of protein synthesis, and only a partial effect on the response to serum, indicates that the inhibition of the insulin response is due to the loss of part of the insulin signalling pathway and is not a result of inhibition of all responses due to impaired cell viability, since protein synthesis is a good indicator of this parameter. The partial abolition of the stimulation of protein synthesis by serum may be due to the loss of the insulin component of this response, although other components may be lost under these conditions. Thus it seems as if there are multiple pathways by which protein synthesis can be activated, and, in the case of the some of the components of foetal calf serum, these pathways do not involve PKC.

3.3. *Effects of the PKC inhibitors, Ro-31-7549, Ro-31-8220 and Ro-31-8425, on the stimulation of protein synthesis by insulin and serum*

As in the case of the down-regulation studies, the three inhibitors each completely abolished the stimulation of protein synthesis by insulin without affecting control rates of protein synthesis (Fig. 2). The K_i's for this inhibition for all three inhibitors are very low (Table I), suggesting that they are exerting their effects via inhibition of PKC as these concentrations are comparable to those obtained by Nixon et al. [16] for other processes in which PKC has been implicated, such as the inhibition of the PMA-induced phosphorylation of p47 in platelets (4.4, 0.7 and 0.4 μM for Ro-31-7549, Ro-31-8220 and Ro-31-8425, respectively) and the PDBu-induced CD3 down-regulation in T cells (3.1, 0.5, and 0.6 μM for Ro-31-7549, Ro-31-8220 and Ro-

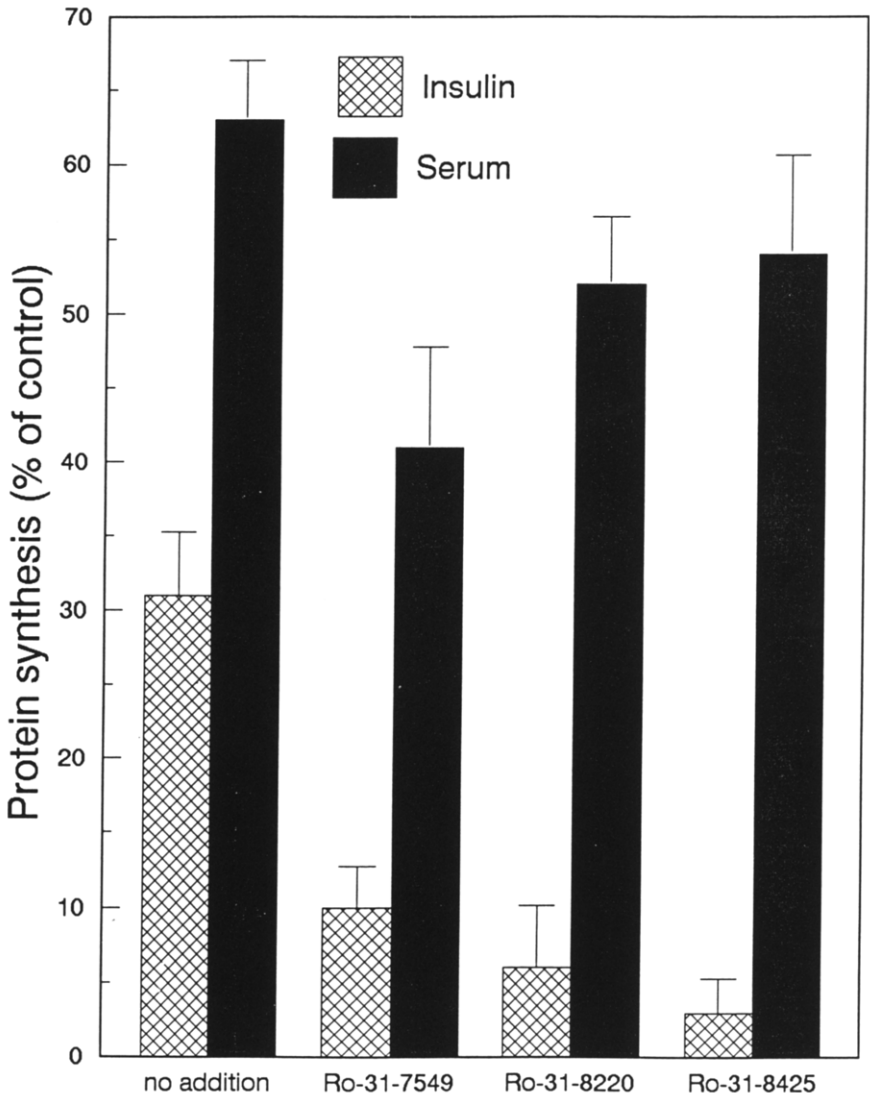

Fig. 2. Effect of PKC inhibitors, Ro-31-7549, Ro-31-8220 and Ro-31-8425, on the stimulation of protein synthesis by insulin. The effect of insulin (20 nM) and serum (10%) on protein synthesis in Swiss 3T3 cells treated at the same time with 1 μM of the PKC inhibitors, Ro-31-7549, Ro-31-8220 or Ro-31-8425, for 20 min. The results are given as means ± S.D. from four independent experiments.

Table I

K_i (μM) for inhibition by PKC inhibitors of the stimulation of protein synthesis by insulin

Agent	K_i (μM) for inhibition of stimulation of protein synthesis by insulin
Ro-31-7549	7.00 ± 1.1
Ro-31-8220	0.50 ± 0.13
Ro-31-8425	0.40 ± 0.15

Protein synthesis was measured as described in section 2. Values represent means ± S.D. for at least four independent experiments with at least eight inhibitor concentrations for each determination. The data were analyzed by non-linear least-squares analysis.

31-8425, respectively). Although the potency of these inhibitors on the stimulation of protein synthesis by insulin is lower than for the inhibition of purified PKC in vitro [15,16], it is likely that this is a result of the higher cellular concentration of ATP compared to those used in in vitro assays, which leads to reduced potency of these compounds which are competitive inhibitors of PKC with respect to ATP.

These inhibitors also completely blocked the stimulation of protein synthesis by phorbol esters (data not shown), but had only limited effects on the degree of stimulation by 10% serum (Fig. 2). These data support the idea that these compounds do not affect cell viability since there is no inhibition of control levels of protein synthesis and the cells still respond markedly to serum.

Other compounds used as PKC inhibitors (e.g. sphingosine and staurosporine) have been reported to affect cell viability adversely [22]. Therefore the effects of the agents used here on a number of other parameters indicative of cell viability were also tested: the PKC-inhibitors, Ro-31-7549, Ro-31-8220 and Ro-31-8425, were without effect on cell attachment, evaluated microscopically, or on Trypan blue exclusion, even after 90 min at a concentration of 10 μM, which is 10–20-times the concentration used in the protein synthesis experiments (Table II).

3.4. *Effect of the PKC inhibitors on eIF-2B activity*

We have previously shown that phorbol esters and insulin each activate eIF-2B in Swiss 3T3 cells [4]. We therefore examined the effect of the PKC inhibitors on the stimulation of eIF-2B by these two agents. The effect of phorbol esters is presumably mediated by PKC: Ro-31-8220 and Ro-31-8425 (both at 1 μM) completely abolished the activation of eIF-2B by 2 μM PDBu (Fig. 3) consistent with this idea. In contrast, both Ro-31-8425 and Ro-31-8220 were without effect on the stimulation of eIF-2B by insulin (Fig. 3).

3.5. *Effect of the PKC inhibitors, Ro-31-7549, Ro-31-8220 and Ro-31-8425, on the insulin receptor, tyrosine kinase*

Staurosporine is a very potent but non-specific protein kinase inhibitor which has been shown also to inhibit receptor tyrosine kinases [23,24]. As the inhibitors used here are structurally similar to staurosporine, it was clearly possible that they inhibited the insulin stimulation of protein synthesis, not by affecting PKC, but by inhibiting the insulin receptor, tyrosine kinase. The effects of these inhibitors on insulin receptor tyrosine kinase activity was measured in an in vitro assay. Although all these compounds could inhibit the receptor kinase, they only did so at much higher concentrations than those used in our protein synthesis studies (Table II). The K_i for the inhibition of the insulin receptor kinase by Ro-31-7549 and Ro-31-8220 in vitro was 10–20-times the K_i for the inhibition of protein synthesis by these compounds in vivo and over 100-times that for Ro-31-8425 (Table II). Furthermore, the K_i's for the in vivo inhibition of the insulin receptor kinase by these competitive inhibitors are likely to be significantly higher than those obtained in vitro, as is the case with

Table II

Effects of the PKC inhibitors on various cellular processes

Agent	Effect on cell attachment	Effect on cell viability	Effect on control levels of protein synthesis (% of untreated control)	K_i (μM) insulin receptor kinase
Staurosporine	Yes	Yes	74 ± 5	n.d.
Ro31-7549	No	No	98 ± 4	15 ± 3
Ro31-8220	No	No	101 ± 3	4 ± 2
Ro31-8425	No	No	100 ± 4	200 ± 11

The effect of staurosporine, Ro31-7549, Ro31-8220 and Ro31-8425 on cell attachment and cell viability was evaluated microscopically and by Trypan blue exclusion, respectively, after treatment of the cells with 10 μM of each inhibitor for 90 min. The effect of the PKC inhibitors on control levels of protein synthesis was measured after treatment of the cells for 60 min with 10 μM of each inhibitor. Results are given as means ± S.D. for three separate experiments. The effect of the PKC inhibitors on the insulin receptor kinase was determined by measurement of inhibition of insulin-dependent phosphorylation of exogenous substrate. Values represent means ± S.D. for at least three separate experiments with a minimum of seven inhibitor concentrations for each determination. Data was analyzed by non-linear least-squares analysis.

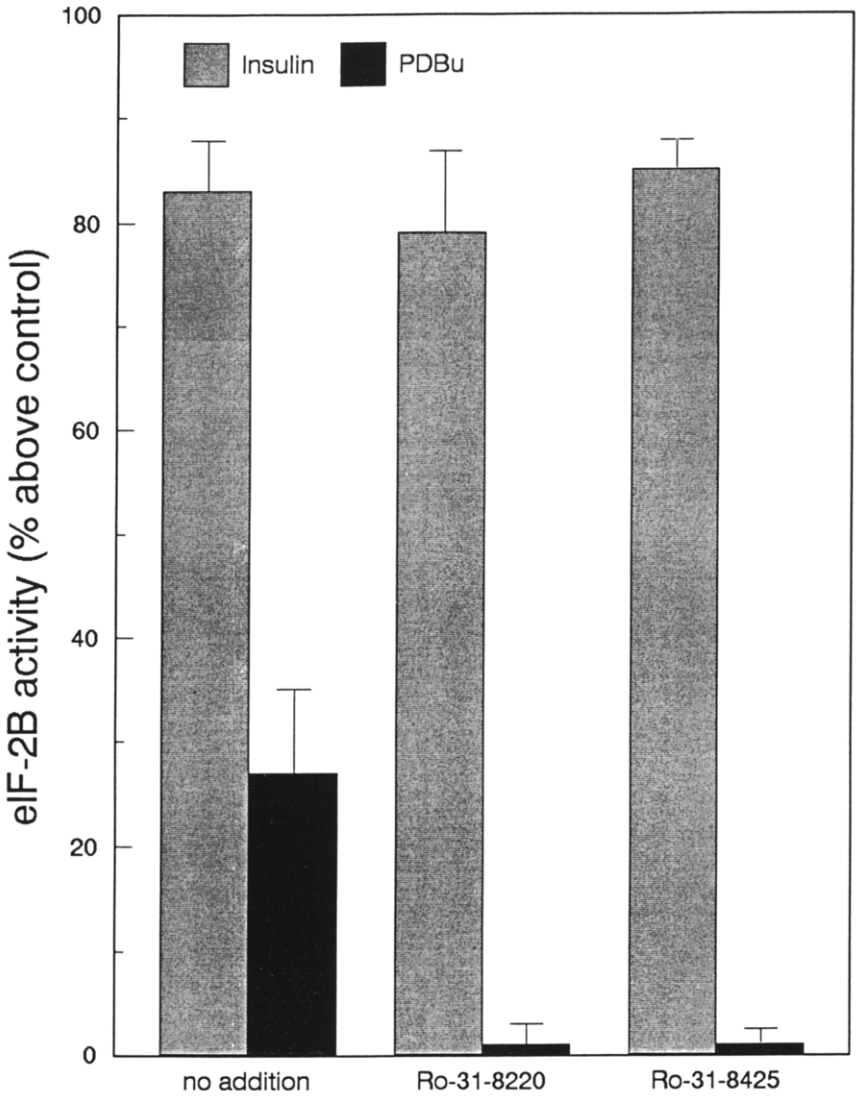

Fig. 3. Effect of PKC inhibitors, Ro-31-8220 and Ro-31-8425, on the stimulation of eIF-2B activity by insulin and PDBu. The effect of insulin (20 nM) and PDBu (2 μM) on the stimulation of eIF-2B activity in Swiss 3T3 cells treated either with no addition or at the same time with 1 μM of the PKC inhibitors, Ro-31-8220 or Ro-31-8425, for 20 min was examined. The results are given as means ± S.D. from four independent experiments.

their inhibition of PKC [16], since the in vivo concentration of ATP is at least 10-fold higher than that used in the tyrosine kinase assays which was 0.25 mM. Thus, it seems unlikely that these inhibitors are working by inhibiting the insulin receptor, tyrosine kinase, although due to their hydrophobicity it is possible that they tend to become concentrated in the plasma membrane. The observation that the PKC inhibitors do not block the activation of eIF-2B by insulin provides evidence that in the intact cell they are not acting simply by inhibiting the function of the insulin receptor.

4. DISCUSSION

The results above describe the use of the new and potent PKC inhibitors, Ro-31-7549, Ro-31-8220 and Ro-31-8425, to investigate the role of PKC in the stimulation of protein synthesis by insulin. In contrast to other PKC inhibitors available, Ro-31-7549, Ro-31-8220 and Ro-31-8425 possess a much improved specificity for PKC compared to other protein kinases [16] and do not seem to have any effect on cell viability. In protein synthesis assays these compounds (at nanomolar concentrations) totally abolished the stimulation of translation by both insulin and phorbol esters, without affecting control levels of protein synthesis, making it likely that they are doing so by inhibiting PKC. The compounds have much lesser effects on the stimulation of protein synthesis by serum, indicating that the cells are still responsive to certain components of serum and that presumably the mechanisms by which these agents activate protein synthesis are independent of PKC.

The data obtained using these inhibitors, taken together with the results from the phorbol ester down-regulation studies, support the hypothesis that PKC is involved in the stimulation of protein synthesis by insulin, although the possibility that these compounds inhibit other still-uncharacterized protein kinases with PKC-like properties cannot be ruled out. The findings that down-regulation of PKC by prolonged treatment with phorbol esters or inhibition of PKC using Ro-31-7549, Ro-31-8220 and Ro-31-8425 totally abolished the insulin effect indicates that there is an essential PKC-dependent component in the stimulation of protein synthesis by insulin. However, the observations that phorbol esters increase protein synthesis, but to a lesser extent than insulin [4], and that the PKC inhibitors block the activation of eIF-2B by PDBu but not by insulin suggest that there is also an additional PKC-independent pathway involved in the insulin response. These two pathways appear to be distinct but overlapping. The first (PKC-dependent) pathway is common to both phorbol esters and insulin. However, other routes also have to be taken to get the full insulin effect, although, if PKC is down-regulated or inhibited these other mechanisms are not sufficient in themselves to bring about an increase in protein synthesis. Thus activation of PKC seems to be necessary but not sufficient to obtain the full stimulation of protein synthesis by insulin.

Two further inferences can be drawn from these results: firstly, activation of eIF-2B is not in itself sufficient for stimulation of overall protein synthesis and, secondly, as a corollary, there are other processes required for the activation of translation by insulin which are PKC dependent. They could include, for example, the phosphorylation of the cap-binding initiation factor, eIF-4E, which has been reported to be enhanced by insulin [25] in a PKC-dependent manner [14]. Phosphorylation of eIF-4E appears to play an important positive role in the control of protein synthesis (reviewed in [5]).

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