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# Insulin stimulates the translocation of protein kinase C in rat adipocytes

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Insulin-induced changes in protein kinase C were examined in cytosol and membrane fractions of rat adipocytes enzymatically after Mono Q column chromatography and by immunoblotting. During a 5–20 min period of insulin treatment, cytosolic protein kinase C decreased by approximately 50%, whereas membrane protein kinase C increased nearly 2-fold. These findings suggest that insulin stimulates the translocation of protein kinase C in rat adipocytes.

Insulin; Protein kinase C

## 1. INTRODUCTION

Protein kinase C (PKC) plays an important role in transmembrane signalling in various cell types [1–3]. PKC is activated by membrane-associated diacylglycerol (DAG), which causes PKC to translocate from cytosol to membranes, and this translocation serves as important evidence for activation of the enzyme [1–4]. Insulin increases DAG production by stimulating the synthesis of phosphatidic acid (PA) de novo [5–9], and the hydrolysis of phosphatidylcholine [9,10] and a glycan derivative of phosphatidylinositol [11]. Thus, it might be expected that the DAG-PKC signalling is important during insulin action, and studies of PKC in BC3H-1 myocytes [12] and rat diaphragm [13] are in accord with this possibility. However, in rat adipocytes, a model tissue for studies of insulin action, insulin did not alter the subcellular distribution of PKC enzyme activity in one study [14], whereas, in two other studies, insulin provoked: (i) increases in cytosolic PKC, as judged from phorbol ester binding [15,16]; and (ii) increases in PKC enzyme activity of cytosol and membrane fractions [16]. Because of these differences and the importance attached to the translocation response, we re-examined the effects of insulin on subcellular distribution of PKC in rat adipocytes.

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*Abbreviations:* PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide; BSA, bovine serum albumin

## 2. MATERIALS AND METHODS

Phosphatidylserine, diolein, histone (type III-S), phenylmethylsulfonyl fluoride (PMSF), leupeptin, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and bovine serum albumin (BSA) were purchased from Sigma. We purchased [$\gamma$-$^{32}$P]ATP from ICN, collagenase from Worthington, and porcine zinc insulin (25.7 IU/mg) from Elanco Products. PKC antiserum, raised to synthetic peptides specific to rat brain type II PKC (see [17]), was provided by Bryan L. Roth and John P. Mehegan, Naval Medical Research Institute, Bethesda, MD. Type II PKC is the major isozyme in rat adipocytes, as determined by hydroxyapatite column chromatography (unpublished observations).

Epididymal fat pads were obtained from fed male Holtzman rats (150–200 g). Free adipocytes were obtained by collagenase digestion [18] of fat pads in Krebs-Ringer-phosphate buffer (KRP) (pH 7.4) containing 3% BSA and 2.5 mM glucose. Adipocytes were incubated for 30 min at 37°C in glucose-free KRP containing 1% BSA. (Glucose-free KRP was used to duplicate conditions generally employed in studies of glucose transport, and we routinely observed 3–8-fold increases in 2-deoxy-[$^3$H]glucose uptake after 15–30 min insulin treatment. Adipocytes remain fully responsive for 6 h in this medium.) Insulin in buffer, TPA in DMSO (0.005%), or vehicle (controls) were then added in a retrograde sequence over the course of a 20-min (unless indicated otherwise) period, which was constant for all samples.

Reactions were terminated by addition of 10 ml ice-cold buffer I (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM PMSF, 20 $\mu$g/ml leupeptin, 20 mM 2-mercaptoethanol). The adipocytes were washed twice and homogenized in buffer I. Homogenates were centrifuged at 1000 × *g* for 2 min, and floating fatty materials were removed. Resultant homogenates were centrifuged at 100000 × *g* for 60 min to obtain cytosol and pellets. Pellets were homogenized in buffer I containing 1% Triton X-100 and centrifuged at 100000 × *g* for 60 min to obtain the solubilized membrane fractions. Equal amounts of the cytosol or solubilized membrane fractions from each treatment group, approximately 2 and 0.6 mg protein, respectively, were applied to a Mono Q column (0.5 × 5 cm, Pharmacia HR 5/5) in a Pharmacia fast protein liquid chromatography system equilibrated with buffer II (20 mM Tris-HCl, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol). PKC was eluted with a 20 ml linear gradient of NaCl (0–0.7 M) in buffer II at a flow rate of 1 ml/min. Fractions of 1 ml were collected. PKC was assayed by measuring the

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phosphorylation of histone [19]. The reaction mixture (250 μl, total volume) contained 20 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 0.02% histone, 10 μM [$\gamma$-$^{32}$P]ATP ($12 \times 10^4$ cpm/nmol), 10 μg phosphatidylserine, 0.1 μg diolein, 0.5 mM $CaCl_2$, and 100 μl column fraction. After incubation for 5 min at 30°C, the reaction was stopped with 25% trichloroacetic acid. Acid-precipitable materials were collected on membrane filters and counted for radioactivity. Basal activity was measured in the presence of 0.5 mM EGTA instead of $Ca^{2+}$, phosphatidylserine and diolein, and was subtracted from the total protein kinase activity to determine PKC activity.

PKC was also analyzed by immunoblotting (see [20]). Equal amounts of cytosol or membrane fractions (5–15 μg of protein) were subjected to SDS-PAGE, transferred quantitatively (as per silver stain) to nitrocellulose membranes, incubated for 1 h with 3% gelatin in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.5, containing 500 mM NaCl), washed with TBS containing 0.05% Tween (TTBS), incubated for 2 h with antiserum to PKC, washed with TTBS, and incubated for 1 h with goat anti-rabbit $\gamma$-globulin antibody coupled either to horseradish peroxidase or alkaline phosphatase. Immunoreactive proteins were visualized by staining with 4-chloro-1-naphthol and $H_2O_2$ [20], or *O*-dianisidine tetrazotized and $\beta$-naphthyl acid phosphate [21]. In preliminary experiments, we documented that staining intensity was directly proportional to the PKC content of the sample subjected to the entire procedure, including SDS-PAGE, transfer and immunoblotting. Protein was determined by the method of Bradford [22].

## 3. RESULTS

During Mono Q column chromatography of cytosol and membrane fractions from rat adipocytes, phospholipid/$Ca^{2+}$-dependent protein kinase activity eluted as shown in fig.1. This elution profile is similar to that of rat brain PKC [23]. Addition of either $Ca^{2+}$ or phospholipids alone to these assays did not stimulate histone phosphorylation significantly above that observed with EGTA. Thus, simple $Ca^{2+}$-dependent, protein kinase activity was not found in PKC-containing fractions. Further cAMP-dependent protein kinase activity eluted prior to PKC (data not shown). It may be noted that there were slight differences between PKC elution profiles of cytosol and membrane fractions, and from experiment to experiment. This may reflect differences in the phosphorylation state of the enzyme, or bound lipids or other ligands which may influence binding of PKC to the Mono Q column. In experiments described below, 'PKC enzyme activity' was determined by summing the phospholipid/$Ca^{2+}$-dependent enzyme activity found in all column fractions (i.e. 'total elutable PKC activity').

Cytosolic PKC enzyme activity decreased rapidly and progressively by 53% and 63% over a 20-min period upon stimulation of adipocytes with either insulin (10 nM) or TPA (100 nM), respectively (fig.2). Increases in membrane PKC enzyme activity were evident at 5–20 min of insulin treatment, but the timing of the peak was variable. Nevertheless, increases were observed in each of the 4 experiments of fig.2, and, if all insulin-stimulated membrane values at 5, 10 and 20 min are considered collectively, the overall mean ± SE percent increase was 92 ± 31 ($n = 12$; $P < 0.02$; paired *t*-test). With TPA treatment, increases in membrane enzyme activity were observed less consistently. Glynn et al. [14] also did not observe consistent increases in membrane PKC activity during TPA treatment of rat adipocytes.

Fig.3 shows an immunoblot of cytosol and membrane fractions from control, and insulin- or TPA-treated tissues. As in the BC3H-1 myocyte [20], only one major band of immunoreactivity was found, and this co-migrated on SDS-PAGE and blotted identically with highly purified 80 kDa rat brain PKC. Cytosol immunoreactivity decreased, whereas membrane immunoreactivity increased during treatment with either 10 nM insulin or 100 nM TPA. In 4 similar experiments in which blots were evaluated by densitometry scanning [20], the decrease in cytosolic immunoreactivity at 20 min of insulin treatment was 47 ± 7% (mean ± SE; $P < 0.01$, paired *t*-test). Comparable immunoblot results were obtained with another antiserum (see [20]), and which recognizes types I, II and III PKC.

We also evaluated changes in PKC with more prolonged insulin treatment (fig.4). After 60 min of insulin

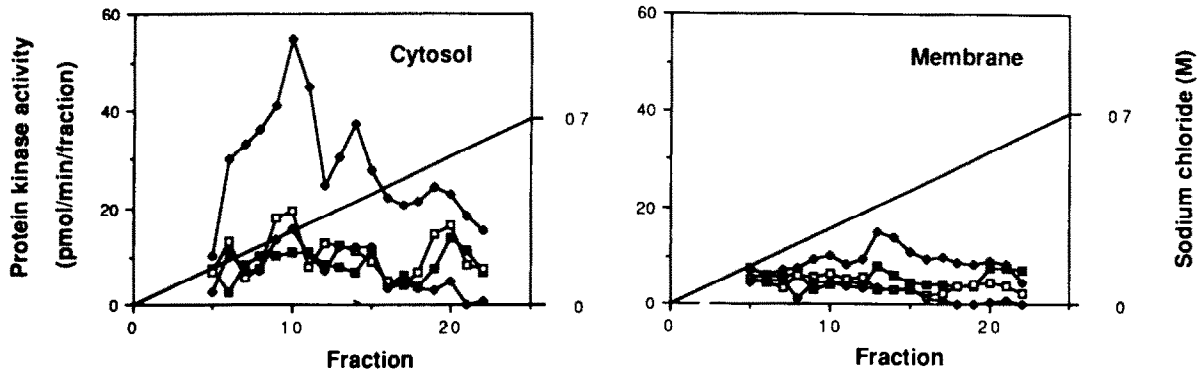


Fig.1. Elution patterns of protein kinase activity of cytosol and membrane fractions from rat adipocytes during purification by Mono Q column chromatography. Cytosol and membrane fractions were applied to the column, and fractions were assayed for protein kinase activity, in the presence of phosphatidylserine (40 μg/ml), diolein (0.4 μg/ml) and 0.5 mM $CaCl_2$ (◆—◆), 0.5 mM EGTA (■—■), 0.5 mM $CaCl_2$ (□---□), or phosphatidylserine (40 μg/ml) (◇---◇).

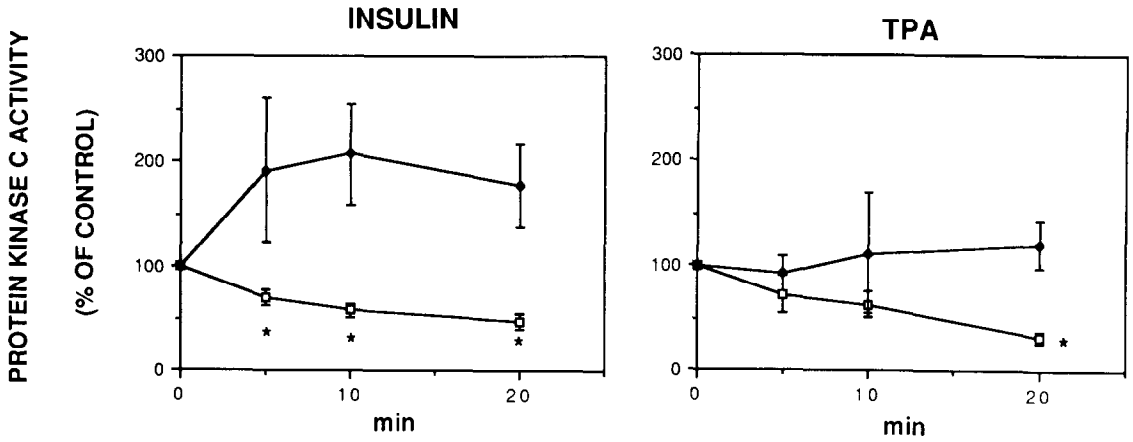


Fig.2. Time course of effects of insulin (10 nM) (left) and TPA (100 nM) (right) on cytosolic and membrane-associated protein kinase C. Total elutable protein kinase C activity was determined after purification of the enzyme by chromatography on the Mono Q column (see figs 1 and 4). Mean ± SE results of 4 insulin and 3 TPA experiments are shown, expressed as % of control activity. * $P < 0.05$ (paired $t$-test).

treatment, there was a profound loss in cytosolic PKC enzyme activity. Membrane PKC enzyme activity remained elevated, but, as is evident, total PKC enzyme activity decreased. Similarly, changes in cytosol and membrane PKC were observed at 30 and 120 min of insulin treatment (data not shown).

## 4. DISCUSSION

In this study, we found that insulin provoked rapid changes in rat adipocyte PKC, suggestive of translocation of the enzyme from the cytosol to the membrane fraction. Using chromatography on Mono Q columns to purify PKC, we observed decreases in cytosol enzyme activity, and increases in membrane-associated enzyme activity, during 5–20 min of insulin treatment. Similar changes in immunoreactive PKC were also observed with insulin treatment. It therefore seems likely that our enzyme assays primarily reflected changes in enzyme content, and, moreover, by two independent methods, we were able to observe evidence of subcellular redistribution of PKC during insulin treatment of adipocytes. Interestingly, similar evidence for PKC translocation was observed during insulin treatment of BC3H-1 myocytes [20].

Insulin-induced changes in adipocyte cytosolic PKC were comparable to those of TPA, which binds to and activates PKC [1,3,4] and provokes the translocation of PKC from the cytosol to the plasma membrane [1,3,4]. Our findings with TPA are similar to those observed by others in rat adipocytes [14] and other tissues, although

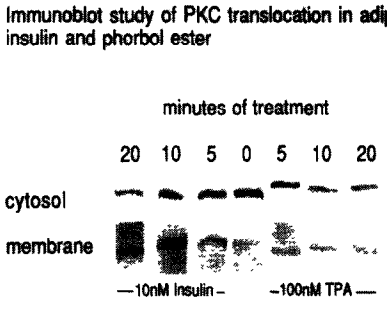


Fig.3. Immunoblot analysis of insulin (10 nM)- and TPA (100 nM)-induced changes in protein kinase C content of cytosol (5 $\mu$g protein) and membrane (10 $\mu$g protein) fractions of rat adipocytes. Times of insulin or TPA treatment are depicted in the figure, with the control (○) shown in the center. Similar results were observed in at least 3 other experiments for each agonist.

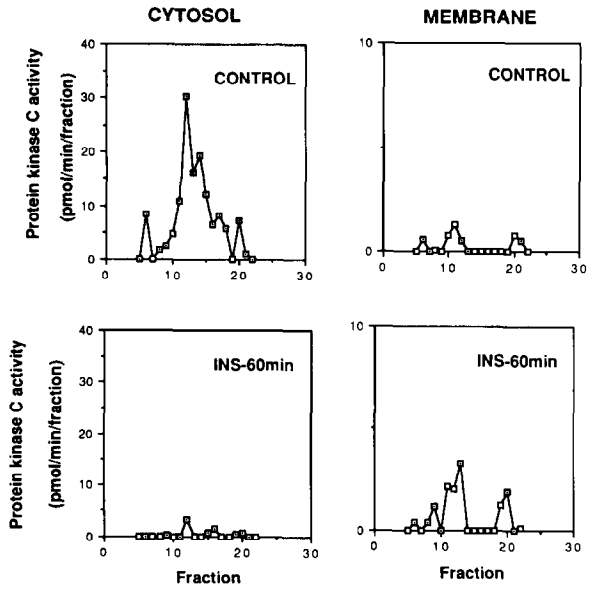


Fig.4. Effects of more prolonged insulin treatment on rat adipocyte protein kinase C enzyme activity. Adipocytes were incubated for 60 min with or without insulin (10 nM). Protein kinase C enzyme activity was determined after chromatography of cytosol (left) and membrane (right) fractions on the Mono Q column (see figs 2 and 3).

proteolytic conversion of PKC to M-kinase and other products [25,26] may have limited the accumulation of the holoenzyme in the membrane fraction. However, during insulin treatment, this proteolysis may have been less intense than with TPA, as more prominent increases in membrane PKC were observed.

The subcellular redistribution and apparent translocation of PKC observed presently provides further evidence (see [15,16]) that insulin activates PKC in rat adipocytes. Nevertheless, our findings differ from those of previous studies, probably because of the following. Most importantly, we purified PKC by chromatography on Mono Q columns, and this removes DAG, and possibly other endogenous activators and inhibitors, more effectively than DEAE-cellulose columns (submitted for publication). We also employed optimal concentrations of diolein and our enzyme assays probably primarily reflected changes in content, rather than enzymatic activity, of PKC. In support of this, we observed similar changes in immunoreactive PKC. In previous studies [14,16] of PKC enzyme activity, cytosols were used without purification, or after chromatography on DEAE-cellulose columns. Since these cytosols contain substantial amounts of lipids (see above), including DAG, increases in enzyme activity may have obscured decreases in enzyme content. In studies using PKC binding assays [15,16], increases in cytosol activity may have reflected the translocation of PKC to the membrane fraction, followed by proteolytic cleavage, and release to the cytosol of regulatory fragments, which may bind labeled phorbol esters more effectively than the holoenzyme.

In summary, using two independent methods, we have found that insulin provokes rapid changes in cytosol and membrane PKC in rat adipocytes. Our findings suggest that PKC is translocated from the cytosol to the membrane fraction during insulin action, and this suggests that insulin activates PKC in rat adipocytes.

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