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# Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes

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Effects of insulin and phorbol esters on subcellular distribution of protein kinase C (PKC) isoforms were examined in rat adipocytes. Both agonists provoked rapid decreases in cytosolic, and/or increases in membrane, immunoreactive PCK-$\alpha$, PKC-$\beta$, PKC-$\gamma$ and PKC-$\epsilon$. Effects of phorbol esters on PCK-$\alpha$ redistribution to the plasma membrane, however, were much greater than those of insulin. In contrast, insulin, but not phorbol esters, stimulated the translocation of PKC-$\beta$ to the plasma membrane, and provoked changes in PKC-$\zeta$ redistribution. Neither agonist altered subcellular distribution of PKC-$\delta$, which was detected only in membrane fractions. Our findings indicate that insulin and phorbol esters have overlapping and distinctly different effects on the subcellular redistribution of specific PKC isoforms.

## INTRODUCTION

Insulin and phorbol esters provoke rapid increases in membrane-associated protein kinase C (PKC) in rat adipocytes [1,2]. These increases appear to be due to translocation of PKC from cytosol to the membrane fraction, a process that may reflect activation of the enzyme. Insulin-induced increases in membrane-associated PKC-dependent phosphorylation of histone IIIs [1] and a synthetic peptide fragment of glycogen synthase [2] have been observed in the above-cited adipocyte studies, but these enzyme assays do not provide insight into which PKC isoform(s) is (are) translocated. We have previously reported [1] that immunoreactive PKC-$\beta$ is translocated in response to treatment of rat adipocytes with insulin and phorbol 12-myristate 13-acetate (PMA). In the present paper, we have characterized the PKC profile in rat adipocytes and report on the effects of insulin and PMA on the subcellular distribution of PKC isoforms, namely $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$ and $\zeta$.

## EXPERIMENTAL

As described previously [1], rat adipocytes were prepared by collagenase digestion, suspended (10% by volume) in glucose-free Krebs–Ringer phosphate buffer containing 1% BSA, equilibrated for 30 min at 37 °C, and then incubated over a 30 min period (constant for all samples), during which 3 nM-insulin or 500 nM-PMA was added in a retrograde sequence and was present for the indicated times of treatment. Control adipocytes were treated similarly with vehicle (Krebs–Ringer phosphate buffer) alone for 30 min.

After incubation (also see ref. [1]), adipocytes were rinsed and homogenized in ice-cold 0.25 M-sucrose containing 20 mM-Tris/HCl (pH 7.5), 1.2 mM-EGTA, 1 mM-phenylmethanesulphonyl fluoride, 20 mM-2-mercaptoethanol and 20 $\mu$g of leupeptin/ml (buffer I). Cytosol (100000 *g* supernatants) and solubilized membrane fractions (in buffer I containing 1% Triton X-100 and supplemented with 5 mM-EGTA and 2 mM-EDTA) were obtained, stored at −70 °C in Laemmli buffer [2a] (without prior boiling), and analysed by Western blotting [1]. In some experiments, plasma membranes and microsomes were isolated as described previously [3]. Equal amounts of cytosol (2 $\mu$g of protein for PKC-$\beta$ and 40–60 $\mu$g of protein for all other PKC isoforms) or membrane (10 $\mu$g of protein for PKC-$\beta$ and 40–60 $\mu$g of protein for all other PKC isoforms) proteins from each of the samples in a given experiment were subjected to SDS/PAGE, electrolytic transfer to nitrocellulose membranes, blocking with gelatin, incubation with isoform-specific anti-PKC serum, washing, incubation with mouse anti-(rabbit $\gamma$-globulin) antibody linked to alkaline phosphatase, subsequent colour development with tetrazotized *o*-dianisidine and $\beta$-naphthyl acid phosphate, and laser densitometric scanning [1]. Mixtures of rat brain PKC isoforms used as standard were purified either partially by Mono Q column chromatography [1] or to apparent homogeneity by a five-step procedure [4] (results were the same). Immunoreactivity was directly proportional to the amounts of standards (Fig. 1) and samples subjected to immunoblotting. However, note that it is possible to saturate the immunodetection system, and it is important to use samples that contain PKC in amounts that fall on the linear portion of the dose–response curves (Fig. 1).

The antiserum used for PKC-$\beta$ (isoforms I and II) immunoblotting (obtained from J. Mehegan and B. Roth, Bethesda Naval Medical Research Institute, Bethesda, MD, U.S.A.) was the same as that used previously [1,5]. [Similar results (not shown) were obtained with an anti-(PKC-$\beta$) serum.] The antisera used for immunoblotting PKC-$\alpha$, PKC-$\delta$ and PKC-$\zeta$ were obtained from Gibco (see ref. [6] for peptides used as antigens and blotting results). Antisera used for immunoblotting PKC-$\epsilon$ and PKC-$\gamma$ were prepared [7]. All antisera were raised by immunization of rabbits with synthetic peptides specific for variable regions in catalytic domains of each PKC isoform. Specificity of immunoreactive rat adipocyte PKC isoforms was verified by: (1) comparison with a standard mixture of rat brain PKC isoforms (each form has a slightly different mobility on SDS/PAGE); (2) loss of immunoreactivity of both brain standards and samples when assays were conducted in the presence of an excess of the specific synthetic peptide which had been used for immunization; (3) time-dependent agonist effects that were different for each PKC isoform; (4) failure of isoform-specific antisera to cross-react with other PKC isoforms [e.g. using these

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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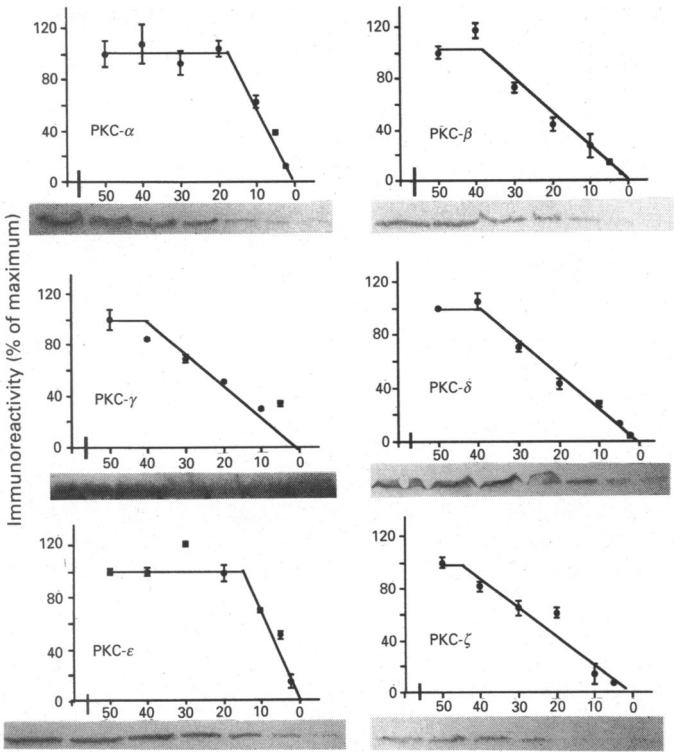


**Fig. 1. Quantification of PKC isoforms by immunoblotting**

Rat brain PKCs were partially purified as a mixture by Mono Q column chromatography and placed into Laemmli buffer. Various amounts (0–50 μl) were subjected to SDS/PAGE, incubated with specific PKC antibodies, as described in the Experimental section and quantified using an LKB Ultroscan XL densitometer. Results are means ± S.E.M. of values obtained from three scans of separate adjacent areas of each blot (this provides an indication of the precision of the assay). Corresponding blots are shown beneath graphs of each PKC isoform. Comparable results were obtained in other experiments using graded amounts of both rat brain PKC standards and cellular extracts (cytosol and membrane fractions).

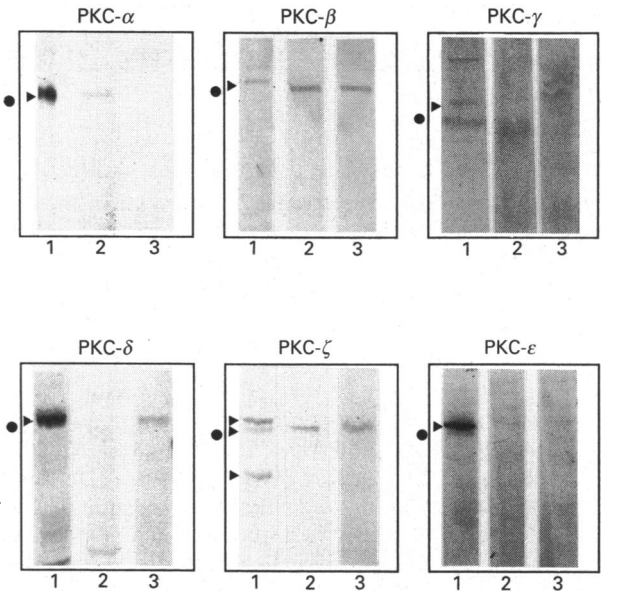


**Fig. 2. Immunoreactive PKC isoforms in cytosol (lane 2) and membrane (lane 3) fractions of rat adipocytes and rat brain (lane 1)**

The positions of an 84 kDa molecular-mass standard are depicted by closed circles (●). Arrowheads (▶) indicate those bands that were no longer evident when immunoassays were conducted in the presence of an excess of antigenic peptide.

antisera, we have found (unpublished work) that: BC3H-1 myocytes contain immunoreactive PKC-α, PKC-β and PKC-ζ, but no detectable PKC-γ, PKC-δ or PKC-ε; rat soleus muscle contains PKC-α, PKC-β, PKC-ε and PKC-ζ, but no detectable PKC-γ or PKC-δ; rat adipocyte cytosols contain PKC-α, PKC-β, PKC-γ, PKC-ε and PKC-ζ, but no detectable PKC-δ; control rat adipocyte membranes contain PKC-β, PKC-γ, PKC-δ, PKC-ε and PKC-ζ, but little discernible PKC-α; acute PMA-treated rat adipocyte membranes contain PKC-α, PKC-β, PKC-γ, PKC-δ and PKC-ζ]; and (5) treatment of rat adipocytes with PMA for 20 h provoked losses (50–90%) of all PKC isoforms, except PKC-ε, in which no change was observed (results not shown). From these comparisons, it is clear that the antisera that were used did not cross-react non-specifically with other PKC isoforms that were studied here. However, we cannot rule out the possibility that these antisera would recognize epitopes of PKC isoforms that were not studied here.

## RESULTS

Fig. 2 shows the comparison of immunoreactive PKC isoforms in rat adipocyte cytosol and membrane fractions with rat brain PKC standards. Also indicated are the positions of an 80 kDa molecular-mass standard and the immunoreactive bands that were no longer evident when assays were conducted in the presence of an excess of specific peptides used as antigens to raise antisera (only these 'adsorption-sensitive' bands were considered as specific PKC bands). Note that: (1) all PKC isoforms migrated with molecular-mass standards of 80–90 kDa (the slightly higher apparent molecular masses of some PKC isoforms observed here may reflect the fact that samples in Laemmli buffer were not heated); (2) 80–90 kDa PKC-ζ in rat brain cytosol was a double with a third lower band at approx. 60 kDa (these three bands have also been noted by others [6]), whereas rat adipocyte PKC-ζ was essentially a single 85 kDa band, with a faint higher-molecular-mass band observed only in some blots; and (3) only one PKC-γ band was absorption-sensitive when assayed in the presence of excess peptide antigen. Immunoreactive PKC-β, PKC-γ, PKC-ε and PKC-ζ were detectable in both cytosol and membrane fractions of control and insulin-treated adipocytes (Figs. 2–4). PKC-α was detectable in control cytosol, only weakly or not evident in control membranes, but readily apparent in PMA-treated membranes. PKC-δ was detectable in membrane, but not cytosol fractions.

Figs. 3–5 show time-dependent effects of 3 nM-insulin and 500 nM-PMA (maximally stimulating doses) on cytosolic and membrane-associated PKC-β, PKC-α, PKC-δ and PKC-ζ. Insulin and PMA provoked comparable decreases in cytosolic, and increases in membrane PKC-β. In the case of PKC-α, PMA provoked striking decreases in cytosolic, and increases (on a relative basis, infinite, at times, as basal activity was frequently unmeasurable) in membrane PKC-α immunoreactivity at all times of treatment; in contrast, insulin provoked small, but significant ($P < 0.05$; $n = 4$, paired $t$ test), biphasic decreases in cytosolic PKC-α immunoreactivity at 1 min and 20 min, but membrane changes were not discernible. In contrast with PKC-β and PKC-α, neither insulin nor PMA altered membrane PKC-δ significantly. In the case of PKC-ζ, insulin provoked sizeable decreases in cytosolic, and increases in membrane, immunoreactivity: PMA, on the other hand, at least acutely, provoked little or no change in PKC-ζ. Although detailed time-course experiments for PKC-ε and PKC-γ were not conducted,

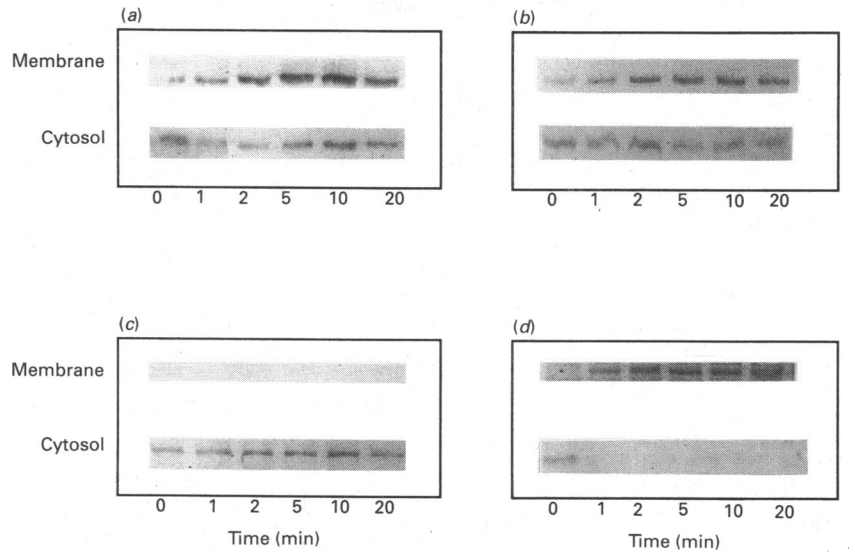


**Fig. 3. Time-dependent changes in immunoreactive PKC-$\beta$ (*a*, *b*) and PKC-$\alpha$ (*c*, *d*) in cytosol and membrane fractions of rat adipocytes treated with insulin (*a*, *c*) or PMA (*b*, *d*)**

Representative immunoblots are shown.

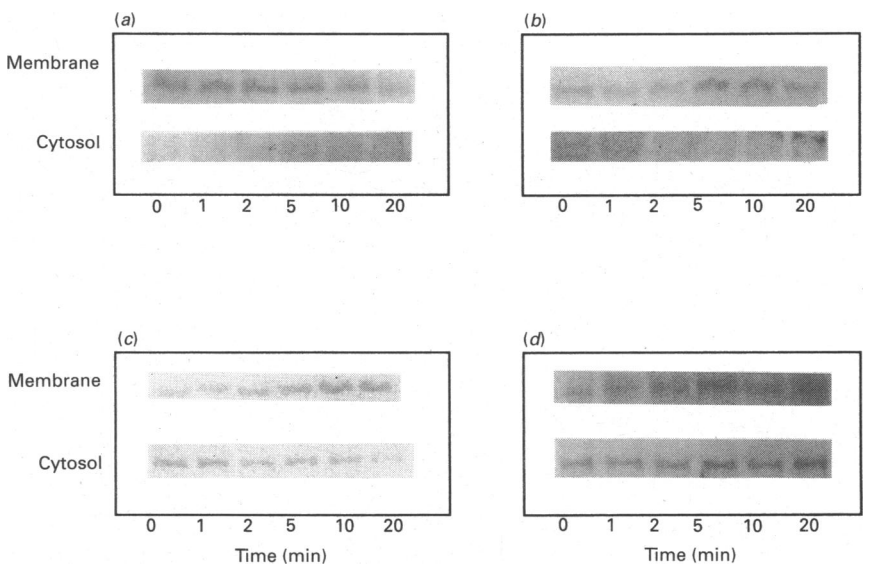


**Fig. 4. Time-dependent changes in immunoreactive PKC-$\delta$ (*a*, *b*) and PKC-$\zeta$ (*c*, *d*) in cytosol and membrane fractions of rat adipocytes treated with insulin (*a*, *c*) and PMA (*b*, *d*)**

Representative immunoblots are shown.

in experiments in which treatments were for 10–15 min (Fig. 6), insulin and PMA (respectively) provoked: (a) $36\pm9\%$ ($P < 0.01$; $n = 6$) and $18\pm2\%$ ($P < 0.001$; $n = 5$) decreases in cytosolic PKC-$\epsilon$ (membrane changes were not observed); (b) $46\pm2\%$ ($P < 0.001$; $n = 4$) and $51\pm8\%$ ($P < 0.01$; $n = 4$) decreases in cytosolic PKC-$\gamma$; and (c) $105\pm31\%$ ($P < 0.05$; $n = 3$) and $240\pm59\%$ ($P < 0.025$; $n = 4$) increases in membrane PKC-$\gamma$.

As shown in Fig. 7, insulin provoked $60\pm10\%$ ($n = 6$; $P < 0.005$) increases in plasma-membrane PKC-$\beta$ within 1 min, whereas PMA was without effect in seven experiments (not shown). PMA, however, provoked a marked increase in plasma-membrane PKC-$\alpha$ at 10 min ($718\pm30\%$; $n = 4$), whereas insulin was without effect (Fig. 7). Both insulin and PMA increased microsomal PKC-$\beta$ after 10 min, but not at 1 min, of treatment.

## DISCUSSION

Although insulin and PMA have been reported to stimulate comparably the apparent translocation of PKC-dependent histone-phosphorylating activity and immunoreactive PKC-$\beta$ from the cytosol to membrane fractions in rat adipocytes [1], these agonists were here found to have different effects on subcellular redistribution of other specific PKC isoforms. Among the more notable differences, PMA was greatly superior to insulin in provoking PKC-$\alpha$ redistribution to the plasma membrane, but less effective than insulin in provoking acute changes in the redistribution of PKC-$\zeta$, in particular, and PKC-$\epsilon$. Also, although both insulin and PMA stimulated PKC-$\beta$ translocation to microsomal membranes, only insulin stimulated the translocation of PKC-$\beta$ to plasma membranes, at least at the times examined.

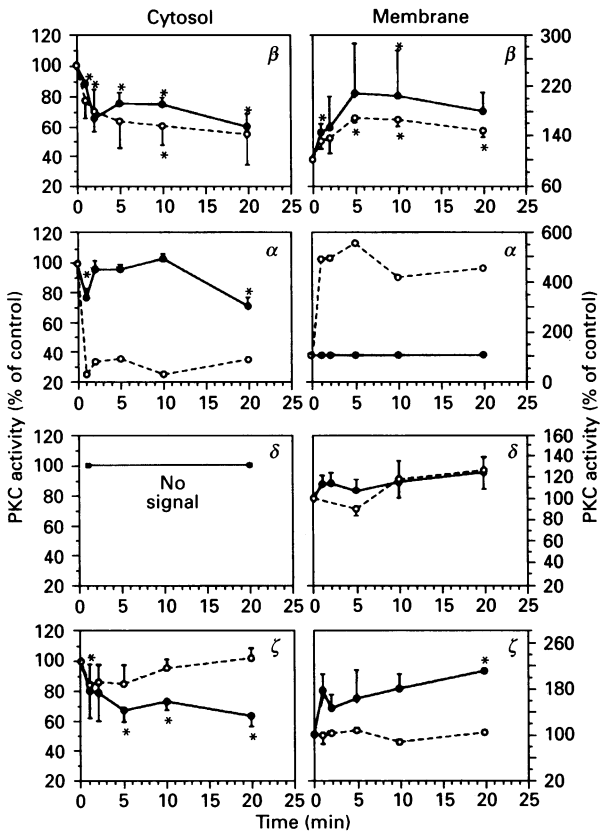


**Fig. 5. Time-dependent changes in cytosolic (left) and membrane (right) immunoreactive PKC isoforms in rat adipocytes treated with insulin (●) or PMA (○)**

Values are means ± S.E.M. of relative changes (expressed as % of control) in three to five separate experiments. $*P < 0.05$ (paired $t$ test). In the case of PKC-$\alpha$, only one experiment with PMA treatment is shown: in other repeat ($n = 4$) experiments, PKC-$\alpha$ was not measurable at all in control membranes, and relative increases after PMA treatment were 'infinite'.

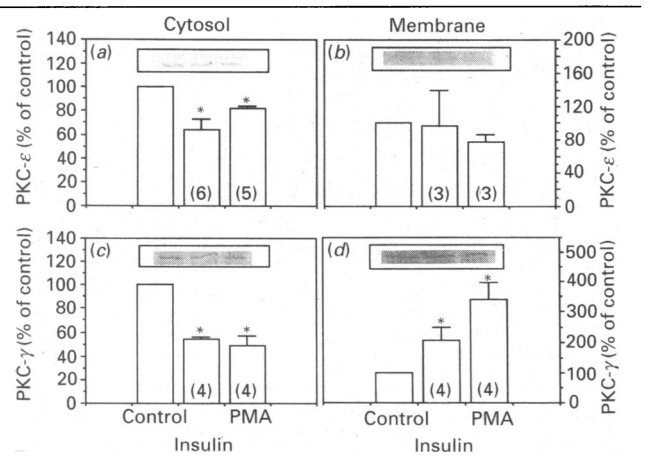


**Fig. 6. Effects of insulin and PMA on cytosolic (*a*, *c*) and membrane (*b*, *d*) immunoreactive PKC-ε (*a*, *b*) and PKC-γ (*c*, *d*) in rat adipocytes**

Treatments were for 10–15 min. Representative immunoblots are shown in insets. The numbers of experiments are shown in parentheses. $*P < 0.05$ (paired $t$ test).

It may therefore be surmised that there are both overlapping and distinctly different effects of insulin and PMA on the subcellular redistribution of specific PKC isoforms in rat adipocytes. We have also observed (unpublished work) overlapping and distinct

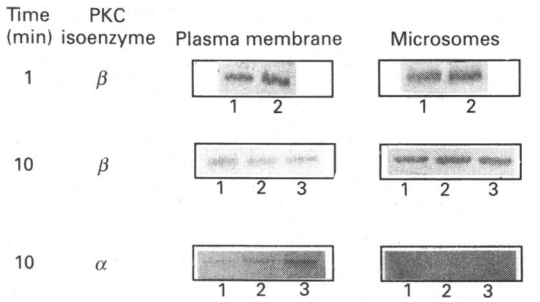


**Fig. 7. Effects of insulin and PMA on immunoreactive PKC-α and PKC-β in isolated plasma-membrane and microsomal fractions of rat adipocytes**

Representative immunoblots are shown. Lane 1, control; lane 2, +insulin; lane 3, +PMA.

effects of insulin and PMA on PKC isoform redistribution in other cell types, but the PKC isoform-specific redistribution patterns in these cells differ from that found in rat adipocytes, e.g. in the rat soleus, insulin is more effective than PMA in stimulating the redistribution of both PKC-$\alpha$ and PKC-$\beta$; in BC3H-1 myocytes, PMA is much more effective than insulin in stimulating PKC-$\alpha$ and PKC-$\zeta$ redistribution, but much less effective than insulin in stimulating PKC-$\beta$ translocation.

It was of interest to find that rat adipocytes contain PKC-$\alpha$, PKC-$\gamma$, PKC-$\delta$, PKC-$\epsilon$ and PKC-$\zeta$, in addition to PKC-$\beta$, which had previously been reported [1]. To date, PKC-$\gamma$ has been found almost exclusively in neural tissues [8], but there are no published studies of this isoform in rat adipose tissue. Our observation of immunoreactive PKC-$\gamma$ in adipocytes correlates with a peak of $Ca^{2+}$/phospholipid/diolein-dependent histone-phosphorylating enzyme activity in rat adipocyte cytosol that is co-eluted (i.e. at relatively low ionic strength) with rat brain PKC-$\gamma$ from hydroxyapatite columns (R. V. Farese, M. L. Standaert, A. J. Francois, K. Ways, T. P. Arnold, H. Hernandez & D. R. Cooper, unpublished work). Also, in concert with the present finding of multiple immunoreactive PKC isoforms in rat adipocytes, we have observed multiple (five or more) peaks of PKC-dependent protein kinase enzyme activity on hydroxyapatite column chromatography.

In keeping with other reports [9], we have found that immunoreactive PKC-$\delta$ was present primarily in the membrane fraction, with little or no immunoreactivity in the cytosol. It was therefore not surprising that insulin and PMA did not acutely alter the subcellular distribution of PKC-$\delta$. Nevertheless, 'chronic' treatment of adipocytes with PMA for 20 h partially depleted membrane PKC-$\delta$ (see above).

The fact that insulin, but not PMA, provoked significant acute changes in the subcellular redistribution of PKC-$\zeta$ in rat adipocytes is of interest for several reasons. First, this failure of acute PMA treatment to alter PKC-$\zeta$ in adipocytes is tissue-specific, as PMA acutely enhances translocation of 80 kDa PKC-$\zeta$ in platelets [10] and rat soleus muscle and BC3H-1 myocytes (unpublished work). Second, the present findings raise the possibility that, in rat adipocytes, PKC-$\zeta$ may respond to an insulin-stimulated pool of diacylglycerol, or another membrane-associated ligand that is relatively inaccessible to, or not mimicked by, acutely added PMA [note, however, that with prolonged (20 h) PMA treatment in rat adipocytes, there is substantial loss of PKC-$\zeta$]. Third, PKC-$\zeta$ translocation responses to agonist and/or PMA observed in platelets [10], rat adipocytes, rat soleus muscles and BC3H-1 myocytes suggest that 80–85 kDa PKC-$\zeta$ in these intact tissues contains diacylglycerol/PMA (or other ligand)-binding sites, which were not evident in studies *in vitro* of

a 64 kDa PKC-$\zeta$ subspecies that was obtained from COS-7 cells transfected with a PKC-$\zeta$ cDNA construct [11]. (It should be noted that the amino acid sequence of the PKC-$\zeta$ peptide used as the antigen for raising antiserum for immunoblotting in ref. [11] is identical with that used here).

It is of interest that insulin increased plasma-membrane PKC-$\beta$ at 1, but not at 10, min and, on the other hand, increased microsomal PKC-$\beta$ at 10, but not at 1, min. This may reflect early changes in the hydrolysis of plasma-membrane phospholipids, followed by later increases in phospholipid synthesis *de novo* in the endoplasmic reticulum [12].

In summary, insulin provoked rapid decreases in cytosolic, and/or increases in membrane-associated immunoreactive PKC-$\alpha$, PKC-$\beta$, PKC-$\gamma$, PKC-$\epsilon$ and PKC-$\zeta$ in rat adipocytes. PMA also provoked rapid changes in the subcellular redistribution of PKC-$\alpha$, PKC-$\beta$, PKC-$\gamma$ and PKC-$\epsilon$, but the effects of PMA on PKC-$\alpha$ were much greater than those of insulin, and PMA had little or no acute effect on PKC-$\zeta$. Insulin, but not PMA, stimulated the translocation of PKC-$\beta$ to the plasma membrane, whereas PMA stimulated the translocation of PKC-$\alpha$ to the plasma membrane. Both insulin and PMA stimulated the translocation of PKC-$\beta$ to the microsomes (endoplasmic reticulum). Our findings suggest that insulin and PMA have both overlapping and distinctly different effects on the subcellular redistribution of specific PKC isoforms in rat adipocytes. These findings may explain why insulin and PMA have similar and dissimilar effects on biological processes.

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