Effects of insulin and phorbol esters on MARCKS (myristoylated alanine-rich C-kinase substrate) phosphorylation (and other parameters of protein kinase C activation) in rat adipocytes, rat soleus muscle and BC3H-1 myocytes

Thomas P. ARNOLD,* Mary L. STANDAERT,* Herman HERNANDEZ,* James WATSON,* Harald MISCHAK,† Marcelo G. KAZANIETZ,† LiMing ZHAO,* Denise R. COOPER* and Robert V. FARESE*‡

*James A. Haley Veterans' Hospital, Departments of Internal Medicine and Biochemistry and Molecular Biology, University of South Florida, Tampa, FL 33612, and †Laboratories of Genetics and Carcinogenesis, NCI, NIH, Bethesda, MD 20892, U.S.A.

To evaluate the question of whether or not insulin activates protein kinase C (PKC), we compared the effects of insulin and phorbol esters on the phosphorylation of the PKC substrate, i.e. myristoylated alanine-rich C-kinase substrate (MARCKS). In rat adipocytes, rat soleus muscle and BC3H-1 myocytes, maximally effective concentrations of insulin and phorbol esters provoked comparable, rapid, 2-fold (on average), non-additive increases in the phosphorylation of immunoprecipitable MARCKS. These effects of insulin and phorbol esters on MARCKS phosphorylation in intact adipocytes and soleus muscles were paralleled by similar increases in the phosphorylation of an exogenous, soluble, 85 kDa PKC substrate (apparently a MARCKS protein) during incubation of post-nuclear membrane fractions *in vitro*. Increases in the phosphorylation of this 85 kDa PKC substrate *in vitro* were also observed in assays of both plasma membranes and microsomes obtained from rat adipocytes that had been treated with insulin or phorbol esters. These insulin-induced increases in PKC-dependent phosphorylating activities of adipocyte plasma membrane and microsomes were associated with increases in membrane contents of diacylglycerol, PKC-β_1 and PKC-β_2. Our findings suggest that insulin both translocates and activates PKC in rat adipocytes, rat soleus muscles and BC3H-1 myocytes.

INTRODUCTION

Considerable evidence suggests that insulin activates the diacylglycerol (DAG)/protein kinase C (PKC) signalling system in many target tissues. For example, insulin stimulates the translocation of PKC from cytosol to membrane, and/or increases the enzymic activity of PKC, in rat adipocytes [1–3], rat diaphragm [4], rat soleus muscle [5–7], rat gastrocnemius muscle [6], BC3H-1 myocytes [8–10], rat hepatocytes [11], CHO-R cells [12], fetal chick neurons [13] and H4IIE hepatoma cells [14]. In the above-mentioned cells that have been examined, increases in DAG production have also been observed during insulin treatment [5–8,11,12,15–17]. With respect to the phosphorylation of PKC substrates, insulin mimics phorbol esters and stimulates the phosphorylation of: (*a*) a 15 kDa protein (and trypsin-derived peptides) in rat diaphragm [18]; (*b*) eukaryotic initiation factors eIF-4F P25 and eIF-3 P120 in 3T3/L1 cells [19]; (*c*) 40 kDa proteins in rat adipocytes [20], CHO-R cells [12] and BC3H-1 myocytes [21]; and (*d*) acidic 80 kDa proteins in BC3H-1 myocytes [21] and rat soleus muscles [6]. In some of these studies [12,19], these acute phosphorylation effects of both insulin and phorbol esters are lost after phorbol-ester-induced PKC depletion. On the other hand, in certain cells insulin was reported to have a relatively small or no effect on the phosphorylation of a specific, well-accepted, PKC substrate, i.e. the 80–87 kDa myristoylated alanine-rich C-kinase substrate (MARCKS), and the role of DAG/PKC signalling during insulin action was therefore questioned [22]. We have now compared the effects of insulin and the PKC activator phorbol 12-myristate 13-acetate (PMA) on MARCKS phosphorylation in intact rat adipocytes, rat soleus muscles and BC3H-1 myocytes. We also examined: (*a*) whether insulin or PMA provoked increases in PKC activity in assays *in vitro* (with an apparent MARCKS protein as substrate) of total post-nuclear membranes of adipocytes and soleus muscles, and in isolated plasma membrane and microsomal membranes of adipocytes; and (*b*) whether there were insulin-induced increases in the contents of DAG and PKC in plasma membranes and microsomes of adipocytes.

MATERIALS AND METHODS

Incubations and ^{32}P-labelling of intact cells

Rat adipocytes were prepared by collagenase digestion of epididymal fat-pads of 150–200 g male Holtzman rats as described previously [2]. In each experiment, 20–30 ml of cells was batch-incubated for 120 min at 37 °C in 2 vol. of glucose-free Krebs–Ringer bicarbonate buffer (KRBHA) containing 30 mM Hepes (pH 7.4), 1% BSA and 10 mCi of [^{32}P]P$_i$ (NEN), and then divided into batches in plastic tubes and (unless stated otherwise) treated with 10 mM insulin (Elanco), 500 nM PMA (Sigma) or vehicle (controls) for the designated times. In the time course as well as the dose–response experiments, the total duration of the incubation was held constant for all samples (i.e. 150 min) by adding treatments in a retrograde sequence (i.e. at 30, 20, 10, 5, 2 and 1 min) during the last 30 min of incubation (the 'treatment period'). For controls (designated as 0 min of agonist treatment), vehicle alone was added at 30 min before the end of this treatment period (this, or other times of vehicle addition, did not alter

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; MARCKS, myristoylated alanine-rich C-kinase substrate.

† To whom correspondence should be addressed.

results). Since all samples were incubated for a total of 150 min and had attained comparable levels of basal protein phosphorylation, the only experimental variable was the duration of insulin or PMA treatment, and the control could therefore be compared with each of the treated samples. Reactions were stopped by adding ice-cold BSA-free KRBH. The adipocytes were washed twice with this buffer, and then lysed by freeze–thawing in a small volume of hypotonic buffer containing 30 mM Hepes (pH 7.4), 1 mM $NaVO_4$, 1 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM EGTA, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1000 units/ml aprotinin, 5000 units/ml bacitracin, 2 μM pepstatin and 2 μM leupeptin. Lysates were centrifuged at 105000 ***g*** at 4 °C for 1 h to obtain clear cytosol fractions. (Note: the lysing buffer contained phosphatase inhibitors, and the efficacy of the kinase-stopping procedures was verified by conducting experiments in which [γ-^{32}P]ATP was added at the time of lysis of unlabelled cells; no ^{32}P labelling of protein was observed in these lysates that were carried through all procedures.) In a few experiments, where designated, the cell lysis procedure was carried out in the above-described buffer containing 1 % Triton X-100 to obtain samples with both cytosol and detergent-solubilized membrane proteins. Although labelled MARCKS was primarily found in the cytosol (see below), this detergent cell-lysis procedure would obviate potential discrepancies due to differential release of labelled MARCKS from membrane to cytosol (see [23,24]) resulting from insulin or PMA treatment.

Soleus muscles from each rat were paired and used for control and stimulated samples as described previously [5,6]. Muscle ends were ligated, stretched, incubated at 37 °C for 120 min under O_2/CO_2 (19:1) in 5 ml of Krebs–Ringer bicarbonate buffer (KRB) containing 12 mM Hepes (pH 7.4), 5 mM glucose, 2 mM sodium pyruvate, 0.1 % BSA, and 330 μCi of [^{32}P]P_i, and then treated for 15 min with 10 nM insulin, 500 nM PMA or vehicle (controls). After incubation, soleus muscles were washed with ice-cold BSA-free KRBH and homogenized with a Brinkman Polytron (see [5,6]) in buffer (see [21]) containing 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), 2.5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 1.2 mM EGTA, 1 mM $NaVO_4$, 5 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM PMSF, 4000 units/ml bacitracin, 2 μM leupeptin and 2 μM pepstatin (Buffer A). Homogenates were centrifuged at 105000 ***g*** for 1 h at 4 °C to obtain cytosol fractions.

BC3H-1 myocytes were cultured as described [8], washed, incubated for 120 min at 37 °C under air/CO_2 (9:1) in 5 ml of serum-free Dulbecco's modified Eagle's medium containing 0.1 % BSA and 330 μCi of [^{32}P]P_i, and then treated with 100 nM insulin, 500 nM PMA or vehicle (controls) for 15 min (see [21]). After incubation, myocytes were washed with ice-cold Dulbecco's PBS, scraped, homogenized (10 strokes, Potter–Elvejhem) in Buffer A and centrifuged at 105000 ***g*** for 1 h at 4 °C to obtain cytosol fractions.

In each of the cell types, insulin did not significantly alter the ^{32}P labelling of trichloroacetic acid-soluble and -insoluble preparations of the cytosolic fractions. {We have previously documented that (*a*) insulin, if anything, slightly diminishes [^{32}P]ATP specific radioactivity during incubations of rat adipose tissue, and (*b*) within 60–120 min of incubation, ATP specific activity appears to be near or at equilibrium, as evidenced by the fact that the labelling of phosphatidylinositol and its monophosphate and bisphosphate has reached a plateau (see [25–27]).} Whereas we readily detected ^{32}P labelling of immunoprecipitable 80 or 85 kDa proteins, i.e. MARCKS, in all three cytosolic preparations, we could detect only slight, if any, labelling ($<$ 10 % of cytosolic) of immunoprecipitable 80 or 85 kDa protein in membrane fractions, despite the fact that these fractions contained appreciable amounts of immunoreactive MARCKS, as determined by immunoblotting (see below). This is in keeping with the concept that membrane-bound MARCKS is released to the cytosol as it is phosphorylated [23,24]. (Nevertheless, as alluded to above, some adipocyte experiments were conducted in which both cytosolic and membrane-associated MARCKS were analysed in Triton X-100 extracts of the whole cell.) In each of the three cell types, we documented that insulin provokes excellent increases in hexose transport in the presently used incubation conditions: in rat adipocytes, 20–40-fold increases in 3-*O*-methylglucose uptake and 5–10-fold increases in 2-deoxyglucose uptake; in rat solei, 2–4-fold increases in 2-deoxyglucose uptake; and in BC3H-1 myocytes, 2–3-fold increases in 2-deoxyglucose uptake.

Immunoprecipitation of MARCKS

Equal amounts of cytosolic protein (200–250 μg) or Triton X-100-solubilized cytosol plus membrane-associated protein (500–600 μg) were incubated at 4 °C, first for 16 h with an excess (1:20 dilution) of immune serum (kindly supplied by Dr. Ivar Walaas, Dr. Otto Walaas and Dr. Paul Greengard) obtained from rabbits inoculated with bovine or rat [results were identical with both antisera, although only results with anti-(bovine MARCKS) antiserum are reported here] brain MARCKS protein (see [28,29]), and second for 4–8 h with sheep anti-(rabbit IgG) antiserum (Sigma). Resultant immunoprecipitates were centrifuged at 100000 ***g*** for 30 min, washed three times, resuspended in Laemmli buffer, boiled for 10 min, subjected to one-dimensional SDS/PAGE (10.5 % polyacrylamide reducing gel), and analysed by autoradiography and densitometry scanning. The completeness of immunoprecipitation was verified by showing that: (*a*) higher antibody concentrations did not increase the recovery of immunoprecipitable MARCKS; (*b*) a second immunoprecipitation failed to yield significant immunoprecipitable ^{32}P-labelled MARCKS; and (*c*) there was complete immunoprecipitation of the ^{32}P-labelled 85 kDa PKC substrate (presumably MARCKS; see below), as demonstrated by its complete removal from the assay cytosol fraction (see below) after immunoprecipitation. The specificity of the immunoprecipitation was verified by showing that: (*a*) only a small fraction of total ^{32}P-labelled cytosolic proteins was immunoprecipitated, particularly in rat adipocytes, which contained many heavily labelled cytosolic proteins (see below), and (*b*) non-immune rabbit serum (Sigma) failed to immunoprecipitate ^{32}P-labelled 80 or 85 kDa (MARCKS) proteins.

Membrane-dependent phosphorylation of a soluble exogenous 85 kDa PKC substrate *in vitro*

Adipocytes or soleus muscles were incubated (without [^{32}P]P_i) and treated with 10 nM insulin, 500 nM PMA or vehicle (controls) as described above. After incubation, adipocytes and soleus muscles were washed three times with ice-cold BSA-free incubation medium, and, as described [30], cells were lysed in a small volume (1.5–3 ml) of hypotonic buffer, containing 1 mM $NaHCO_3$, 5 mM $MgCl_2$ and 100 μM PMSF (pH 7.5) (note that disruption of soleus muscle required a 30 s Polytron burst). A $\frac{1}{10}$ vol. of ice-cold Tris/HCl buffer (500 mM; pH 7.5) was added, nuclei were removed by centrifugation for 5 min at 500 ***g***, and post-nuclear membranes were recovered by centrifugation at 100000 ***g*** for 1 h. In some experiments, microsomal and plasma-membrane fractions were obtained from control and insulin- or PMA-treated adipocytes, by methods exactly as described by Weber et al. [31]. Membrane fractions were washed twice, and 150 μg of membrane protein was suspended in assay buffer

containing 1 mM $NaHCO_3$, 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 200 μM $NaVO_4$, 200 μM $Na_4P_2O_7$, 2 mM NaF, 200 μM PMSF and 2 μM $CaCl_2$, and then incubated in a final volume of 250 μl for 10 min at 37 °C with 20 μM [γ-^{32}P]ATP (4000 c.p.m./pmol) and boiled cytosol (5 μg of protein) obtained from S49T-lymphoma cells, which are rich in a soluble 85 kDa PKC substrate (kindly supplied by Dr. Balu R. Chakravarthy; see [30]). {Note: this heat-stable acidic (see [30]) 85 kDa PKC substrate is immunoprecipitated by (see above), and detected by immunoblotting with (see below) the Walaas–Greengard anti-MARCKS antiserum, and therefore appears to be a MARCKS protein. In further support of this inference, antiserum raised against the partially purified 85 kDa protein (kindly provided by Dr. B. R. Chakravarthy), like each of the Walaas–Greengard antisera, was very effective in immunoprecipitating adipocyte 85 kDa MARCKS phosphoprotein.} After incubation, an ice-cold EGTA solution was added (final concn. 1 mM), membranes were removed by centrifugation at 4 °C for 1 h at 100000 *g*, soluble proteins were resolved by SDS/PAGE (10.5% reducing gels), and the ^{32}P-labelled 85 kDa PKC substrate contained therein was quantified by autoradiography and laser densitometric scanning. ^{32}P-labelling of this soluble 85 kDa PKC substrate (which was added in excess; see Figure 4) was linear with respect to time and the concentration of either membrane protein or other sources of PKC; e.g. we used column-purified recombinant PKC-α obtained from a baculovirus–insect-cell expression system, and this PKC-α provoked marked increases in ^{32}P labelling of the 85 kDa substrate. No labelling of the soluble 85 kDa substrate was observed in the absence of membranes or other PKC sources, and there was no release of endogenous 85 kDa ^{32}P-labelled protein from membranes that had been incubated without the exogenous soluble 85 kDa PKC substrate (see Figure 4). Further, labelling of the 85 kDa substrate was completely inhibited by the PKC-(19–36) pseudosubstrate (Bachem), a specific PKC inhibitor [32], in concentrations similar to those found to inhibit a variety of other PKC-dependent phosphorylations *in vitro* (see [33]) (i.e. half-maximal and maximal or near-maximal at 5–10 and 10–100 μM respectively). As reported previously [30], ^{32}P labelling of the 85 kDa PKC substrate is not stimulated by calcicalmodulin or cyclic AMP, and the substrate is not PKC itself.

Immunoblot analysis of MARCKS

Proteins were resolved by SDS/PAGE (reducing gels) and electrolytically transferred to nitrocellulose membranes, which, after blocking non-specific sites with gelatin, were incubated first for 16–20 h with the Walaas–Greengard anti-MARCKS antiserum (diluted 1:200) and then incubated for 2 h with goat anti-(rabbit γ-globulin) antiserum, coupled to alkaline phosphatase for subsequent colour development (see [2,5,6] for further details).

PKC translocation experiments

As described above, adipocytes were incubated and treated for 1–30 min with vehicle (controls) or 3 or 10 nM insulin, after which purified plasma membranes, microsomes and cytosol were obtained and analysed for immunoreactive PKC-β_1 and PKC-β_2, by using methods described previously [2,5,6,34]. Antisera for PKC-β_1 and PKC-β_2 (obtained from Research and Diagnostic Antibodies) were raised by immunizing rabbits with synthetic peptides contained in variable (V5) regions of the catalytic domains of these isoforms. Specificity of immunoreactive PKC bands was verified by: (*a*) comparison with purified rat brain PKC standards that migrated on SDS/PAGE at 78–80 kDa; (*b*) loss of immunoreactivity when assays were conducted in the presence of an excess of immunogenic synthetic peptide; and (*c*) testing against recombinant PKC-β_1 and PKC-β_2 obtained from baculovirus–insect-cell expression systems.

Measurement of DAG

DAG was measured by the method of Preiss et al. [35].

RESULTS

MARCKS phosphorylation in rat adipocytes

In preliminary experiments, we found that many cytosolic proteins were labelled with [^{32}P]P_i during the 150 min incubation of rat adipocytes. Bands of ^{32}P-labelled proteins that migrated at 80 and 85 kDa on SDS/PAGE were consistently observed in these cytosolic preparations, and the phosphorylation of these proteins appeared to be stimulated by both insulin and PMA (Figure 1). To determine whether the 80 or 85 kDa phosphoproteins were MARCKS proteins, we used immunoblot analysis (see below) and purification by immunoprecipitation. Upon immunoprecipitation of ^{32}P-labelled cytosolic proteins with anti-MARCKS antiserum, the major band of ^{32}P-labelled immunoprecipitable protein migrated at 85 kDa during SDS/PAGE (Figure 2). Moreover, insulin and PMA provoked comparable rapid increases in the ^{32}P-labelling of the immunoprecipitated 85 kDa protein [the relationship of other, lesser, ^{32}P-labelled

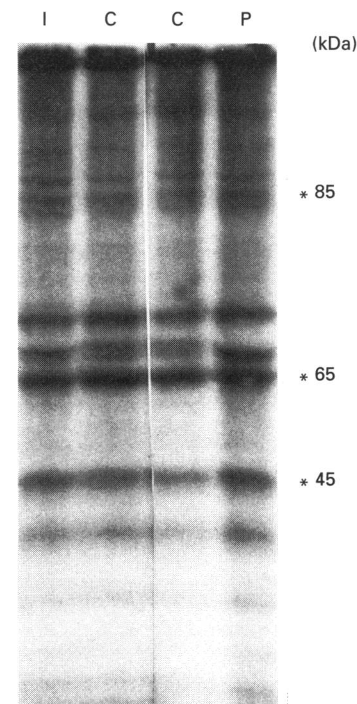

Figure 1 ^{32}P labelling of cytosolic proteins in rat adipocytes

As described in the Materials and methods section, adipocytes were labelled for 135 min and then treated over a 15 min period with 10 nM insulin (I), 500 nM PMA (P), or vehicle (controls, C) (total incubation time = 150 min). Cytosols were obtained, and 25 μg of protein of each sample was analysed by SDS/PAGE and autoradiography (shown here). Positions of protein standards are shown in kDa in this and subsequent Figures.

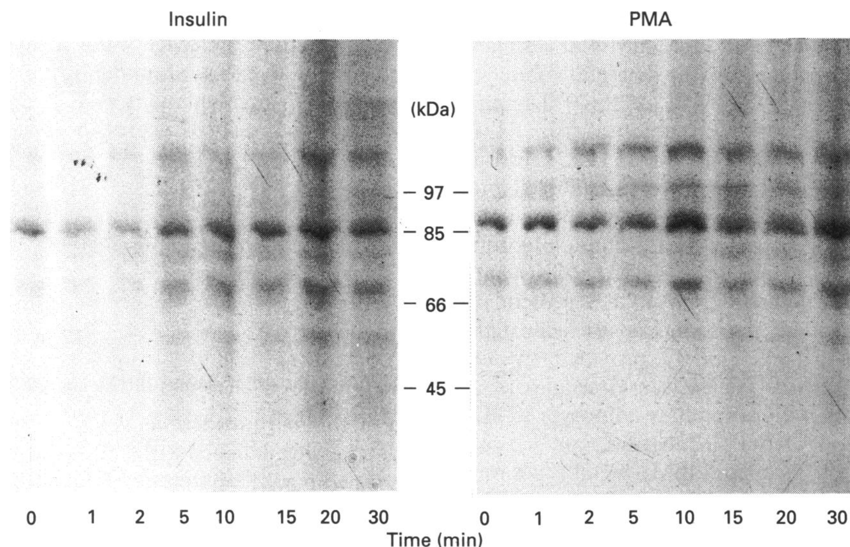

Figure 2 Time-dependent effects of insulin and PMA on ^{32}P labelling of immunoprecipitable MARCKS protein in cytosolic preparations of rat adipocytes

As described in the Materials and methods section, cells were labelled for 120 min and then treated over a 30 min period with vehicle (controls, designated as 0 min of agonist treatment) or with 10 nM insulin or 500 nM PMA for the indicated times. The total incubation time for all samples was 150 min. Cytosolic proteins (200 μg) were subjected to immunoprecipitation by anti-MARCKS antiserum, and precipitates wee analysed by SDS/PAGE and autoradiography (shown here).

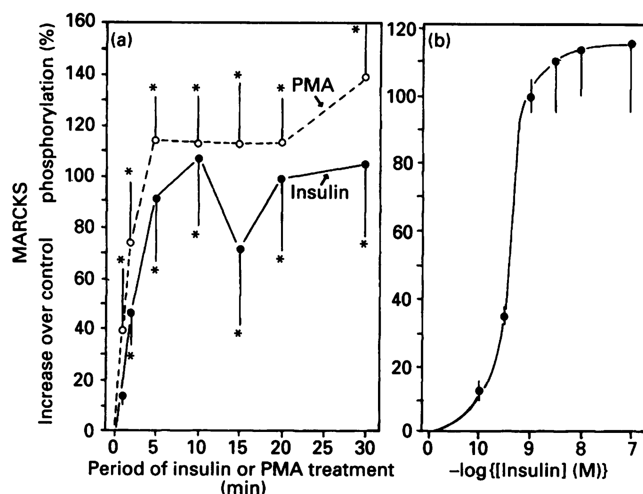

Figure 3 Effects of insulin and PMA on ^{32}P labelling of immunoprecipitable MARCKS protein in cytosolic preparations of rat adipocytes

(**a**) Time-course experiments. These were conducted as described in Figure 1. Shown here are laser-densitometric scan results from autoradiograms of seven experiments in which the time-dependent effects of insulin (10 nM) and PMA (500 nM) were simultaneously compared in the same adipocyte preparation. Results are shown as mean ($\pm$S.E.M.) percentage increases over vehicle-treated controls: $^{*}P < 0.05$ (paired t test). (**b**) Insulin dose–response experiments. These were conducted as in (**a**) except that indicated treatments were present during the last 15 min of the treatment period. Results are the mean$\pm$range of two experiments, each analysed in duplicate.

immunoprecipitated proteins to MARCKS is uncertain, but other molecular sizes of MARCKS have been noted, and immunoblots (see below) revealed traces of immunoreactivity at approx. 70 kDa and 95 kDa].

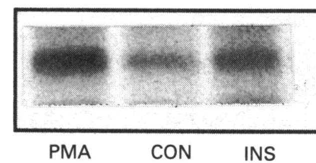

Figure 4 Effects of insulin and PMA on ^{32}P labelling of Triton X-100-solubilized cytosol + membrane-associated immunoprecipitable MARCKS in rat adipocytes

Adipocytes were prelabelled for 120 min and then incubated for 15 min, as described in Figures 1–3, with 10 nM insulin (INS) or 500 nM PMA (CON, control). Cells were homogenized in buffer containing 1% Triton X-100, and cytosolic plus membrane-associated MARCKS (500 μg of protein) was subjected to immunoprecipitation, SDS/PAGE and autoradiography, as described in the Materials and methods section. A representative autoradiogram of immunoprecipitable 85 kDa MARCKS is shown here.

The results of a total of seven completely separate time-course experiments in which both 10 nM insulin and 500 nM PMA were directly compared in the same adipocyte preparation are portrayed in Figure 3 (these concentrations were used, as they provoke maximal stimulation of hexose transport). As is apparent, stimulatory effects of PMA and insulin on ^{32}P labelling of immunoprecipitated cytosolic MARCKS were statistically significant within 1 and 2 min respectively, and persisted during the 30 min treatment period. In these seven unselected experiments, the mean increases in cytosolic MARCKS phosphorylation were approx. 2-fold during insulin and PMA treatment, but, in selected experiments as much as 4-fold increases were observed with both treatments. In dose–response experiments (Figure 3), insulin was found to provoke maximal effects on cytosolic MARCKS phosphorylation at 1–10 nM, and half-maximal effects at approx. 0.3–0.5 nM. We also conducted experiments in which 10 nM insulin and 500 nM PMA were used separately and in combination, and found that there was no

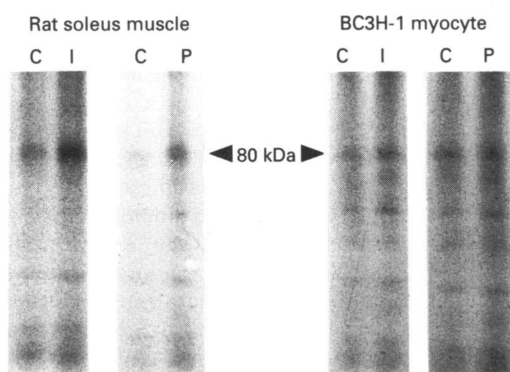

Figure 5 Effects of insulin and PMA on ^{32}P labelling of immunoprecipitable MARCKS protein in cytosolic preparations of the rat soleus (left) and BC3H-1 myocytes (right)

As described in the Materials and methods section, solei or myocytes were labelled with [^{32}P]P_i for 120 min, and then treated without (control) or with 10 nM (solei) or 100 nM (myocytes) insulin or 500 nM PMA for 15 min. Cytosolic proteins were subjected to immunoprecipitation with anti-MARCKS antiserum, and precipitates were analysed by SDS/PAGE, autoradiography, and scanning laser densitometry. Shown here are representative autoradiograms from experiments in which an insulin (I)- or PMA (P)-treated soleus muscle was compared with corresponding controls (C).

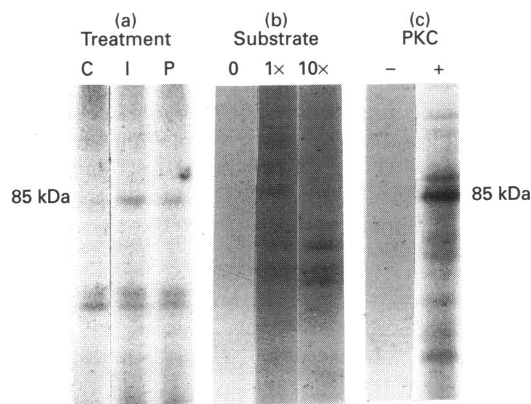

Figure 6 Phosphorylation of 85 kDa PKC substrate *in vitro* by rat adipocyte post-nuclear membrane preparations

After treatment of intact rat adipocytes, total post-nuclear membranes were isolated and assayed *in vitro*, by using exogenous soluble 85 kDa PKC substrate, as described in the Materials and methods section. After assay, membranes were removed by centrifugation, and soluble ^{32}P-labelled proteins were analysed by SDS/PAGE and autoradiography (shown here). (**a**) Intact adipocytes were treated without (i.e. control, indicated as C) or with 10 nM insulin (I) or 500 nM PMA (P) for 10 min, before obtaining post-nuclear membranes. (**b**) Membranes from insulin-treated adipocytes were incubated with no (0), 5 μg (1 ×) or 50 μg (10 ×) of boiled cytosolic protein from S49T-lymphoma cells containing 85 kDa PKC substrate. (**c**) Assay was conducted in the absence (−) of membranes or a PKC source, or in the presence (+) of baculovirally derived PKC-α, instead of membranes.

additivity of maximal stimulatory effects of these agonists on ^{32}P labelling of immunoprecipitated cytosolic MARCKS (results not shown).

In some experiments, we examined the effects of 15 min treatments with 10 nM insulin and 500 nM PMA on the phosphorylation of Triton X-100-solubilized cytosol plus membrane-associated immunoprecipitable MARCKS: increases of 77 ± 19 % (mean ± S.E.M.; $n = 5$; $P < 0.025$) and 110 ± 27 % (mean ± S.E.M.; $n = 4$; $P < 0.005$) respectively were observed (see Figure 4). These effects of insulin and PMA on cytosolic plus membrane-associated MARCKS were therefore similar to those observed with cytosolic preparations.

Table 1 Effects of insulin and PMA treatment on the phosphorylation of exogenous soluble 85 kDa PKC substrate by rat adipocyte membrane preparations *in vitro*

Rat adipocytes were equilibrated for 30 min in glucose-free KRBHA, and then treated for 1 or 10 min with 10 nM insulin, 500 nM PMA, or vehicle (controls). After incubation, membrane fractions were obtained and incubated with [γ-^{32}P]ATP and exogenous 85 kDa PKC substrate as described in the Materials and methods section. ^{32}P labelling of the soluble 85 kDa PKC substrate was measured after removal of membranes by ultracentrifugation, purification of soluble proteins by SDS/PAGE, autoradiography and scanning laser densitometry. Results are given as percentage increase (mean ± S.E.M. for the numbers of experiments shown in parentheses) over the corresponding control. Shown in brackets are P values, as determined by paired t testing.

Membrane fraction (duration of treatment)	85 kDa PKC substrate phosphorylation (% increase versus control)	
	Insulin treatment	PMA treatment
Total post-nuclear membranes (10 min)	130 ± 20 (4) [$P < 0.01$]	168 ± 7 (4) [$P < 0.001$]
Plasma membranes (1 min)	105 ± 14 (5) [$P < 0.005$]	127 ± 20 (4) [$P < 0.01$]
Plasma membranes (10 min)	84 ± 7 (3) [$P < 0.01$]	107 ± 8 (3) [$P < 0.01$]
Microsomes (1 min)	46 ± 2 (5) [$P < 0.001$]	74 ± 4 (4) [$P < 0.001$]
Microsomes (10 min)	195 ± 23 (3) [$P < 0.025$]	171 ± 22 (3) [$P < 0.025$]

MARCKS phosphorylation in rat soleus muscles

The phosphorylation of total cytosolic proteins in the rat soleus muscle and stimulatory effects of insulin and PMA on the phosphorylation of an acidic 80 kDa protein have been reported previously [6]. Upon immunoprecipitation of ^{32}P-labelled cytosolic proteins of the rat soleus muscle by anti-MARCKS antiserum, we found a major band of immunoprecipitable ^{32}P-labelled protein that migrated at 80 kDa on SDS/PAGE (Figure 5). Both insulin (10 nM) and PMA (500 nM) stimulated the phosphorylation of the immunoprecipitated 80 kDa MARCKS protein by approx. 2-fold: insulin- and PMA-induced increases (means ± S.E.M.) were + 99 ± 14 % ($n = 7$; $P < 0.001$, paired t test) and + 112 ± 11 % ($n = 8$; $P < 0.001$) respectively.

MARCKS phosphorylation in BC3H-1 myocytes

The phosphorylation of total cytosolic proteins in BC3H-1 myocytes and the stimulatory effects of insulin and PMA on the phosphorylation of an acidic 80 kDa protein have been published previously [21]. Upon immunoprecipitation of ^{32}P-labelled cytosolic proteins from BC3H-1 myocytes with anti-MARCKS antiserum, precipitates contained a prominent band of ^{32}P-labelled protein that migrated at 80 kDa on SDS/PAGE (Fig. 5). As in rat adipocytes and soleus muscles, both insulin (100 nM) and phorbol ester (500 nM) provoked 2-fold increases in the phosphorylation of the immunoprecipitated 80 kDa MARCKS protein: insulin- and PMA-induced increases (mean ± S.E.M.) were + 95 ± 8 % ($n = 4$; $P < 0.005$, paired t test) and + 129 ± 14 % ($n = 3$; $P < 0.025$) respectively.

Phosphorylation *in vitro* of the 85 kDa PKC substrate by rat adipocyte and soleus-muscle membrane preparations

Figure 6(a) portrays changes in phosphorylating activity of total post-nuclear membrane preparations that were obtained from control or insulin- or PMA-treated adipocytes, and then assayed *in vitro* with an exogenous soluble 85 kDa PKC substrate. At 10 min of treatment (Figure 6a and Table 1), insulin- and PMA-induced increases in total post-nuclear membrane-dependent phosphorylation of this soluble 85 kDa PKC substrate were (means ± S.E.M.) +130±20% ($n = 4$; $P < 0.01$, paired t test) and +168±7% ($n = 4$; $P < 0.001$) respectively. In other experiments (results not shown), comparable stimulatory effects of insulin and PMA on total post-nuclear membranes were noted at 5, 10 and 20 min of treatment. Also shown in Figure 6 are results that demonstrate that in this assay of PKC enzyme activity *in vitro*, (*a*) the 85 kDa PKC substrate was present in excess (a 10-fold increase in substrate concentration did not increase the level of 85 kDa PKC substrate phosphorylation; see Figure 6b), and (*b*) the phosphorylation of soluble 85 kDa protein was dependent on the addition of both exogenous substrate (Figure 6b) and either membrane protein as a PKC source (cf. Figures 6c and 6a), or PKC itself (Figure 6c).

We also examined the inductive effects of insulin and PMA treatment in intact rat adipocytes on the subsequent phosphorylation of the 85 kDa PKC substrate *in vitro* by highly purified (see [31]) plasma membranes and microsomal membranes from rat adipocytes. As shown in Figure 7 and Table 1, both insulin and PMA provoked rapid (i.e. at 1 and 10 min) 2–3-fold increases in PKC enzyme activity of both plasma membrane and microsomal fractions, as measured by 85 kDa PKC substrate phosphorylation in the assay system *in vitro*. As shown in time-course experiments (Figure 8), insulin effects were found to persist for at least 30 min in each subcellular fraction. These findings may reflect the activation of two processes during insulin action, i.e. phospholipid hydrolysis in the plasma membrane and phospholipid synthesis *de novo* in the endoplasmic reticulum (also see below).

Significant stimulatory effects of insulin and PMA on the 85 kDa PKC substrate-phosphorylating activity of total post-nuclear membrane preparations of the rat soleus were also observed (albeit less than that observed in adipocytes): increases of +37±6% ($n = 3$; $P < 0.05$, paired t test) and +46±1% ($n = 3$; $P < 0.025$) were observed after 15 min treatment with 10 nM insulin or 500 nM PMA respectively.

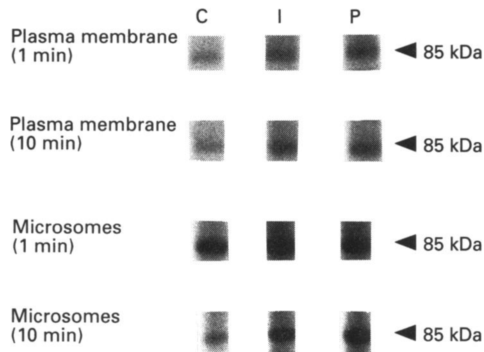

Figure 7 Effects of insulin and PMA on the phosphorylation of 85 kDa PKC substrate *in vitro* by various rat adipocyte membrane preparations

Adipocytes were treated for 1 or 10 min without (controls, C) or with 10 nM insulin (I) or 500 nM PMA (P), as indicated. Total post-nuclear membranes, plasma membranes or microsomal membranes were isolated and assayed *in vitro* with exogenous soluble 85 kDa PKC substrate (see Figure 6 and Table 1 for other details). Shown here are representative autoradiograms of ^{32}P-labelled 85 kDa soluble proteins (purified by SDS/PAGE).

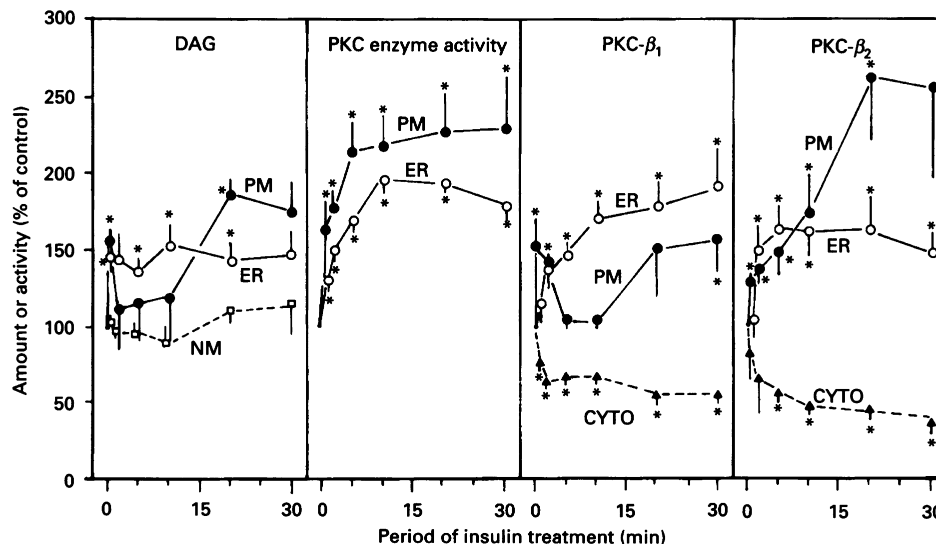

Figure 8 Time course of insulin-induced changes in DAG content, PKC enzyme activation and redistribution of PKC-β_1 and PKC-β_2 in subcellular fractions of rat adipocytes

Adipocytes were treated for indicated times with 3 nM or 10 nM insulin, as described in the Materials and methods section. Cytosol (CYTO; ▲), plasma membrane (PM; ●), microsomal (endoplasmic reticulum, ER; ○), and combined nuclear–mitochondrial (NM; □) subcellular fractions were then obtained and analysed for DAG content, PKC enzyme activity (85 kDa PKC-substrate phosphorylation *in vitro*) and/or immunoreactive PKC-β_1 and PKC-β_2 as described in the Materials and methods section. Results, expressed as percentage of control, are mean ± S.E.M. from 3–7 experiments. Asterisks indicate $P < 0.05$, as determined by paired t test.

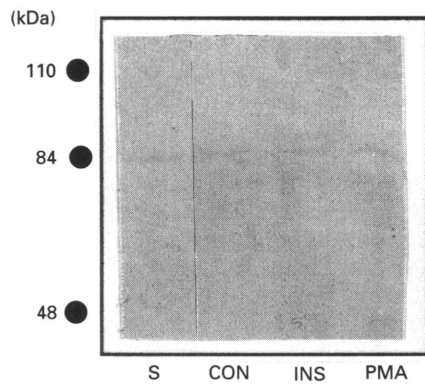

Figure 9 Recovery of immunoreactive MARCKS in boiled cytosol of S49T-lymphoma cells (S) and the cytosol fractions of rat adipocytes treated without (CON) or with 10 nM insulin (INS) or 500 nM PMA

Adipocytes were incubated for 15 min with the indicated treatments, cytosols were isolated, and immunoblots were developed with 40 μg of cytosolic protein, as described in the Materials and methods section. Shown here is a representative immunoblot.

Effects of prolonged PMA treatment on MARCKS phosphorylation in BC3H-1 myocytes

We questioned whether prolonged PMA treatment and partial depletion of PKC in BC3H-1 myocytes (see [36]) would alter the effects of insulin and PMA on the phosphorylation of immunoprecipitable 80 kDa MARCKS protein. After 48 h of treatment of BC3H-1 myocytes with 5 μM PMA (see [36]), there was a 2-fold increase in 'basal' phosphorylation of immunoprecipitable 80 kDa protein (in comparison with non-PMA-treated myocytes), but no further increase during subsequent acute treatment with either 100 nM insulin or 500 nM PMA (results not shown). These findings suggested that residual PKC in these chronic PMA-treated cells was stimulated 'constitutively', and further acute stimulation by either insulin or PMA was unable to enhance phosphorylation of immunoprecipitable 80 kDa MARCKS protein. In keeping with this possibility, PKC-β, which is acutely translocated only very slowly and weakly by PMA in BC3H-1 myocytes, is not significantly depleted by chronic PMA treatment in these particular cells [36,37]. Alternatively, the 'basal' phosphorylation may have been elevated for other reasons, and there may have been significant depletion of PKC isoforms that mediate the phosphorylation of MARCKS. In either case, acute effects of insulin and PMA on phosphorylation of immunoprecipitable 80 kDa MARCKS appeared to be PKC-dependent, as they required the availability of 'open' PKC-specific phosphorylation sites and/or specific PKC isoforms.

Immunoblot analysis of MARCKS

Distinct bands of immunoreactivity that migrated on SDS/PAGE at 85 kDa were observed, both in the cytosol (Figure 9) and in membrane preparations (results not shown) of rat adipocytes, and in the boiled S49T-lymphoma cytosol that was used as the source of exogenous 85 kDa PKC substrate in the PKC enzyme-activity *in vitro* assay system (see above). Traces of immunoreactive proteins that migrated at approx. 70 kDa and 95 kDa were also present in the rat adipocyte cytosol, and this may be relevant to the lesser bands of ^{32}P-labelled proteins found in cytosolic immunoprecipitates (see above). Importantly, neither insulin nor PMA treatment altered the level of immunoreactive MARCKS either in the rat cytosol or in the immunoprecipitates obtained from the cytosol (Table 2). Thus it is clear that insulin- and PMA-induced changes in cytosolic MARCKS phosphorylation were not simply due to changes in the availability of cytosolic MARCKS, or its release from membrane sites to the cytosol. Further support for this conclusion is derived from studies of Triton X-100-solubilized, cytosolic plus membrane-associated, immunoprecipitable MARCKS in rat adipocytes (see above).

Table 2 Failure of insulin and PMA to alter the recovery of immunoreactive MARCKS protein in cytosolic preparations of rat adipocytes

Rat adipocytes were treated for 15 min without (controls) or with the indicated additions. Cytosols were prepared, and equal amounts of protein were used directly (40 μg of protein) or subjected to immunoprecipitation (300 μg of protein) with anti-MARCKS antiserum (see the Materials and methods section). Cytosols and immunoprecipitates were dissolved in Laemmli buffer, and immunoblot analyses for 85 kDa MARCKS protein were conducted as described in the Materials and methods section. Blots were scanned by laser densitometry to obtain relative changes in immunoreactivity.

	Immunoreactive MARCKS protein (% of control)	
Treatment	Cytosol	Immunoprecipitates
Insulin (3 nM)	102 ± 6 (6)	105 ± 18 (3)
PMA (500 nM)	95 ± 13 (6)	118 ± 20 (3)

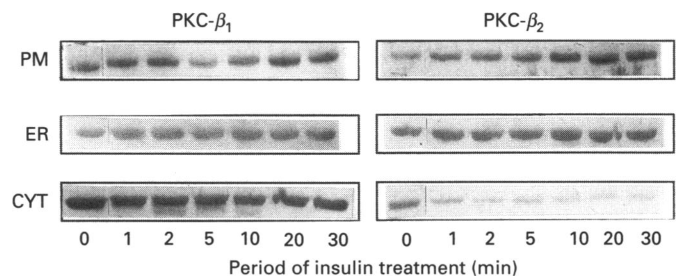

Figure 10 Time course of insulin-induced redistribution of PKC-β_1 and PKC-β_2 in subcellular fractions of rat adipocytes

As in Figure 8, rats were treated with 3 nM or 10 nM insulin for the indicated times, and immunoreactive PKC-β_1 and PKC-β_2 were measured in the cytosol (CYT), plasma membrane (PM) and microsomes (ER), 2, 20 and 20 μg of protein respectively being used for Western-blot analysis. Representative immunoblots are shown here.

Translocation of immunoreactive PKC-β_1 and β_2 in rat adipocytes

We questioned whether insulin-induced increases in adipocyte membrane PKC enzyme activity were associated with increases in PKC content. Similar to findings of a previous study in which we measured total immunoreactive PKC-β (i.e. both β_1 and β_2 isoforms) with an antiserum raised against the V_3 region common to both isoforms [34], we have now found that insulin provoked rapid changes in the subcellular redistribution of both PKC-β_1 and PKC-β_2 (Figures 8 and 10). Levels of both PKC-β isoforms decreased by 40–60 % in the cytosol, and increased by 50–100 % in microsomal membranes. However, the changes in PKC-β_1 and PKC-β_2 in plasma membranes were more divergent: thus, increases in PKC-β_1 (approx. 50 %) were biphasic, whereas

increases in PKC-β_2 appeared to be progressive or more cumulative (increasing to approx. 150% above control) during the 30 min insulin treatment period. The reason for these differences between PKC-β_1 and PKC-β_2 levels in the plasma membrane during insulin treatment is at present uncertain, but may reflect differences in the actual translocation and/or trafficking and degradative turnover of the isoforms.

Effects of insulin on DAG levels in plasma membrane and microsomal preparations of rat adipocytes

We questioned whether the insulin-induced increases in adipocyte membrane PKC content and enzyme activity were associated with increases in DAG. Insulin (10 nM) provoked 50–90% biphasic increases in the DAG content of plasma membranes, and steady 50% increases in the DAG content of microsomes, during the 30 min treatment period (Figure 8). The increases in both plasma and microsomal membranes were significant within 1 min of insulin treatment. (Note that mean levels of DAG in control plasma membranes and microsomes were approx. 13 and 27 nmol/mg of protein respectively.) In contrast, there were no changes in DAG content of the combined nuclear–mitochondrial-membrane fraction during insulin treatment (Figure 8).

DISCUSSION

Using two well-characterized [28,29] anti-MARCKS antisera, we were able to immunoprecipitate an 85 kDa phosphoprotein in rat adipocyte cytosol preparations, and 80 kDa phosphoproteins in cytosol preparations of the rat soleus muscle and BC3H-1 myocytes. In each of these cell types, these 85 and 80 kDa immunoprecipitable proteins were rapidly and comparably phosphorylated in response to both insulin and PMA treatment. In adipocytes, the effects of maximally effective levels of insulin and PMA on the phosphorylation of immunoprecipitable 85 kDa protein were non-additive. It seems most likely that these 85 and 80 kDa phosphoproteins that were immunoprecipitated by the anti-MARCKS antiserum were in fact MARCKS proteins. This conclusion is based not only on the fact that these phosphoproteins were immunoprecipitated by at least two (and probably three) anti-MARCKS antisera, but, we have also reported that insulin and PMA comparably phosphorylate acidic 80 kDa proteins in both rat soleus muscles [6] and BC3H-1 myocytes [21]. In addition, in BC3H-1 myocytes, after prolonged PMA treatment, both acute insulin and PMA treatments failed to stimulate the phosphorylation of the immunoprecipitable 80 kDa protein.

In addition to the changes in MARCKS phosphorylation in intact cells, insulin and PMA stimulated the phosphorylating activity of adipocyte and soleus-muscle post-nuclear membrane preparations, when these membranes were assayed *in vitro* in a system that utilizes an exogenous soluble 85 kDa PKC substrate (apparently itself a MARCKS protein). In the rat adipocyte, using highly purified membrane preparations, we were further able to show that increases in this PKC-substrate-phosphorylating activity were evident in both plasma membranes and microsomal membranes within 1 min of either insulin or PMA treatment, and insulin effects in both membranes persisted for at least 30 min. It should be noted that these increases in total post-nuclear and purified plasma and microsomal-membrane PKC enzyme activities (i.e. for phosphorylation of the soluble 85 kDa PKC substrate *in vitro*) were evident in membranes that were assayed in a more 'native' state than is generally the case in most assays of PKC enzyme activity: i.e. there was no exogenously added phospholipid or DAG, and membranes were not solubilized by detergents in the present assay. Thus the changes in membrane PKC enzyme activity observed in the assay system *in vitro* presently used most probably reflect insulin- and PMA-induced changes in the content of PKC and/or 'endogenous PKC activators', as they existed in membranes in intact cells. Along these lines, associated insulin-induced increases in the contents of DAG, PKC-β_1 and PKC-β_2 in adipocyte plasma membranes and microsomal fractions were observed, but there was not a strict correlation between insulin-induced changes in DAG content and alterations in either total PKC-β_1 plus PKC-β_2 content, or PKC enzyme activity. The lack of strict correlation between changes in DAG content with changes in PKC content and/or enzyme activity may reflect the following: (*a*) PKC enzyme activity and/or translocation may be very sensitive to small, difficult-to-measure, changes in DAG content; (*b*) PKC enzyme activity is dependent on both the content, as well as the activation state, of membrane PKC; (*c*) there may be PKC activators in the membranes other than DAG; (*d*) PKC translocation and enzyme activation may outlive increases in DAG [38], perhaps through the action of PKC receptor proteins that stabilize the interaction of activated specific PKC isoforms with membrane binding sites [39]; and (*e*) DAG measurements include a variety of molecular species of variable potency for activating PKC.

Whatever the explanation for the differences between relative changes in PKC enzyme activity, PKC and DAG contents within each membrane preparation, it is likely that the differences observed here between changes in plasma-membrane and microsomal-membrane DAG/PKC signalling in rat adipocytes reflect insulin-induced activation of two or more separate processes. This conclusion is primarily based on the fact that the membrane preparations are very pure (e.g., as we have verified in our preparations, GLUT4 levels are high in microsomal membranes, but very low in control plasma membranes; also see below), and it is unlikely that there is significant cross-contamination between plasma membranes and microsomes. It is tempting to speculate that these insulin-induced processes include the initial hydrolysis of phospholipids in the plasma membrane, followed rapidly by phospholipid synthesis *de novo* in the endoplasmic reticulum. In support of this speculation, we have found that rapid glucose-induced increases in phospholipid synthesis *de novo* in rat adipocytes are attended by increases in DAG and PKC-β contents in microsome, but not in plasma membranes (R. V. Farese, M. L. Standaert, T. P. Arnold and D. R. Cooper, unpublished work). These findings with glucose provide confirmatory evidence that plasma membranes are not contaminated with microsomes.

The fact that we were able to show that insulin and PMA provoked similar increases in (*a*) MARCKS phosphorylation in intact adipocytes, and (*b*) 85 kDa PKC-substrate phosphorylation *in vitro*, as mediated by derived membrane preparations, is important for several reasons. First, the assay *in vitro* is conducted in the presence of phosphatase inhibitors, and observed alterations in the phosphorylation of the 85 kDa PKC substrate can be attributed to changes in kinase, rather than phosphatase, activity. Second, the assay *in vitro* utilizes an excess of exogenously added 85 kDa PKC substrate, the alterations in phosphorylation of this substrate in response to insulin or PMA treatment are clearly not due to changes in the level or availability of phosphorylatable substrate. Third, the similarity of findings in intact cells and derived membranes assayed *in vitro* suggests that both sets of findings truly reflect the same phenomena, i.e. the activation states of cellular PKC during insulin and PMA treatment.

The mean stimulatory effects of both insulin and PMA on MARCKS phosphorylation in each of the presently studied cells were approx. 2-fold, but, in selected experiments the increases

with both agonists were as much as 4-fold on repeated occasions. However, even the latter degree of stimulation is much less than that reported with PMA treatment in some (but not all) cultured cell lines that were previously studied [22]. Further, for insulin-induced changes in cells studied previously [22], small increases (e.g. in NIH3T3 cells), or no increases at all (e.g. in HIRcB cells), in MARCKS phosphorylation were noted. The reasons for these differences in (and between) insulin- and PMA-stimulated MARCKS phosphorylation in the previous [22] and the present studies are uncertain, but several possibilities should be considered. First, insulin may not activate PKC in all cell lines, despite activating other signalling systems. Second, insulin effects on PKC in some cell lines may be small or delayed, relative to PMA. This, 'basal' PKC activity may be particularly low in certain cells, and this would allow for greater relative changes in MARCKS phosphorylation during agonist treatment. Fourth, the level of MARCKS in various cell lines may vary considerably, and the stoichiometry of MARCKS phosphorylation may vary, depending not only on the cellular concentration of MARCKS, but also on its cellular location (particularly in cells over-expressing MARCKS), the mechanism and cellular site of PKC activation, and the PKC isoform composition of the cell line in question. Along these lines, we have found that insulin and PMA have decidedly different effects on the translocation of PKC-α and PKC-β, major isoforms present in the three cells that were studied here. Thus, in rat adipocytes, insulin promotes translocation of PKC-β to the plasma membrane more strongly than do phorbol esters, whereas PMA translocates PKC-α more strongly than does insulin [34]; in the rat soleus muscle, insulin translocates both PKC-α and PKC-β more strongly than does PMA (R. V. Farese, M. L. Standaert, T. P. Arnold, D. R. Cooper and K. Yamada, unpublished work); and in BC3H-1 myocytes, PMA translocates PKC-α very rapidly and strongly, but has lesser and slow-to-develop effects on PKC-β, whereas insulin strongly and rapidly translocates PKC-β, but has little or no effect on PKC-α [37]. Further, it has been reported [40] that in some cells, PKC activation due to phosphatidylcholine hydrolysis may differ considerably from PKC activation provoked by PMA or hydrolysis of inositol lipids: in that previous study [40] of differential PKC activation, apparent MARCKS phosphorylation was stimulated by PMA and by inositol-lipid hydrolysis, but not by phosphatidylcholine hydrolysis, which nevertheless clearly activated PKC, as deduced from changes in several other PKC-dependent parameters (note that insulin stimulates the hydrolysis of phosphatidylcholine [41] rather than of phosphatidylinositol 3,4-bisphosphate [42]). Thus cellular compartmentalization or isoform-specific translocation of PKC undoubtedly occurs, and therefore may account for some differences in MARCKS phosphorylation that have been observed in some cells in response to insulin and PMA treatment. Clearly, however, the potential problem of differences between insulin- and PMA-stimulated MARCKS phosphorylation was not a significant factor in the present studies of rat adipocytes, rat soleus muscle and BC3H-1 myocytes.

At present, there is no apparent reason to explain why Blackshear et al. [22] failed to detect insulin effects on MARCKS phosphorylation in BC3H-1 myocytes, although it may be noted that this group (unlike Cooper et al. [8,10]) did not observe any changes in PKC enzyme activity or cellular distribution during insulin actin in their cultured myocytes [43]. Unfortunately, Blackshear et al. [22] did not show that insulin activated hexose transport in their BC3H-1 myocytes, as a measure of a process that might require PKC activation.

In summary, in rat adipocytes, rat soleus muscle and BC3H-1 myocytes, insulin and PMA provoked comparable increases in the phosphorylation of MARCKS proteins, as measured both in intact cells and in assays of derived membrane preparations *in vitro*. Our findings suggest that insulin both translocates and activates PKC in these cells.

This work was supported by funds from the Research Service of the Department of Veterans' Affairs and by National Institutes of Health Grant DK38079.

REFERENCES

1 Draznin, B., Leitner, J. W., Sussman, K. E. and Sherman, N. A. (1988) Biochem. Biophys. Res. Commun. **156**, 570–575
2 Ishizuka, T., Cooper, D. R. and Farese, R. V. (1989) FEBS Lett. **257**, 337–340
3 Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A. and Londos, C. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1052–1056
4 Walaas, S. I., Horn, R. S., Adler, A., Albert, K. A. and Walaas, O. (1987) FEBS Lett. **220**, 311–318
5 Ishizuka, T., Cooper, D. R., Hernandez, H., Buckley, D., Standaert, M. and Farese, R. V. (1990) Diabetes **39**, 181–190
6 Yu, B., Standaert, M. L., Arnold, T., Hernandez, H., Watson, J., Ways, K., Cooper, D. R. and Farese, R. V. (1992) Endocrinology (Baltimore) **130**, 3345–3355
7 Chen, K. S., Heydrick, S., Kurowski, S. and Ruderman, N. B. (1991) Trans. Assoc. Am. Physicians **104**, 206–212
8 Cooper, D. R., Konda, T. S., Standaert, M. L., Davis, J. S., Pollet, R. J. and Farese, R. V. (1987) J. Biol. Chem. **262**, 3633–3639
9 Acevedo-Duncan, M., Cooper, D. R., Standaert, M. L. and Farese, R. V. (1989) FEBS Lett. **244**, 174–176
10 Cooper, D. R., Ishizuka, T., Watson, J. E., Standaert, M. L., Nair, G. and Farese, R. V. (1990) Biochim. Biophys. Acta **1054**, 95–102
11 Cooper, D. R., Hernandez, H., Kuo, J. Y. and Farese, R. V. (1990) Arch. Biochem. Biophys. **276**, 486–494
12 Cherqui, G., Reynet, C., Caron, M., Melin, B., Wicek, D., Clauser, E., Capeau, J. and Picard, J. (1990) J. Biol. Chem. **265**, 21254–21261
13 Heidenreich, K. A., Toledo, S. P., Brunton, L. L., Watson, M. J., Daniel-Issakani, S. and Strulovici, B. (1990) J. Biol. Chem. **265**, 15076–15082
14 Messina, J. L., Standaert, M. L., Ishizuka, T., Weinstock, R. S. and Farese, R. V. (1992) J. Biol. Chem. **267**, 9223–9228
15 Boggs, K. P., Farese, R. V. and Buse, M. G. (1991) Endocrinology (Baltimore) **128**, 636–637
16 Sowell, M. O., Boggs, K. P., Robinson, K. A., Dutton, S. L. and Buse, M. G. (1991) Am. J. Physiol. **260**, E247–E256
17 Hoffman, J. M., Ishizuka, T. and Farese, R. V. (1991) Endocrinology (Baltimore) **128**, 2937–2948
18 Walaas, O., Horn, R. S. and Walaas, S. I. (1991) Biochim. Biophys. Acta **1094**, 92–102
19 Morley, S. J. and Traugh, J. A. (1990) J. Biol. Chem. **265**, 10611–10616
20 Graves, C. B. and McDonald, J. M. (1985) J. Biol. Chem. **260**, 11286–11292
21 Vila, M., Cooper, D. R., Davis, J. S., Standaert, M. L. and Farese, R. V. (1989) FEBS Lett. **244**, 177–180
22 Blackshear, P. J., Haupt, D. M. and Stumpo, D. J. (1991) J. Biol. Chem. **266**, 10946–10952
23 Thelen, M., Rosen, A., Nairn, A. C. and Aderem, A. (1991) Nature (London) **351**, 320–322
24 Manenti, S., Sorokine, O., Van Dorsselaer, A. V. and Taniguchi, H. (1992) J. Biol. Chem. **267**, 22310–22315
25 Farese, R. V., Sabir, M. A., Larson, R. E. and Trudeau, W., III (1983) Cell Calcium **4**, 195–218
26 Farese, R. V., Kuo, J. Y., Babischkin, J. S. and Davis, J. S. (1986) J. Biol. Chem. **261**, 8589–8592
27 Farese, R. V., Davis, J. S., Barnes, D. E., Standaert, M. L., Babischkin, J. S., Hock, R., Rosic, N. K. and Pollet, R. J. (1985) Biochem. J. **231**, 269–278
28 Albert, K. A., Walaas, S. I., Wang, J. K. and Greengard, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2822–2826
29 Ouimet, C. C., Wang, J. K., Walaas, S. I., Albert, K. A. and Greengard, P. (1990) J. Neurosci. **10**, 1683–1698
30 Chakravarthy, B. R., Franks, D. J., Whitfield, J. F. and Durkin, J. P. (1989) Biochem. Biophys. Res Commun. **160**, 340–345
31 Weber, T. M., Hoost, H. G., Simpson, I. A. and Cushman, S. W. (1988) Recept. Biochem. Methodol. **12B**, 171–187
32 House, C. and Kemp, B. E. (1987) Science **238**, 1726–1728
33 Standaert, M. L., Sasse, J., Cooper, D. R. and Farese, R. V. (1991) FEBS Lett. **282**, 139–142
34 Farese, R. V., Standaert, M. L., Francois, A., Ways, K., Arnold, T. P., Hernandez, H. and Cooper, D. R. (1992) Biochem. J. **288**, 319–323

35 Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. and Bell, R. M. (1986) J. Biol. Chem. **261**, 8597–8600
36 Cooper, D. R., Watson, J. E., Acevedo-Duncan, M., Pollet, R. J., Standaert, M. L. and Farese, R. V. (1989) Biochem. Biophys. Res. Commun. **161**, 327–334
37 Standaert, M. L., Cooper, D. R., Hernandez, H., Arnold, T. P. and Farese, R. V. Endocrinology (Baltimore) **132**, 689–692
38 Bazzi, M. D. and Nelsestuen, G. L. (1989) Biochemistry **28**, 3577–3585
39 Mochly-Rosen, D., Khaner, H., Lopez, J. and Smith, B. L. (1991) J. Biol. Chem. **266**, 14866–14868
40 Laviada, I. D., Larrodera, P., Nieto, J. L., Cornet, M. E., Meco, M. T., Sanchez, M. J., Guddal, P. H., Johansen, T., Haro, A. and Moscat, J. (1991) J. Biol. Chem. **266**, 1170–1176
41 Hoffman, J., Standaert, M. L., Nair, G. P. and Farese, R. V. (1991) Biochemistry **30**, 3315–3321
42 Farese, R. V., Davis, J. S., Barnes, D. E., Standaert, M. L., Babischkin, J. S., Hock, R., Rosic, N. K. and Pollet, R. J. (1985) Biochem. J. **231**, 269–278
43 Spach, D. H., Nemenoff, R. A. and Blackshear, P. J. (1986) J. Biol. Chem. **261**, 12750–12753

Received 18 January 1993/5 May 1993; accepted 24 May 1993