Insulin increases mRNA levels of protein kinase C-α and -β in rat adipocytes and protein kinase C-α, -β and -θ in rat skeletal muscle

Antoine AVIGNON, Mary L. STANDAERT, Kouji YAMADA, Harald MISCHAK, Barbara SPENCER and Robert V. FARESE*

Departments of Internal Medicine and Biochemistry, J. A. Haley Veterans' Hospital, University of South Florida College of Medicine, University of South Florida, Tampa, FL 33612, U.S.A.

Effects of insulin on levels of mRNA encoding protein kinase C (PKC)-α, PKC-β, PKC-ε and PKC-θ were examined by ribonuclease protection assay in primary cultures of rat adipocytes *in vitro*, and in rat adipose tissue and gastrocnemius muscle *in vivo*. In all cases, insulin increased the levels of PKC-α mRNA and PKC-β mRNA, and, in muscle, insulin also increased the level of PKC-θ mRNA. PKC-ε mRNA levels, on the other hand, were not altered significantly. Insulin also stimulated the apparent translocation of PKC-α, -β, -ε and -θ, to the membrane fractions of adipocytes, adipose tissue and gastrocnemius muscles, and, in some instances, total PKC levels were diminished, e.g. PKC-α and PKC-β in cultured adipocytes *in vitro* and/or whole adipose tissue *in vivo*, and PKC-α and PKC-θ in the gastrocnemius muscle. Thus, insulin-induced increases in PKC mRNA may have been partly compensatory in nature to restore PKC levels following translocation and proteolytic losses. However, much more severe depletion of PKC-α and PKC-β by phorbol ester treatment in cultured rat adipocytes *in vitro* resulted in, if anything, smaller increases in PKC-α mRNA and PKC-β mRNA, and it therefore appears that insulin effects on PKC mRNA levels were not simply due to decreases in respective PKC levels. In addition, effects of insulin, particularly on PKC-β mRNA, could not be attributed to increased glucose metabolism, which alone decreased PKC-β mRNA in cultured adipocytes *in vitro*. We conclude that insulin-induced translocation and degradation of PKC-α, PKC-β and PKC-θ are attended by selective increases in their mRNAs. This mechanism of increasing mRNA may be important in maintaining PKC levels during the continued action of insulin.

INTRODUCTION

Insulin rapidly increases diacylglycerol (DAG) production and activates protein kinase C (PKC) in rat adipocytes [1–4] and rat skeletal muscles [4–8]. In both tissues, insulin stimulates the translocation of PKC-α, PKC-β and PKC-ε [1,3,4,6,8,9] to membrane fractions, as well as the phosphorylation of specific PKC substrates [4,10]. In muscle, insulin also stimulates the translocation of PKC-θ to the membrane fraction (see accompanying paper [9]). Translocation of PKC to membranes frequently results in the degradation of PKC, particularly during phorbol ester treatment, apparently via activation and exposure of the hinge region of activated PKC to proteolytic enzymes [11]. The extent of degradation of translocated PKC, however, varies, depending upon the tissue, the agonist and the PKC isoform that is translocated. Conceivably, PKC translocation and/or degradation may or may not be attended by compensatory or non-compensatory changes in PKC mRNA production, but there is little information on this subject, particularly in cells or tissues of the mature rat, as stimulated by agonists other than phorbol esters. In this study, we questioned whether insulin regulates PKC mRNA levels in two important target tissues of the rat, i.e. fat and muscle. Accordingly, in both rat adipocytes cultured *in vitro*, and in epididymal fat pads and gastrocnemius muscles of rats treated *in vivo*, we found that insulin provoked increases in the levels of PKC-α mRNA and PKC-β mRNA, but not PKC-ε mRNA. In addition, insulin treatment *in vivo* increased PKC-θ mRNA in the gastrocnemius muscle. We also compared the effects of insulin with those of other reported [1–3,12–14] PKC activators in cultured rat adipocytes (i.e. phorbol esters and 25 mM glucose) to determine whether insulin effects on PKC mRNA could be secondary to degradative loss of PKC, as provoked by phorbol ester treatment, or, if insulin effects could be explained by an increase in glucose metabolism, as occurs with higher medium glucose levels. Accordingly, phorbol esters increased PKC-α mRNA and PKC-β mRNA only modestly relative to insulin; furthermore, 25 mM glucose increased PKC-α mRNA mildly, but diminished PKC-β mRNA. The effects of insulin on PKC-β mRNA, in particular, therefore appeared to be relatively specific.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies, Inc. Electrophoresis and protein assay reagents were from Bio-Rad. [γ-^{32}P]UTP and 2-deoxy[^{3}H]glucose (2-DOG) were from DuPont/New England Nuclear. Insulin was purchased from Elanco. Collagenase was purchased from Worthington. Antiserum for PKC-β was a gift from Drs. Bryan Roth and John Mehegan (see [1,3,4,14,15]). Polyclonal antisera for PKC-α, -δ, -ε and -ζ were purchased from Life Technologies, Inc. Monoclonal antibodies directed against PKC-θ were purchased from Transduction Laboratories. cDNA clones encoding

Abbreviations used: DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; 2-DOG, 2-deoxyglucose; KRP, Krebs–Ringer phosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RPA, ribonuclease protection assay.

*To whom correspondence should be addressed.

rat PKC-α, -β_1, -β_2 and -ϵ were kindly provided by Dr. Y. Ono (Kobe University, Kobe, Japan) (for sequence data, see refs. [16–18]). The cDNA clone encoding mouse PKC-θ has been described previously [19]. Other chemicals and biochemicals were purchased from Sigma, except those indicated below in specific procedures.

Primary cultures of adipocytes

Rat adipocytes were prepared from fed, 200 g male Holtzman Sprague–Dawley rats, and cultured, as described previously [3]. Briefly, epididymal fat pads were minced in Krebs–Ringer phosphate (KRP) buffer (pH 7.4) containing 1 mg/ml collagenase, 3% (w/v) BSA and 2.5 mM glucose, and incubated for 30 min at 37 °C. Following collagenase treatment, isolated fat cells were obtained by sieving through a pre-wetted nylon filter, washed several times in KRP containing 1% BSA, and then cultured for 20 h at a 1:20 dilution (v/v) in DMEM containing 5 mM glucose, 10 mM Hepes, 0.5% BSA and, where indicated, 10 nM insulin, supplemental (10–25 mM, final concentration) glucose, or 500 nM phorbol 12-myristate 13-acetate (PMA).

In vivo studies

Rats were injected: (a) with 3 units of NPH insulin (Lilly) subcutaneously; in which case they were given 5% (w/v) dextrose in their drinking water to maintain serum glucose levels, which were indistinguishable from untreated rats at the time of killing 16–17 h later (long-term experiment); or (b) as described previously [8] and in the accompanying paper [9], intramuscularly with 0.25 unit of regular insulin in saline every 30 min for 0.5 to 4 h, along with 1.5 ml of 10% glucose in saline given intraperitoneally every 30 min to maintain serum glucose levels (see [8]) (short-term experiment). Control rats were injected identically with saline at the same times as the longest-treated group. At the indicated time intervals, rats were killed by decapitation, and tissues were rapidly removed for analyses.

RNA isolation

RNA was prepared directly at the end of the adipocyte cultures, using RNAzol B (Tel-Test, Inc., Texas, U.S.A.), as specified by the manufacturer. RNA was quantified spectrophotometrically by absorbance at 260 nm and stored in 75% (v/v) ethanol at −70 °C. For *in vivo* experiments, epididymal fat pads and gastrocnemius muscles were removed and snap-frozen in liquid nitrogen, stored at −70 °C, and extracted for RNA by the same method.

Ribonuclease (RNase) protection assay (RPA)

Riboprobes were prepared as follows. For PKC-α, a 370 bp *Eco*RI–*Pst*I fragment of a full-length rat PKC-α cDNA was subcloned in a PGEM-4Z transcription vector (Promega), linearized by digestion with *Sau*96I, and transcribed with T7 polymerase (see below), yielding a ^{32}P-labelled RNA probe protecting a 155 nucleotide sequence of rat PKC-α mRNA. For PKC-β, four probes were used: (a) a 515-bp *Hin*dIII–*Pst*I fragment of full-length rat PKC-β cDNA was subcloned in PGEM-4Z, linearized by digestion with *Sau*96I and transcribed with T7 polymerase, yielding a ^{32}P-labelled RNA probe (β-Probe A) protecting a 100 nucleotide sequence of the C-4 region, common to all rat PKC-β mRNA forms, including spliced forms of PKC-β_1 and PKC-β_2 mRNA; (b) a 446-bp *Eco*RI–*Pst*I fragment of full-length rat PKC-β cDNA was subcloned in PGEM-4Z, linearized by digestion with *Pst*I and transcribed with SP6 polymerase (the 3′ overhang left by *Pst*I digestion was removed with T4 DNA polymerase before transcription), yielding a ^{32}P-labelled RNA probe (β-Probe B) protecting a 258 nucleotide sequence of the 3′ untranslated region, common to all rat PKC-β forms including spliced forms of PKC β_1 and PKC β_2 mRNA (see [18]); (c) a 714-bp *Pst*I fragment of full-length rat PKC-β_1 cDNA was subcloned in PGEM-4Z, linearized by digestion with *Sph*I and transcribed with SP6 polymerase (the 3′ overhang left by *Sph*I digestion was removed with T4 DNA polymerase before transcription), yielding a ^{32}P-labelled RNA probe (β-Probe C) protecting a 266 nucleotide sequence of rat PKC-β_1 mRNA; and (d) a 619-bp *Pst*I–*Eco*RI fragment of full-length rat PKC-β_2 cDNA was subcloned in PGEM-4Z, linearized by digestion with *Sph*I and transcribed with SP6 polymerase, yielding a ^{32}P-labelled RNA probe (β-Probe D) protecting a 171 nucleotide sequence of the rat PKC-β_2 mRNA. For PKC-ϵ, a 513-bp *Pst* I–*Eco*RI fragment of full-length rat PKC-ϵ cDNA was subcloned in PGEM-4Z, linearized with *Sau*96I and transcribed with T7 polymerase, yielding a ^{32}P-labelled RNA probe protecting a 206 nucleotide sequence of rat PKC-ϵ mRNA. For PKC-θ, a 318-bp *Hin*dIII fragment from the mouse full-length PKC-θ cDNA was subcloned in PGEM 42, linearized by digestion with *Pvu*II and transcribed with T7 polymerase, yielding a probe protecting a 318 nucleotide sequence of the rat PKC-θ mRNA under the digestion conditions specified below. A mouse actin cDNA probe (Ambion, Inc., Austin, TX, U.S.A.) was linearized with *Hin*dIII and was used to generate a ^{32}P-labelled riboprobe protecting a 250 nucleotide sequence of rat β-actin mRNA (N. B. the complementary sequence of the antisense RNA contained 12 mismatches due to the differences between mouse and rat β-actin, and the digestion conditions of the RPA gave a main protected fragment of 250 nucleotides.) Each of the antisense RNA probes was labelled using 800 to 1000 Ci/mmol [γ-^{32}P]UTP, along with other components of the Ambion Maxiscript kit. RPAs were carried out simultaneously on 10 μg of RNA from each sample being compared, as per the instructions of the manufacturer (Ambion RPA II kit), using a 1/100 dilution of a mixture of 250 units/ml RNase A and 10000 units/ml RNase T_1 for the digestion step of each PKC mRNA duplex, except in the case of PKC-θ, in which RNase T_1 was used without RNase A. After digestion, samples were subjected to SDS/PAGE, autoradiography and scanning laser densitometry to quantify ^{32}P-labelling of protected fragments. In each case, the difference in length between duplex-protected sequences and the undigested labelled probe was predictable from the size of the plasmid fragment contained in the probe (see Figure 1). No signals were observed in control yeast RNA samples.

Western-blot analysis

Details of this method have been described previously [1,3,4,14]. In brief, after cell culturing as described above, adipocytes were washed and resuspended in 0.5 ml of ice-cold buffer (Buffer A) containing 20 mM Tris/HCl (pH 7.4), 1.2 mM EGTA, 20 mM 2-mercaptoethanol, 250 mM sucrose, 2 mM PMSF, 100 μg/ml leupeptin and 3000 units/ml aprotinin, and then sonicated at power setting 45 (VibraCell Sonicator) for 15 s. Tissues from *in vivo* studies were homogenized with a Brinkman Polytron, but otherwise processed identically. Cytosolic fractions were obtained by centrifugation at 100000 ***g*** for 60 min. Membrane pellets were resuspended by sonication in Buffer A supplemented with 5 mM EGTA, 2 mM EDTA and 1% Triton X-100, and then centrifuged at 100000 ***g*** for 30 min to remove insoluble residue. Protein levels were determined by the Bio-Rad assay, and samples were stored,

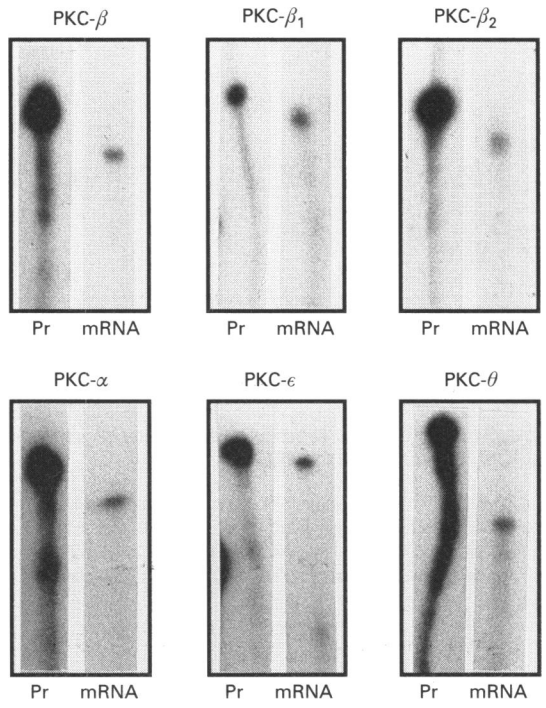


Figure 1 Ribonuclease protection assays of PKC mRNA

The ^{32} P-labelled undigested probes (Pr) and the protected fragments (mRNA) are depicted in the autoradiograms.

without boiling, at −70 °C in Laemmli buffer (see [3,4,14]). Equal amounts of cytosolic or solubilized membrane protein were subjected to SDS/PAGE (8.5% gels) and Western-blot analysis, as previously described [1,3,4,14]), except that, in some cases, ECL (Amersham kit) was used to improve immunodetection. The epitope specificities of antisera recognizing PKC-α, -β and -ε were verified by loss of immunoreactivity when assays of brain standards and tissue samples were conducted in the presence of the competing peptide that was used for the immunization procedures (also see accompanying paper). Antisera specificities were also verified by blotting recombinant PKCs (α, β, γ, δ, ε, ζ), as described previously [3,14]. The specificity of the anti-PKC-θ antibody was verified by failure to observe a signal with extracts of rat brain, adipocytes, liver and heart.

[^{3}H]2-DOG uptake

Cultured adipocytes were washed three times, equilibrated for 30 min at 37 °C in glucose-free KRP buffer containing 1% BSA and [^{3}H]2-DOG uptake, during a 1-min period, was determined after a 30-min treatment period with or without 10 nM insulin or 500 nM PMA, as described previously [20].

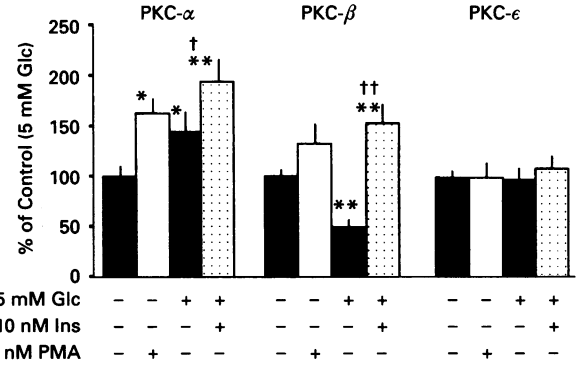
Statistical analysis

RPA samples from treated and control cell or tissue preparations were analysed simultaneously, and the densitometric scan value of each sample was calculated relative to the mean control value (set at 100%) of 4–6 fully separate control samples. Statistical differences between mean (±S.E.M.) values were calculated by unpaired Student's *t*-test and were considered significant when $P < 0.05$. Western-blot samples were also analysed simultaneously, and the densitometric scan value of each treated sample was compared with the corresponding control sample for each immunoblot, and relative differences of mean (±S.E.M.) values were analysed by paired *t*-test.

RESULTS

Effects of insulin, glucose and PMA on PKC mRNA levels in cultured rat adipocytes

Increasing the medium glucose concentration from 5 to 25 mM provoked a 43% increase in PKC-α mRNA, a 47% decrease in PKC-β mRNA and no significant change in either PKC-ε mRNA (Figures 2 and 3) or β-actin mRNA (results not shown) in adipocytes cultured for 20 h.



*P < 0.05 versus 5 mM Glc; **P < 0.001 versus 5 mM Glc; †P < 0.05 versus 25 mM Glc; ††P < 0.001 versus 25 mM Glc.

Figure 2 Effects of glucose (Glc), insulin (Ins), and PMA on levels of PKC-α mRNA, PKC-β mRNA and PKC-ε mRNA in cultured rat adipocytes

Adipocytes were cultured for 20 h in the presence of 5 mM glucose control, 25 mM glucose (Glc), 10 nM insulin plus 25 mM glucose (Ins), or 500 nM PMA plus 5 mM glucose (PMA), as indicated. The RPAs were conducted as described in the Materials and methods section using 10 μg of RNA. β-Probe A and β-probe B were used in these experiments to measure PKC-β mRNA and gave similar results, which were pooled. Shown here are mean values ± S.E.M. of 12 determinations. *P* values are also shown.

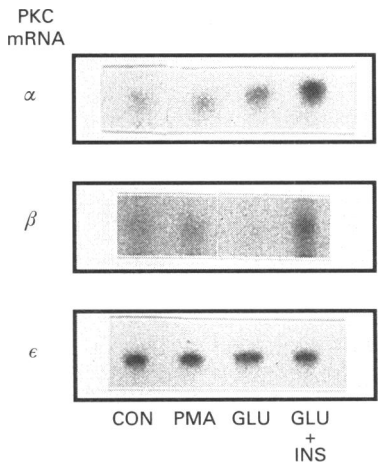


Figure 3 Representative autoradiograms of protected ^{32}P-labelled fragments of riboprobes detecting mRNAs of PKC-α, PKC-β and PKC-ε in rat adipocytes

Treatments are described in Figure 2. Abbreviations: CON, control; GLU, 25 mM glucose.

Table 1 Effects of insulin on PKC-β mRNA levels in adipocytes cultured in medium containing different glucose concentrations

Adipocytes were cultured for 20 h in medium containing the indicated glucose and insulin concentrations. Values were calculated relative to the mean control value at 5 mM glucose (Group A), arbitrarily set at 100.

Group	Medium glucose Concn. (mM)	Insulin addition (nM)	PKC-β mRNA (%) [mean ± S.E.M. (n)]
A	5.0	0	100 ± 20 (5)
B	5.0	10	202 ± 23 (5)
C	12.5	10	253 ± 16 (5)
D	25.0	10	153 ± 18 (5)

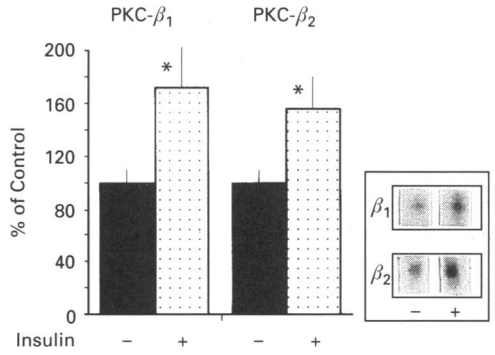


Figure 4 Effects of insulin on PKC-β_1 mRNA and PKC-β_2 mRNA levels in cultured rat adipocytes

Adipocytes were cultured for 20 h in medium containing 5 mM glucose (solid bars), or 10 nM insulin plus 25 mM glucose (stippled bars). β-Probes C and D were used to specifically measure PKC-β_1 mRNA and PKC-β_2 mRNA respectively. See Figure 2 for other details. Representative autoradiograms are shown in the inset to the right. Bargram values are mean ± S.E.M. of 12 (β_1) and eight (β_2) determinations. Asterisks indicate $P < 0.05$.

The addition of 10 nM insulin along with 25 mM glucose to the 20-h culture of rat adipocytes further increased PKC-α mRNA to a level 2-fold greater than that observed with the 5 mM glucose and, moreover, increased PKC-β mRNA by 1.5- and 2.4-fold more than that observed with 5 and 25 mM glucose respectively (Figures 2 and 3). In contrast, insulin did not alter the levels of PKC-ϵ mRNA (Figures 2 and 3) or β-actin mRNA (results not shown).

The concentration of medium glucose appeared to be important for determining the relative change in PKC-β mRNA in insulin-treated adipocytes. As stated above, 25 mM glucose decreased PKC-β mRNA and insulin effects in this medium on PKC-β mRNA were greater, both with respect to absolute increment and percentage change, than those observed in relation to the 5 mM glucose control (Figure 2). On the other hand, insulin effects on PKC-β mRNA were, if anything, greater in the presence of 5 and 12.5 mM glucose than in the presence of 25 mM glucose (Table 1). Obviously, insulin effects on PKC-β mRNA were not dependent upon the co-presence of a supraphysiological glucose level.

The β-probe A was used in most of the above experiments to measure PKC-β mRNA. However, results obtained with β-probe B were virtually the same, and results of β-probes A and B, which should measure all forms of PKC-β mRNA (see [18]), were therefore pooled. Further studies were conducted to confirm

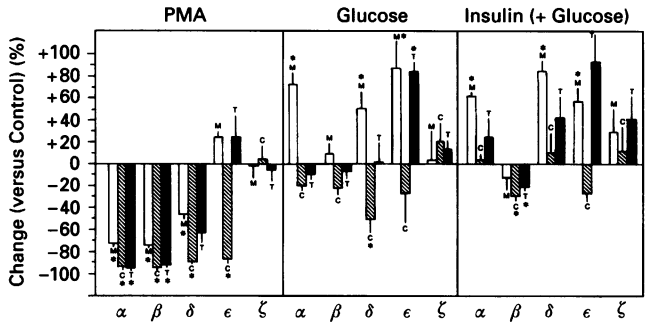


Figure 5 Effects of treatment of cultured rat adipocytes for 20 h with 500 nM PMA (left), 25 mM glucose alone (middle), or 10 nM insulin plus 25 mM glucose (right) on the subcellular distribution of immunoreactive PKC-α, PKC-β, PKC-δ, PKC-ϵ and PKC-ζ

Effects of treatments on PKC were compared to PKC levels in control adipocytes cultured in the presence of 5 mM glucose. Bargram values are mean ± S.E.M. of 4–6 experiments (also see Figure 6 for representative blots), with cytosolic PKC levels (C) depicted as open bars, membrane PKC levels (M) as solid bars, and total PKC levels (T) as hatched bars. Asterisks indicate $P < 0.05$.

that insulin provoked increases in PKC-β mRNA, and to rule out the possibility that the above-described increases in PKC-β mRNA, as measured by β-probes A and B, were not simply the result of a change in the splicing of the PKC-β mRNA, coupled with differences in the stability of PKC-β_1 mRNA and/or PKC-β_2 mRNA. We therefore measured changes in PKC-β_1 mRNA and PKC-β_2 mRNA splice products, using β-probes C and D respectively, that overlapped their specific splice sites (see [18]) and therefore protected specific mRNA fragments, the sizes of which were dependent upon the presence of both contiguous parts of the mature splice product. Accordingly, insulin provoked increases of 73 and 57 % in PKC-β_1 mRNA and PKC-β_2 mRNA respectively (Figure 4). Thus, it may be surmised that insulin increased total PKC-β mRNA levels, including both β_1 and β_2 splice products.

As shown in Figures 2 and 3, treatment of adipocytes with PMA for 20 h provoked: (a) a 63 % increase in PKC-α mRNA; (b) a relatively small, statistically insignificant, increase in PKC-β mRNA; and (c) no changes in either PKC-ϵ mRNA (Figures 2 and 3) or β-actin mRNA (data not shown).

Effects of insulin, glucose and PMA on immunoreactive PKC-α, PKC-β, PKC-ε, PKC-δ and PKC-ζ, in cultured rat adipocytes

Similar to findings in previous studies [20,21], 20-h treatment of rat adipocytes with PMA markedly decreased cytosolic, membrane and total PKC-α, PKC-β and PKC-δ, as measured by Western-blot analysis (Figures 5 and 6). In contrast, PKC-ϵ was translocated, but was not decreased in total amount, and PKC-ζ did not change in either amount or subcellular distribution in response to 20-h PMA treatment. (N.B. membrane contents of PKC-ϵ and PKC-δ, unlike PKC-α, PKC-β and PKC-ζ, exceeded the cytosolic contents of PKC-ϵ and PKC-δ, and the total amounts of each PKC were more reflective of the subcellular fraction that predominated.)

Treatment of adipocytes with 10 nM insulin plus 25 mM glucose for 20 h provoked: (a) increases in membrane, but no significant changes in cytosolic or total PKC-α; (b) modest (20–30 %), but significant, decreases in total and cytosolic PKC-β, along with small, but statistically insignificant, decreases in membrane PKC-β; (c) increases in membrane levels of PKC-ϵ

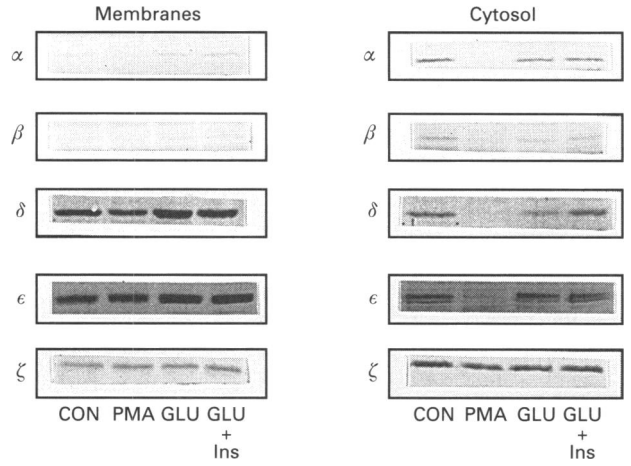


Figure 6 Effects of treatment of cultured rat adipocytes for 20 h with 500 nM PMA, 25 mM glucose (GLU), 10 nM insulin plus 25 mM glucose (GLU + INS) or 5 mM glucose (control, CON) on the subcellular distribution of PKC-α, PKC-β, PKC-δ, PKC-ε and PKC-ζ

Representative immunoblots are shown here (also see Figure 5 for experimental details and results of a series of experiments).

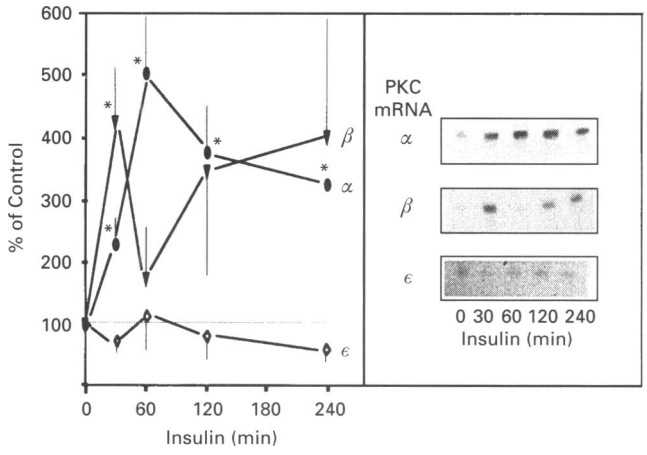


Figure 7 Effects of short-term insulin treatment *in vivo* on levels of PKC-α mRNA, PKC-β mRNA and PKC-ε mRNA in rat epididymal fat pads

Rats were treated as described in the Materials and methods section. At the indicated times, rats were killed and their fat pads were removed and subjected to RNA extraction and PKC mRNA measurement. Values shown at the left are mean ± S.E.M. of 3–4 determinations, and effects of insulin at eact time point are represented as percentages of the mean control value set a 100%. Asterisks indicate $P < 0.05$. Representative autoradiograms are shown at the right.

and PKC-δ (total levels also increased, although not significantly); and (d) no significant changes in total, membrane or cytosolic levels of PKC-ζ (Figures 5 and 6). Thus, insulin provoked a modest depletion of PKC-β while translocating and increasing membrane levels of PKC-α, PKC-δ and PKC-ε.

Treatment of adipocytes with 25 mM glucose for 20 h provoked: (a) significant increases in membrane contents of PKC-α, PKC-δ and PKC-ε; (b) significant decreases in cytosolic PKC-δ and PKC-ε; (c) increases in total PKC-ε; and (d) no significant changes in PKC-ζ, cytosolic PKC-α or PKC-β (although PKC-β showed a tendency to translocate) (Figures 5 and 6).

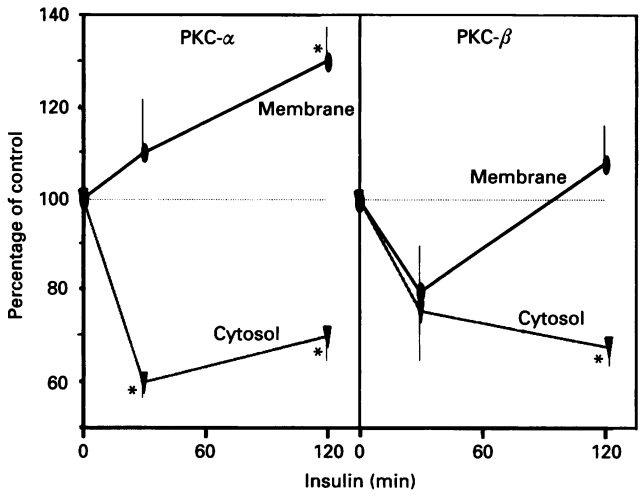


Figure 8 Effects of short-term insulin treatment *in vivo* on levels of immunoreactive PKC-α and PKC-β in cytosol and membrane fractions of rat epididymal fat pads

Experiments were conducted as in Figure 7, except that there were controls at both 30 and 120 min time points. Values are mean ± S.E.M. of 3–4 determinations, in which effects of insulin are plotted relative to the control set at 100%. Asterisks indicate $P < 0.05$.

Effects of 20-h treatment of cultured adipocytes with PMA (500 nM), insulin (10 nM) or glucose (25 mM) on [^{3}H]2-DOG uptake

After 20-h culture in the absence of any treatment, acute insulin treatment for 30 min induced a 3–4-fold increase in [^{3}H]2-DOG uptake, and comparable acute PMA treatment induced a lesser response (data not shown). These increases in transport were only slightly less than those observed in fresh adipocytes (data not shown). Control [^{3}H]2-DOG uptake was not affected significantly by any of the 20-h treatments. On the other hand, acute insulin- and/or PMA-stimulated [^{3}H]2-DOG uptake was diminished by 60–80% after 20-h PMA pretreatment, and by 30–40% after 20-h pretreatment with 10 nM insulin plus 25 mM glucose, but was affected little, if at all, by 25 mM glucose pretreatment.

Effects of *in vivo* insulin treatment on PKC mRNA and PKC protein levels in rat epididymal adipose tissue

In short-term studies of adipose tissue *in vivo*, insulin treatment induced a 2.3-fold increase in PKC-α mRNA within 30 min, a 5-fold increase at 60 min, and 3–4-fold increases at later time points (Figure 7). Increases in adipose tissue PKC-α mRNA were associated with decreases in cytosolic, and increases in membrane, PKC-α, as determined by Western-blot analysis (Figure 8). With respect to adipose tissue PKC-β mRNA, the increase induced by insulin was maximal (4.2-fold) within 30 min (Figure 7), and this was initially associated with decreases in both cytosolic and membrane levels of adipose tissue PKC-β (Western-blot analysis), followed by restoration of the membrane level of this PKC isoform (Figure 8). These findings suggested that PKC-α and PKC-β were translocated, partially depleted, and partially restored during continued insulin treatment in adipose tissue. In contrast to PKC-α mRNA and PKC-β mRNA, adipose tissue PKC-ε mRNA was not altered by insulin in these short-term

Table 2 Effects of long-term insulin treatment *in vivo* on PKC-α mRNA and PKC-β mRNA in rat epididymal fat pads

Rats were treated as described in the Materials and methods section. At 16–17 h after insulin injection, rats were killed, and fat pads were analysed for PKC mRNA. Values are means ± S.E.M. of 3–4 determinations, with the mean control set at 100%. *P* (in brackets) was determined by *t*-test. Abbreviation: N.S., not significant.

	Percentage of mean control	
Treatment	PKC-α mRNA	PKC-β mRNA
None	100 ± 23	100 ± 24
Insulin	79 ± 29 [N.S.]	217 ± 29 [$P < 0.05$]

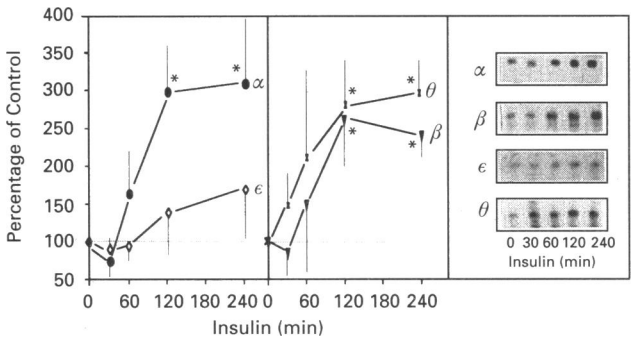


Figure 9 Effects of short-term insulin treatment *in vivo* on PKC-α mRNA, PKC-β mRNA and PKC-ε mRNA levels in rat gastrocnemius muscles

Rats were treated as described in the Materials and methods section and in Figure 7. At indicated times, rats were killed and their gastrocnemius muscles were removed and subjected to RNA extraction and measurement of PKC mRNA levels. Values shown at the left are mean ± S.E.M. of 3–4 determinations, and insulin effects at each time point are represented as percentages of the mean control value set at 100%. Representative autoradiograms are shown at the right. Asterisks indicate $P < 0.05$.

experiments (Figure 7) (changes in immunoreactive PKC-ε levels were not measured).

In long-term *in vivo* experiments, adipose tissue PKC-β mRNA was significantly increased within 16–17 h of insulin injection (Table 2). In contrast, PKC-α mRNA was not changed significantly in these experiments (Table 2).

Insulin treatment had no effect on adipose tissue β-actin mRNA levels in the short-term *in vivo* experiments, and this RNA was therefore used as a recovery correction factor in these experiments. In the long-term experiments, however, adipose tissue β-actin mRNA was increased by insulin and thus could not be used for recovery corrections. However, the insulin effect on adipose tissue PKC-β mRNA was validated by the absence of an effect on PKC-α mRNA in the long-term *in vivo* experiments.

Effects of *in vivo* insulin treatment on PKC mRNA and PKC protein levels in gastrocnemius and other muscles

In the short-term *in vivo* experiments, insulin provoked increases in PKC-α mRNA, PKC-β mRNA and PKC-θ mRNA in the gastrocnemius muscle, with 2–3-fold increases observed at 2 and 4 h (Figure 9). The time courses for increases in PKC-α mRNA and PKC-β mRNA in the gastrocnemius muscle were different from those observed in adipose tissue, with significant increases in muscle PKC mRNA levels becoming apparent only after 1 h of insulin treatment. PKC-ε mRNA showed a tendency to increase at later time points of insulin treatment, but this change was not statistically significant (Figure 9). As in adipose tissue, insulin had no effect on gastrocnemius β-actin mRNA in the short-term experiments, and this was therefore used as a recovery correction factor. At the protein level in the gastrocnemius muscle, as described in the accompanying paper [9], membrane levels of PKC-α, PKC-β, PKC-ε and PKC-θ were increased at 30 and/or 120 min of insulin treatment *in vivo*, and, except for a transient decrease in PKC-α at 30 min and a decrease in PKC-θ at 120 min, cytosolic PKC levels were maintained near control levels. In long-term *in vivo* experiments, we did not observe any significant changes in gastrocnemius PKC-β mRNA 16–17 h after insulin injection, but, 16–17 h after a second insulin injection, PKC-β mRNA was increased approximately 2-fold (data not shown). In contrast, PKC-α mRNA and PKC-ε mRNA did not increase with either one or two insulin injections. In limited studies of the soleus and quadriceps muscles *in vivo*, at 16–17 h after insulin injection, PKC-β mRNA levels were increased by 65 ± 20% (mean ± S.E.M.; $n = 9$; $P < 0.05$) and 34 ± 9% ($n = 8$; $P < 0.05$) respectively, with no apparent changes in PKC-α mRNA. (We did not study PKC-ε mRNA or PKC-θ mRNA in these studies.)

DISCUSSION

In this study, we questioned whether insulin-induced PKC translocation in rat adipocytes and rat muscles is accompanied by changes in the levels of PKC mRNA and total immunoreactive PKC. These questions seemed important, since translocative activation of PKC, particularly if strong and prolonged, might reasonably be attended by proteolytic decreases in translocated PKC. We also currently compared the effects of insulin on PKC mRNA with those of glucose and PMA in cultured rat adipocytes. Accordingly, we found that insulin provoked increases in both PKC-α mRNA and PKC-β mRNA, but had little or no effect on PKC-ε mRNA, in rat adipocytes and gastrocnemius muscles. We also found that insulin provoked increases in PKC-θ mRNA in the gastrocnemius muscle. Glucose and PMA also provoked lesser increases in PKC-α mRNA in rat adipocytes, but, in the case of PKC-β mRNA, PMA-induced increases were small and not statistically significant, and glucose was inhibitory. Thus, although each of these three agonists acutely stimulates the translocation of PKC-α, PKC-β and PKC-ε in rat adipocytes, their effects on levels of mRNAs that encode these PKC isoforms are at least partly different, particularly in the case of PKC-β.

Presumably, increases in PKC-β mRNA served to offset or minimize the decreases in total immunoreactive PKC-β that were observed after 20-h insulin treatment of cultured rat adipocytes *in vitro* and whole adipose tissue *in vivo*. It may therefore be speculated that losses of PKC-β during continued insulin treatment would have been greater in the absence of an increase in PKC-β mRNA. Similarly, it seems likely that insulin-induced increases in PKC-α mRNA may have diminished or prevented the development of decreases in PKC-α, and may have therefore contributed to sustaining membrane increases of this PKC isoform in adipocytes and adipose tissue during ongoing insulin treatment. The same may be said of the gastrocnemius muscle, i.e. increases in PKC-α mRNA, PKC-β mRNA and PKC-θ mRNA may have maintained both membrane increases and cytosolic levels of these PKC isoforms.

Unlike insulin, 20-h PMA treatment of cultured rat adipocytes provoked profound losses of immunoreactive PKC-α and PKC-β. Obviously, the modest increases in mRNA during PMA

treatment were not effective in offsetting increases in PKC-α and PKC-β degradation. In addition, it may be surmised, from the failure of PMA to provoke more substantial increases in adipocyte PKC-α mRNA and PKC-β mRNA, that simple increases in proteolytic degradation and subsequent depletion of PKC-α and PKC-β are not followed by proportional obligatory increases in their mRNAs. This would further imply that insulin effects on PKC-α mRNA and PKC-β mRNA were not simply obligatory or compensatory in nature.

The failure of immunoreactive PKC-ϵ to diminish in response to prolonged treatment of adipocytes with either PMA, insulin, or glucose, coupled with the lack of appreciable change in PKC-ϵ mRNA following these treatments, suggested that this PKC isoform, at least in rat adipocytes, was relatively resistant to proteolytic degradation, despite the fact that it was effectively translocated to adipocyte membranes by each of these agonists. At present, there is no simple explanation for the resistance of this PKC isoform to proteolytic degradation following its translocation. Also, it is uncertain why there were actual increases in total PKC-ϵ in insulin- and glucose-treated adipocytes, in the absence of an increase in PKC-ϵ mRNA. The latter findings raise the possibility that there may have been an alteration in the synthesis of this PKC at the translational level, or a decrease in its degradation rate, during insulin or glucose treatment.

The fact that insulin provoked decreases in PKC-β and possibly PKC-α protein contents and increases in their mRNA levels in cultured rat adipocytes *in vitro* and in fat pads *in vivo* provides evidence that there is considerable turnover of these PKC isoforms during insulin action in rat adipose tissue. The same reasoning seems applicable to PKC-α, PKC-β and PKC-θ in rat skeletal muscle as well. Such turnover would be expected with translocative activation. Thus the present findings of increases in PKC-α mRNA, PKC-β mRNA and PKC-θ mRNA provide further evidence to support the notion that insulin translocates and activates these PKC isoforms in rat adipocytes and skeletal muscles.

Although it is widely recognized that prolonged phorbol ester treatment frequently results in PKC depletion, it is becoming increasingly clear that significant depletion of PKC and specific PKC isoforms may also occur during the prolonged actions of other agonists, e.g. as observed currently with insulin and previously with bombesin [22], α-adrenergic agents [23], and synthetic DAGs [22,24]. Also, increases in certain PKC isoforms may occur even when other PKC isoforms are concomitantly depleted, e.g. as observed in the present study and previously with bombesin [22]. Thus, the continued presence of agonists that increase DAG can deplete or augment levels of specific PKC isoforms, and, given the substrate specificities of PKC isoforms, this may serve as a regulatory mechanism. Presumably, alterations in PKC mRNA availability, as currently observed, are important in controlling tissue levels of specific PKC isoforms.

In summary, we have found that insulin provokes: (a) increases in PKC-α mRNA and PKC-β mRNA in cultured rat adipocytes *in vitro* and rat epididymal fat pads *in vivo*; and (b) increases in PKC-α mRNA, PKC-β mRNA and PKC-θ mRNA in rat gastrocnemius muscle *in vivo*. These effects of insulin on mRNA levels were associated with the translocation and, in some instances, partial depletion of these PKC isoforms. Thus, insulin action is attended by increases in the degradative turnover and resynthesis of these PKC isoforms. These findings therefore support the notion that these isoforms are translocated and activated by insulin in rat adipocytes and skeletal muscle. We speculate that this mechanism may be important to maintain levels of these PKC isoforms that may otherwise be depleted during sustained insulin action. Additionally, these increases in PKC mRNA during continued action of insulin may be important for sustaining increases in membrane contents of these PKC isoforms, and this continuous activation of PKC may result in feedback inhibition of insulin receptor tyrosine kinase activity [25,26] or other signalling factors that mediate acute insulin effects on glucose metabolism. Further studies will be required to test these possibilities.

Funds for this research were provided by the Veterans' Affairs Merit Review Program; National Institutes of Health DK38079; and 'Region Languedoc-Roussillon' and 'Institut Servier du Diabetes', Montpelier, France.

REFERENCES

1 Ishizuka, T., Cooper, D. R. and Farese, R. V. (1989) FEBS Lett. **257**, 337–340
2 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
3 Farese, R. V., Standaert, M. L., Francois, A. J., Ways, K., Arnold, T. P., Hernandez, H. and Cooper, D. R. (1992) Biochem. J. **288**, 319–323
4 Arnold, T. P., Standaert, M. L., Hernandez, H., Watson, J., Mischak, H., Kazanietz, M. G., Zhao, L., Cooper, D. R. and Farese, R. V. (1993) Biochem. J. **295**, 155–164
5 Walaas, S. I., Horn, R. S., Adler, A., Albert, K. A. and Walaas, O. (1987) FEBS Lett. **220**, 311–318
6 Ishizuka, T., Cooper, D. R., Hernandez, H., Buckley, D., Standaert, M. and Farese, R. V. (1990) Diabetes **39**, 181–190
7 Chen, K. S., Heydrick, S., Kurowski, T. and Ruderman, N. B. (1991) Trans. Assoc. Am. Physicians **CIV**, 206–212
8 Yu, B., Standaert, M. L., Arnold, T. P., Hernandez, H., Watson, J., Ways, K., Cooper, D. R. and Farese, R. V. (1992) Endocrinology **130**, 3345–3355
9 Yamada, K., Avignon, A., Standaert, M. L., Cooper, D. R., Spencer, B. and Farese, R. V. (1995) Biochem. J. **308**, 177–180
10 Walaas, O., Horn, R. S. and Walaas, S. I. (1991) Biochim. Biophys. Acta **1094**, 92–102
11 Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. **58**, 31–44
12 Draznin, B., Leitner, J. W., Sussman, K. E. and Sherman, N. A. (1988) Biochem. Biophys. Res. Commun. **156**, 570–575
13 Ishizuka, T., Hoffman, H., Cooper, D. R., Watson, J. E., Pushkin, D. B. and Farese, R. V. (1989) FEBS Lett. **249**, 234–238
14 Farese, R. V., Standaert, M. L., Arnold, T. P., Yamada, K., Musunuru, K., Hernandez, H., Mischak, H. and Cooper, D. R. (1994) J. Clin. Invest. **93**, 1894–1899
15 Roth, B. L., Mehegan, J. P., Jacobowitz, D. M., Robey, F. and Iadarola, M. J. (1989) J. Neurochem. **52**, 215–221
16 Ono, Y., Fujii, T., Igarashi, K., Kikkawa, U., Ogita, K. and Nishizuka, Y. (1988) Nucleic Acids Res. **16**, 5199–5200
17 Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. **263**, 6927–6932
18 Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. and Nishizuka, Y. (1987) Science **23**, 1116–1120
19 Mischak, H., Goodnight, J., Henderson, D. W., Osada, S., Ohno, S. and Mushinski, J. F. (1993) FEBS Lett. **326**, 51–55
20 Ishizuka, T., Cooper, D. R., Arnold, T. P., Hernandez, H. and Farese, R. V. (1991) Diabetes **40**, 1274–1281
21 Standaert, M. L., Cooper, D. R., Hernandez, H., Arnold, T. P. and Farese, R. V. (1992) Endocrinology **132**, 689–692
22 Olivier, A. R. and Parker, P. J. (1994) J. Biol. Chem. **269**, 2758–2763
23 Hu, Z., Azhar, S. and Hoffman, B. B. (1992) J. Cardiovasc. Pharmacol. **20**, 982–989
24 Brooks, G., Goss, M. W., East, J. A. and Hart, I. R. (1993) J. Biol. Chem. **268**, 23868–23875
25 Haring, H., Kirsch, D., Obermaier, B., Ermel, B. and Machicao, F. (1986) J. Biol. Chem. **261**, 3869–3875
26 Chin, J. E., Liu, F. and Roth, R. A. (1994) Mol. Endocrinol. **8**, 51–58

Received 12 September 1994/19 December 1994; accepted 5 January 1995