Effects of insulin on the translocation of protein kinase C-θ and other protein kinase C isoforms in rat skeletal muscles

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Protein kinase C (PKC)-θ is a newly recognized major PKC isoform in skeletal muscle. In this study we found that insulin provoked rapid biphasic increases in membrane-associated immunoreactive PKC-θ, as well as PKC-α, PKC-β and PKC-ϵ, in rat soleus muscles incubated *in vitro*. Effects of insulin on PKC isoforms in the soleus were comparable in magnitude with those of phorbol esters. Increases in membrane-associated PKC-θ, PKC-α, PKC-β and PKC-ϵ were also observed in rat gastrocnemius muscles after insulin treatment *in vivo*. Our findings suggest that PKC-θ, like other diacylglycerol-sensitive PKC isoforms (α, β and ϵ), may play a role in insulin action in skeletal muscles.

INTRODUCTION

Skeletal muscle is a major target tissue for insulin. A role for diacylglycerol (DAG)/protein kinase C (PKC) signalling during insulin action in skeletal muscle has been suggested by a number of studies [1–6]. PKC activation, however, may involve one or more PKC isoforms, and, save for limited early studies of PKC-β and -ϵ [2,3], there is relatively little information on which PKC isoforms are translocated in response to insulin treatment in skeletal muscles of the mature rat. Importantly, there are no reports on PKC-θ, a newly recognized major PKC isoform in skeletal muscle [7], nor on PKC-α, another relatively abundant PKC isoform in this tissue. In this study, we evaluated the effects of insulin and/or phorbol esters on the subcellular distribution of PKC-θ, PKC-α, PKC-β and PKC-ϵ in rat soleus (red fibres), gastrocnemius (mixed fibres) and tensor fascia lata (white fibres) muscles.

MATERIALS AND METHODS

In vitro studies

As described previously [2,3], soleus muscles from fed 150 g male Holtzmann Sprague–Dawley rats were ligated, stretched and equilibrated at 37 °C under 95% O_2/5% CO_2 in glucose-free Krebs–Ringer bicarbonate (KRB) buffer containing 0.1% BSA and 2 mM sodium pyruvate. Insulin (100 nM) (Elanco) or phorbol 12-myristate 13-acetate (PMA) (500 nM) (Sigma) was then added and incubation was continued for the indicated times. In each case, one treated soleus muscle was directly compared with the contralateral control soleus muscle, and the change in PKC due to treatment was calculated as a percentage of the control. In some experiments, metabolic integrity and responsiveness to insulin were evaluated by incubating solei for 60 min in KRB medium containing 5 mM glucose, [U-^{14}C]D-glucose (1 μCi/ml) (NEN/Dupont) and [6-^{3}H]L-glucose (1 μCi/ml) (NEN/Dupont) to correct for non-specific uptake. Total uptake of D-glucose and incorporation into glycogen and lipids were calculated on the basis of the specific radioactivity of medium D-glucose (the latter incorporation rates are probably underestimated because of isotope dilution by intracellular metabolites).

In vivo studies

As described previously [3], rats were injected intramuscularly every 30 min with 0.25 unit of insulin in saline, and intraperitoneally with 1.5 ml of 10% (w/v) glucose in saline to increase serum insulin to maximally effective levels (see [3]) and to maintain blood sugar, over a 30–120 min period. Control rats were injected identically with saline at the same times.

Western-blot analyses

Cytosol and membrane fractions were prepared and subjected to immunoblot analysis as described previously [8], except that non-specific binding sites were blocked with polyvinyl alcohol (see [9]) for studies of PKC-α, -β, -δ, and -ϵ; manufacturer's instructions (Transduction Laboratories) and ECL (Amersham) were used for the PKC-θ assay. In all cases, equal amounts of protein from control and treated samples were analysed simultaneously on the same blot. The polyclonal antisera used for detecting PKC-α, -ϵ, and -δ were obtained from Life Sciences Lab. Anti-PKC-β serum was obtained from Drs. Bryan Roth and John Mehegan (see [2–4,8,10]). The monoclonal antibody used for detecting PKC-θ was from Transduction Labs. As described previously [8], specificities for PKC-α, -β, -δ, and -ϵ antisera were verified (a) by loss of immunoreactivity when assays were conducted with added immunogenic peptide (see below), and (b) by blotting recombinant PKCs α, β, γ, δ, ϵ and ζ obtained from stably transfected NIH3T3 cells or baculovirus-infected insect cells. Specificity of the PKC-θ antibody was verified by failure to observe a signal in extracts of rat brain, adipocytes, liver and heart, and by detection of a single 80 kDa band in muscle extracts. As described previously [2–4,8], immunoblots were quantified by scanning laser densitometry and treated samples were compared with the corresponding control.

RESULTS

The presence of immunoreactive PKCs α, β and ϵ in cytosol and membrane preparations of rat skeletal muscle was verified by demonstrating the loss of immunoreactivity when samples were

Abbreviations used: DAG, diacylglycerol; KRB, Krebs–Ringer bicarbonate; PC, phosphatidylcholine; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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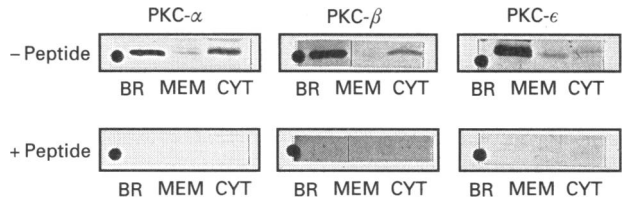

Figure 1 Verification of the presence of PKC-α, PKC-β and PKC-ε in cytosol (CYT) and membrane (MEM) fractions of the rat soleus muscle

Western-blot analyses were conducted in the absence (−) or presence (+) of immunogenic peptide. Brain PKC (BR) is shown for comparison.

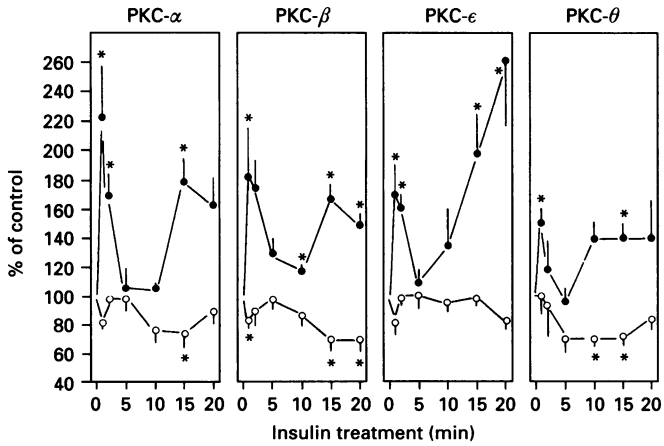

Figure 2 Time-dependent effects of insulin on levels of immunoreactive PKC-α, PKC-β, PKC-ε and PKC-θ in membrane (solid circles) and cytosolic (open circles) fractions of rat soleus muscles incubated *in vitro*

See Materials and methods section for experimental details and Figure 3 for representative immunoblots. Shown here are mean ± S.E.M. values of 4–6 comparisons of insulin-treated versus control muscles at each time point. Asterisks indicate $P < 0.05$ (paired *t*-test).

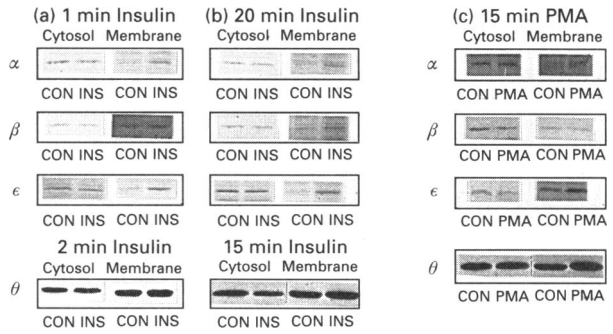

Figure 3 Effects of insulin and PMA on subcellular distribution of immunoreactive PKC-α, PKC-β, PKC-ε and PKC-θ in rat soleus muscles incubated *in vitro*

The duration of treatment is indicated in the Figure. The respective amounts of cytosolic and membrane protein that were analysed were: for PKC-α, PKC-β and PKC-ε, 100 and 125 μg; for PKC-θ, 60 and 30 μg. Shown here are representative immunoblots.

Table 1 Effects of PMA on subcellular distribution of PKC isoforms in rat soleus muscles incubated *in vitro*

Solei were incubated with or without 500 nM PMA for 15 min. Cytosol and membrane fractions were prepared and analysed for immunoreactive PKC isoforms. See the Materials and methods section for experimental details and Figure 3 for representative immunoblots. *P* was determined by paired *t*-test. Abbreviation: N.S., not significant.

	Percentage change, PMA-treated versus Control [mean ± S.E.M (*n*)]	
PKC isoform	Cytosol	Membrane
α	−20 ± 5 (6) ($P < 0.025$)	+72 ± 12 (6) ($P < 0.005$)
β	−37 ± 6 (6) ($P < 0.005$)	−5 ± 8 (6) (N.S.)
ε	−55 ± 6 (6) ($P < 0.001$)	+93 ± 32 (6) ($P < 0.05$)
θ	−21 ± 20 (6) (N.S.)	+49 ± 17 (5) ($P < 0.05$)

assayed in the presence of the immunizing peptide (Figure 1). Peptide competition studies were not possible for PKC-θ; however, only a single 80 kDa immunoreactive band was found in muscle, but not in rat brain, adipocytes, liver and heart. Approximate apparent molecular masses (relative to the migration of protein standards) of PKCs α, β, ε and θ on SDS/PAGE were 80, 80, 95 and 80 kDa respectively. Approximate ratios of total cytosolic immunoreactive PKC to total membrane immunoreactive PKC in control muscles were 3–4:1 for PKCs α, β and ε, and 1:1 for PKC-θ. The presence of mRNAs for PKC-α, -ε and -θ in rat skeletal muscle has been reported previously [7]. The presence of PKC-β mRNA in rat skeletal muscle, albeit in amounts apparently less than other PKC mRNAs (in general, exposure times for autoradiography were approximately 4-fold longer for PKC-β mRNA analyses, as compared with PKC-α mRNA and PKC-ε mRNA using probes of similar specific radioactivities), was confirmed, both by Northern-blot analysis (data not shown) and by ribonuclease protection assay (see accompanying paper i.e. ref. [11]).

In vitro studies

In the soleus muscle, insulin provoked rapid, biphasic increases in membrane contents of PKC-α, -β, -ε and -θ, with initial peaks at 1–2 min, followed first by transient nadirs and, subsequently, by secondary increases at 10–20 min (Figures 2 and 3). Concomitantly, cytosolic levels of PKC-α, -β, -ε and -θ decreased, although not significantly at all time points. PKC-δ was localized in the membrane fraction in control samples and was therefore not studied in translocation experiments. PMA treatment for 15 min provoked changes in the distribution of PKC-α, -β, -ε and -θ that were, in most respects, similar to those of insulin, except that increases in membrane PKC-β were apparently not sustained (Figure 3 and Table 1).

As shown in Table 2, the soleus muscle preparations that were used in all the present experiments *in vitro* responded well to insulin, in terms of total glucose uptake and incorporation into lipids and glycogen. For comparison, results from incubations of rat hemi-diaphragms are shown, and similar uptake and incorporation rates were observed (Table 2). Also, in other *in vivo* studies (data not shown) in which rats were studied during euglycaemic–hyperinsulinaemic clamp conditions, total glucose uptake rates into the soleus and diaphragm were remarkably similar to those observed *in vitro*, i.e. approximately 3000 nmol/g of tissue per 60 min. In similar euglycaemic–hyperinsulinaemic clamp studies, the uptake of non-metabolizable 2-deoxyglucose was also found to be approximately 3400 nmol/g of tissue per 60 min as compared with the basal uptake rate of approximately 1200 nmol/g of tissue per 60 min [12].

Table 2 Effects of insulin on D-glucose uptake and incorporation into lipids and glycogen in soleus and hemi-diaphragm muscles incubated *in vitro*

Hemi-diaphragms, attached to ribs, were incubated in the same conditions as soleus muscles (see the Materials and methods section). The muscles were equilibrated for 20 min and then incubated for 60 min with [U-^{14}C]D-glucose and [6-^{3}H]L-glucose, as described in the Materials and methods section. Each insulin-stimulated muscle was incubated in parallel with its contralateral control muscle obtained from the same rat. Soleus values are means ± S.E.M. with the number of comparisons (insulin versus control) shown in parentheses. Diaphragm values are means of two comparisons.

		D-Glucose metabolism (nmol/g of tissue per 60 min)		
Muscle type	Treatment ...	Total glucose uptake	Glucose incorporated into lipids	Glucose incorporated into glycogen
(A) Soleus muscle				
	None (control)	1117 ± 145 (4)	37 ± 6 (4)	216 ± 20
	Insulin (100 nM)	2569 ± 219 (4)	84 ± 13 (4)	656 ± 49
(B) Hemi-diaphragm				
	None (control)	1237	70	253
	Insulin (100 nM)	2900	195	520

Table 3 Effects of insulin treatment *in vivo* on membrane contents of PKC-α, PKC-β, PKC-ε and PKC-θ in rat gastrocnemius muscles

Rats were treated for indicated times, and membrane fractions were analysed for immunoreactive PKC isoforms as described in the Materials and methods section. *P* values (in brackets) was determined by paired *t*-test. Abbreviation: N.S., not significant.

	Percentage increase, Insulin-treated versus Control muscles [mean ± S.E.M. (*n*)]			
Treatment time	PKC-α	PKC-β	PKC-ε	PKC-θ
30 min	+23 ± 16 (13) (N.S.)	+108 ± 29 (10) ($P < 0.005$)	+48 ± 9 (8) ($P < 0.005$)	+54 ± 8 (5) ($P < 0.005$)
120 min	+48 ± 14 (14) ($P < 0.005$)	+65 ± 28 (10) ($P < 0.05$)	+46 ± 12 (12) ($P < 0.005$)	+3 ± 6 (4) (N.S.)

In vivo studies

Membrane contents of PKC-α, -β, -ε and -θ in the gastrocnemius muscle were increased at 30 and/or 120 min of insulin treatment (Table 3). Insulin effects on membrane PKC-α at 30 min, and on membrane PKC-θ at 120 min, may have been limited by the fact that total amounts of these PKC isoforms were decreased by 20–30% at these time points, presumably reflecting losses, secondary to translocation and proteolysis of activated PKC. In another series of experiments, comparable with effects of insulin at 30 min shown in Table 3, PKC-θ levels in gastrocnemius muscle membrane fractions were increased by 57 ± 11% (mean ± S.E.M.; $n = 5$; $P < 0.01$) at 60 min insulin treatment. Thus, membrane PKC-θ was increased at 30 and 60 min, but not at 120 min, of insulin treatment *in vivo*. In most cases (i.e. except for PKC-α at 30 min and PKC-θ at 120 min), cytosolic PKC levels in the gastrocnemius muscle were only mildly decreased or maintained near the control level in these *in vivo* experiments (data not shown). However, it should be noted that (see accompanying paper, ref. [11]) insulin provoked increases in mRNA levels for PKC-α, -β, -ε and -θ within this time frame (60–120 min) in the gastrocnemius muscle, and this, along with a generalized insulin-induced increase in muscle protein synthesis in skeletal muscles, may have offset losses of cytosolic PKC levels. In limited studies of the soleus muscle *in vivo*, insulin provoked 40 ± 10% ($n = 5$; $P < 0.025$) increases in the membrane content of PKC-θ at 120 min, but, at 30 min of insulin treatment, membrane PKC-θ levels were not different from control levels, although cytosolic PKC-θ levels were decreased by 35 ± 5% ($P < 0.001$) which suggests an early transient depletion of an insulin-sensitive PKC-θ pool. In limited studies of the tensor fascia lata muscle *in vivo*, insulin treatment for 30 min provoked increases in membrane PKC-α and PKC-ε of 71 ± 20% ($n = 5$; $P < 0.025$) and 83 ± 25% ($n = 4$; $P < 0.05$) respectively, and decreases in cytosolic PKC-α and PKC-ε of 39 ± 9% ($n = 4$; $P < 0.025$) and 15 ± 8% ($n = 4$; not significant); on the other hand, changes in the subcellular distribution of PKC-β or PKC-θ were relatively small and were not statistically significant at this particular time point in this muscle.

DISCUSSION

In soleus, gastrocnemius and tensor fascia lata muscles, insulin provoked increases in membrane contents of PKC-α, -β, -ε and/or -θ, apparently via translocation from the cytosol. Since DAG contents and membrane PKC enzyme activities of the soleus and gastrocnemius muscles are also increased by comparable insulin treatments *in vitro* and/or *in vivo* [2,3], it is likely that the presently observed changes in PKC are triggered by increases in membrane-associated contents of DAG and are therefore reflective of a true activation of the enzyme. On the other hand, there were differences in membrane levels of each PKC isoform at various time points, and factors other than DAG may have influenced specific PKC isoform translocation to, and persistence at, various membrane sites.

The biphasic increases in membrane contents of PKC isoforms presently observed in insulin-treated solei *in vitro* are similar, in many respects, to those observed in other insulin-sensitive cells. Most notably, biphasic increases in membrane PKC and/or DAG have been observed in rat adipocytes ([4,8]; K. Yamada, A. Avignon, M. L. Standaert, D. R. Cooper, B. Spencer and R. V. Farese, unpublished work) and BC3H-1 myocytes [13–15]. The initial increases in membrane PKC are most likely due to increases in DAG (and possibly other lipids) in the plasma membrane (see [4,8]), that occur secondary to hydrolysis of

phosphatidylcholine (PC) and, to a lesser extent, phosphatidylinositol (PI)-glycans (see [13,15]). The secondary increases in membrane PKC probably reflect both *de novo* phospholipid-DAG synthesis in the endoplasmic reticulum, as well as replenishment and continued hydrolysis of PC and PI-glycans, thus generating DAG (and possibly other lipids) in the plasma membrane (see [8,13–15]). The multiple sources of DAG during insulin action, along with other factors, may contribute to compartmentalization and differences in specific PKC isoform translocation.

Although insulin-induced increases in membrane-associated PKC-α, -β, -ϵ and -θ were similar in certain circumstances (e.g. in the soleus incubated *in vitro*), greater variability in changes of these isoforms was observed in other circumstances (i.e. in certain of the more prolonged *in vivo* studies). The latter variability may reflect greater depletion of insulin-sensitive PKC pools and/or time-dependent changes in signalling through multiple phospholipid pathways during ongoing insulin treatment *in vivo*. More detailed studies will be needed to resolve these issues.

It should be noted that the presently observed effects of insulin on apparent PKC translocation in the soleus muscle *in vitro*, as well as those observed previously in isolated adipocytes [4,8], occurred in the absence of extracellular glucose. It is therefore clear that these effects of insulin on PKC cannot be due to glucose transport-induced increases in DAG production. This conclusion also follows from the fact that the initial peaks in membrane PKC at 1–2 min of insulin treatment would be expected to precede changes in glucose transport. It should also be noted that insulin increased membrane levels of 'novel', Ca^{2+}-independent PKC isoforms (θ and ϵ), as well as 'conventional', Ca^{2+}-dependent PKC isoforms: this rules out the possibility that simple alterations in Ca^{2+} can account for insulin-induced PKC translocation.

The stimulatory effects of insulin on the apparent translocation of PKC-θ are noteworthy, as this PKC isoform is particularly abundant in, and is relatively specific for, skeletal muscle [6]. From the present results, it would appear that this PKC isoform, as well as PKC-α, -β and -ϵ, may play an important role in insulin action in muscle. Further studies are needed to determine which biological processes are altered by insulin-sensitive PKC isoforms.

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