Translocation of protein kinase C isoforms in rat muscle in response to fasting and refeeding

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Weanling rats were offered food *ad libitum*, or fasted for 18 h, or fasted and refed for times ranging from 5 to 30 min. Five protein kinase C (PKC) isoforms (α, ϵ, ζ, θ and μ) were detected in the hindlimb muscles by Western immunoblotting. PKC forms ϵ and θ were abundant in plantaris, but not in soleus muscle, and no difference in localization was detected between fed rats and those fasted for 18 h. PKC forms α and μ were affected by fasting and refeeding. PKC-μ was found only in the cytosolic fraction of the plantaris muscle of the fasted animal, but in the fully-fed animals it was also associated with the membrane fraction. The pattern of localization observed in the fully-fed state was restored in the fasted rats by 20 min refeeding. In contrast, PKC-α was not detected in the cytosolic fraction of the plantaris in fasted animals but rapidly reappeared there on refeeding, being restored to 20 % and 80 % of the fed value within 5 and 30 min of refeeding respectively. The timing of these changes was correlated with the increase in serum insulin concentration, which was significantly elevated above the fasted value by 5 min and at subsequent times. These data suggest a possible role for PKC isoforms α and μ in the metabolic changes that occur in skeletal muscle on transition between the fasted and the fed state.

Fasting: Protein kinase C: Skeletal muscle

Phasic skeletal muscles of the rat, e.g. the plantaris and gastrocnemius, are sensitive to an overnight fast, which results in a decrease in protein synthesis which is rapidly restored by refeeding (Garlick *et al*. 1983; Millward *et al*. 1983). In contrast, protein metabolism in the tonic or 'red' skeletal muscles such as the soleus is less sensitive to nutritional manipulation. Insulin is believed to be involved in these changes in muscles such as the plantaris, because infusion of this hormone alone restores protein synthesis rates to those observed in the fully fed animal within 20 min (Garlick *et al*. 1983). The signal transduction cascade induced by occupancy of the insulin receptor initially involves activation of phospholipase A_2 and cyclo-oxygenase (*EC* 1.14.99.1), leading to arachidonic acid release and prostaglandin production (Reeds & Palmer, 1983). How early events in the signal transduction cascade subsequently lead to increases in translation has been investigated *in vitro* (Thompson *et al*. 1997). This work has implicated the protein kinase C (PKC) family of enzymes. Six isoforms were detected (α, δ, ε, ζ, ι and μ), of which one or more of a group of three, PKC-α, -δ and -ε were implicated in the control of the rapid increases in translation observed in L6 myoblasts. Inactive PKC forms appear to be present as soluble components of the cytosolic fraction of cell protein; treatment with agonists such as hormones and neurotransmitters results in translocation of the enzymes to cellular membranes and subsequent activation (Kraft & Anderson, 1983). Thus, their localization within the cell can provide important clues as to their involvement in specific metabolic control mechanisms. The fasted–refed rat model was used in the present study to test the physiological role of PKC by determining, first, which isoforms are present in rat plantaris and soleus muscles and second, which of the isoforms present translocate in response to fasting and refeeding.

Materials and methods

Male Rowett Hooded rats, 22 d old, 3 d post-weaning and weighing 51 ± 1 g were divided into six groups of three. One group was allowed food, a choice of stock pellets (CRM, Special Dietary Services, Witham, Essex, UK) or PW3 diet (Pullar & Webster, 1977) *ad libitum*; the remaining five groups had food removed at 16.00 hours, but were allowed free access to water. After 18 h the rats fed *ad libitum* and one group of three fasted animals were killed and soleus and plantaris muscles were removed from both hindlimbs. The remaining animals were given a choice of

Abbreviations: PKC, protein kinase C.

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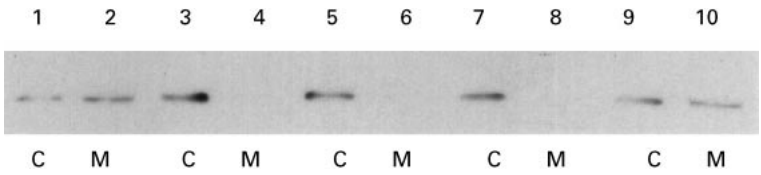

Fig. 1. Western immunoblot of rat plantaris muscle with an antibody to protein kinase C-μ. Cytosolic (C) and membrane (M) protein fractions from plantaris muscle of rats that were fully fed (lanes 1, 2), fasted for 18 h (lanes 3, 4) or fasted and refed for 5 min (lanes 5, 6), 10 min (lanes 7, 8) or 20 min (lanes 9, 10) are shown.

food as detailed earlier. Feeding began immediately and all animals sampled both diets, the more familiar stock pellets being preferred. Precisely 5, 10, 20 or 30 min after refeeding was observed, animals were killed and their soleus and plantaris muscles removed. All muscles were immediately frozen in liquid N_2 and stored in liquid N_2 until analysed. Blood samples were also collected at slaughter and were stored on ice and centrifuged at 10 000 ***g*** for 30 min. Serum insulin was measured by radioimmunoassay (MacRae *et al.* 1991).

Muscle extraction procedure and Western immunoblotting

Cytosolic and membrane-bound protein fractions of plantaris and soleus muscles were extracted and Western immunoblotting was performed as described previously (Palmer *et al.* 1998). Protein content of the extracts was measured by the method of Bradford (1976). The same amount of protein was loaded on to each lane of a single gel, between gels the amount of protein loaded was 20–30 μg. The nitrocellulose (PVDF) membrane used for immunoblotting was from Millipore Corporation (Bedford, Beds., UK). Monoclonal antibodies to nine PKC isoforms (α, β, γ, δ, ε, ζ, θ, ι and μ) were from Affiniti Research Laboratories (Exeter, Devon, UK). PKC immunoreactivity was visualized by chemiluminescence (ECL, Amersham International, Bucks., UK) on a Packard Instant Imager. Duplicate or triplicate measurements were made of each isoform. All other chemicals used were from BDH (Poole, Dorset, UK) or Sigma (Poole, Dorset, UK).

Results

PKC-μ was present in both the cytosolic and membrane protein fractions of the plantaris muscles from the fully-fed animals (Fig. 1). Following an 18 h fast, this isoform appeared to be exclusively cytosolic and this localization persisted on refeeding for 5 or 10 min (one of triplicate blots is shown, Fig. 1). After 20 min refeeding the pattern in the fully-fed muscle was restored, with PKC-μ distributed between both cytosolic and membrane fractions (Fig. 1). A similar translocation occurred in the soleus muscle where cytosolic PKC-μ was apparent in fasted rats and in those refed for 5 and 10 min, but was not detected in the groups refed for 20 min or 30 min or the fully-fed group (Fig. 2)

In contrast to the situation with PKC-μ, PKC-α in the plantaris appeared to translocate in the opposite direction; cytosolic PKC-α disappeared on fasting and reappeared rapidly on refeeding (Fig. 3). In the rats fasted for 18 h the cytosolic fraction contained 90 % less PKC-α than in the fully-fed rat. Refeeding for only 5 min significantly increased ($P < 0{\cdot}01$) the cytosolic PKC-α to 20 % of the fed level. At 10, 20 and 30 min after consuming food, the proportion in the cytosol had increased to 50, 65 and 80 % of the value for fully-fed rats ($P < 0{\cdot}001$; Fig. 3). The anticipated decrease on refeeding in the membrane-associated PKC-α was not quantifiable. The reason for this was the large amount of PKC-α present in the membrane of the

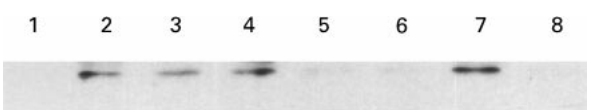

Fig. 2. Western immunoblot of rat soleus muscle with an antibody to protein kinase C-μ. Cytosolic proteins of soleus muscle from rats that were fully fed (lanes 1, 8) fasted (lanes 2, 7) or fasted and refed for 5, 10, 20 or 30 min (lanes 3–6) are shown.

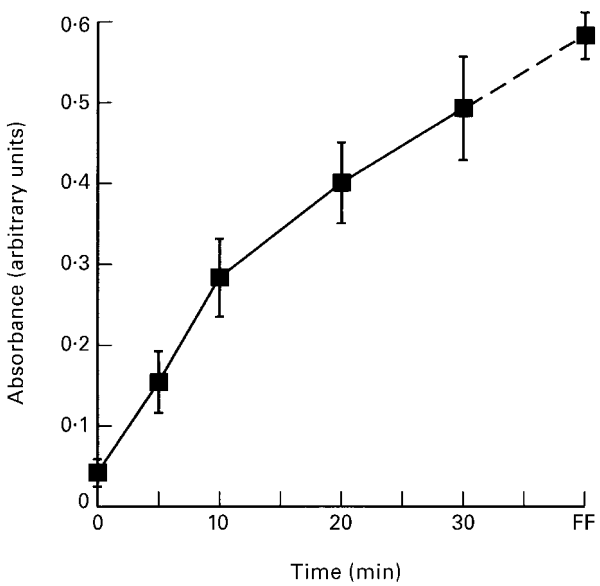

Fig. 3. Quantification of Western immunoblot of plantaris muscle with an antibody to protein kinase C-α. The Fig. shows cytosolic protein kinase C-α (arbitrary absorbance units/μg protein loaded) in fasted animals (time 0) or animals that had been refed for 5–30 min. FF, value for fully-fed animals. Values are means for three animals with their standard errors represented by vertical bars. All values for refed rats were significantly greater than the fasted (time 0) value; 5 min ($P < 0{\cdot}01$); 10, 20 and 30 min ($P < 0{\cdot}001$).

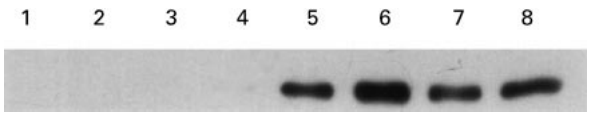

Fig. 4. Western immunoblot of membrane proteins of rat soleus and plantaris muscles with an antibody to protein kinase C-ε. Lanes 1–4: membrane proteins of soleus muscle from rats that were fasted, fed, refed for 5 min and refed for 30 min respectively; lanes 5–8 plantaris muscle in the same order. No protein kinase C-ε was detected in the cytosol.

fasted animals and the correspondingly small change in abundance. For example, in two Western blots of the membrane-associated PKC-α, values of 3·7 and 2·9 arbitrary absorbance units were obtained in the fasted state and 3·3 and 2·7 absorbance units respectively after 30 min refeeding. PKC-α also appeared in the soleus muscle, where it was also largely membrane-associated. No change in the location of PKC-α with intake was detected in this muscle (results not shown).

PKC-ε was found in the membrane of plantaris muscle, but was not detected in the cytosol in fed, fasted or refed rats (Fig. 4). PKC-ε appeared to be absent in the soleus and was not found even after prolonged (30 min) exposure of the Western blot.

PKC-θ appeared to be similar to PKC-ε in that it was abundant in the membrane of plantaris, but absent from the cytosolic fraction from rats on any dietary treatment. Also, like PKC-ε, PKC-θ was virtually undetectable in soleus, although a trace was detected after prolonged exposure of the Western blot (Fig. 5). A trace of PKC-ζ was detected in both muscles; again no translocation between the cytosolic and membrane fractions was observed (results not shown). PKC isoforms β, γ, δ and ι were not detected in either muscle.

The concentration of insulin in serum was low in the fasted animals (Table 1) but rose rapidly on refeeding and was significantly greater than the fasted value at all times after refeeding.

Discussion

The various PKC isoforms are involved in a diverse range of processes within the cell, including differentiation, smooth-muscle contraction and growth (for reviews see Liu, 1996 and Jaken, 1996). In both soleus and plantaris muscles of the rat, total PKC activity falls as the metabolic rate declines

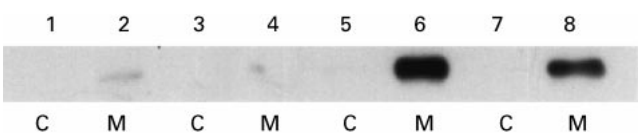

Fig. 5. Western immunoblot of rat soleus and plantaris muscles with an antibody to protein kinase C-θ. Cytosolic (C) and membrane-bound (M) protein fractions of soleus from fasted rats (lanes 1, 2), soleus from fed rats (lanes 3, 4), plantaris from fasted rats (lanes 5, 6), and plantaris from fed rats (lanes 7, 8) are shown.

Table 1. Serum insulin concentrations in fasted rats, fasted rats refed for 5, 10, 20 or 30 min and fully-fed rats†

(Mean values with their standard errors for four estimations)

	Insulin (μU/ml)	
	Mean	SE
Fasted	9·1	3·5
Refed 5 min	76·7**	14·9
10 min	64·3***	4·2
20 min	57·2*	21·2
30 min	70·0**	14·8
Fully fed	38·4**	7·5

Mean values were significantly different from those for fasted rats: *$P<0.05$, **$P<0.01$, ***$P<0.001$ (Student's t test).
† For details of procedures, see p. 154.

with age (Richter & Nielsen, 1991). The present study was designed, first, to identify which isoforms of PKC are present in muscles of different fibre proportions and physiological functions. A second aim was to investigate the effect of nutritional status on the localization of these isoforms within the cell. One of the rationales behind this approach was the well established observation that a reversible suppression of protein synthesis occurs in response to an overnight fast followed by refeeding for <30 min (Garlick *et al*. 1983).

Isoforms present in muscle

Of the nine PKC isoforms examined, five (α, ε, ζ, θ and μ) were detected in plantaris muscle of the weanling rat. The same isoforms were found in muscles of the fetal sheep (Palmer *et al*. 1998), whilst a study by Thompson *et al*. (1997), using the same antibodies, additionally found PKC isoforms δ and ι. Surprisingly, that study failed to find PKC-θ, an isoform originally identified from muscle (Osada *et al*. 1992). Thus, there is some consensus as to the isoforms present in muscle and muscle-derived cell lines. Two other isoforms which have been detected in previous studies, PKC-β (Yamada *et al*. 1995; Avignon *et al*. 1996) and PKC-δ (Avignon *et al*. 1996; Thompson *et al*. 1997), were not detected in the present study, possibly because their expression was below the limits of detection. Of the isoforms that were detected, three (ε, ζ and θ) did not appear to alter their location in response to dietary manipulation. PKC-θ appeared to be exclusively membrane-bound in both the fasted and refed rat plantaris. Yamada *et al*. (1995) reported PKC-θ to be present in both soleus and gastrocnemius muscles, but in the present study, in sharp contrast to plantaris, where PKC-θ appeared to be present in large amounts, this isoform initially appeared to be absent from the soleus after a 10 min visualization. On more prolonged (30 min) exposure of the Western blot, a trace of PKC-θ was observed in the membrane. As the soleus muscle of the rat is a slow-twitch muscle composed predominately of type I fibres with only 13 % type II fibres, whilst the plantaris contains over 90 % type II fibres (Kobzik *et al*. 1994), the presence of PKC-θ predominately in plantaris may indicate a type II fibre-specific pattern for this isoform. PKC-ε, which was also found in plantaris but

not in soleus muscle, and also appeared to be predominately membrane associated, may have a similar fibre type specificity.

Nutritionally-induced translocation

Translocation of PKC isoforms to the membrane is believed to enhance their biological activity; thus the localization of PKC, particularly the Ca^{2+}-dependent isoforms, α, β and γ, may provide important clues as to their activity (Kraft & Anderson, 1983; Bell, 1986). However the precise biological roles of the various isoforms and their natural substrates are only now beginning to be elucidated (Liu, 1996). Of the two PKC isoforms which did translocate in response to fasting and refeeding, α and μ, the former has been implicated as a mediator of insulin action in previous studies. Thus, in response to high concentrations of insulin, PKC isoforms α, β, ε and θ all translocated from the cytosol to the membrane in both isolated soleus and gastrocnemius muscles (Yamada *et al*. 1995). Furthermore, diabetic rats exhibited higher levels of membrane-bound PKC-α, -β, -δ and -ε, with a corresponding decrease in the amount in the cytosol compared with control animals (Avignon *et al*. 1996). These effects were associated with an increase in membrane diacylglycerol and the authors proposed that this persistent translocation may be associated with impaired glycogen synthesis and insulin resistance. The data presented here, however, show that, in the fully-fed state, when plasma insulin concentration is high, some PKC-α is present in the cytosol. The isoform leaves the cytosol after an overnight fast, when insulin levels in the serum are low, and returns there rapidly when food is restored. In the case of PKC-μ, the changes are in the opposite direction; this isoform is distributed between the cytosol and membrane in the fully-fed rats, and translocation to the cytosol occurs on fasting. The re-establishment of the fully-fed distribution pattern of both PKC-α and PKC-μ on refeeding occurs over a period of time consistent with their possible involvement in a process that is sensitive to the fasting–refeeding cycle. Among such events is a change in the rate of translation and an increase in the fractional rate of protein synthesis (Garlick *et al*. 1983). In a recent experiment involving an identical feeding regimen, and using the same strain of rats, but weighing 80 g (LM Birnie, RM Palmer and GE Lobley, unpublished results), the fasting-induced suppression in protein synthesis was 40 %, greater than that reported by Garlick *et al*. (1983). Direct evidence for the involvement of these PKC isoforms in changes in the rate of muscle protein synthesis is lacking and there are other metabolic changes, induced by fasting and refeeding, in which PKC isoforms have been implicated. Thus, it has been proposed that a PKC may mediate the inhibition of glycogen synthase (*EC* 2.4.1.11), a manifestation of insulin resistance (Ahmad *et al*. 1984), and may regulate glucose transport in skeletal muscle (Henriksen *et al*. 1989). Although it is clear that the current study does not prove a direct link between PKC translocation and changes in protein metabolism, there are several mechanistic routes through which PKC isoforms may participate in such a process. These include, for example, action on ribosomal protein S6 (Hei *et al*. 1993, 1994) or on the mRNA cap binding protein, eIF-4E, which is thought to be a limiting factor in the control of initiation (Pause *et al*. 1994), or by phosphorylation and a reduction in tyrosine kinase (*EC* 2.7.1.112) activity of the insulin receptor (Bollag *et al*. 1986). Regardless of any involvement with aspects of protein metabolism, the current study has shown that at least two PKC isoforms are nutrient-sensitive in skeletal muscle, and their change in cellular distribution may mediate some of the important metabolic events associated with fasting and refeeding.

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