Growth and metabolism of fetal and maternal muscles of adolescent sheep on adequate or high feed intakes: possible role of protein kinase C-α in fetal muscle growth

Robert M. Palmer*, Michael G. Thompson, Chrystel Meallet, Amanda Thom, Raymond P. Aitken and Jacqueline M. Wallace

Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

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From days 4–104 of pregnancy, adolescent sheep, weighing 43·7 (SE 0·87) kg were offered a complete diet at two different intakes (approximately 5 or 15 kg/week) designed to meet slightly, or well above, maternal maintenance requirements. The fetal and maternal muscles were taken on day 104 of pregnancy and analysed for total DNA, RNA and protein. Ewes offered a high intake to promote rapid maternal weight gain, weighed more (76·5 (SE 4·5) *v.* 50·0 (SE 1·7) kg) and had muscles with a greater fresh weight, whilst their fetuses had smaller muscles, than those fed at a lower intake. Plantaris muscle of the ewes fed at the high intake contained more RNA and protein; again the opposite situation was found in the fetal muscle. On the higher maternal intakes, the DNA, RNA and protein contents of the fetal plantaris muscle were less than in fetuses of ewes fed at the lower intake. To investigate the possible mechanisms involved in this decrease in fetal muscle mass, cytosolic and membrane-associated muscle proteins were subjected to Western immunoblotting with antibodies to nine isoforms of protein kinase C (PKC), a family of enzymes known to play an important role in cell growth. Five PKC isoforms (α, ε, θ, μ and ζ) were identified in fetal muscle. One of these, PKC-α, was located predominantly in the cytosolic compartment in the smaller fetuses of the ewes fed at a high plane of nutrition, but was present to a greater extent in the membranes of the more rapidly growing fetuses of the ewes fed at the lower intake. This was the only isoform to demonstrate nutritionally related changes in its subcellular compartmentation suggesting that it may mediate some aspects of the change in fetal growth rate.

Pregnancy: Protein kinase C: Sheep

During pregnancy, the mother must alter the partitioning of available nutrients to allow for maintenance of her own body tissues and the increasing demand for macronutrients by the gravid uterus. In the adolescent ewe, a high plane of nutrition led to increased maternal weight gain and a reduction in the weight of both placenta and fetus at 95 d gestation and at term (Wallace *et al.* 1996). Changes in maternal body weight could be primarily due to increased fat deposition, or could involve other tissues such as muscle, where small changes may be masked by a considerable increase in fat reserves. Thus, the first objective of the present study was to examine changes in the DNA, RNA and protein contents of maternal and fetal muscle of adolescent ewes on different levels of feed intake. Alterations in muscle DNA, RNA and protein content may involve changes in a number of signalling pathways. Evidence from muscle cell cultures suggests that protein kinase C (PKC) plays a role in the cascade of events by which changes in activity of phospholipases affect accretion of RNA and protein (Morrison *et al.* 1995; Thompson *et al.* 1997) and that one or more of a group of three PKC isoforms (α, δ and/or ε) may mediate changes in ribosomal activity and protein synthesis in muscle cells *in vitro* (Thompson *et al.* 1997). This may involve changes in the activity of PKC or in the intracellular redistribution of the enzyme, i.e. translocation from the cytosol to the particulate fraction (Kraft & Anderson, 1983), possibly reflecting their activation by lipid binding at such sites

Abbreviations: PHAS-1, phosphorylated heat- and acid-stable protein-1; PKC, protein kinase C.

***Corresponding author:** Dr Robert Palmer, fax +44 (0) 1224 716629, email rmp@rri.sari.ac.uk

(Bell, 1986). Thus, the second aim of the present study was to investigate changes in abundance or translocation of PKC isoforms in skeletal muscle of fetal lambs exhibiting different rates of muscle protein accretion.

Materials and methods

Animals and nutritional treatments

Embryos from superovulated ewes, inseminated by a single sire, were recovered on day 4 after oestrus. They were synchronously transferred in singleton into the uterus of eight recipient adolescent ewes as described previously (Wallace *et al.* 1996).

The adolescent recipients were peripubertal, 200 (SEM 1·8) d old and weighed 43·7 (SE 0·87) kg at the time of induction of ovulation. Following embryo transfer the adolescent ewes were housed in individual pens and offered two different levels of a complete diet which had previously been shown (Wallace *et al.* 1996) to promote either rapid or normal maternal weight gain. In the former case, the aim was to achieve a rapid growth rate of between 250 and 350 g/d, whilst in the latter case, the aim was to maintain a moderate growth rate of approximately 60 g/d until the 104th day of gestation (Wallace *et al.* 1996).

The diet contained (g/kg DM): barley 500, finely milled hay 300, molasses 100, fishmeal 90, vitamin–mineral mix 20, NaCl 30, dicalcium phosphate 50. It had a DM content of 855 g/kg and provided 10·2 MJ metabolizable energy and 137 g crude protein/kg DM.

On the day of embryo transplantation all ewes were given similar intakes. The moderate intake group were offered their entire ration from the day of embryo transplant, and the level of feed offered was reviewed weekly and adjusted on the basis of live-weight gain (Fig. 1). The level of feed offered to the high intake group was increased gradually over the first 2 weeks of gestation until the level of the daily feed refusal was approximately 15% of the total offered; this was taken to be the *ad libitum* intake. The level of feed offered was reviewed three times per week, and adjusted for individual animals when appropriate on the basis of the gain in weight and the amount of feed refused. Both groups of animals received their ration in two equal feeds at 08.00 and 16.00 hours.

On day 104 of pregnancy ewes received their morning feed and were weighed, condition scored (Russel *et al.* 1969) and killed by jugular injection with an overdose of sodium pentobarbitone (20 ml Euthasate; 200 mg pentobarbitone/ml; Willows Francis Veterinary, Crawley, Sussex, UK). The main vessels of the neck were severed, the gravid uterus removed and the fetus killed using 2 ml Euthasate administered by cardiac puncture. Muscles for Western blotting analysis were removed first. One fetal limb was removed, the plantaris muscle was excised, and weighed, and samples (100–200 mg) were frozen and stored in liquid N_2. The plantaris muscle from the contralateral limb and the gastrocnemius and tibialis anterior from both limbs of the fetuses were dissected intact, weighed and frozen in liquid N_2. Maternal muscles, the plantaris, soleus and gastrocnemius from both limbs and vastus muscle from one limb were dissected and weighed, and samples were frozen in liquid N_2. Samples from all these muscles were stored at −70°. Muscle samples (100–150 mg) were homogenized and prepared for analysis as described by Garlick *et al.* (1980). Briefly, samples were homogenized, using an Ultra Turrax homogenizer fitted with a 6·1 mm rotor diameter shaft capable of homogenizing volumes as small as 0·3 ml (A. & J. Beveridge Ltd, Edinburgh, UK), in ice-cold 0·2 M-$HClO_4$ and centrifuged at 3000 ***g*** for 15 min. The pellet was dissolved in 5 ml 0·3 M-NaOH by incubation for 30 min at 37° and a sample (20 μl) taken for protein estimation (Lowry *et al.* 1951). The remainder of the solution was reprecipitated by adding 1·5 ml 2 M-$HClO_4$. Total RNA in the supernatant fraction was measured by the method of Ashford & Pain (1986). Finally total DNA was measured by the method of Burton (1956).

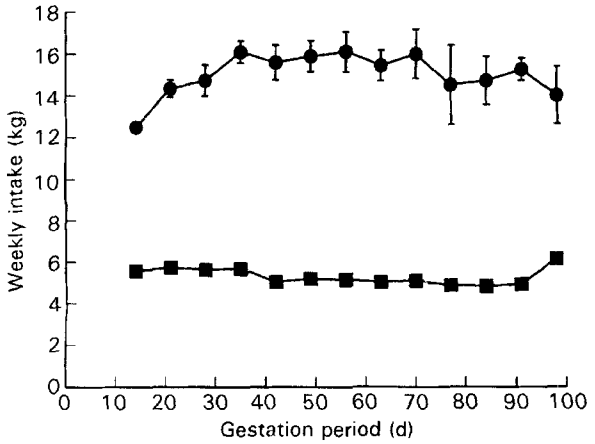

Fig. 1. Weekly food intakes of groups of four ewes offered two different levels of the same diet (low, ■; high, ●) from day 4 to day 104 of pregnancy. Values are means with their standard errors represented by vertical bars.

Western blotting for protein kinase C isoforms

Samples of fetal plantaris muscle (100–150 mg) were homogenized (Ultra Turrax homogenizer) in 500 μl 20 mM-Tris, pH 7·5, 0·25 mM-sucrose, 10 mM-ethylene glycol tetraacetic acid, 2 mM-EDTA, 1 mM-phenylmethylsulphonyl fluoride and 20 μg/ml leupeptin (Berry *et al.* 1990) to extract cytosolic proteins. After standing on ice for 1 h and centrifugation at 100 000 ***g***, the pellet was re-homogenized in the same buffer containing 10 ml/l Triton X-100 to release membrane-bound proteins, recentrifuged at 100 000 ***g***, after a further 1 h on ice. Cytosolic and membrane-associated proteins were stored, without boiling, in gel electrophoresis buffer containing (g/l): SDS 50, glycerol 130, 60 mM-Tris-HCl (pH 6·8), bromophenol blue 2, and mercaptoethanol 50. Equal amounts (20–30 μg protein) were loaded onto SDS–polyacrylamide gels (4·5% stacking gel and 10% running gel) and electrophoresed at 20 mA per gel. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose

membrane at constant current (65 mA) for 90 min. The membrane was blocked with a dried-milk solution (50 g/l Cadbury's Marvel in 10 mM-Tris pH 7·5, 10 mM-NaCl and 1 ml/l Tween 20) and then probed (Lisanti *et al.* 1994; Smart *et al.* 1995) with monoclonal antibodies to nine PKC isozymes (Affiniti, Exeter, Devon, UK), which have been used successfully in a wide range of species (human, dog, rat, mouse, chick and frog). Antibodies were dissolved in the same dried-milk-powder solution at dilutions of: α, 1 : 5000; β, 1 : 2500; δ, μ and ζ, 1 : 1000; ε, 1 : 500; θ, ι and λ, 1 : 250. Following conjugation to a second antibody (polyclonal goat anti-mouse immunoglobulin G labelled with horseradish peroxidase) PKC immunoreactivity was visualized by chemiluminescence (ECL, Amersham International, Bucks., UK).

Statistics

Significance of differences between the two intake groups was assessed by Student's *t* test; all values quoted are means with their standard errors. The mean weights of the muscles from left and right limbs of the ewes and fetuses were used in Table 1, following a two-way ANOVA where no difference between limbs was detected.

Results

Mean dietary intakes of the two groups of ewes are shown in Fig. 1. Ewes fed at the lower intake gained 62 (SE 9) g/d and weighed 50·0 (SE 1·7) kg on day 104 of pregnancy, whilst those fed at the high intake gained 327 (SE 31) g/d and weighed 76·5 (SE 4·5) kg, significantly ($P < 0.001$) more than the lower intake animals, at slaughter.

All the maternal muscles examined (soleus, plantaris, gastrocnemius and vastus) weighed more in the high intake animals (Table 1), although the increase in the weight of the gastrocnemius was not significant. The maternal plantaris muscle contained 24 % more protein, 29 % more RNA and 16 % more DNA than the plantaris muscle of the lower intake group, but the increase in DNA was not significant (Table 2). Maternal soleus muscle showed similar trends; on the high level of nutrition, total protein in soleus was 63 % greater and total RNA was 60 % higher. Again the increase in DNA (30 %) was smaller than the changes in protein and RNA (Table 2).

Fetuses from the high intake ewes were smaller than those from the mothers fed at a lower intake (1·35 (SE 0·18) *v.* 1·57 (SE 0·07) kg). Although this difference was not significant, the fetal tibialis anterior muscle was significantly smaller (Table 1) and the plantaris muscle in the fetuses of the high intake ewes contained 42 % less protein, 29 % less RNA and 33 % less DNA than in the same muscle of fetuses from ewes fed at lower intakes (Table 3).

Body condition score was also greater in the ewes fed at high intakes (3·1 (SE 0·2) *v.* 1·9 (SE 0·1); $P < 0.01$), an indication that the animals were fatter. This was confirmed by expressing maternal muscle weight as a proportion of body weight. Vastus muscle weight was 7·6 (SE 0·4) g/kg body weight in ewes fed at the lower intake, but only 6·1 (SE 0·4) g/kg ($P < 0.05$) body weight in the high-intake animals. Corresponding values for plantaris were 2·42 (SE 0·3) and 1·95 (SE 0·2) g/kg ($P < 0.05$) respectively.

Protein kinase C isozymes in fetal muscle

Plantaris muscles from fetal lambs on each dietary treatment were examined by Western immunoblotting. This showed the presence of five PKC isoforms (with approximate molecular masses), PKC-μ (115 kDa), α (81 kDa), ε (88 kDa), θ (79 kDa) and ζ (74 kDa). PKC-ζ (Fig. 2(a)) and PKC-θ (Fig. 2(b)) appeared exclusively in the cytosol whilst PKC-ε was mainly membrane-associated (Fig. 2(c)). PKC-μ was distributed in approximately equal amounts between the two subcellular fractions (Fig. 2(d)). In duplicate blots of each of these isoforms no intake-dependent changes in cellular location were observed. PKC-α was found in both protein fractions, three animals were examined for this isoform and duplicate blots are shown (Fig. 2(e)). In contrast to the other isoforms, an intake-dependent change was observed. PKC-α was more

Table 1. Weight (g) of maternal and fetal muscles from ewes fed on moderate (low) or high intakes of the same diet†

(Mean values with their standard errors for four observations)

Intake...	Low		High	
	Mean	SE	Mean	SE
Maternal muscles				
vastus	381	18	468*	14
gastrocnemius	121	10	149	12
plantaris	46·6	2·3	57·4*	3·7
soleus	2·0	0·2	3·2*	0·3
Fetal muscles				
gastrocnemius	3·55	0·25	3·05	0·20
plantaris	1·24	0·05	0·95	0·14
tibialis anterior	1·76	0·09	1·38*	0·18

Mean values were significantly different from those for the lower intake, *$P < 0.05$ (Student's *t* test).

† Mean values for left and right limb muscle from each animal are presented, except for maternal vastus where muscle from only one limb was dissected.

Table 2. Protein, RNA and DNA contents of maternal plantaris and soleus muscles from ewes fed at low or high intakes of the same diet (Mean values with their standard errors for four observations)

Muscle...	Plantaris				Soleus			
Intake...	Low		High		Low		High	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Protein (g)	7·89	0·46	9·78*	0·63	0·19	0·03	0·33**	0·14
RNA (mg)	40·2	2·9	52·1*	2·2	1·53	0·22	2·45*	0·30
DNA (mg)	19·6	1·8	22·8	1·1	0·30	0·02	0·39	0·03

Mean values were significantly different from the corresponding values in the lower intake ewes: $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ (Student's *t* test).

Table 3. Protein, RNA and DNA contents of fetal plantaris muscles from ewes fed at a low or high intake of the same diet

(Mean values with their standard errors for four observations)

Intake...	Low		High	
	Mean	SE	Mean	SE
Protein (g)	91·98	6·68	53·72*	11·05
RNA (mg)	2·20	0·17	1·57*	0·32
DNA (mg)	5·00	0·37	3·36*	1·79

Mean values were significantly different from those for the lower intake group, $^{*}P < 0{\cdot}05$ (Student's *t* test).

abundant in the cytosol than in the membrane-bound extract in the slower growing fetuses of the high-intake ewes. In the more rapidly growing fetuses of the ewes fed at lower intakes, the amounts of PKC-α in the cytosol and membrane were similar.

Discussion

Dietary-induced changes in maternal and fetal muscle growth were examined in adolescent ewes fed at two levels of nutrition. The adolescent ewe was chosen because of the particular susceptibility of adolescent animals to produce smaller offspring than normal. This has been observed in man (Scholl & Hediger, 1993) and in the rat (Palmer *et al.* 1996). In a previous study using the adolescent ewe, mean birth weights at term were reduced from 4·34 kg in the normally growing dam, to 2·74 kg in the rapidly growing mother on a high plane of nutrition (Wallace *et al.* 1996). The differences observed here, approximately 40 d before parturition, were much smaller, but some reduction in fetal muscle growth in the rapidly growing ewes was evident even at this earlier stage of pregnancy.

Ewes fed at a high plane of nutrition were almost 60 % heavier than those fed at lower intakes. The body condition score, which has been described as an acceptable estimate of the proportion of fat in a live animal (Russel *et al.* 1969) was greater in the animals fed at the higher intakes, suggesting that fat deposition may have accounted for much of this increase in weight. The increase in muscle mass was smaller, typically about 23 %. The fact that maternal muscles of ewes fed at the higher intake were larger in absolute terms, but represented a smaller proportion of total body weight, is a further indication of the increased fatness in these animals. The exception was the maternal soleus, where the difference in weight was almost 60 %, similar to the weight increase of the whole animal. The soleus muscle, however, is vestigial in the sheep, being only approximately 4 % of the size of the plantaris, and changes in such a small muscle cannot be considered to be of great relevance to the whole body. Changes in much larger muscles such as the vastus, which represents up to 0·75 % of the total body weight are likely to be more representative of changes in whole body musculature. The increases observed in maternal muscle appear to involve some degree of hyperplasia, since there was an increase in DNA in the muscle of high-intake ewes. However, this was not significant and was smaller than the changes in RNA and protein, indicating that some hypertrophy of the muscles had also occurred. Unlike the changes in the mother, those in the plantaris of fetuses from high-intake ewes appeared to be largely due to hypoplasia, since the decreases in the RNA and DNA contents were proportionately similar.

It was not possible to examine identical muscles in the mother and the fetal lamb. The vastus muscle was damaged in the rapid initial dissection of the lamb to enable removal and storage of major organs. Also, the soleus was too small to be dissected accurately in the fetus and therefore the tibialis anterior was chosen instead. Taken together, data from the four maternal and three fetal muscles suggest that a high intake appears to favour skeletal muscle growth in the dam, whilst compromising the growth of the fetus, since the three fetal muscles were 17–32 % smaller in the fetuses of the larger, high intake ewes.

The onset of a catabolic state as pregnancy progresses (Naismith, 1969), resulting in the loss of maternal muscle protein, must involve a change in the balance between protein synthesis and degradation. Previous studies in the

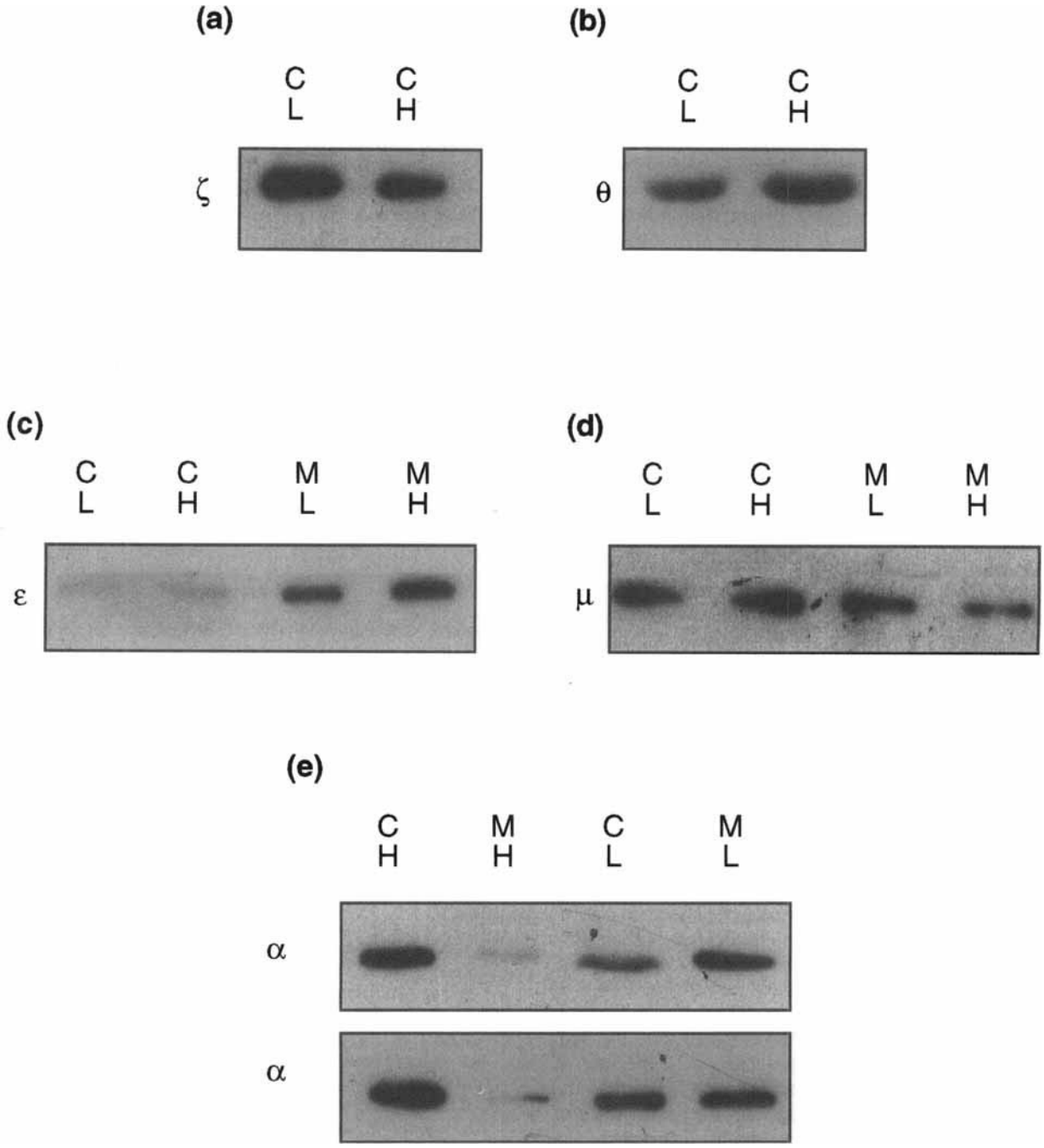

Fig. 2. Western blots of proteins from 104 d fetal plantaris muscle. The figure shows cytosolic (C) and membrane-bound (M) protein extracts from plantaris muscles from fetuses of ewes fed at a high (H) or lower (L) level of nutrition. (a) Protein kinase C (PKC)-ζ; (b) PKC-θ; (c) PKC-ε; (d) PKC-μ; (e) PKC-α shown in duplicate.

rat have shown the importance of maternal hormone levels by demonstrating that a decrease in maternal growth hormone and insulin-like growth factor-I in the circulation of the mother is associated with a loss of maternal muscle and larger fetuses (Palmer *et al.* 1996). The intracellular mechanism by which alterations in the growth rate of the mother and her fetus occurs has not been investigated in detail and the final objective of the present study was to examine a part of this intracellular signalling cascade. A mechanism by which very-short-term events such as diacylglycerol and inositol phosphate release can ultimately lead to sustained cellular responses such as growth and differentation has recently been proposed (Nishizuka, 1995). Central to these events are the PKC isoforms, which are amenable to measurement in the whole animal, unlike some of the membrane phospholipid metabolites whose changes are very rapid and transient.

Whilst in an inactive state, most PKC isoforms are located in the cytosol; translocation of PKC to the membrane is believed to convert the enzyme to an active

form; thus the localization of the PKC provides important clues as to its activity (Kraft & Anderson, 1983; Bell, 1986). A recent study (Godson *et al.* 1996) has shown that activated PKC-α is concentrated in highly specific sites around the periphery of the cell. Of the numerous studies of PKC, a few have involved investigation of physiological events affecting skeletal muscle. Zappelli *et al.* (1996) have demonstrated that selective expression of PKC-θ is part of the mechanism of fetal myoblast differentiation induced by transforming growth factor-β. Hong *et al.* (1995) found three PKC isoforms, α, δ and ζ in cultured L8 rat muscle cells. Yamada *et al.* (1995) detected PKC-α, β, ε and θ in muscles of the rat, and demonstrated that translocation of all these isoforms was induced by high concentrations of insulin in soleus and gastrocnemius muscle. An increase in membrane bound PKC-α, β, ε and δ, with a corresponding decrease in the amount in the cytosol and an increase in activity (diacylglycerol release) in diabetic rats has also been reported recently (Avignon *et al.* 1996). The authors suggest that this persistent translocation may be associated with insulin resistance. The antibodies used here have been employed in a previous study in L6 myoblasts in which six isoforms (PKC-α, μ, ε, δ, ι and ζ) were detected (Thompson *et al.* 1997). The present study has identified five isoforms, PKC-α, μ, ε, θ and ζ; thus there is considerable consensus as to the isoforms present in skeletal muscle. Importantly, all these studies agree that the classical Ca^{2+}-, diacylglycerol- and phospholipid-dependent PKC-α is present.

In addition to the growing body of information concerning the presence and location of the many isoforms in different tissues, numerous substrates which are phosphorylated by PKC have now been identified (for recent reviews, see Liu, 1996; Jaken, 1996). However, to date few studies have linked one specific isoform to its natural substrate. One or more of a group of three isoforms, PKC-α, δ and ε, have been proposed as possible mediators of translational control in L6 myoblasts (Thompson *et al.* 1997). One of these, PKC-δ, is either absent or was undetected by the antibody in the present study, but the other two isoforms were detected and one of these, PKC-α, appears to a greater extent in the membrane in a situation where fetal muscle growth and development are affected. Whether this event is related to a change in the rate of protein synthesis is as yet unknown, indeed whether the alteration in fetal growth relates to a change in the rates of protein synthesis, degradation or both, has yet to be examined. However, the observation that inhibition of PKC-α expression inhibits the release of arachidonic acid (Godson *et al.* 1993), coupled with the much earlier demonstration that prostaglandin-$F_2\alpha$, a metabolite of arachidonic acid, is involved in the stimulation of translation by insulin (Reeds & Palmer, 1983) suggests the possibility of involvement of this PKC isoform in the control of protein synthesis. If this is the case, there are several possible mechanisms in which a PKC isoform, possibly PKC-α, could participate as part of the signal transduction cascade. The first is by action on ribosomal protein S6; two S6 kinases which are part of the insulin-activated protein kinase cascade have been identified in rat skeletal muscle (Hei *et al.* 1993). Second, the mechanism may involve the mRNA cap binding protein, eIF-4E, which is thought to be a limiting factor in the control of initiation and whose activity is controlled by the translational regulator, phosphorylated heat- and acid-stable protein-1 (PHAS-1). When unphosphorylated, PHAS-1 binds tightly to eIF-4E and inhibits translation (Pause *et al.* 1994). On phosphorylation, following administration of insulin and other growth factors, eIF-4E is dissociated from PHAS-1 and is capable of initiating translation (Hei *et al.* 1994). The third possibility is an inhibitory mechanism, involving either the down-regulation of protein synthesis by the phosphorylation of eIF-2-α (de Haro *et al.* 1996), or blocking the insulin-induced autophosphorylation of the β-subunit of the insulin receptor (Chin *et al.* 1993), a known consequence of overexpression of PKC-α. These possibilities await further investigation.

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