Effects of calcium ionophore on vitamin E-deficient rat muscle

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Damage to skeletal muscles may be mediated via free radicals or intracellular calcium overload. To look for inter-relationships between these pathways we have examined the effect of intracellular Ca overload on muscles from rats fed on either a vitamin E-deficient or vitamin E-sufficient diet and assessed the non-enzymic lipid peroxidation in these muscles by examining the production of thiobarbituric acid reactive substances by homogenates. Vitamin E-deficient muscles were more susceptible to Ca-induced intracellular enzyme efflux and this was acutely corrected by supplementation of the external medium with 230 μmol α-tocopherol/l. Vitamin E-deficient muscles showed increased levels of basal lipid peroxides and were more susceptible to iron-catalysed lipid peroxidation. Addition of the Ca ionophore A23187 increased lipid peroxidation in vitamin E-deficient muscle homogenates, but had the opposite effect in vitamin E-sufficient muscles. These results demonstrate that vitamin E-deficient muscle has an increased susceptibility to intracellular Ca overload, but that this effect cannot be explained by a direct stimulatory effect of the ionophore on non-enzymic lipid peroxidation.

Calcium ionophore: Vitamin E deficiency: Muscle: Rat

Nutritional myodegeneration (also known as nutritional myopathy, nutritional muscular dystrophy and white muscle disease) occurs in animals maintained on vitamin E-deficient diets (Allen *et al.* 1974) and may be precipitated by stress factors such as sudden increase in exercise, exposure to inclement weather and also erratic feeding and pregnancy (Gitter *et al.* 1978). Myopathy may also be precipitated in deficient animals by feeding them on diets rich in polyunsaturated fatty acids (McMurray *et al.* 1980). Skeletal muscle in affected animals shows characteristic whitish-yellow lesions predominantly involving type 1 fibres.

The most widely quoted function of vitamin E in vivo is as an antioxidant acting to scavenge lipid-soluble free radical species (Tappel, 1962) and it therefore seems reasonable to assume that muscle damage in vitamin E-deficient animals is due to increased free radical activity. Such a mechanism is supported by the work of Patterson & Allen (1972) who found that vitamin E-deficient piglets were highly susceptible to iron-induced myo-degeneration. In other situations skeletal muscle damage has been suggested to be mediated by calcium and Wrogemann & Pena (1976) suggested that the basis of a final common pathogenic mechanism for muscle necrosis in a wide variety of muscle disorders is an increased net influx of Ca ions. Jones *et al.* (1984) have also demonstrated that external Ca is an important factor involved in the damage leading to enzyme release from normal skeletal muscle in vitro and that there is a dramatic increase in total muscle Ca during damaging protocols (Claremont *et al.* 1984). These results suggest that in some situations damage to skeletal muscle is mediated by an increase in intracellular Ca concentration which then initiates further pathological changes. Inhibitor studies have helped elucidate the series of processes involved (Jackson *et al.* 1984, 1987) and a scheme for the biochemical mechanisms underlying muscle damage has been proposed (Jackson *et al.* 1985*a*).

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In order to investigate whether such mechanisms occur in vitamin E-deficient muscles we have studied the effect of increased intracellular Ca concentration (induced by the Ca ionophore A23187) on vitamin E-deficient muscles.

EXPERIMENTAL

Nutritional manipulation of animals

Weanling female Wistar rats were fed on a Hoffman-La Roche vitamin E-deficient diet (no. 814) obtained from Dyets Inc. (Pennsylvania, USA) for 16–20 weeks. Control rats were fed on the same diet supplemented with 100 μg α-tocopheryl acetate/g. Efficacy of the diet was confirmed at death by plasma and tissue vitamin E analysis. The vitamin E-deficient diet had no significant effect on the weights of the rats, or on the muscles used in the incubation experiments.

Plasma and tissue vitamin E analysis

Plasma levels of vitamin E were analysed using the high-performance liquid chromatography (HPLC) method described by Catignani & Bieri (1983) modified by reconstitution of the sample in hexane and use of methanol–acetonitrile–chloroform (47:47:6, by vol.) as the mobile phase. The equipment used was a Shimadzu SPD – 6A HPLC with u.v. detection at 292 nm and sensitivity set at 0·002 A. A spherisorb 5 OD S-2 250 mm × 4·6 mm reverse-phase column was used. Tissues were homogenized on ice in 3·5 ml Tris–hydrochloric acid, pH 7·4 (containing (mmol/l): Tris–HCl, 10; sodium chloride 150; dithioerythreitol 1, EDTA, 1). Homogenate (2 ml) was extracted for 2 min on a vortex mixer with 3·5 ml hexane–propan-2-ol (3:2, v/v) containing 0·25 g butylated hydroxy-toluene/l. Tubes were centrifuged at 4° for 5 min at 1000 rev/min. The hexane phase was removed, concentrated and reconstituted as described previously.

Muscle incubation studies

Rats were killed by intoxification via an intraperitoneal injection of Nembutal and the soleus muscles were carefully and rapidly removed. The muscles were mounted in special holders and incubated in 4 ml bicarbonate-buffered mammalian Ringer solution at 37° as previously described (Jones et al. 1983). After 30 min pre-incubation period, muscles were treated for 30 min with the Ca ionophore A23187 (20 μmol/l) solubilized in ethanol. The medium was then replaced and renewed every 30 min for a period of 2 h. The creatine kinase (EC 2.7.3.2; CK) activity of the incubation media was assayed as previously described (Jones et al. 1983). The effect of α-tocopherol (230 μmol/l) in this system was investigated by dissolving in ethanol and adding to the incubation medium throughout the experiment. An equal amount of ethanol (10 ml) was added to the medium surrounding control muscles and in all cases one of the pair of muscles from each animal provided the control tissue.

Assay of CK activities

CK activities were assayed as previously described (Jones et al. 1983) in plasma (20 μl), diluted muscle homogenate (20 μl) and incubation fluid (50 μl). Muscles (quadriceps) were homogenized in Tris buffer, pH 7·3 (containing (mmol/l): Tris, 50; disodium EDTA, 100; β-mercaptoethanol, 10).

Plasma pyruvate kinase (EC 2.7.1.40) levels were also measured using an enzyme-linked assay following the reduction of NADH to NAD$^+$ with a corresponding decrease in absorbance at 334 nm (Fujii & Miwa, 1983).

Table 1. *Plasma, muscle and liver vitamin E analysis of rats receiving vitamin E-deficient and vitamin E-sufficient diets**

(Mean values with their standard errors for ten rats)

	Plasma (μg/ml)		Gastrocnemius (μg/g wet wt)		Quadriceps (μg/g wet wt)		Liver (μg/g wet wt)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Vitamin E-sufficient	14·9	0·9	25·6	1·7	20·9	1·6	197·6	16
Vitamin E-deficient	ND		1·3	0·7	0·4	0·1	27·9	6

* For details, see p. 246.
ND, none detectable.

Muscle cation content

At the end of the incubation experiments, soleus muscles were freeze-dried and analysed for Ca, magnesium, potassium and sodium ion contents as described previously (Jackson *et al.* 1985*b*).

Analysis of prostaglandin E_2 (PGE$_2$) content of muscle incubation media and plasma

Eluents from the muscle incubation studies were immediately frozen and stored at $-20°$ before analysis for PGE$_2$ content as previously described (Phoenix *et al.* 1989).

Non-enzymic lipid peroxidation

The susceptibility of the gastrocnemius muscles from the rats of differing vitamin E status to lipid peroxidation was investigated as previously described (Jackson *et al.* 1983*a*), but with the addition of 1 mmol EDTA/l to the reaction mixture. The effects of boiling, increasing Ca (calcium chloride) or of increasing Ca ionophore A23187 concentration were investigated in both vitamin E-deficient and vitamin E-sufficient gastrocnemius muscle homogenates. Experiments investigating concentration dependence were carried out on the same day.

Statistics

All results are expressed as means with their standard errors, and statistical significance of results was assessed using Student's unpaired t test with $P > 0.05$ being considered non-significant.

RESULTS
Vitamin E status of animals

There were no overt signs of vitamin E deficiency but analysis of plasma, gastrocnemius and quadriceps muscles and liver indicated that the diet used was extremely effective in producing deficiency in the rats (Table 1). Plasma pyruvate kinase levels were also grossly elevated in vitamin E-deficient rats but there was no significant difference in plasma CK levels ($P = 0.33$) (Table 2). Analysis of muscle CK levels in quadriceps revealed a significantly greater CK content in vitamin E-deficient muscle ($P = 0.0012$) with approximately 1·5 times the level found in vitamin E-sufficient muscle.

Effect of vitamin E status on response to stimulation with Ca ionophore

Addition of the Ca ionophore A23187 (20 μmol/l) to the incubation media produced the expected rise in efflux of intracellular CK. There was no significant difference in the resting

Table 2. *Plasma and muscle enzyme activities in rats receiving vitamin E-deficient and vitamin E-sufficient diets*†

(Mean values with their standard errors for ten rats)

	Plasma CK (units/l)		Muscle CK (units/g wet wt)		Plasma PK (units/l)	
	Mean	SE	Mean	SE	Mean	SE
Vitamin E-sufficient	139·29	23·5	2068·47	140·9	133·93	13·3
Vitamin E-deficient	170·8	20·5	3042·2*	203·1	1589·69**	281·5

CK, creatine kinase (*EC* 2.7.3.2); PK, pyruvate kinase (*EC* 2.7.1.40).
Mean values were significantly different from those for vitamin E-sufficient: * $P < 0.05$, ** $P < 0.01$.
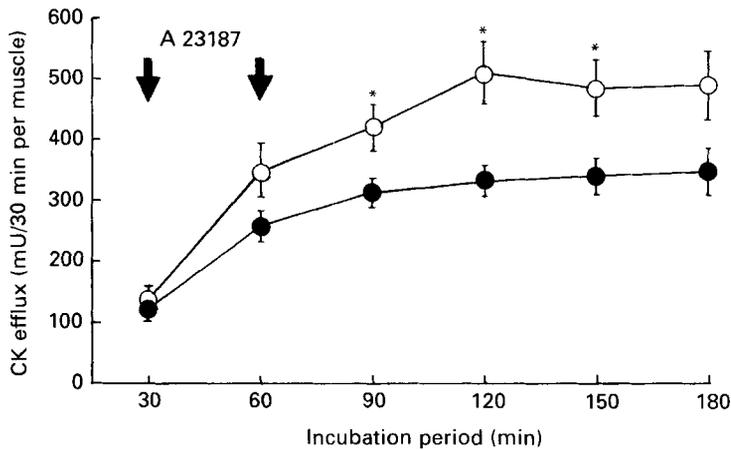† For details, see p. 246.

Fig. 1. Creatine kinase (*EC* 2.7.3.2; CK) efflux from soleus muscles of rats fed on either vitamin E-deficient (○) or vitamin E-sufficient (●) diets. Muscles were treated with the calcium ionophore A23187 (20 μmol/l) for 30 min. Points are means, with their standard errors represented by verical bars, for twelve muscles. * Mean values were significantly different from vitamin E-sufficient values ($P < 0.05$). For details of diets and procedures, see pp. 246–247.

levels of enzyme efflux from vitamin E-deficient and vitamin E-sufficient muscles, but after treatment with ionophore the deficient muscles exhibited significantly greater enzyme efflux than the sufficient muscles (Fig. 1).

Vitamin E status of the soleus muscles used in the incubation studies did not influence the rise in total Ca in response to ionophore treatment. Fresh rat soleus muscles have a total Ca content of approximately 4·5 μmol/g dry weight, and all muscles analysed after Ca ionophore treatment have values significantly higher than this (Phoenix *et al.* 1989). There was no significant difference in the concentration of any of the cations analysed in the deficient and sufficient muscles (Table 3).

To investigate whether the greater enzyme efflux from vitamin E-deficient muscles could be reduced by supplementing with extracellular vitamin E, 230 μmol α-tocopherol/l were added to the incubation medium (Fig. 2). This protected against the Ca-induced damage, with α-tocopherol-treated muscles having significantly lower enzyme efflux at all sampling points from treatment with ionophore to the end of the incubation 2 h later. The same effect

Table 3. *Cation concentration in calcium ionophore A23187-treated soleus muscle of rats receiving vitamin E-deficient and vitamin E-sufficient diets**

(Mean values with their standard errors for eight rats)

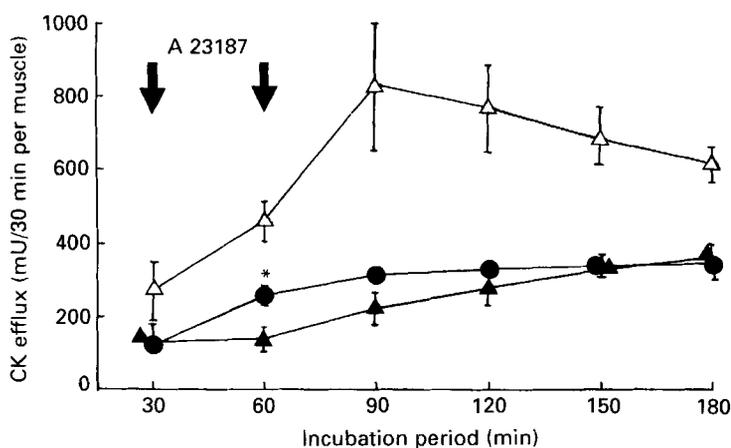| | Cation concentration (μM/g dry wt) | | | | | | | |
| | Calcium | | Magnesium | | Potassium | | Sodium | |
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Vitamin E-sufficient	11·4	0·2	50·7	6·7	289·5	12·7	389·9	56·1
Vitamin E-deficient	13·0	0·9	35·4	1·7	287·8	12·8	417·3	24·8

* For details, see pp. 246–247.

Fig. 2. Creatine kinase (*EC* 2.7.3.2; CK) efflux from soleus muscles of rats fed on a vitamin E-deficient diet, treated for 30 min with 20 μmol calcium ionophore A23187/l in the presence (▲) and absence (△) of 230 μmol α-tocopherol/l. Control muscles had an equivalent volume of ethanol added to the incubation medium. The response of vitamin E-sufficient soleus muscles to ionophore A23187 (●) without added ethanol has been included to allow comparisons to be drawn. *Mean value was significantly different from that obtained in vitamin E-deficient muscles in the presence of α-tocopherol ($P = 0.023$). Points are means, with their standard errors represented by vertical bars, for four muscles. For details of diets and procedures, see pp. 246–247.

was observed with vitamin E-sufficient muscle. Comparison of the data from vitamin E-sufficient muscles treated with A23187 with that from vitamin E-deficient muscles treated in the presence of 230 μmol α-tocopherol/l revealed that the addition of extracellular vitamin E to deficient muscles results in a response to Ca ionophore which is comparable to that of vitamin E-sufficient muscle (Fig. 2), with the results only being significantly different ($P = 0.023$) immediately after ionophore treatment.

During these experiments, it was observed that ethanol, present in the incubation medium at 42·9 mmol/l, appeared to exacerbate enzyme efflux and this exacerbation, compared with muscles incubated in the absence of ethanol, became significant during the final 90 min of an experiment. However, the efficacy of extracellular α-tocopherol at inhibiting enzyme efflux was further demonstrated as it overcame both the ethanol effect and the ionophore-induced enzyme efflux from vitamin E-sufficient muscles (Fig. 3).
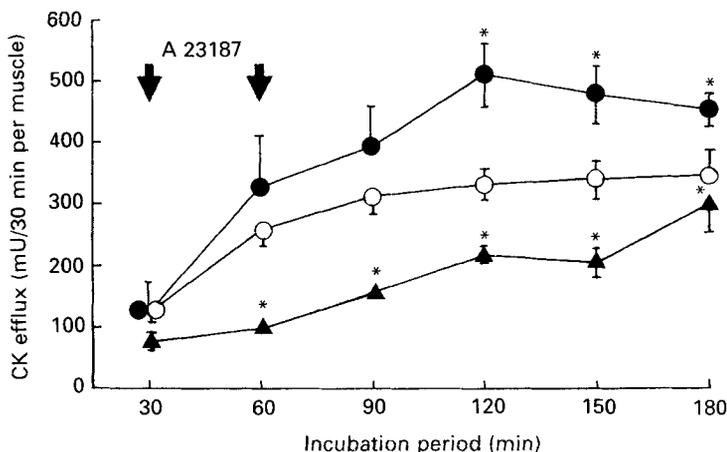
Fig. 3. The effect of ethanol on creatine kinase (EC 2.7.3.2; CK) efflux from calcium ionophore A23187 (23 μmol/l)-stimulated soleus muscles from rats fed on a vitamin E-sufficient diet. ($\bigcirc$), Vitamin E-sufficient muscle without additions; ($\bullet$), sufficient muscle + 10 μl ethanol; ($\blacktriangle$), sufficient muscle + 230 μmol α-tocopherol/l. Points are means, with their standard errors represented by vertical bars, for four muscles. * Mean values were significantly different from those of vitamin E-sufficient muscle without additions ($P < 0.05$). For details of diets and procedures, see pp. 246–247.

Table 4. *Effect of vitamin E deficiency on prostaglandin E_2 (PGE_2) content of rat plasma*

(Mean values with their standard errors for ten rats)

	PGE_2 (pg/ml)	
	Mean	SE
Vitamin E-sufficient	229	32
Vitamin E-deficient	258	36

Effect of vitamin E deficiency on PGE_2 content of plasma and efflux from incubated muscles

Vitamin E status had no effect on circulating PGE_2 levels (Table 4). Similarly, there was no significant difference in the PGE_2 release from incubated deficient and sufficient muscles stimulated with ionophore A23187 (Fig. 4) at all time points except immediately after ionophore stimulation ($P = 0.038$) when the vitamin E-sufficient muscles released more PGE_2.

Non-enzymic lipid peroxidation in skeletal muscle homogenates of differing vitamin E status

To investigate whether increased enzyme efflux from vitamin E-deficient muscles was associated with increased lipid peroxidation, the content of thiobarbituric acid-reacting substances (TBARS) in muscle homogenates was evaluated.

Basal content of TBARS in homogenates was significantly higher in vitamin E-deficient muscles ($P = 0.0001$), and incubation for 2 h at 37° increased the production of TBARS in deficient muscles compared with the increase found in sufficient muscle (Fig. 5).
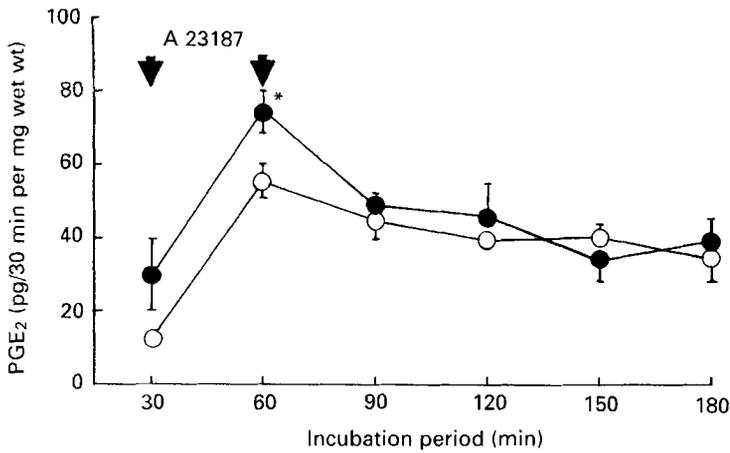
Fig. 4. Prostaglandin E_2 (PGE$_2$) efflux from soleus muscles of rats fed on either vitamin E-deficient (○) or vitamin E-sufficient (●) diets. Muscles were treated with calcium ionophore A23187 (20 μmol/l) for 30 min. Points are means, with their standard errors represented by vertical bars, for four muscles. Samples were assayed in duplicate. * Mean value was significantly different from vitamin E-deficient value ($P = 0.038$). For details of diets and procedures, see pp. 246–247.
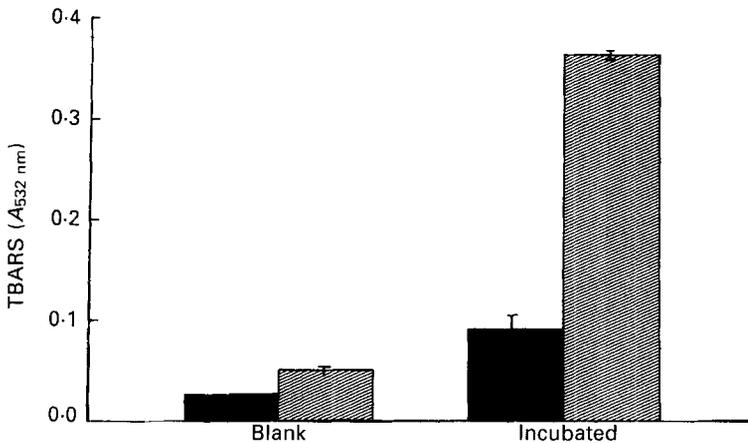
Fig. 5. Production of thiobarbituric acid-reactive substances (TBARS) in vitamin E-deficient (▨) and vitamin E-sufficient (■) gastrocnemius muscle homogenates. Blank values indicate TBARS measured in non-incubated sample. Values shown are the means, with their standard errors represented by vertical bars, for sixteen analyses. For details of procedures, see pp. 246 247.

Addition of increasing amounts of A23187 to vitamin E-sufficient muscle homogenates resulted in a decrease ($P < 0.05$) in TBARS production compared with non-ionophore A23187-treated homogenates. However, addition of ionophore A23187 to deficient-muscle homogenates produced the opposite effect, with an increase in TBARS, which became significantly greater with each increase in concentration of ionophore A23187 (Fig. 6).

To investigate whether these effects of the ionophore A23187 were mimicked by Ca, differing concentrations of CaCl$_2$ were incorporated in the assay. This had no significant
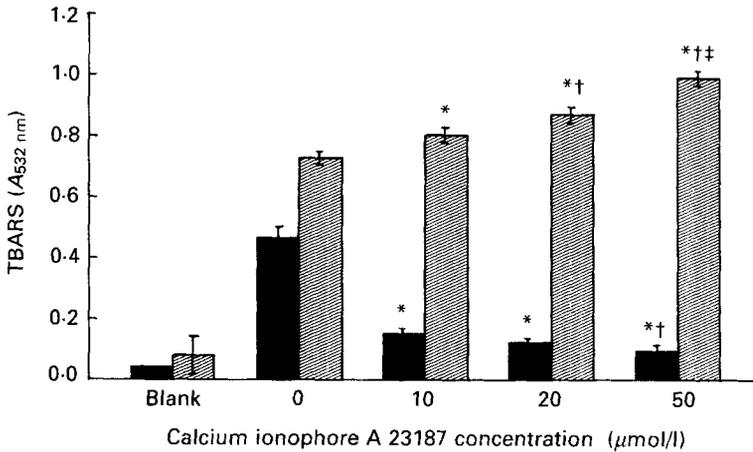
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Fig. 6. Effect of increasing calcium ionophore A23187 concentration on production of thiobarbituric acid-reactive substances (TBARS) in vitamin E-deficient (▨) and vitamin E-sufficient (■) gastrocnemius muscle homogenates. Values shown are the means, with their standard errors represented by vertical bars, for twenty-four analyses. * Mean values were significantly different from the corresponding value in the absence of Ca ionophore A23187 (0 μmol/l) ($P < 0.05$). † Mean values were significantly different from those for 10 μmol A23187/l ($P < 0.05$). ‡ Mean values were significantly different from those for 20 μmol Ca ionophore A23187/l ($P < 0.01$). For details of procedures, see pp. 246–247.

effect on vitamin E-sufficient muscle and showed no consistent trend with vitamin E-deficient muscle, although at 500 μmol $CaCl_2$/l production of TBARS was significantly reduced compared with normal ($P = 0.036$) (Fig. 7). This was further reduced at higher concentrations (5 mmol/l) of $CaCl_2$ (results not shown) and it is thought that this effect may be due to the formation of insoluble Ca soaps at high concentrations.

To investigate the possibility that the effects of the ionophore on production of TBARS in muscle were mediated by an enzymic process, the homogenates were boiled before incubation. This significantly reduced TBARS production in both muscle types ($P < 0.001$) although the TBARS production in vitamin E-deficient muscles was still greatly elevated ($A_{532\,nm}$ boiled 0.304 (SE 0.007); $A_{532\,nm}$ normal 0.364 (SE 0.005)), the production in vitamin E-sufficient muscle was reduced to a level not significantly different from control levels ($P = 0.063$).

DISCUSSION

It appears that there is an increased susceptibility of vitamin E-deficient muscles to Ca-induced enzyme efflux. Although the increased muscle CK content of deficient muscles suggests this may be the underlying cause to the greater enzyme efflux, the fact that there was no significant difference in either plasma CK activity or initial resting enzyme efflux from incubated deficient and sufficient muscles suggests that the muscle CK content is unrelated to Ca-induced enzyme efflux. The findings presented in Fig. 2 also indicate that deficiency of vitamin E is responsible, since the increased release of CK is acutely corrected by the presence of extracellular α-tocopherol in the incubation medium. Rapid replenishment of vitamin E has also been demonstrated in erythrocytes from deficient animals (Nakamura & Masugi, 1979).

To investigate non-enzymic lipid peroxidation in vitamin E-deficient and vitamin E-sufficient muscles, the TBARS assay was used. It is known that the TBARS test is an indirect method of measuring lipid peroxidation since many other decomposition products
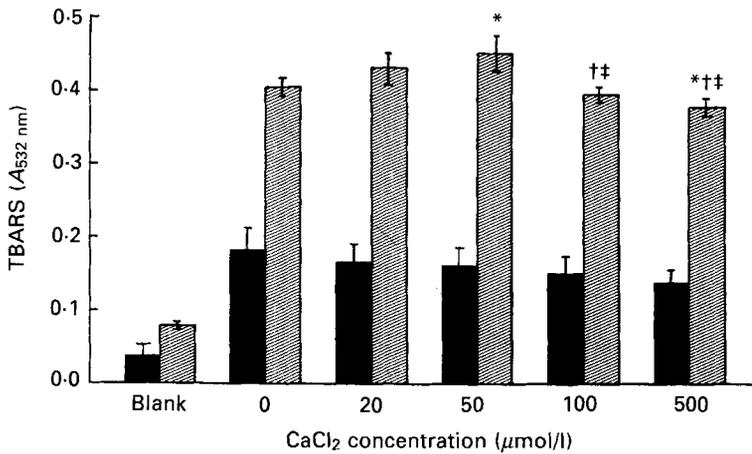
Fig. 7. Effect of increasing calcium chloride concentration on thiobarbituric acid-reactive substances (TBARS) production in vitamin E-deficient (▨) and vitamin E-sufficient (■) gastrocnemius muscle homogenates. Values shown are the means, with their standard errors represented by vertical bars, for sixteen analyses. * Mean values were significantly different from the corresponding value in the absence of $CaCl_2$ (0 μmol/l) ($P < 0.05$). † Mean values were significantly different from those for 20 μmol $CaCl_2$/l ($P < 0.05$). ‡ Mean values were significantly different from those for 50 μmol $CaCl_2$/l ($P < 0.001$). For details of procedures, see pp. 246–247.

apart from malondialdehyde (MDA) are produced that will react with thiobarbituric acid (TBA), but it does provide a semi-quantitative guide to free-radical mediated damage when used in a closed in vitro system such as that used in the present series of experiments. Lee & Csallany (1987), utilizing a HPLC technique (Csallany *et al.* 1984), found that conventional TBA test results were much higher than free MDA levels, although the same trends were found with both methods. When they investigated rats of differing vitamin E status, they found significantly higher free MDA levels in the livers of vitamin E-deficient rats compared with vitamin E-sufficient rats. The findings presented here also indicate significantly higher basal concentrations of TBARS in vitamin E-deficient gastrocnemius compared with sufficient tissue. This is in contrast to findings of McMurray & Dormandy (1974) who failed to find any relationship between MDA production in human skeletal muscle homogenates and vitamin E content over a narrow range, and also Jackson *et al.* (1983*b*) working with rats, although the discrepancy in the latter case is probably due to the greater efficacy of the vitamin E-deficient diet used in the present study.

Ca has been reported to enhance free radical-induced damage (Braughler *et al.* 1985) and has been shown to increase the sensitivity of erythrocytes to lipid peroxidation (Jain & Shohet, 1981). To demonstrate such a link in the present case, ionophore A23187 needs to be demonstrated as being capable of stimulating lipid peroxidation in both vitamin E-deficient and vitamin E-sufficient muscle since it increases enzyme efflux in both types. However, when this was investigated (Fig. 6) conflicting patterns were observed. Although ionophore A23187 did stimulate lipid peroxidation in deficient muscles, the opposite effect was seen in sufficient muscles.

Results on boiling muscle homogenates suggest that effects on lipid peroxidation in vitamin E-sufficient muscle may be due to an enzyme-mediated pathway, whilst the main effect in deficient muscle may be due to non-enzymic lipid peroxidation. From these findings, a link between free radical damage and Ca does seem apparent in vitamin E-deficient muscles. The failure of direct supplementation with Ca in the assay to promote

lipid peroxidation in deficient homogenates may be due to inadequate availability. It may be that the ionophore is required to present the Ca in a utilizable form to some site in the homogenate.

If Ca is the mediator of damage in the system investigated here and not free radicals, then what is the protective mechanism provided by vitamin E? Table 3 illustrates that the initial rise in total cell Ca is unaffected by vitamin E status. Ca is required in the activation of phospholipase A_2 (EC 3.1.1.4), which has been implicated in the processes of enzyme leakage from damaged muscle (Jackson et al. 1984, 1985a) by its action on membranes to produce free fatty acids (including arachidonic acid) which then act as substrates for cyclo-oxygenase (EC 1.14.99.1) and lipoxygenase (EC 1.13.11.12) enzymes. Vitamin E has been shown to inhibit purified phospholipase A_2 (Douglas et al. 1986) and also to inhibit PGE_2 synthesis in rat blood (Hope et al. 1975) and brain (Meydani et al. 1985). However, in the present study no effect of vitamin E on PGE_2 production in vitro or circulating levels in vivo could be demonstrated (Table 4, Fig. 4). The lipoxygenase pathway leads to the production of membrane-damaging leukotrienes, and inhibition of lipoxygenase enzymes by α-tocopherol has been well-documented in some cell types (Grossman & Waksman, 1984; Reddanna et al. 1985), although the localization of lipoxygenase to the cytosolic fraction of cells (Nugteren, 1975; Narumiya et al. 1981; Ruzicka et al. 1983; Morgan et al. 1984) makes an inhibition of this enzyme an unlikely mode of action for the lipophilic α-tocopherol molecule in skeletal muscle damage. The results of the present study suggest that the proposed mechanisms of damage (Jackson et al. 1985a) are incomplete when one considers the importance of vitamin E.

Recent work has shown that extracellular α-tocopherol is protective against Ca-induced enzyme efflux in normal rat skeletal muscle (Phoenix et al. 1989) and that this may be due to a non-antioxidant function of the molecule.

The reason for the increased CK efflux caused by ethanol is unclear, but effects of ethanol have been extensively studied in liver and it has been associated with increased activity of free radicals and production of lipid peroxides (Litov et al. 1981; Fink et al. 1985). Ethanol has also been reported to reduce hepatocyte α-tocopherol content (Bjorneboe et al. 1987a, b). In the present studies (values not presented in detail) ethanol did not act to promote lipid peroxidation in skeletal muscle homogenates. It is possible that the effects seen in the incubation studies are due to a 'leaching' effect of the ethanol on the tissue α-tocopherol content, which again is acutely corrected by the addition of extracellular α-tocopherol (Fig. 2), although other mechanisms are possible.

In conclusion, vitamin E-deficient muscle is more susceptible to Ca-induced enzyme efflux using the Ca ionophore A23187 and is more susceptible to lipid peroxidation in vitro. Experiments to determine whether there is any link between action of ionophore and free radical activity revealed conflicting results with vitamin E-deficient and vitamin E-sufficient muscles. In speculation, a structural-membrane-stabilizing role for vitamin E in skeletal muscle damage may provide a possible explanation for the different responses observed in vitamin E-deficient and vitamin E-sufficient muscle.

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