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Inhibition of Ca^{2+}-induced cytosolic enzyme efflux from skeletal muscle by vitamin E and related compounds

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1. Efflux of an intracellular enzyme (creatine kinase) from normal rat skeletal muscles was induced by treatment with the Ca^{2+} ionophore A23187. Addition of α-tocopherol (230 μM) to the incubation medium was found to significantly diminish this efflux, and this effect was mimicked by α-tocopherol acetate, phytol and isophytol, but not by Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). 2. Analysis of muscle cation content has shown that these protective effects of α-tocopherol etc. are not due to an inhibition of the Ca^{2+} accumulating effects of the ionophore. 3. Non-enzymic lipid peroxidation of skeletal-muscle homogenates was found to be inhibited by α-tocopherol and Trolox C, partially inhibited by phytol and isophytol, but unaffected by α-tocopherol acetate. 4. The activity of lipoxygenase enzymes was partially inhibited by α-tocopherol, phytol and isophytol, but not by α-tocopherol acetate or Trolox C. 5. Prostaglandin E_2 efflux from isolated skeletal muscles was stimulated by treatment with the Ca^{2+} ionophore, but this was unaffected by α-tocopherol treatment.

INTRODUCTION

Skeletal-muscle damage occurs in a number of different physiological and pathological situations such as after excessive exercise and in patients with various degenerative neuromuscular diseases. This damage is associated with ultrastructural changes to the muscle and gross elevations of the activity of muscle-derived enzymes, such as creatine kinase (CK), in blood plasma. Various workers have studied the mechanisms underlying muscle damage (Dawson, 1966; Duncan, 1978; Publicover *et al.*, 1978; Rodemann *et al.*, 1981; Suarez-Kurtz, 1981; Jackson *et al.*, 1984, 1987), and previous studies from this group suggest that an accumulation of intracellular Ca^{2+} is a key event in the damaging processes (Jones *et al.*, 1984). Overt vitamin E deficiency results in a severe myopathy in many animal species, associated with changes in the muscle ultrastructure (Molenaar *et al.*, 1968) and high plasma CK activities (Anderson *et al.*, 1977). Vitamin E-depleted but morphologically normal animal muscles also show an increased release of intracellular enzymes induced by excessive contractile activity (Jackson *et al.*, 1983*a*), suggesting that vitamin E may be an important factor in the modulation of muscle damage after stress or exercise.

The aim of the present work was to examine the possibility that an increase in the extracellular vitamin E concentration could influence the response of skeletal muscles to damage induced by elevation of the intracellular Ca^{2+} content. Results obtained suggest that this indeed occurs, and the possible mechanisms of action of vitamin E have been explored.

EXPERIMENTAL

Muscle-incubation studies

Male Wistar rats (100–200 g) fed on a standard laboratory diet (CRM, Lab Sure, Cambridge, U.K.) *ad libitum* were killed by cervical dislocation and the soleus muscles carefully and rapidly removed. The muscles were mounted in special holders and incubated in 3.5 ml of bicarbonate-buffered mammalian Ringer solution maintained at 37 °C as previously described (Jones *et al.*, 1983). After a 30 min preincubation period, muscles were treated for 30 min with the Ca^{2+} ionophore A23187 (20 μM) solubilized in 100% ethanol. The medium was then replaced and renewed every 30 min for the next 2 h. The CK activity of the incubation media was assayed as previously described (Jones *et al.*, 1983), and all putative protective agents were tested for any direct inhibitory effects on the CK assay. In all cases one of the pair of muscles from each animal provided the control tissue. α-Tocopherol and related compounds were dissolved in 100% ethanol and added to the incubation media; an equal amount of ethanol (10 μl) was added to the media surrounding control muscles.

Analysis of muscle cation content

At the end of the incubation experiment, muscles were freeze-dried and analysed for Ca, Mg, K and Na content as previously described (Jackson *et al.*, 1985).

Non-enzymic lipid peroxidation

The effect of all compounds used in the muscle-incubation system on non-enzymic lipid peroxidation was investigated in autoxidizing mouse skeletal-muscle homogenates as previously described (Jackson *et al.*, 1983*b*), but with the addition of 1 mM-EDTA to the reaction mixture. This method involves the measurement of thiobarbituric acid-reactive substances (TBARS) as an index of lipid peroxidation. In brief, homogenates (2%, w/v) of fresh mouse skeletal muscle in 100 mM-potassium phosphate buffer, pH 7.4, were incubated with ascorbate (50 μM) and $FeSO_4$ (50 μM) at 37 °C in the absence or presence of tocopherol or other components. After 2 h the reaction was stopped and the TBARS

Abbreviations used: CK, creatine kinase; TBA(RS), thiobarbituric acid(-reactive substances); PG, prostaglandin; NDGA, nordihydroguaiaretic acid; Trolox C, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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measured by addition of an equivalent volume of trichloroacetic acid (0.61 M), thiobarbituric acid (55.5 mM) and disodium EDTA (1 mM) solution. The resultant mixture was heated at 100 °C for 12 min, cooled, and the TBARS/TBA chromagen extracted into butan-1-ol and the A_{532} read against appropriate blanks.

Lipoxygenase assay

Any possible inhibition of lipoxygenase enzyme activity by α-tocopherol and related compounds was investigated using purified soya-bean type 1 lipoxygenase (EC 1.13.11.12; Sigma Chemical Co.). Lipoxygenase activity was determined spectrophotometrically by monitoring the formation of conjugated dienes at 234 nm by the method of Ben Aziz *et al.* (1970), modified by the use of sodium deoxycholate in order to maintain optically clear solutions and by use of a phosphate buffer (Nishikimi *et al.*, 1980). Final concentrations in the cuvette (1.2 ml) were: linoleic acid, 2.5×10^{-4} M; sodium deoxycholate, 7.2×10^{-3} M; lipoxygenase, 800 units (all in 0.05 M-potassium phosphate buffer, pH 8.6). Compounds to be tested were solubilized in dimethyl sulphoxide and preincubated with the lipoxygenase for 10 min at 37 °C. The reaction was started by addition of the enzyme.

Analysis of the prostaglandin E_2(PGE_2) content of muscle incubation media

Eluents from the muscle-incubation studies were immediately frozen and stored at −20 °C. PGE_2 determination was undertaken within 1 month by competitive-binding radioimmunoassay using an antibody donated by Dr. F. Carey and Dr. R. A. Forder (ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, U.K.) and radioimmunoassay procedures described in detail elsewhere (Bailey *et al.*, 1983). The highest cross-reactivities of the PGE_2 antibody were observed with PGE_1 (36%), PGA_2 (5%), PGB_2 (2.7%) and 6-oxo-PGE_1 (1.2%); all other eicosanoids gave less than 0.05% cross-reactivity. Samples were assayed in duplicate, and duplicates were within 25% of each other.

All reagents used were of Analar grade or the highest grade commercially available, DL-α-tocopherol and Trolox C were obtained from Hoffman–La Roche and Co., Basle, Switzerland, phytol was from the Sigma Chemical Co., and isophytol was kindly given by Dr. J. F. Pennock, Department of Biochemistry, University of Liverpool, Liverpool, U.K. The statistical significance of results was assessed by using Student's *t* test, a *P* value of > 0.05 being considered non-significant.

RESULTS

Effect of vitamin E and related compounds on intracellular enzyme efflux from Ca^{2+} ionophore-treated muscles

Addition of the Ca^{2+} ionophore A23187 (20 μM) to the incubation media produced the expected release of intracellular CK (Fig. 1). Addition of α-tocopherol (230 μM) to the medium during the preincubation period and throughout the experiment completely inhibited this rise in enzyme efflux and maintained low levels of creatine kinase release throughout the 3 h period of study (Fig. 1*a*). To eliminate the possibility that the extracellular vitamin E was interfering with the action of the ionophore, the experiment was repeated with α-tocopherol added to the incubation medium immediately after treatment with the ionophore (Fig. 1*b*). Although an initial rise in CK efflux was seen, the ensuing increase in CK efflux was prevented by the presence of α-tocopherol and was significantly less than in controls after 1 h of tocopherol treatment (Fig. 1*b*).

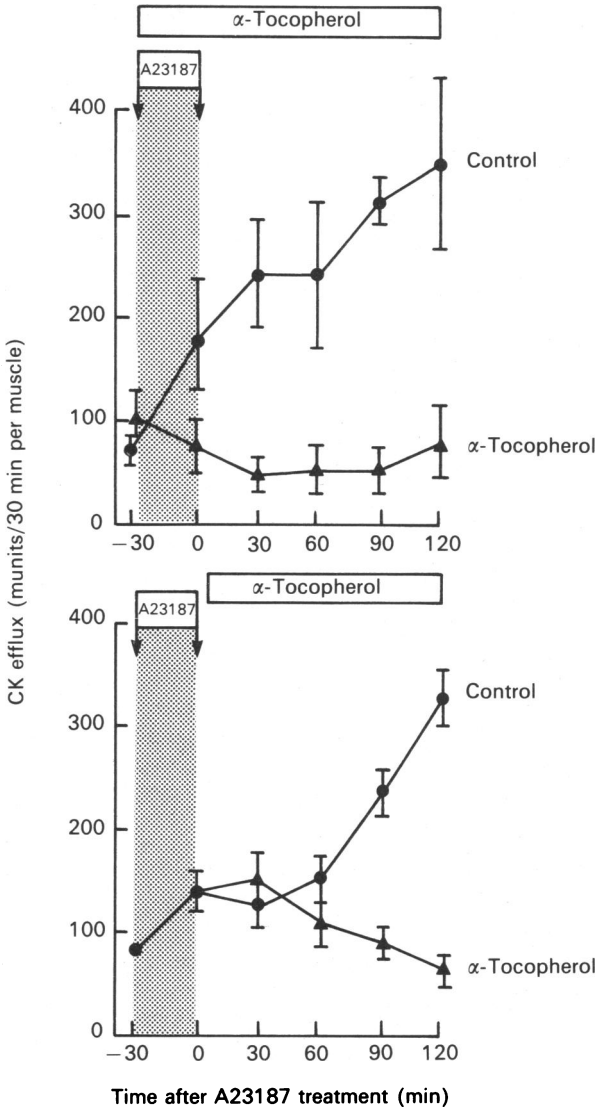

Fig. 1. Efflux of CK from rat soleus muscles treated with Ca^{2+} ionophore (A23187) in the presence (▲) and absence (●) of α-tocopherol (230 μM)

(*a*) α-Tocopherol present throughout the experiment; (*b*) α-tocopherol present after ionophore treatment.

To investigate the possible mechanisms by which α-tocopherol was preventing Ca^{2+}-ionophore-induced efflux of intracellular enzymes, these experiments were repeated with α-tocopherol acetate (230 μM). Unhydrolysed α-tocopherol acetate has little or no antioxidant ability, but added to the incubation media throughout the experiment was found to prevent the CK efflux following Ca^{2+}-ionophore treatment (Fig. 2*a*) and to diminish the efflux when added immediately after the ionophore (Fig. 2*b*).

Further information on the mechanism of action

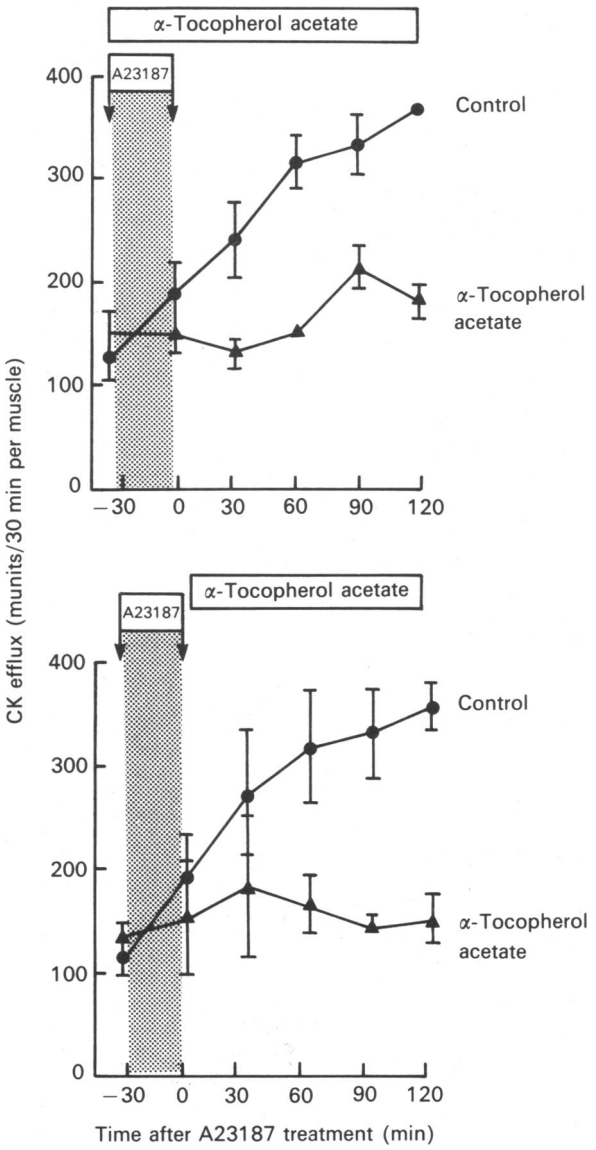

Fig. 2. Efflux of CK from rat soleus muscles treated with Ca^{2+} ionophore (A23187) in the presence (▲) and absence (●) of α-tocopherol acetate (230 μM)

(*a*) α-Tocopherol acetate added throughout the experiment; (*b*) α-tocopherol acetate added after ionophore treatment.

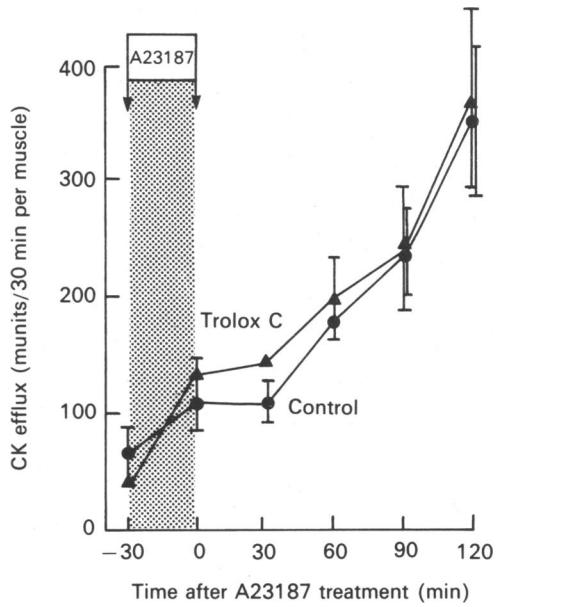

Fig. 3. Efflux of CK from rat soleus muscles treated with Ca^{2+} ionophore (A23187) in the presence (▲) or absence (●) of Trolox C (540 μM) added throughout the experiment

of the α-tocopherol was obtained by addition of Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at concentrations up to 540 μM, to the media surrounding the muscles. This compound is an effective antioxidant having the same chromanol ring structure as α-tocopherol, but not having the phytyl side chain, and was found to have no effect on the CK efflux from Ca^{2+}-ionophore-treated muscles (Fig. 3).

Since Trolox C did not inhibit Ca^{2+}-induced enzyme efflux, phytol and isophytol were tested as representing other parts of the tocopherol molecule. These were both found to be effective inhibitors of the release of enzymes (Fig. 4*a* and 4*b*). The structures of the various compounds tested are shown in Fig. 5. All of the agents tested had a minor inhibitory effect on the CK assay, but these were insufficient to account for the dramatic decreases seen in cytosolic enzyme efflux.

Effect of α-tocopherol and related compounds on muscle cation content

The possibility that α-tocopherol and the other compounds were inhibiting the rise in muscle Ca^{2+} content seen in this type of damage was assessed by analysis of muscle cation content at the end of the experiments (Table 1). Fresh rat soleus muscles have a total calcium content of approx. 4.5 μM/g dry wt., and all the muscles analysed after Ca^{2+}-ionophore treatment have values significantly higher than this (Table 1). The rise in total Ca^{2+} content after Ca^{2+}-ionophore treatment was found to be somewhat variable, and α-tocopherol and isophytol appeared to curtail the increase by significant amounts, but the other agents found to be effective inhibitors of CK efflux (α-tocopherol acetate and phytol) had no effect. No consistent effects of the various agents on muscle Mg, K or Na content were seen. The Na content of the ionophore-treated muscles was found to be consistently higher than those of the unincubated controls.

Effect of α-tocopherol and related compounds on non-enzymic lipid peroxidation in muscle homogenates

The effect of all the compounds tested in the incubation system on the production of TBARS by skeletal-muscle homogenates is shown in Table 2, together with the basal TBARS content of the homogenate immediately after homogenization. Nordihydroguaiaretic acid (NDGA) was included as a known antioxidant for comparison. Trolox C and α-tocopherol markedly decreased the amount of TBARS produced, demonstrating their known ability to act as antioxidants. Both phytol and isophytol also had some effect on lowering TBARS production, but, as expected, α-tocopherol acetate had very little antioxidant activity.

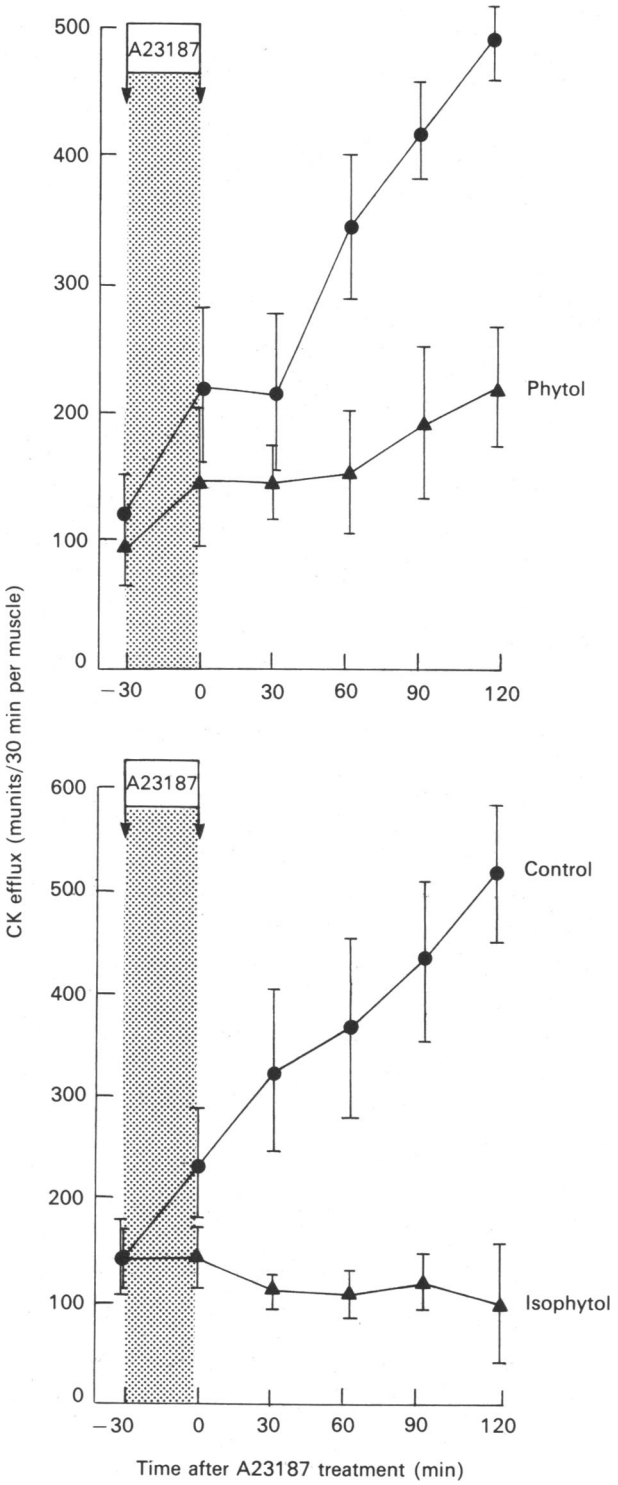

Fig. 4. Efflux of creatine kinase from rat soleus muscles treated with Ca^{2+} ionophore (A23187) (*a*) in the presence of phytol (230 μM) throughout the experiment or (*b*) isophytol (230 μM) throughout the experiment

Effect of α-tocopherol and related compounds on the activity of lipoxygenase enzymes

The effect of vitamin E and related compounds on soya-bean lipoxygenase activity, together with the effect of NDGA, a known inhibitor, are shown in Table 3.

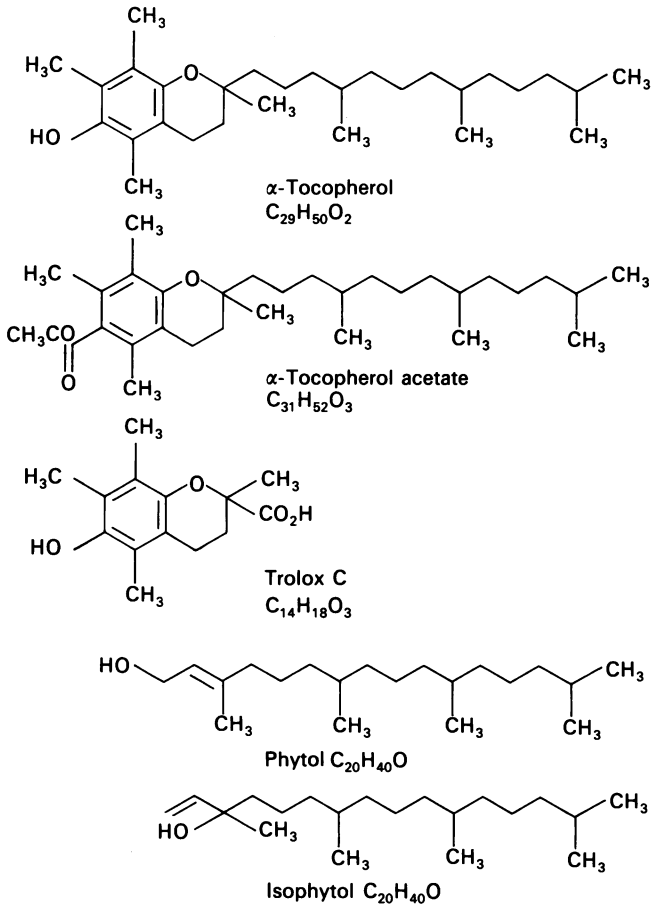

Fig. 5. Structures of the compounds tested

α-Tocopherol, phytol and isophytol inhibited lipoxygenase by about 50% at a concentration of 230 μM, whereas α-tocopherol acetate and Trolox C were essentially ineffective.

Effect on PGE_2 release

The concentration of PGE_2 in the muscle eluents from the incubation studies is shown in Table 4. This demonstrates the expected rise in PGE_2 efflux during and after ionophore treatment (Jackson *et al.*, 1987), but there was no significant influence of α-tocopherol on this.

DISCUSSION

A knowledge of the mechanisms by which damage occurs to skeletal muscle and of the ways in which this damage might be prevented could be of considerable importance in preserving muscle-cell viability in patients with various degenerative neuromuscular disorders and in normal subjects after excessive exercise. Previous work has shown that removal of the extracellular Ca^{2+} from the incubation medium surrounding isolated muscles diminishes the intracellular enzyme efflux and ultrastructural damage induced by excessive contractile activity (Jones *et al.*, 1984) and the intracellular enzyme efflux induced by both low concentrations of deoxycholate (Jones *et al.*, 1984) and by metabolic inhibitors such as sodium cyanide and 2,4-dinitrophenol (Jackson

Table 1. Cation content of incubated muscle after treatment with the Ca^{2+} ionophore A23187 in the presence or absence of various agents

Muscles were assayed at the end of the experiment, i.e. 120 min after the end of calcium-ionophore treatment. Results are means ± S.E.M. for four to seven muscles.

Treatment	Cation...	Cation content (μM/g dry wt.) Ca^{2+}	Mg^{2+}	K^+	Na^+
Fresh muscle		4.5±0.5	33.2±0.9	324.0±8.4	176.8±8.1
A23187-treated		12.5±1.3	41.7±2.2	285.6±23.4	388.5±36.6
+α-Tocopherol		7.8±0.4	40.2±1.9	281.9±19.6	326.1±13.2
A23187-treated		15.7±1.9	36.1±4.3	364.2±38.2	398.6±40.2
+α-Tocopherol acetate		12.3±0.9	35.8±2.9	382.0±48.0	325.5±19.6
A23187-treated		28.3±6.7	32.4±1.8	444.4±16.8	472.9±34.6
+Trolox C		25.6±1.6	27.9±1.0	423.5±11.7	438.1±27.4
A23187-treated		26.0±1.7	26.4±1.0	450.5±15.9	405.0±26.2
+Phytol		21.0±2.6	29.0±0.7	538.8±15.0	383.1±29.3
A23187-treated		21.2±1.8	21.8±1.2	468.1±10.7	500.8±21.0
+Isophytol		13.5±1.0	24.0±2.9	441.2±47.8	414.3±43.7

Table 2. Effect of α-tocopherol and related compounds on TBARS produced by normal skeletal-muscle homogenates

Results shown are means ± S.E.M. for four to seven incubations. Compounds were dissolved in 100% ethanol and tested at a final concentration equivalent to that tested in the muscle-incubation studies.

Conditions	TBARS (A_{532}/g wet wt.)	Mean inhibition (%)
No incubation	0.35±0.01	–
No additions	1.88±0.02	–
NDGA	0	100
α-Tocopherol	0.26±0.01	85.9
α-Tocopherol acetate	1.58±0.01	16.0
Trolox C	0.02±0.01	98.7
Phytol	0.96±0.01	48.7
Isophytol	1.28±0.02	31.9

Table 3. Inhibition of soya-bean lipoxygenase activity by various compounds

Inhibitor	A_{234}/min (mean ± S.E.M.)	Mean inhibition (%)
Dimethyl sulphoxide	0.108±0.0058	–
NDGA	0.005±0.0071	94.4
α-Tocopherol	0.048±0.0017	46.7
α-Tocopherol Acetate	0.098±0.0017	–
Trolox C	0.103±0.0026	–
Phytol	0.038±0.0052	57.8
Isophytol	0.049±0.0077	45.6

et al., 1984). This work, combined with measurements of the increase in total muscle Ca^{2+} found after excessive contractile activity and treatment of muscles with metabolic inhibitors (Claremont *et al.*, 1984), has suggested that an accumulation of intracellular Ca^{2+} is a key step in the damaging process after a number of different stresses. Treatment of muscles with the Ca^{2+} ionophore A23187 appears to mimic this damage, causing release of cytosolic enzymes (Jones *et al.*, 1984) and ultrastructural damage to the muscle similar to that seen in certain degenerative neuromuscular disorders (Publicover *et al.*, 1978), and has been used as the damaging agent in the present work to be certain that any protective effects of tocopherol and other compounds were due to a diminution in Ca^{2+}-mediated degenerative processes.

A protective effect of vitamin E in the process of cytosolic enzyme efflux from damaged tissue was suggested by studies which demonstrated an exacerbation of contractile activity-induced enzyme efflux from isolated vitamin E-depleted muscles (Jackson *et al.*, 1983*a*), although addition of normal plasma concentrations of α-tocopherol to the incubation media surrounding normal muscles had no effect on the response to a given amount of stress induced by contractile activity. The results reported here demonstrate a decrease in the toxic effects of the Ca^{2+} ionophore A23187 on normal muscle by α-tocopherol at approx. 5 times the normal plasma vitamin E concentration (i.e. 230 μM). The mechanism by which vitamin E prevents the damaging membrane effect of Ca^{2+} ionophore does not appear to involve prevention of the Ca^{2+}-accumulating effects of the A23187, since addition of the α-tocopherol or α-tocopherol acetate after ionophore treatment was protective to the muscles (Figs 1*b* and 2*b*). Also the Ca content of muscles treated with tocopherol, tocopherol acetate, phytol and isophytol was grossly elevated compared with controls (Table 1), despite all these compounds having significant protective effects against intracellular enzyme efflux.

Considerable variation in the cation content of the A23187-treated muscles was found (Table 1), although the reproducibility of measurements of the cation content of fresh muscle was acceptable. This is likely to be either a true variation between different animals or due to analysis of variable amounts of extracellular fluid with

Table 4. Release of PGE_2 by isolated muscles

Values for α-tocopherol treated muscles were not significantly different from muscles treated with ionophore alone at all time points. Results are means ± S.E.M. A23187, Ca^{2+} ionophore A23187.

	PGE_2 release (pg/30 min per mg wet wt. of muscle)					
	Pretreated	During A23187 treatment	Time after treatment (min)... 0–30	30–60	60–90	90–120
A23187-treated	1.62±0.27	8.11±2.80	5.13±1.15	5.84±1.51	9.04±2.23	10.32±3.13
+α-Tocopherol	1.64±0.90	7.00±3.26	4.37±1.36	5.71±1.64	7.31±1.44	7.90±1.95

the muscle samples, since no attempt to wash the muscles was made. However, the magnitude of the elevations in muscle Ca^{2+} is considerably greater than the differences seen for other cations.

Protection of the Ca^{2+}-induced release of intracellular enzymes by α-tocopherol would normally be taken as evidence that some form of free-radical-mediated process had been initiated by the Ca^{2+} overload, but the experiments reported here with α-tocopherol acetate, Trolox C, phytol and isophytol (Fig. 2, 3 and 4) demonstrate the importance of the phytyl chain of the tocopherol molecule in this action (see Fig. 5). Previous studies have also suggested that lipid peroxidation via lipoxygenase enzymes might be involved in the mechanisms of enzyme efflux induced by Ca^{2+} overload (Jackson *et al.*, 1987), and we have therefore used the molecules described above to examine the role of the phytyl chain in the inhibition of both non-enzymic lipid peroxidation and lipoxygenase activity by tocopherol.

α-Tocopherol and Trolox C were both found to be effective inhibitors of non-enzymic lipid peroxidation in skeletal-muscle homogenates (Table 2), confirming previous work concerning the importance of the phenolic part of the molecule on its free-radical-scavenging abilities (Burton *et al.*, 1983). α-Tocopherol acetate, as expected, had virtually no effect in this system, demonstrating that muscle is unable to convert α-tocopherol acetate into α-tocopherol, whereas phytol and isophytol were relatively poor antioxidants. By comparison with the abilities of these compounds to inhibit Ca^{2+}-induced intracellular enzyme efflux, it is therefore clear that a simple antioxidant role cannot explain the effects of these compounds in the muscle-incubation system. In particular, α-tocopherol acetate had virtually no antioxidant effect, but was an effective agent in prevention of intracellular enzyme efflux, whereas with Trolox C the situation was reversed.

Inhibition of lipoxygenase enzymes by α-tocopherol has been well documented (Grossman *et al.*, 1984; Reddanna *et al.*, 1985), and the results in Table 3 demonstrate that this effect on soya-bean lipoxygenase is partially mimicked by phytol and isophytol, but not by α-tocopherol acetate or Trolox C. Soya-bean lipoxygenase activity was studied because considerable similarities have been demonstrated between the mechanisms of action of this enzyme and mammalian lipoxygenases (Gardner, 1980). The results obtained are in disagreement with those of both Reddanna *et al.* (1985) and Grossman *et al.* (1984), who describe inhibition of lipoxygenase activity by α-tocopherol acetate, although both utilized more sensitive polarographic techniques than the spectrophotometric assays used here. The mechanism of action of peroxidation via lipoxygenase enzymes involves production of a lipid free-radical intermediate, and it is therefore not surprising that α-tocopherol is inhibitory in such a system, although the lack of effect of Trolox C suggests a relatively specific interaction of the tocopherol with the lipoxygenase or its substrate. The mechanisms of the inhibitory effects of phytol and isophytol in this system are currently unexplained.

Lucy and Diplock (Lucy, 1972; Diplock & Lucy, 1973) have proposed a hypothesis for the mode of action of vitamin E suggesting that the molecule has a physicochemical role in the stabilization of biological membranes and that interactions of the methyl groups of the phytyl chain and the *cis* double bonds of fatty acids act to form a complex which promotes the stability of the membrane. Lucy (see Lucy & Dingle, 1964) had also previously commented on the ability of compounds with long isoprenoid chains to stabilize membranes. However, this theory has been criticized, since it is thought that there is only approximately one molecule of α-tocopherol per 1000 polyunsaturated fatty acid molecules in the membrane, which would be insufficient to confer protection; however, if α-tocopherol inhibits lipoxygenase in a specific manner, this could account for the protective effects of relatively small amounts of the molecule.

Weber *et al.* (1958) have also noted the biological activity of the isoprene side chain of vitamin E in restoration of normal mitochondrial enzyme activities after extraction with organic solvents.

Other possible mechanisms of protection by α-tocopherol and related compounds include inhibition of phospholipase A_2 enzymes, since Ca^{2+} activation of this enzyme has been implicated in the processes of enzyme leakage from damaged muscle (Jackson *et al.*, 1984), and α-tocopherol has been reported to inhibit phospholipase A_2 in platelets (Douglas *et al.*, 1986). The results in Table 4 suggest that, in this case, this has not occurred, since muscle PGE_2 release was increased by Ca^{2+} ionophore, presumably reflecting an increase in phospholipase activity, but this was unaffected by α-tocopherol treatment.

Pascoe and co-workers (Pascoe & Reed, 1987; Pascoe *et al.*, 1987*a*, *b*) have recently suggested that α-tocopherol and α-tocopherol acetate protect hepatocytes against various toxins by preservation of cellular thiols. They do not appear to have assessed the role of phytyl compounds in their system, but the possibility that preservation of cellular thiol content is involved in the actions seen here requires further investigation.

Our results therefore indicate that vitamin E and

certain related compounds can inhibit the muscle sarcolemmal changes induced by intracellular Ca^{2+} overload, which leads to intracellular enzyme efflux. The mechanism by which this occurs is at least partially dependent upon the phytyl chain of the tocopherol molecule rather than its antioxidant ability. Some results suggest that this effect may be mediated by an ability of phytyl compounds to inhibit lipoxygenase enzymes, although a lack of inhibitory effect of α-tocopherol acetate in the system used here argues against this. It therefore seems likely that, at least in this case, the protective effects of vitamin E against cytotoxic agents may not be solely mediated by its antioxidant effects.

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