Action of long-chain fatty acids *in vitro* on Ca²⁺-stimulatable, Mg²⁺-dependent ATPase activity in human red cell membranes

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Human red cell membrane Ca²⁺-stimulatable, Mg²⁺-dependent adenosine triphosphatase (Ca²⁺-ATPase) activity and its response to thyroid hormone have been studied following exposure of membranes *in vitro* to specific long-chain fatty acids. Basal enzyme activity (no added thyroid hormone) was significantly decreased by additions of 10⁻⁹–10⁻⁴ M-stearic (18:0) and oleic (18:1 *cis*-9) acids. Methyl oleate and elaidic (18:1 *trans*-9), palmitic (16:0) and lauric (12:0) acids at 10⁻⁶ and 10⁻⁴ M were not inhibitory, nor were arachidonic (20:4) and linolenic (18:3) acids. Myristic acid (14:0) was inhibitory only at 10⁻⁴ M. Thus, chain length of 18 carbon atoms and anionic charge were the principal determinants of inhibitory activity. Introduction of a *cis*-9 double bond (oleic acid) did not alter the inhibitory activity of the 18-carbon moiety (stearic acid), but the *trans*-9 elaidic acid did not cause enzyme inhibition. While the predominant effect of fatty acids on erythrocyte Ca²⁺-ATPase *in situ* is inhibition of basal activity, elaidic, linoleic (18:2) and palmitoleic (16:1) acids at 10⁻⁶ and 10⁻⁴ M stimulated the enzyme. Methyl elaidate was not stimulatory. These structure-activity relationships differ from those described for fatty acids and purified red cell Ca²⁺-ATPase reconstituted in liposomes. Thyroid hormone stimulation of Ca²⁺-ATPase was significantly decreased by stearic and oleic acids (10⁻⁹–10⁻⁴ M), but also by elaidic, linoleic, palmitoleic and myristic acids. Arachidonic, palmitic and lauric acids were ineffective, as were the methyl esters of oleic and elaidic acids. Thus, inhibition of the iodothyronine effect on Ca²⁺-ATPase by fatty acids has similar, but not identical, structure-activity relationships to those for basal enzyme activity. To examine mechanisms for these fatty acid effects, we studied the action of oleic and stearic acids on responsiveness of the enzyme to purified calmodulin, the Ca²⁺-binding activator protein for Ca²⁺-ATPase. Oleic and stearic acids (10⁻⁹–10⁻⁴ M) progressively inhibited, but did not abolish, enzyme stimulation by calmodulin (10⁻⁹ M). Double-reciprocal analysis of the effect of oleic acid on calmodulin stimulation indicated noncompetitive inhibition. Addition of calmodulin to membranes in the presence of equimolar oleic acid restored basal enzyme activity. Oleic acid also reduced ¹²⁵I-calmodulin binding to membranes, but had no effect on the binding of [¹²⁵I]T₄ by ghosts. The mechanism of the decrease by long chain fatty acids of Ca²⁺-ATPase activity *in situ* in human red cell ghosts thus is calmodulin-dependent and involves reduction in membrane binding of calmodulin.

INTRODUCTION

The activity of human red cell membrane Ca²⁺-ATPase is regulated by several factors, including membrane phospholipid and fatty acid content (Niggli *et al.*, 1981; Sarkadi *et al.*, 1982). Unsaturated fatty acids, such as oleic and linoleic acids, have been shown *in vitro* to enhance the activity of Ca²⁺-ATPase in purified enzyme preparations (Niggli *et al.*, 1981) and in membrane enzyme models (Pine *et al.*, 1983; Wetzker *et al.*, 1983). Thyroid hormone analogues are hydrophobic molecules that have specific (Botta & Fariás, 1985) and nonspecific (Davis *et al.*, 1984) interactions with cell membranes. We (Davis & Blas, 1981; Davis, F. B., *et al.*, 1982) and others (Galo *et al.*, 1981; Costante *et al.*, 1986) have reported that thyroid hormone at physiological concentrations stimulates human and animal red cell membrane Ca²⁺-ATPase activity. The mechanism of this extranuclear action of iodothyronines is incompletely understood

(Davis, P. J., *et al.* 1982; Davis *et al.*, 1987). As with thyroid hormone stimulation of thymocyte plasma membrane sugar transport (Segal & Ingbar, 1981), thyroid hormone action on cell membrane Ca²⁺-ATPase requires calmodulin, a soluble Ca²⁺-binding activator protein for Ca²⁺-ATPase (Davis *et al.*, 1983b). In the present studies we have examined the effects of additions *in vitro* of fatty acids on basal, thyroid hormone- and calmodulin-stimulated human red cell Ca²⁺-ATPase activity.

MATERIALS AND METHODS

Reagents

All fatty acids were obtained from Sigma, as were disodium ATP and L-thyroxine (T₄). Fatty acids were reported to be > 98.2% pure by gas chromatography (Sigma). The purity of myristic and elaidic acids, and

Abbreviations used: Ca²⁺-ATPase, Ca²⁺-stimulatable, Mg²⁺-dependent adenosine triphosphatase; C-kinase, calcium-stimulated, phospholipid-dependent protein kinase; T₄, L-thyroxine.

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Fig. 1. Effect of oleic acid on basal, thyroid hormone- and calmodulin-stimulated Ca^{2+} -ATPase activity

Conditions of assay were as described in the Materials and methods section. The solvent for oleic acid was 1% dimethyl sulphoxide, which was also in control samples. The T_4 concentration in selected samples (●) was 10^{-10} M, and the exogenous calmodulin concentration (■) was 6×10^{-9} M, or 100 ng/mg of membrane protein. Basal activity (○) was that without added T_4 or calmodulin. T_4 -stimulatable enzyme activity is indicated by the distance between ● and ○, and calmodulin-stimulatable activity the distance between ○ and ■. Inhibition of basal activity was significant at oleic acid concentrations $\geq 10^{-7}$ M ($P < 0.05$, paired t test) while T_4 and calmodulin effects were inhibited by oleic acid concentrations as low as 10^{-9} M ($P < 0.01$) and 10^{-8} M ($P < 0.001$), respectively.

oleic acid methyl ester, was verified by gas chromatography by Dr. Todd Hennessey, State University of New York at Buffalo. Calmodulin was purified in our laboratory from bovine brain by the method of Charbonneau *et al.* (1983), using phenothiazine-Sepharose affinity chromatography. The phenothiazine, 2-chloro-10-(3-aminopropyl)phenothiazine hydrochloride, for calmodulin purification was generously provided by Dr. Stephen Kennedy (NIH, Bethesda, MD, U.S.A.). ^{125}I -Calmodulin and ^{125}I - T_4 were obtained from Amersham International.

Erythrocyte membranes

Human red cells were obtained from healthy volunteers who provided their informed consent. The cells were washed and hypo-osmotically lysed, as previously described (Davis & Blas, 1981). Membranes were stored at -70°C until used within 24–72 h of preparation.

Membrane Ca^{2+} -ATPase assay

Components of the assay medium were: 25 mM-Tris/HCl, pH 7.45, 75 mM-NaCl, 25 mM-KCl, 1 mM- MgCl_2 , 1 mM-ATP and 0.1 mM-EGTA, with or without 0.15 mM- CaCl_2 . Ca^{2+} -ATPase activity was determined as the difference in ATP hydrolysis in the presence and absence of Ca^{2+} (Davis & Blas, 1981). With the added Ca^{2+} , a free

Ca^{2+} concentration of 20 μM in the Tris-buffered assay medium was confirmed by ion-specific electrode and by calculation from a computer program kindly provided by Dr. John T. Penniston (Mayo Clinic, Rochester, MN, U.S.A.). Assay results were expressed as μmol of P_i liberated/90 min per mg of membrane protein. P_i was measured by the Fiske-Subbarow method (1925) and membrane protein by the Lowry *et al.* (1951) technique, with bovine serum albumin as standard. Control incubations provided corrections for nonenzymic hydrolysis of ATP and for membrane content of endogenous P_i . All assays were conducted in duplicate and results are means $\pm$ S.E.M. of three or more experiments.

Effect of thyroid hormone and fatty acids on membrane Ca^{2+} -ATPase activity

The action of iodothyronine on membrane Ca^{2+} -ATPase was measured by determining enzyme activity in the presence and absence of 10^{-10} M- T_4 . Enzyme activity in the absence of hormone was termed basal activity. A stock solution of T_4 , 10^{-3} M in 4% propylene glycol/0.04 M-KOH, was diluted in 10 mM-Tris for use in the assays. Incubations of hormone and membranes were carried out for 60 min at 37°C before enzyme assay. Samples for basal activity contained hormone diluent, but no T_4 .

Fatty acids were added simultaneously with hormone or hormone diluent in the preincubation. Fatty acid diluent (1% dimethyl sulphoxide) was used in control samples. Neither 1% dimethyl sulphoxide nor the diluent for T_4 have been shown to have any significant effect on enzyme activity.

In other studies, unlabelled purified bovine brain calmodulin was added to incubations of membranes, with or without fatty acid, to determine whether fatty acid effects on enzyme activity were reversible with the addition of exogenous calmodulin.

Effect of oleic acid on binding of ^{125}I - T_4 and ^{125}I -calmodulin by red cell membranes

The effect of oleic acid on membrane binding of radiolabelled T_4 and calmodulin was determined by our previously published method (Warnick *et al.*, 1986). Red cell membranes were incubated in 10 mM-Tris buffer for 60 min at 37°C with either ^{125}I - T_4 (10^{-11} M) or ^{125}I -calmodulin (5.9×10^{-9} M), in the presence or absence of 10^{-7} – 10^{-5} M-oleic acid. Membranes were sedimented at 20000 g for 20 min and distribution of radioactivity between pellet and supernatant was determined to estimate bound/free separation.

Statistical analysis

Effects of fatty acids on Ca^{2+} -ATPase activity and on binding of hormone and calmodulin to red cell membranes were analysed by analysis of variance (ANOVA) and paired t test.

RESULTS

Effect of oleic acid *in vitro* on basal Ca^{2+} -ATPase activity and T_4 -stimulatable Ca^{2+} -ATPase activity

As shown in Fig. 1, human red cell basal Ca^{2+} -ATPase activity (without added hormone) was $0.44 \mu\text{mol}$ of P_i /90 min per mg of membrane protein; 10^{-10} M- T_4 increased enzyme activity to 0.50 (14%, $P < 0.001$, paired

Fig. 2. Effect of several fatty acids on basal Ca^{2+} -ATPase activity

Fatty acids were dissolved in dimethyl sulphoxide, and final dilutions in the assay, as well as in control samples without fatty acid, contained 1% dimethyl sulphoxide. Results shown are the means $\pm$ S.E.M. of the percentage of control enzyme activity obtained with the fatty acids 20:4 (arachidonic), 16:0 (palmitic), 18:3 (linolenic), 18:1 *cis*-9 (oleic) and 18:0 (stearic). There was significant ($P < 0.001$, ANOVA) inhibition of basal activity by 18:1 *cis*-9 and 18:0 only.

t test). Oleic acid, over a concentration range of 10^{-10} – 10^{-4} M, progressively decreased basal ($P < 0.01$, ANOVA) and hormone-responsive ($P < 0.001$, ANOVA) enzyme activities (Fig. 1). Reduction in basal activity became significant at 10^{-7} M ($P < 0.05$, paired *t* test) and T_4 -responsive Ca^{2+} -ATPase activity was significantly decreased by 10^{-9} M-oleic acid ($P < 0.01$). The IC_{50} of oleic acid for basal and T_4 -responsive activities was 10^{-9} M. At an oleic acid concentration of 10^{-4} M, the basal and T_4 -stimulatable Ca^{2+} -ATPase activities were 71 ± 1 and $6 \pm 10\%$ of control, respectively. Although oleic acid decreased membrane Ca^{2+} -ATPase activity, the fatty acid (10^{-6} M) studied under conditions of varying Ca^{2+} concentrations did not alter the free Ca^{2+} optimum ($20 \mu\text{M}$) for enzyme activity (results not shown).

Effect of other fatty acids on basal and T_4 -stimulatable human red cell Ca^{2+} -ATPase activity

The effect of fatty acids of varying length and degree of saturation on basal and T_4 -stimulatable Ca^{2+} -ATPase activity was determined, and the results are shown in Figs. 2 and 3 and Table 1. At 10^{-6} M concentrations, only stearic (18:0) and oleic (18:1 *cis*-9) acids significantly inhibited basal enzyme activity (Fig. 2 and Table 1). At 10^{-4} M, myristic acid (14:0) was, like stearic and oleic acids, inhibitory. In contrast, elaidic (18:1 *trans*-9), linoleic (18:2) and palmitoleic (16:1) acids, at 10^{-6} and

10^{-4} M, had modest but significant stimulatory effects on basal activity.

Thyroid hormone action on Ca^{2+} -ATPase was susceptible to inhibition by a broader range of fatty acids (Fig. 3 and Table 1). Stearic, oleic, elaidic, linoleic, palmitoleic and myristic acids significantly decreased hormone effect on the enzyme.

Arachidonic acid, oleic and elaidic acid methyl esters, linolenic, palmitic, myristoleic and lauric acids were without significant effect on basal and hormone-responsive Ca^{2+} -ATPase activity.

Effect of fatty acids on response of Ca^{2+} -ATPase activity to exogenous purified calmodulin

In the absence of exogenous oleic acid, purified calmodulin, at a concentration of 6×10^{-9} M (100 ng/mg of membrane protein) increased enzyme activity above basal levels comparably to T_4 (Fig. 1). In parallel with its effects on basal and T_4 -stimulatable Ca^{2+} -ATPase activities, oleic acid progressively decreased calmodulin-stimulatable activity of the enzyme (Fig. 1, $P < 0.001$, ANOVA). However, 61% of control calmodulin responsiveness persisted even in the presence of 10^{-4} M-oleic acid. Stearic acid also significantly inhibited enzyme stimulation by exogenous calmodulin (34% of control calmodulin effect at a stearic acid concentration of 10^{-5} M). Arachidonic acid, 10^{-4} M, eliminated calmodulin stimulation of Ca^{2+} -ATPase activity, while palmitic acid

Fig. 3. Effect of selected fatty acids on T_4 stimulation of Ca^{2+} -ATPase activity

Enzyme activity was determined in the presence and absence of 10^{-10} M- T_4 and the increase in enzyme activity with T_4 in samples without fatty acid served as the control value. The same fatty acids were used as shown in Fig. 2. Results are the means $\pm$ S.E.M. of the percentage of control T_4 effect seen with each fatty acid concentration. Significant inhibition of the T_4 effect occurred only with 18:1 *cis*-9 and 18:0 ($P < 0.005$, ANOVA).

Table 1. Effect of fatty acids on basal and thyroid hormone-stimulated human erythrocyte Ca^{2+} -ATPase activity

Fatty acid	Ca^{2+} -ATPase activity (% of control)					
	Basal			T_4 -stimulatable		
	10^{-6} M	10^{-4} M	<i>P</i>	10^{-6} M	10^{-4} M	<i>P</i>
Arachidonic (20:4)*	104 ± 3†	115 ± 16	NS	78 ± 7‡	74 ± 22	NS
Stearic (18:0)	81 ± 4	65 ± 1	< 0.001	41 ± 15	30 ± 7	< 0.005
Oleic (18:1 <i>cis</i> -9)	77 ± 3	71 ± 1	< 0.001	18 ± 7	6 ± 10	< 0.001
Elaidic (18:1 <i>trans</i> -9)	122 ± 1	132 ± 2	< 0.001	59 ± 7	30 ± 6	< 0.001
Oleic acid methyl ester	96 ± 2	99 ± 1	NS	90 ± 6	93 ± 8	NS
Elaidic acid methyl ester	103 ± 2	101 ± 2	NS	80 ± 14	99 ± 13	NS
Linoleic (18:2)	107 ± 2	129 ± 5	< 0.001	101 ± 17	3 ± 35	< 0.003
Linolenic (18:3)	95 ± 3	98 ± 2	NS	103 ± 22	49 ± 11	NS
Palmitic (16:0)	102 ± 2	105 ± 2	NS	108 ± 10	125 ± 18	NS
Palmitoleic (16:1)	115 ± 2	126 ± 4	< 0.001	33 ± 3	36 ± 8	< 0.001
Myristic (14:0)	92 ± 2	74 ± 4	< 0.001	79 ± 6	46 ± 7	< 0.001
Myristoleic (14:1)	97 ± 1	97 ± 1	NS	119 ± 12	99 ± 13	NS
Lauric (12:0)	99 ± 1	101 ± 1	NS	111 ± 8	99 ± 7	NS

* Number of carbon atoms: number of double bonds.

† Means ± S.E.M. of percentage of control enzyme activity without fatty acid. Results are from three experiments performed in duplicate. *P* value is calculated by ANOVA, comparing samples with fatty acids at each of the concentrations specified with controls without fatty acids. NS, not significant.

‡ Means ± S.E.M. of percentage of control increase in enzyme activity with 10^{-10} M- T_4 .

Fig. 4. Response of Ca^{2+} -ATPase activity to exogenous calmodulin in the presence and absence of 10^{-6} M-oleic acid

Calmodulin was added, in concentrations from 3×10^{-9} to 12×10^{-9} M, to samples with (●) and without (○) 10^{-6} M-oleic acid. The change in enzyme activity with addition of calmodulin is shown on the ordinate. The fatty acid significantly inhibited the enzyme response to calmodulin. The insert shows a double-reciprocal plot which suggests that 18:1 inhibits the calmodulin (CaM) effect in a noncompetitive manner.

had no significant effect on calmodulin stimulation (results not shown).

Exogenous purified calmodulin, $(3\text{--}12) \times 10^{-9}$ M, was added to samples with and without oleic acid, 10^{-6} M, and enzyme activity (without thyroid hormone) determined. The increase in enzyme activity with calmodulin is shown in Fig. 4 (open circles). Oleic acid inhibited the calmodulin effect at all calmodulin concentrations (filled circles). Double-reciprocal analysis (insert) showed that oleic acid inhibition of calmodulin stimulation was not competitive. Exogenous calmodulin was also ineffective in countering the inhibition of the T_4 effect on the enzyme by oleic acid (results not shown). In separate studies, 10^{-8} M-calmodulin overcame the inhibitory effect of 10^{-8} M-oleic acid on basal Ca^{2+} -ATPase (results not shown).

Calmodulin and T_4 binding to red cell membranes in the presence of oleic acid

As shown in Table 2, additions of oleic acid (10^{-7} – 10^{-5} M) to red cell membranes resulted in significant reduction in the binding of ^{125}I -calmodulin by ghosts. At 10^{-6} M-oleic acid, for example, calmodulin binding was decreased by 39% ($P < 0.001$, paired *t* test). In contrast with calmodulin binding, $[^{125}\text{I}]\text{T}_4$ binding by membranes was unaffected by the presence of oleic acid at concentrations up to 10^{-5} M (Table 2).

DISCUSSION

The activity of purified human red cell Ca^{2+} -ATPase is readily manipulated by the lipid environment in which it is reconstituted (Niggli *et al.*, 1981). For example, unsaturated *cis* fatty acids, such as oleic, at nanomole/ μg enzyme protein concentrations, enhance activity of purified Ca^{2+} -ATPase, as do acidic phospholipids (Niggli *et al.*, 1981). Purified Ca^{2+} -stimulated, phospholipid-dependent protein kinase (C-kinase) is also activated by unsaturated *cis* fatty acids (Murakami & Routtenberg,

Table 2. Effect of oleic acid on binding of radiolabelled calmodulin and L-thyroxine to human erythrocyte membranes

Results are means $\pm$ S.E.M. of four experiments conducted in duplicate. Significance: * $P < 0.001$ by paired t test compared with control without oleic acid.

Ligand	[Oleic acid] (M) . . .	Tracer bound (%)			
		Control	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵
¹²⁵ I-calmodulin		2.9 $\pm$ 0.3	2.5 $\pm$ 0.3*	1.8 $\pm$ 0.3*	1.3 $\pm$ 0.2*
[¹²⁵ I]T ₄		8.0 $\pm$ 0.5	7.6 $\pm$ 0.6	8.1 $\pm$ 0.6	7.7 $\pm$ 0.5

1985; Murakami *et al.*, 1986). These observations caused us to study the effects of a series of fatty acids on basal red cell Ca²⁺-ATPase activity *in situ*, that is, in red cell membranes, and on thyroid hormone-stimulated Ca²⁺-ATPase activity. The latter has been extensively described by our laboratory (Davis *et al.*, 1983a,b, 1987). The concentrations of fatty acids used include those which circulate endogenously in man during acute illness (Chopra *et al.*, 1985).

In our red cell membrane assay system, selected 18-carbon fatty acids (stearic, oleic) produced inhibition of basal Ca²⁺-ATPase activity, as well as inhibition of the thyroid hormone effect. The structure-activity relationships of fatty acids with the membrane-associated Ca²⁺-ATPase, reported here, are different in certain regards from those described for purified human red cell Ca²⁺-ATPase reconstituted in liposomes. For example, the response of the enzyme *in situ* to long-chain fatty acids is predominantly inhibition, whereas the purified enzyme, in the absence of phospholipid, is reported to be stimulated by fatty acids (Niggli *et al.*, 1981). Stimulation of the purified enzyme in the latter studies, however, occurred at oleic and linoleic acid concentrations of 10⁻⁶–10⁻⁵ M. In the current studies, those fatty acids which were significantly active produced inhibition of enzyme activity at concentrations as low as 10⁻⁷–10⁻⁸ M. It should be pointed out that in some reports of Ca²⁺-ATPase *in situ* in biological membranes, e.g. striated muscle sarcoplasmic reticulum (Munkongke *et al.*, 1985) or human red cell (Wetzker *et al.*, 1983), oleic acid at 10⁻⁶–10⁻⁵ M has previously been found to be inhibitory.

The structure-activity studies reported here have several interesting features. First, the *trans* conformation of the unsaturated elaidic acid caused stimulation of basal enzyme activity, in contrast to the inhibitory effect of oleic acid. The effect of thyroid hormone on the enzyme, however, was inhibited by elaidic acid in our system. Oleic and elaidic acid analogues with the acidic group methylated were ineffective in altering either basal or hormone-stimulated enzyme activity, indicating the absolute requirement for a negatively charged molecule.

Second, while unsaturated and saturated fatty acids have been incompletely compared in the reconstituted, purified Ca²⁺-ATPase model, it has been concluded that unsaturated fatty acids are required for enzyme stimulation (Schmalzing & Kutschera, 1982). In the case of the membrane-associated enzyme reported here, however, it is clear that both saturated (stearic) and unsaturated (oleic) structures of the same chain length may act as inhibitors of basal and hormone-stimulated Ca²⁺-

ATPase. The introduction of additional double bonds (linolenic and linoleic acids versus oleic acid) negated the inhibitory effect of 18-carbon fatty acids on basal enzyme activity, but the action on the T₄ effect persisted, at least with linoleic acid. Fatty acid stimulation of red cell Ca²⁺-ATPase has been reported to increase with the number of double bonds (Schmalzing & Kutschera, 1982), and fatty acid inhibition of Na⁺,K⁺-ATPase in other tissues has been reported to increase with the number of double bonds (Swann, 1984; Kelly *et al.*, 1986). The discrepancy between our data and those of Schmalzing & Kutschera (1982) may reside in either the assay temperature or the molar ratio of fatty acid to tissue phospholipids, as they report both factors to determine a positive or negative fatty acid effect on Mg²⁺-ATPase and Na⁺,K⁺-ATPase in the red cell membrane.

Third, although fatty acid chain length studies are not exhaustive in the current report, only 18-carbon chain length fatty acids inhibited both basal and T₄-stimulated Ca²⁺-ATPase; a saturated 16-carbon moiety, palmitic acid, was inactive, as was the 20-carbon unsaturated arachidonic acid. The addition of multiple double bonds, including *cis*-9, in linoleic acid was associated with fatty acid effect on hormone action only. Palmitoleic acid, with a *cis*-9 double bond in a 16-carbon chain, caused basal enzyme stimulation. Palmitoleic acid has 3-fold the activity of palmitic acid in its effect on Na⁺-Ca²⁺ exchange in cardiac sarcolemma (Philipson & Ward, 1985). The inhibitory action we describe of high concentrations of myristic acid (14:0) on basal and hormone-responsive activity is interesting in light of the potent activity of myristic acid, compared with other saturated long-chain fatty acids, on Na⁺-Ca²⁺ exchange in sarcolemma, but is not explicable in terms of conventional structure-activity relationships of the fatty acids.

A number of mechanisms by which fatty acids might inhibit enzyme activity were considered. Fatty acid effects in other membrane and enzyme models have been attributed to altered membrane fluidity (Usher *et al.*, 1978), altered membrane surface charge (Wojtczak & Nalecz, 1979), or direct action of the enzyme (Niggli *et al.*, 1981). It is well-established that membrane lipids are required for Ca²⁺-ATPase activity (Ronner *et al.*, 1977). Alteration of membrane fatty acid composition by dietary lipid manipulation is known to affect red cell Ca²⁺-ATPase response to thyroid hormone in laboratory animals (Galo *et al.*, 1981).

Finally, it is clear from our studies that fatty acids can decrease calmodulin binding to red cell membranes. Klinger *et al.* (1984) have shown release of endogenous

calmodulin from red cell membranes upon incubation with oleic acid. The same laboratory also reported that depletion of calmodulin from the red cell membrane is associated with susceptibility of Ca^{2+} -ATPase to stimulation by oleate (Wetzker *et al.*, 1983), in contrast to inhibition in the calmodulin-replete membrane. In our assay system, blocking calmodulin action with a variety of calmodulin inhibitors results in decreases of both basal and thyroid hormone-stimulated Ca^{2+} -ATPase activity (Davis & Davis, 1985). Lineweaver-Burk analysis of experiments in which purified calmodulin was added to membranes in the presence of oleic acid (10^{-6} M) indicated that fatty acid inhibition of calmodulin-dependent enzyme activity was noncompetitive. Separate studies to determine the extent of reversibility by calmodulin of the oleic acid effect were carried out at lower concentrations (10^{-8} M) of fatty acid and showed that basal enzyme activity could be completely restored by addition of equimolar amounts of calmodulin. This suggests that fatty acid inhibition of basal enzyme activity is mediated in large measure, if not exclusively, by an anti-calmodulin effect. The T_4 effect, however, was not restored by calmodulin in the presence of oleic acid. It is possible that binding of selected fatty acids to the enzyme, as reported by Lee *et al.* (1982), may be responsible for altered effects of T_4 .

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