Mechanisms of Fatty Acid Effects on Sarcoplasmic Reticulum

III. THE EFFECTS OF PALMITIC AND OLEIC ACIDS ON SARCOPLASMIC RETICULUM FUNCTION—A MODEL FOR FATTY ACID MEMBRANE INTERACTIONS*

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The mechanism by which palmitic and oleic acids modify calcium sequestration by sarcoplasmic reticulum vesicles was investigated by examining the effects of these fatty acids on calcium-dependent ATPase activity, on the phosphoenzyme intermediates found during calcium sequestration reactions, and on passive membrane permeability to calcium. The calcium sequestered in the presence of these fatty acids was also characterized by determining the amount exchangeable with the extravesicular pool or released by the ionophore A23187.

In the presence of 50 μM ATP, 18 μM palmitic acid enhanced and 18 μM oleic acid inhibited calcium sequestration, whereas both fatty acids stimulated ATPase activity. Neither fatty acid had significant effects on the amount or distribution of the phosphoenzyme formed during the calcium transport reaction. Palmitic acid stimulated calcium sequestration only when ATP was present. Oleic acid caused the release of a portion of the accumulated calcium during ATP-supported calcium sequestration and also enhanced the release observed in ATP-depleted reactions. A portion of the calcium sequestered in the presence of palmitic acid appears to be incorporated into a nonexchangeable and ionophore-insensitive calcium pool, although the latter was estimated to be considerably larger than the nonexchangeable pool. These data support the hypothesis that oleic acid inhibits calcium sequestration by increasing membrane permeability to calcium, whereas palmitic acid appears to stimulate calcium sequestration by interacting with a portion of the calcium within the vesicles to form a separate, poorly exchangeable calcium pool.

Calcium sequestration by sarcoplasmic reticulum vesicles isolated from skeletal muscle is coupled to the hydrolysis of ATP by a membrane-bound Ca,Mg-dependent ATPase (1, 2). The transport of calcium from the extravesicular to the intravesicular space involves a reaction sequence whereby calcium and MgATP are rapidly bound to the enzyme and the α-phosphate of ATP is transferred to form an acid-stable phophorylated, enzyme intermediate E,P. When the Ca<sup>2+</sup> gradient across the sarcoplasmic reticulum is reversed, in the presence of ADP and Mg<sup>2+</sup>, E,P can also serve as an intermediate for ATP resynthesis (2–4). During the forward reaction of calcium transport, the ADP-sensitive E,P undergoes a transition to an ADP-insensitive phosphoenzyme, E,P. The transition from E,P to E,P appears to be linked to active calcium translocation (4–6). The subsequent release of calcium to the intravesicular space and decomposition of E,P liberates P<sub>i</sub> into the extravesicular space, allowing the membrane enzyme again to become available to bind calcium and ATP.

Sarcoplasmic reticulum calcium transport and ATPase activity can be modified by manipulation of the lipid environment of the ATPase enzyme (7–15), a property similar to many membrane-bound enzymes (16). Partial hydrolysis of membrane phospholipids by phospholipase, removal of hydrolytic products with albumin, and addition of fatty acids or lysophosphatides to lipid-depleted membranes can alter differently the ATPase activity and calcium transport properties of sarcoplasmic reticulum vesicles. Studies examining the reconstituted ATPase enzyme in synthetic phospholipid bilayers have shown that enzyme activity can be influenced by phospholipid acyl chain length and saturation (12–15). Unsaturated phospholipid acyl chains appear to be required for optimal ATPase activity in reconstituted preparations, suggesting that membrane fluidity is an important determinant of this transport enzyme function (12–14). Calcium transport by sarcoplasmic reticulum vesicles can also be modified by long chain fatty acids (17–20). The effects of fatty acids on sarcoplasmic reticulum vesicles depend upon the molar ratio of fatty acid to membrane lipid, its aliphatic chain length and saturation, and the conditions under which the calcium transport reaction is examined (17–20). In the absence of calcium-precipitating anions, oleic acid (C<sub>18</sub> cis-9 monounsaturated fatty acid) inhibits sarcoplasmic reticulum calcium sequestration, whereas palmitic acid (C<sub>16</sub> saturated), which has a sterically similar aliphatic chain length, can enhance calcium sequestration (17, 20). Saturation-dependent differences in fatty acid effects have also been demonstrated for erythrocyte osmotic fragility (21) and more recently in a lymphocyte capping model (22, 23).

The mechanisms by which free fatty acids produce these complex effects on the sarcoplasmic reticulum remain poorly understood, although exposure of sarcoplasmic reticulum vesicles to micromolar concentrations of palmitic and oleic acids has been found to alter vesicular and membrane structure (24). Observations that micromolar concentrations of palmitic, but not oleic acid, could interact with and lower the ionized Ca<sup>2+</sup> concentrations in solution (25) led us to examine further the effects of these two fatty acids on calcium fluxes across...
the sarcoplasmic reticulum membrane. The effects of palmitic and oleic acids on calcium fluxes and calcium exchange, ATPase activity, and phosphozenzyme levels suggest that palmitic acid enhances calcium sequestration by interacting with a portion of the calcium within the vesicles to form a separate, slowly exchanging, calcium pool. In contrast, oleic acid inhibits calcium sequestration by increasing sarcoplasmic reticulum permeability to calcium.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit white skeletal muscle by the method described previously (26). The "light" sarcoplasmic reticulum fraction was used in all of the experiments described in this report and is referred to as sarcoplasmic reticulum.

Unless otherwise stated, calcium sequestration was determined in 120 mM KCl, 1 mM MgCl₂, 1 mM MgATP, 8-19 μM ⁶⁰CaCl₂, and 40 mM histidine buffer (pH 6.8). Reactions were initiated by the addition of 6 or 12 μg/ml of sarcoplasmic reticulum protein and terminated at selected times by filtering an aliquot of reaction mixture through type HA (0.45 μm pore size) Millipore filters mounted in Swinney adapters. A 50-μl aliquot of the filtrate was added to 2 ml of Biofluor (New England Nuclear) for counting in a Packard or Searle liquid scintillation spectrophotometer. The effects of palmitic and oleic acids on calcium sequestration were examined by adding the fatty acid in ethanol to reaction mixtures with and without MgATP prior to the initiation of the sequestration reaction, or by transferring at a predetermined time an aliquot of the reaction mixture to separate tubes containing the fatty acid in 1:2000 the volume of the transferred aliquot. The final concentration of ethanol, <0.13% (v/v), had no significant effect on calcium sequestration and inhibited ATPase activity less than 10%.

Calcium exchange and calcium influx were determined by a modification of the method of Kata et al. (26) described by Takenaka et al. (5). An aliquot of an ongoing calcium sequestration reaction initiated with ⁶⁰CaCl₂ was transferred to high specific activity, carrier-free ⁴⁰CaCl₂. Sequestration of the labeled calcium was then determined at selected times by the methods described. An identical reaction was initiated with ⁴⁰CaCl₂ to determine the calcium concentration outside the vesicles and the amount of calcium sequestered by the vesicles at the times when calcium exchange and influx were measured.

Sarcoplasmic reticulum ATPase activity was measured under the same conditions as calcium sequestration except that reactions were initiated with either 1 mM MgATP or 50 μM [γ-³²P]ATP. P, liberation was determined after reactions were terminated at appropriate times by addition of an aliquot of the reaction mixture (0.5 ml) to 10% (w/v) ice-cold trichloroacetic acid containing 1 mM Pi as carrier in the reaction mixture, or by transferring at a predetermined time an aliquot of the reaction mixture to separate tubes containing the fatty acid in 1:2000 the volume of the transferred aliquot. The final concentration of ethanol, <0.13% (v/v), had no significant effect on calcium sequestration and inhibited ATPase activity less than 10%.

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The phosphoprotein intermediates (EP) of the ATPase reaction were determined by modification of the methods of Shigekawa et al. (3, 4) and Takenaka et al. (5). The conditions were as in the ATPase determination except that the [γ-³²P]ATP concentration was 50 μM. After termination of the reaction at 1.5 min (a time when the total EP) had reached a plateau level) with 10% (w/v) trichloroacetic acid containing 0.5 mM ATP and 1 mM Pi, as carriers, 100 or 200 μg of sarcoplasmic reticulum protein was added as carrier. The mixture was centrifuged at 1500 × g for 10 min at 4 °C, and the resultant pellet was washed three times in perchloric acid (7%) containing 1 mM Pi. After washing, the final pellet was solubilized by incubation in 1 N NaOH, 2% (w/v) Na₂CO₃, and 0.1 mM KH₂PO₄ at 95 °C for 1 h. Two aliquots were collected, and one was assayed for protein by the method of Lowry et al. (28); approximately 60% of the total protein was consistently recovered. The second aliquot was added to 10 ml of Biofluor and counted for ³⁸P activity. ADP-sensitive EP (E, P) was determined by measuring the phosphoprotein present in the reaction mixture quenched by trichloroacetic acid 4 s after the addition of 1 mM ADP. ADP-insensitive EP (E, P) was calculated as the difference between the total EP and the ADP-sensitive EP.

All reagents used were reagent grade. ATP, purchased as the disodium salt from Sigma, was changed to the potassium salt by passage over Dowex AG 50W-X8 (K⁺ form), neutralized with Tris, and stored at −20 °C with equimolar MgCl₂. [γ-³²P]ATP was prepared by the method of Johnson and Walseth (29) and stored at −20 °C in

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1 The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.
2 μM ATP. Contaminating calcium in a standard reaction mixture was 3 μM, determined by atomic absorption spectroscopy, and was taken into account in all calculations. The fatty acids were purchased from Sigma and stored at −20 °C as stock solutions in ethanol. Each fatty acid produced a single band when examined by thin layer chromatography. 23187 was a gift from Lilly. Deionized water was glass distilled prior to use.

RESULTS

Effects of Palmitic and Oleic Acids on Calcium Sequestration—As previously reported (17) palmitic acid caused a concentration-dependent enhancement of calcium sequestration (Figs. 1 and 2). The stimulatory effect of palmitic acid was observed at 10–15 s, the initial time examined after the onset of the reaction, and the effect persisted for at least 10 min in reactions carried out at high concentrations of ATP (Fig. 1). In the absence of ATP, where 10 nmol of Ca/mg of sarcoplasmic reticulum protein became associated with the sarcoplasmic reticulum vesicles under the standard conditions of these experiments, palmitic acid had no effect on calcium association with the sarcoplasmic reticulum (17). Addition of palmitic acid to sarcoplasmic reticulum vesicles that had sequestered a maximal amount of calcium caused a rapid increase in calcium sequestration (Fig. 3). In contrast, oleic acid (6–24 μM) inhibited calcium sequestration when present from the onset of the reaction (Figs. 1 and 2) and when added to calcium-filled vesicles caused the release of a portion of the sequestered calcium (Fig. 5). At higher concentrations (approximately 36 μM) oleic acid could induce the release of all but approximately 10 nmol/mg of the sequestered calcium, which represents the amount of calcium associated with sarcoplasmic reticulum in the absence of ATP.

Effects of Palmitic and Oleic Acids on ATPase Activity—At concentrations (6–24 μM) that had opposite effects on calcium sequestration, both palmitic and oleic acids stimulated Ca**-activated ATPase activity (Figs. 1 and 2). In the presence of 1 mM ATP, the control ATPase activity was 0.74 ± 0.04 μmol of P/μg of protein per min, whereas in the presence of 18 μM palmitic and oleic acids, ATPase activity increased to 2.12 ± 0.23 and 1.81 ± 0.14 μmol/mg-min, respectively. Neither fatty acid affected the "basal" ATPase activity (approximately 0.04–0.07 μmol/mg-min) determined in the presence of 1 mM EGTA. Furthermore, at concentrations of 6 μM, where palmitic acid was without effect and oleic acid slightly inhibited calcium sequestration, both fatty acids stimulated ATPase activity by more than 75% (Fig. 2). Thus, the ability of these fatty acids to stimulate ATPase activity could be dissociated from their effects on calcium sequestration.

Effects of Palmitic and Oleic Acids on the Phosphoprotein Intermediates of the Sarcoplasmic Reticulum ATPase—To determine if the stimulatory effects of the two fatty acids on Ca**-activated ATPase activity were associated with different alterations in the amount or type of phosphorylated enzyme intermediates, the influence of these compounds on total and ADP-sensitive EP was examined. Thirty seconds after starting the reaction in the presence of 50 μM [γ-32P]ATP, total EP level reached a maximum that was maintained for 2 min. The rate of P i liberation was also virtually constant during

![Graph](http://example.com/graph1.png)

**Fig. 4.** The influence of fatty acids on the time course of P i liberation and the total amount of phosphorylated intermediate during a sarcoplasmic reticulum ATPase reaction. P i liberation (open symbols) and EP levels (closed symbols) in control (upper panel), in the presence of 18 μM palmitic acid (middle panel), and in the presence of 12 μM oleic acid (lower panel) were determined in reactions containing 5 μM added Ca**+, 12 μg/ml of protein and begun with 50 μM Mg[γ-32P]ATP. The data points are mean ± S.E. of three experiments for the fatty acids and the results of a single experiment for the control.

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<td><strong>Effect of palmitic and oleic acids on the ATPase activity, E2P amount, and the E2P decay rate constant</strong></td>
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<td>Reactions were carried out in 10 μM added calcium, with 12 μg/ml of protein and initiated with 50 μM Mg[γ-32P]ATP. The basal ATPase under these conditions is approximately 20–40 nmol of P i/mg-min and was disregarded in the above calculations. The ATPase activity is the mean ± S.E. for 4 experiments.</td>
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**Fig. 3.** The effect of the addition of palmitic and oleic acids to calcium-filled sarcoplasmic reticulum vesicles. Palmitic acid, 12 μM (A; ○—○) and oleic acid, 12 μM (B; □—□), were added to control reactions (●—●) 5 min after onset. Calcium sequestration was determined at the indicated times after addition. Conditions were as in the legend to Fig. 1. Each point is the mean ± S.D. of three experiments.
the initial 2 min of the reaction in the presence or absence of either fatty acid (Fig. 4). Although Ca\(^{2+}\)-activated ATPase activity in the presence of 50 \(\mu\)M ATP is 34% of that determined in 1 mM ATP (Fig. 2), the per cent stimulation of this activity by the fatty acids was the same at both ATP concentrations (Fig. 2 and Table I). There was no significant difference in the total EP 1.5 min after initiation of the reaction in the control (3.51 ± 0.18 nmol/mg), 18 \(\mu\)M palmitic acid (3.28 ± 0.22), or 18 \(\mu\)M oleic acid (3.20 ± 0.22) reactions (Fig. 5). Furthermore, neither fatty acid altered the relative amounts of \(E_1P\) or \(E_2P\) under the steady state conditions examined (Fig. 5). These results demonstrate that these fatty acids neither increased the amount of phosphoprotein intermediate nor altered the distribution of \(E_1P\) and \(E_2P\) during the steady state of the ATPase reaction. Both fatty acids increased the rate constant of \(E_1P\) decay calculated from the ratio of the rate of P liberation: \(E_1P\) amount from 255 to 760 and 528 for palmitic and oleic acids, respectively (Table I).

**Fatty Acid Effects on Sarcoplasmic Reticulum**

**Fig. 5.** The effect of fatty acids on the amount and distribution of the phosphorylated intermediate of the sarcoplasmic reticulum ATPase enzyme. The total phosphorylated intermediate (■), and the distribution between \(E_1P\) (□) and \(E_2P\) (□) were determined as described under "Materials and Methods" in the presence of 10 \(\mu\)M added reaction. Reactions were initiated with 50 \(\mu\)M [\(\gamma\)-\(^32\)P] MgATP. The bars represent the mean ± S.E. for 27, 25, and 19 data points for control, 18 \(\mu\)M palmitic acid (PA) and 18 \(\mu\)M oleic acid, (OA), respectively.

**Fig. 6.** The influence of ATP depletion on the effect of fatty acids to alter membrane calcium sequestration. The time course of a sarcoplasmic reticulum calcium sequestration (■—■) and Pi liberation (□—□) reaction was determined after initiation with 20 \(\mu\)M [\(\gamma\)-\(^32\)P]MgATP. At the arrow, 18 \(\mu\)M palmitic acid (PA) (left, O,— — O) or 18 \(\mu\)M oleic acid (OA) (right, O,— — O) was added to the reaction and subsequent calcium sequestration was determined.

**Fig. 7.** The effect of palmitic acid on calcium influx into calcium-filled sarcoplasmic reticulum vesicles. Control influx (O—O) was determined by adding \(^{45}\)Ca to an ongoing reaction after the sarcoplasmic reticulum vesicles had sequestered a maximal amount of unlabeled calcium (arrow); influx (O— — O) was determined when calcium sequestration has been maximally enhanced by the presence of 18 \(\mu\)M palmitic acid. The closed symbols represent identical reactions initiated in the presence of \(^{46}\)CaCl\(_2\). The conditions were as in the legend to Fig. 1 except the added calcium was 5 \(\mu\)M. Data points are mean ± S.E. for five experiments.

**Effects of Fatty Acids on Spontaneous Calcium Release—** When calcium sequestration and Pi liberation were examined under conditions where a low (20 \(\mu\)M) ATP concentration initiated the reaction, the initial calcium sequestration was followed by a spontaneous calcium release that occurs when more than 90% of the added ATP was hydrolyzed (Fig. 6). Palmitic acid at a concentration of 18 \(\mu\)M had no effect on this spontaneous calcium release (Fig. 6), suggesting that palmitic acid does not increase calcium sequestration by decreasing calcium efflux from the sarcoplasmic reticulum vesicles. However, under similar conditions, 18 \(\mu\)M oleic acid accelerated this calcium release (Fig. 6).

**Effect of Palmitic Acid on Calcium Influx—** As the calcium sequestered by sarcoplasmic reticulum vesicles rapidly exchanges with the extravascular calcium (5, 9, 18), we determined whether the ability of palmitic acid to enhance sequestration resulted from a stimulation of calcium influx. The unidirectional calcium influx rate was examined by adding a small amount of carrier-free \(^{45}\)CaCl\(_2\) during the plateau of calcium content during calcium sequestration reactions started with \(^{45}\)CaCl\(_2\). No significant effects of palmitic acid on this calcium influx were observed (Fig. 7). Calculation of the apparent influx rates (5, 18) from experiments such as those shown in Fig. 7 demonstrated that despite enhanced steady state calcium sequestration, 18 \(\mu\)M palmitic acid did not stimulate calcium influx (657 ± 89 nmol/mg-min) above control values (675 ± 66 nmol/mg-min).\(^2\) There was no difference in the apparent amount of \(^{46}\)Ca sequestered 2 s after its addition to the extravascular calcium pool in the absence (46.6 ± 3.1 nmol/mg, mean ± SE, \(n = 17\)) or presence of 18 \(\mu\)M palmitic acid (48.2 ± 3.7 nmol/mg, mean ± S.E., \(n = 17\)) providing further evidence that palmitic acid had no appreciable effect on steady state calcium influx. The ability of

\[^{2}\text{Calcium influx rates are calculated assuming that the specific activity of the calcium outside the vesicles was constant. Because the amount of calcium sequestered remains constant, the calcium influx and efflux rates are equal, so that a portion of the unlabeled intravesicular calcium will enter the labeled extravascular pool and decrease the specific activity causing an underestimate of the influx rate. When efflux-dependent changes in specific activity were taken into account (see Ref. 5 for details), calculated influx rates remained unaffected by palmitic acid.}\]
palmitic acid to enhance steady state calcium sequestration thus cannot be explained as resulting from stimulation of steady state calcium influx.

Effect on the Exchangeable and A23187-sensitive Calcium Pool within the Sarcoplasmic Reticulum Vesicles—By labeling the extravesicular calcium during a calcium sequestration reaction started with nonradioactive CaCl₂, and assuming that all the calcium present in the reaction was available to distribute with the added label, the amount of sequestered calcium was exchangeable with the extravesicular calcium during a calcium sequestration reaction started with nonradioactive CaCl₂. For both control reactions and reactions containing 18 µM oleic acid, all of the calcium sequestered by the sarcoplasmic reticulum vesicles at 1 min was found to be exchangeable (Fig. 8).

In contrast, after the sarcoplasmic reticulum vesicles sequestered calcium in the presence of 18 µM palmitic acid, a portion (35–40 nmol/mg) of the sequestered calcium remained nonexchangeable up to 4 min after addition of the ⁴⁵Ca label (Fig. 8). To further characterize the state of calcium sequestered by the sarcoplasmic reticulum in the presence of palmitic acid, A23187 was added to sarcoplasmic reticulum vesicles that had accumulated calcium in the presence and absence of fatty acid. Addition of 1 µM A23187 to calcium-filled sarcoplasmic reticulum vesicles 5 min after the start of a control reaction resulted in a rapid release (within 30 s) of all but 5–10 nmol/mg of the sequestered calcium (Fig. 9). The calcium sequestered in the presence of oleic acid was similarly sensitive to the ionophore (Fig. 9), whereas the presence of 18 µM palmitic acid (which enhanced calcium sequestration by 100%) approximately 160 nmol of calcium/mg of protein remained associated with the sarcoplasmic reticulum after A23187 addition (Fig. 9). When the effects of 12, 18, and 24 µM palmitic acid on 12 µg/ml of sarcoplasmic reticulum were examined in a separate series of experiments, the size of the A23187-insensitive calcium pool was dependent on the amount of palmitic acid present and was 52 ± 2 (S.D., n = 3), 114 ± 8 (n = 3), and 161 ± 4 (n = 5) in the presence of 12, 18, and 24 µM fatty acid, respectively. Increasing the amount of added ionophore to 5 µM under similar conditions had no effect on the amount of calcium remaining associated with the sarcoplasmic reticulum, suggesting that the inability of the ionophores to cause calcium release could not be explained by an artefact arising from a decrease in the amount of calcium available to interact with the sarcoplasmic reticulum.

When a calcium sequestration reaction was started in the presence of 5 µM A23187, only approximately 10 nmol of calcium/mg of protein were associated with the membrane. Palmitic acid (18 µM), added at 2 min after the onset of an ionophore-inhibited reaction, did not stimulate calcium sequestration (data not shown). These results can be explained if the ability of palmitic acid to enhance calcium sequestration requires the presence of a high calcium concentration within the sarcoplasmic reticulum vesicles.

**DISCUSSION**

The experiments described above confirm earlier reports (17, 18, 20) that micromolar concentrations of palmitic and oleic acids have different effects on calcium sequestration by isolated sarcoplasmic reticulum vesicles. Palmitic acid enhanced calcium sequestration by sarcoplasmic reticulum vesicles while oleic acid inhibited this reaction (Figs. 1 and 2). The effects caused by both fatty acids were rapid, being fully developed within 10 s after addition of the fatty acids to calcium-filled vesicles (Fig. 3).

The present studies demonstrate that the contrasting ef-
Effects of palmitic and oleic acids on calcium sequestration cannot be explained by different effects on Ca\(^{2+}\)-activated ATPase activity as both fatty acids stimulated steady state ATPase activity to a similar degree at concentrations that have opposite effects on calcium sequestration (Fig. 2). Furthermore, at concentrations that had no effect on calcium sequestration, both fatty acids stimulated ATPase activity (Fig. 2). Examination of the phosphoenzyme intermediates of the ATPase reactions demonstrated that both fatty acids increased the rate of ATP hydrolysis by stimulating the rate of E,P decay without altering significantly either the total amount of EP or the relative proportions of EP and E,P (Fig. 4 and Table 1). These findings demonstrate that the stimulation of the membrane enzyme activity is not due to uncovering of latent calcium pump sites, a mechanism suggested previously to explain amphiphile-induced membrane enzyme stimulation (30, 31). The finding that palmitic acid did not influence the distribution of phosphorylated intermediates during the steady state of the ATPase reaction also indicates that this fatty acid did not enhance calcium sequestration by altering the ratio between the rates of E,P = E,P transitions (1, 2, 5). Furthermore, the effect of these amphiphiles on enzyme-mediated ATP hydrolysis did not discriminate mechanisms of amphiphile-membrane association (32) or incorporation (22-24) that might have depended on aliphatic chain saturation (29).

Addition of oleic acid to sarcoplasmic reticulum vesicles releasing calcium at a time when most of a small amount of added ATP had been consumed, and when EP levels were low, enhanced calcium release (Figs. 4 and 6). This finding suggests that the inhibition of calcium sequestration by oleic acid does not require an ongoing calcium pump reaction, so that “uncoupling” of the ATPase reaction from calcium translocation cannot explain completely the oleic acid effect. It seems more likely that the unsaturated aliphatic chain of oleic acid causes calcium release by disrupting membrane structure and thereby increasing calcium permeability (24, 33, 34). A similar disruptive effect of oleic acid has been observed in red cell membrane (21). Electron microscopic studies of sarcoplasmic reticulum vesicles exposed to micromolar concentrations of oleic acid, under the conditions of the functional studies described above, have shown changes in sarcoplasmic reticulum membrane structure including vesicular “fusison” at low concentrations and disruption of vesicles at higher concentrations (24, 34). These disruptive changes could also explain, at least in part, the inhibition of calcium sequestration by oleic acid (Figs. 1-3). The finding that very low concentrations of oleic acid can stimulate ATPase activity without inhibiting calcium sequestration indicates that stimulation of ATP hydrolysis cannot be explained solely by an effect of oleic acid to reduce intravesicular calcium content and thereby decrease the inhibitory effect of high intravesicular calcium concentration (2). The increased ATPase activity caused by oleic acid may instead be due to stimulation of E,P decay resulting from an oleic acid-induced alteration in the lipid environment of the ATPase protein (11, 12, 14, 15).

Although the effect of palmitic acid to enhance calcium sequestration could, like the effect of oleic acid, be dissociated from a stimulatory effect on sarcoplasmic reticulum ATPase activity (Fig. 2), the evidence presented in this report demonstrates that both ATP-energized calcium transport and high intravesicular Ca\(^{2+}\) concentrations are required for the stimulatory effect of palmitic acid. When ATP was absent from the reaction mixture, the low palmitic acid concentrations examined in the study did not alter the amount of calcium associated with sarcoplasmic reticulum (approximately 10 nmol/mg). Furthermore, palmitic acid did not cause an increase in calcium content after calcium sequestration had been inhibited by A23187. These findings indicate that these low concentrations of palmitic acid do not interact at the outer surface of the membrane to form a membrane/fatty acid complex that traps calcium ions (28). In reactions started with low initial ATP concentrations, sarcoplasmic reticulum vesicles sequestered calcium until most of the ATP was consumed, after which calcium release occurred. This calcium release coincides with a marked decrease in the rate of calcium influx and the amount of EP (Ref. 5 and Figs. 4 and 6). Palmitic acid, added during this late phase of the reaction, had no effect on calcium release (Fig. 6), indicating that palmitic acid does not reduce passive calcium efflux from the sarcoplasmic reticulum vesicles. Together with the effects of A23187 discussed above, these results suggest that active calcium transport is necessary for palmitic acid to exert its stimulatory effect.

When calcium influx was examined in the presence of a palmitic acid concentration that enhanced calcium sequestration, stimulation of the influx rate was not observed (Fig. 7), indicating that palmitic acid did not induce a new steady state in which calcium influx via the pump is stimulated and the calcium efflux rate is increased by the resultant higher intravesicular calcium concentration. The finding that palmitic acid did not stimulate calcium influx at a time when the total sequestered calcium was increased, and the finding that the amount of calcium sequestered at the time of the earliest measurement (10 s, Fig. 7) was increased over control by palmitic acid indicate that the fatty acid may stimulate the calcium sequestration rate at the start of the reaction. This apparent stimulation of initial calcium sequestration rate by palmitic acid might be explained by an indirect effect, possibly related to lowering of the intravesicular Ca\(^{2+}\) concentration by the fatty acid during the initial phase of the reaction (see below).

Characterization of the state of the additional calcium sequestered in the presence of palmitic acid shows that the fatty acid caused a portion of the accumulated calcium to be sequestered into a “pool” that is slowly exchangeable with the extravascular calcium. This calcium pool is also relatively insensitive to A23187 (Figs. 8 and 9). Although it might be expected that the slowly exchanging and ionophore-insensitive calcium pools seen in the presence of palmitic acid would be similar in size as those measured in the absence of the ionophore, the slowly exchangeable pool measured with the ionophore was 100 nmol/mg greater than that estimated by measurement of calcium exchange (Figs. 8 and 9). It is possible that palmitic acid and the ionophore interact in a manner that decreased the amount of ionophore incorporated into, or the effectiveness of the ionophore to move calcium across, the sarcoplasmic reticulum (35, 36). However, the observation that a 5-fold increase in ionophore concentration did not increase the amount of calcium release argues against this explanation. Alternatively, the ionophore could increase the amount of exchangeable calcium through an effect on a calcium-palmitate complex (25).

A number of studies have demonstrated that fatty acids associate with biological membranes (23), including the sarcoplasmic reticulum (8, 18, 24). Freeze-fracture electron microscopic studies of sarcoplasmic reticulum membrane vesicles exposed to high concentrations of palmitic acid have demonstrated protein-free “lakes” in the membrane fracture face that may represent intramembranous accumulation of palmitic acid. However, such lakes were not seen at the low palmitic acid concentrations used in this study. Instead, incubation of sarcoplasmic reticulum vesicles with palmitic acid...
under the conditions of these studies resulted in the replacement of membrane phospholipid with fatty acid (24). These findings are consistent with the view that the added palmitic acid gains access to the environment of high calcium concentration at the intravesicular membrane surface (24). The potential ability of the anionic carboxylic group of palmitic acid to enter the vesicles is of significance in light of studies of the interaction of calcium with a series of fatty acids in aqueous solution. These studies have demonstrated that micromolar concentrations of palmitic acid readily form a calcium-fatty acid complex (25). The calcium sequestered in this calcium-palmitate complex, like the additional calcium sequestered by the sarcoplasmic reticulum vesicles (Fig. 8), is poorly exchangeable with the ionized calcium in the surrounding solution and not rapidly released by A23187.

It would be of interest also to examine calcium influx and phosphoprotein levels at palmitic acid concentrations where enhancement of calcium sequestration was near maximal (Fig. 8 and Ref. 25). However, the potential for calcium-palmitate interaction outside the sarcoplasmic reticulum vesicle at high

![Diagram](https://example.com/diagram.png)

**Fig. 10.** Schematic representation of possible mechanisms of palmitic acid-induced enhancement of sarcoplasmic reticulum calcium sequestration. a, exposure of sarcoplasmic reticulum membrane to palmitic acid (●) could result in the insertion of the aliphatic chain of the fatty acid into the membrane bilayer with the carboxylic acid anionic group (●) residing near the phospholipid head group (●) and exposed to the ionic environment of either the intravesicular or the extravesicular water space. At the extravesicular site the ionized calcium concentration is below that necessary for interaction with the palmitic acid; however, the high ionized calcium concentration within the vesicular space generated by the activity of the calcium pump ATPase results in the complexing of a portion of the sequestered calcium with the carboxylic acid portion of the fatty acid (25). (Although a 2:1 stoichiometry of fatty acid to calcium is suggested by previous studies (25), the possible role of anionic phospholipid headgroups serving as one of the complexing sites cannot be excluded in this membrane scheme). The aliphatic chains of the palmitic acid within and near the membrane surface may act to impart a hydrophobic barrier between the complexed calcium and the intravesicular water space. This isolated calcium would not exchange readily with the noncomplexed intravesicular Ca\(^{2+}\) and could be insensitive to ionophore. Complexing of calcium with only intramembranous palmitic acid (b) would also create a second calcium pool, but this calcium would be expected to reach rapid equilibrium with the remaining intravesicular calcium upon addition of ionophore. c, the fatty acid could form a lamellar or micellar structure within the vesicular space (a structure in equilibrium with intramembranous and intravesicular monomeric fatty acid) whose carboxylic acid portions would interact with and lower the intravesicular ionized calcium concentration (25). The inability to identify such fatty acid structures within the vesicular space and the evidence suggesting that the majority of the fatty acid is in the membrane bilayer argues that if present these structures are small and not likely to account for the total enhancement of calcium sequestration (24).
palmitic acid concentrations would make these determinations and their interpretations unreliable (25). Experiments were therefore carried out at palmitic acid concentrations where enhanced calcium sequestrations was clearly developed and the extravascular calcium-palmitate interaction was minimal (Fig. 8 and Ref. 25).

The phenomenon described here is consistent with the hypothesis that, shortly after the initiation of a calcium transport reaction, intravesicular Ca\(^{2+}\) concentration reaches a level that can form a calcium-palmitate complex. The appearance of this complex would lead to the incorporation of a portion of the calcium, present at high concentrations within the vesicles, into a "pool" that, by lowering intravesicular cal- cium concentration, reduces the inhibitory effect of intravesicular calcium content on calcium transport into the vesicles. Such an effect of palmitic acid is similar to the well known effects of oxalate and phosphate to promote calcium uptake by the sarcoplasmic reticulum (37). Therefore, palmitic acid may promote calcium sequestration by sarcoplasmic reticulum vesicles by acting as a calcium-complexing anion (Fig. 10). If palmitic acid could exert such an effect in vivo, where sarcoplas- matic reticulum calcium content may be submaximal (38), a decrease in the intraluminal free calcium concentration would be expected. The decreased driving force across the sarcoplasm reticulum could reduce the amount and rate of calcium release from the sarcoplasmic reticulum lumen. Were this phenomenon to occur in ischemic heart, a condition where cytosolic fatty acid levels rise (39, 40), it might contribute to the well known decline in myocardial contractility (41).

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