Structural and Related Functional Changes in Sarcoplasmic Reticulum Induced by Long-Chain Fatty Acids

F. M. Munkonge,[1] C. D. Stubbs,[2] and P. J. Quinn[1]

Abstract

The effect of palmitic and oleic acids on Ca^{2+}-ATPase activity in coupled preparations of sarcoplasmic reticulum isolated from rabbit hind leg muscle have been compared with their effects on vesicles uncoupled with Ca^{2+} ionophore, A23187. Palmitate at $2\,\mu M \cdot$ mg protein^{-1} has no significant effect on enzyme activity and does not uncouple catalytic activity from calcium accumulation within the vesicles. Oleic acid at $1\,\mu M \cdot$ mg protein^{-1} uncouples the vesicles, whereas $2\,\mu M \cdot$ mg protein^{-1} completely inhibits Ca^{2+}-ATPase activity. Fluorescence anisotropy of diphenylhexatriene is not significantly altered by palmitate, but a large transient increase in motion of the probe is observed with addition of oleic acid. The effects of oleic acid on enzyme activity are not mediated via an effect on the bulk properties of the hydrophobic domain of the membrane lipids.

Key Words: Ca^{2+}-ATPase; sarcoplasmic reticulum; calcium transport; fatty acids; membrane organization.

Introduction

The sarcoplasmic reticulum has often been used as a model membrane system in which to examine the interaction between intrinsic membrane proteins and phospholipids. The major membrane protein is Ca^{2+}-ATPase which acts to accumulate calcium within the cysternae of the sarcoplasmic reticulum during muscle relaxation (Møller *et al.*, 1982). The catalytic activity of the enzyme and the permeability properties of the membrane are two useful

[1] Department of Biochemistry, King's College London (KQC), Campden Hill Road, London W8 7AH, U.K.
[2] Department of Pathology and Laboratory Medicine, Hahnemann University, Philadelphia, Pennsylvania 19102-1192.

criteria for assessing the functional integrity of the system. A number of agents including anesthetics (De Boland *et al.*, 1975; Heffron and Gronert, 1979), monovalent ions (Martonosi *et al.*, 1968; Fiehn and Hasselbach, 1970; Swoboda *et al.*, 1979; Ng and Howard, 1980), and certain long-chain fatty acids (Hasselbach and Makinose, 1962; Cheah, 1981; Katz *et al.*, 1982; Simmonds *et al.*, 1982) have been shown to influence Ca^{2+}-ATPase activity and/or calcium transport across the membrane. Based on these data a number of models have been proposed to explain the way membrane lipids and other agents interact with the protein.

In the case of long-chain fatty acids it is clear that the particular perturbation depends on the type of fatty acid, i.e., the hydrocarbon chain length and the presence of *cis* unsaturated bonds and their position in the chain. For example, long-chain saturated fatty acids varying in chain length from 14–20 carbon atoms have been shown to produce relatively little effect on the initial calcium uptake into vesicle suspensions containing a precipitating anion (Katz *et al.*, 1982). By contrast, C_{18} and C_{20} *cis*-unsaturated fatty acids markedly inhibit initial calcium uptake. Similar results are obtained with steady-state levels of free calcium accumulated into vesicles in the presence of various fatty acids (Cheah, 1981), but the effect on Ca^{2+}-ATPase appears to differ depending on the system. Thus in coupled systems Ca^{2+}-ATPase activity is stimulated at low concentrations of fatty acids as the calcium gradient across the membrane is collapsed and inhibited at higher concentrations presumably by interaction of the fatty acids directly with the protein. In reconstituted systems inhibition or stimulation of catalytic activity depends on the type of phospholipid into which the enzyme is intercalated, e.g., oleic acid is reported to inhibit Ca^{2+}-ATPase in protein reconstituted into dioleoylphosphatidylcholine, whereas stimulation is observed at similar concentrations in protein reconstituted in dimyristoyl-phosphatidylcholine (Simmonds *et al.*, 1982). The consequences of fatty acid binding have been assessed in these reconstituted systems by measurement of the extent to which fatty acid probes are able to quench the intrinsic trytophan fluorescence of the protein.

The present experiments were undertaken to provide information on the extent to which fatty acids affect the two functional parameters of the membrane, namely, the ability of the vesicles to maintain a gradient of calcium and catalytic activity and to correlate these changes with an index of the motional properties of the hydrophobic domain of the membrane. There is evidence that equilibration of the membrane lipid organization upon addition of fatty acids to sarcoplasmic reticulum vesicles is not immediate, and studies of these time-dependent changes are a particular feature of this investigation.

Experimental

Sarcoplasmic reticulum vesicles were prepared in a tightly coupled configuration by including dithiothreitol in the homogenizing medium and isolating vesicles according to the method of Warren et al. (1974). Addition of ionophore, A23187 (6.4 μM), to coupled vesicles in which Ca^{2+} has accumulated results in an increase in Ca^{2+}-ATPase activity assayed using the enzymic method described by Madden et al. (1979) since ATP hydrolysis is no longer constrained by a calcium gradient across the membrane (Weber, 1971; Madden et al., 1981). The ratio of ATP hydrolysis in the presence and absence of A23187 always exceeded 3.5 when determined at 32°C; the ratio decreased at 37°C due to an increase in the relative rate of ATP hydrolysis in the coupled vesicles.

Palmitic and oleic acids were obtained from Sigma (London) Ltd. and were added to the vesicle suspension (30 or 60 μM, 0.03 mg protein/ml) in ethanol. The final concentration of ethanol did not exceed 0.5% (v/v), and this was found to have no significant effect on ATPase activity of coupled vesicles or on motion of diphenylhexatriene. Fluorescence anisotropy measurements were performed using a single photon counting fluorimeter and a xenon lamp as an excitation source (Kinosita et al., 1976, 1981). All measurements were made at 37°C.

Results and Discussion

A comparison of the effect of palmitate with oleate on Ca^{2+}-ATPase activity in coupled and uncoupled vesicles is shown in Fig. 1. The presence of $2\,\mu M \cdot mg\ protein^{-1}$ palmitate causes a slight increase in enzyme activity over a 2-h incubation period at 37°C, but the vesicles remain coupled. Addition of $2\,\mu M$ oleate $\cdot mg\ protein^{-1}$, on the other hand, causes an immediate and complete inhibition of Ca^{2+}-ATPase activity. The different effects of the two fatty acids is not due to a difference in binding to the membranes because measurement of the association of radioactivity-labeled fatty acids to vesicles isolated by centrifugation methods in our laboratory, and consistent with reports by other groups (Katz et al., 1982), show that between 60 and 80% of the free fatty acids bind to the membrane over a wide range of concentrations. Nearly complete binding of oleate is reported to occur in reconstituted Ca^{2+}-ATPase systems (Simmonds et al., 1982), but there is no evidence to suggest that, in intact or reconstituted membranes, different fatty acids partition differently between the aqueous and membrane phases. There is some evidence, however, that higher concentrations of palmitate $(3\,\mu M \cdot mg\ protein^{-1})$ may extract phospholipid from the membrane and form mixed lipid micelles (Herbette et al., 1984).
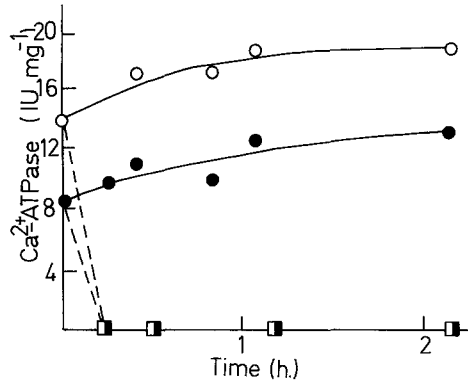
Fig. 1. Ca^{2+}-ATPase activity of sarcoplasmic reticulum vesicles assayed at 37°C during incubation in the presence of 2 μM fatty acid · mg^{-1} protein. Palmitic acid (O, ●) and oleic acid (□, ■) were added to coupled vesicles (●, ■) or vesicles uncoupled by addition of 6.4 μM A23187 (O, □).

The effect of oleic acid on sarcoplasmic-reticulum functions depends on the concentration of fatty acid. Figure 2 compares the effect of 1 and 2 μM oleic acid · mg protein^{-1} during incubation at 37°C on the activity of Ca^{2+}-ATPase. As in the first experiment, 2 μM oleic or higher concentrations up 6 μM fatty acid cause a complete inhibition of Ca^{2+}-ATPase and this inhibition is not reversed during 2-h incubation. By contrast, 1 μM oleic acid causes an increase in activity of the enzyme reaching a steady-state value
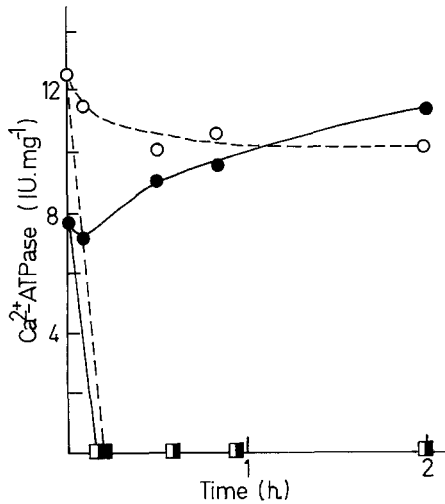

Fig. 2. Ca^{2+}-ATPase activity of sarcoplasmic reticulum vesicles incubated for varying periods of time at 37°C in the presence of 1 μM oleic acid · mg^{-1} protein (O, ●) and 2 μM oleic acid · mg^{-1} protein (□, ■). The vesicles were in a coupled configuration (solid curve) or uncoupled by addition of 6.4 μM A23187 (dashed curve).

slightly lower than that observed in uncoupled vesicles. When oleic acid is added to uncoupled vesicles, there is a slight inhibition of Ca^{2+}-ATPase activity which is presumably due to a direct effect of the fatty acid on the enzyme. This result is in agreement with the report of Cheah (1981) who showed that oleic acid concentrations less than about $0.5\,\mu M \cdot mg\ protein^{-1}$ increased catalytic activity in coupled vesicles when assayed immediately after addition of fatty acid. Together with arachidonic acid, the concentration at which maximal stimulation is observed coincides with a concentration that completely inhibits calcium transport suggesting that stimulation arises from release of constraints on enzyme activity imposed by a gradient of calcium across the membrane. Similar findings have been reported by Messineo et al. (1984) who concluded that oleic acid in concentrations of up to $2\,\mu M \cdot mg\ protein^{-1}$ caused a breakdown of membrane permeability to calcium, but, in contrast to our results, they reported enhanced Ca^{2+}-ATPase activity under these conditions.

Changes in the physical state of hydrophobic domains of the sarcoplasmic reticulum due to the presence of different fatty acids have been probed using fluorescence polarization of diphenylhexatriene intercalated into the membrane. The results of addition of palmitic or oleic acids to a vesicle suspension on the steady-state fluorescence anisotropy of the probe at intervals during incubation at 37°C are shown in Fig. 3. This shows that palmitic acid in a concentration of $2\,\mu M \cdot mg\ protein^{-1}$ causes only a small perturbation of the hydrophobic domain of the membrane which remains unchanged throughout the incubation period. The minimal effect of the saturated fatty acid on the physical properties of the membranes is consistent with the results in Fig. 1 showing no significant effect on Ca^{2+}-ATPase activity. Oleic acid causes a marked decrease in fluorescence anisotropy of the probe, suggesting the creation of a more disordered mobile hydrophobic domain which is gradually restored to a state comparable to that of the native membrane over a period of 1–2 h. Higher concentrations of oleic acid ($> 4\,\mu M \cdot mg\ protein^{-1}$) have been shown to cause a much greater decrease in fluorescence anisotropy, and the fluorescence properties do not recover during subsequent incubation (Stubbs et al., 1984).

Perturbation of the hydrophobic domain in the vicinity of the Ca^{2+}-ATPase by fatty acids and phospholipids has been monitored by changes in intrinsic fluorescence of the tryptophan residues of the protein (London and Feigenson, 1981; East and Lee, 1982; Simmonds et al., 1982). Loss of enzyme activity caused by increasing concentrations of oleic acid in the membrane was found to result in a progressive decrease in fluorescence intensity from the trytophan, reaching a limiting quenching value of about 70%. Based on changes in fluorescence quenching of Ca^{2+}-ATPase reconstituted into a brominated phospholipid it was shown that oleic acid binds more weakly to
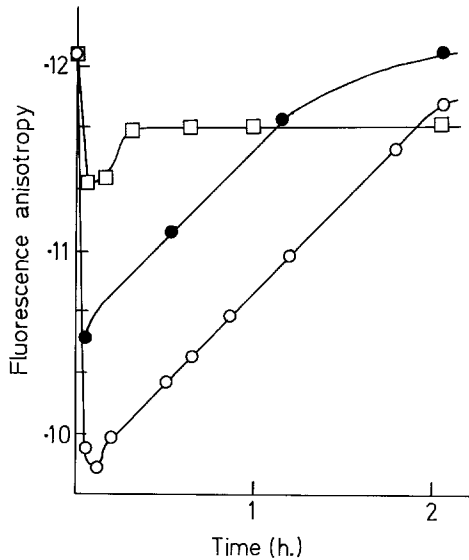
Fig. 3. Fluorescence anisotropy of diphenylhexatriene probe intercalated into sarcoplasmic reticulum vesicles incubated at 37°C after addition of 2 μM palmitic acid ($\square$), 1 μM oleic acid ($\bullet$), and 2 μM oleic acid ($\circ$). Concentrations of fatty acids were expressed as equivalents per mg protein.

the protein than does the phospholipid, and there was evidence to support the existence of fatty acid binding sites to which phospholipids do not have access (Simmonds *et al.*, 1982). Comparison of the effects of oleic acid on Ca^{2+}-ATPase and motion in the hydrophobic domain of the membrane presented in this work suggests that at low concentrations ($< 1 \mu$M $\cdot$ mg protein^{-1}) the fatty acid causes uncoupling of catalytic activity from accumulation of calcium within the vesicles. The uncoupling effect is presumably related to the perturbation of the hydrophobic domain, and because the average probe motion is eventually restored to values observed in the coupled state, an alteration in the arrangement of the native membrane lipid organization is implied. In addition to uncoupling the Ca^{2+}-ATPase, the effect of high concentrations of oleic acid ($> 2 \mu$M $\cdot$ mg protein^{-1}) is to inhibit catalytic site activity, and this is likely to be mediated via a direct interaction of the fatty acid with the enzyme. It is noteworthy that the average motional properties reported by the fluorescence probe are very similar in preparations that are fully active and uncoupled and those that are completely inhibited and presumably leaky to Ca^{2+}. Clearly the effects of oleic acid are not mediated via the bulk lipid properties of the molecules into which the enzyme protein is inserted.

Acknowledgments

References

Cheah, A. M. (1981). *Biochim. Biophys. Acta* **648**, 113–119.

De Boland, A., Jilka, R. L., and Martonosi, A. (1975). *J. Biol. Chem.* **250**, 7501–7510.

East, J. M., and Lee, A. G. (1982). *Biochemistry* **21**, 4144–4151.

Fiehn, W., and Hasselbach, W. (1970). *Eur. J. Biochem.* **13**, 510–518.

Hasselbach, W., and Makinose, M. (1962). *Biochem. Biophys. Res. Commun.* **7**, 44–47.

Heffron, J. J. A., and Gronert, G. A. (1979). *Biochem. Soc. Trans.* **7**, 44–47.

Herbette, L. G., Farreau, C., Segalman, K., Naapolitano, C. A., and Watras, J. (1984). *J. Biol. Chem.* **259**, 1325–1335.

Katz, A. M., Nash-Adler, P., Watras, J., Messineo, F. C., Takenaka, H., and Louis, C. F. (1982). *Biochim. Biophys. Acta* **687**, 17–26.

Kinosita, K., Mitaku, S., Ikegami, A., Ohbo, N., and Kunu, T. L. (1976). *Jpn. J. Appl. Phys.* **15**, 2433–2440.

Kinosita, K., Kataoka, R., Kimura, Y., Gotoh, O., and Ikegami, A. (1981). *Biochemistry* **20**, 4270–4277.

London, E., and Feigenson, G. W. (1981). *Biochemistry* **20**, 1939–1948.

Madden, T. D., Chapman, D., and Quinn, P. J. (1979). *Nature (London)* **279**, 588–541.

Madden, T. D., King, M. D., and Quinn, P. J. (1981). *Biochim. Biophys. Acta* **641** 265–269.

Martonosi, A., and Feretos, R. (1964). *J. Biol. Chem.* **239**, 648–658.

Martonosi, A., Donley, J., and Halpin, R. A. (1968). *J. Biol. Chem.* **243**, 61–70.

Messineo, G. C., Rathier, M., Farreau, C., Watras, J., and Takenaka, H. (1984). *J. Biol. Chem.* **259**, 1336–3343.

Møller, J. V., Anderson, J. P., and Le Maire, M. (1982). *Mol. Cell. Biochem.* **42**, 83–107.

Ng, R. H., and Howard, B. D. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1346–1350.

Simmonds, A. C., East, J. M., Jones, O. T., Rooney, E. K., McWhirler, J., and Lee, A. G. (1982). *Biochim. Biophys. Acta* **693**, 398–406.

Stubbs, C. D., Kinosita, K., Munkonge, F., Quinn, P. J., and Ikegami, A. (1984). *Biochim. Biophys. Acta* **775**, 374–380.

Swoboda, G., Fritzsche, J., and Hasselbach, W. (1979). *Eur. J. Biochem.* **95**, 77–88.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 622–626.

Weber, A. (1971). *J. Gen. Physiol.* **57**, 50–63.