Modulation by fatty acids of Ca^{2+} fluxes in sarcoplasmic-reticulum vesicles

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The fatty acids palmitic ($\text{C}_{16:0}$), stearic ($\text{C}_{18:0}$), arachidic ($\text{C}_{20:0}$) and arachidonic ($\text{C}_{20:4}$) acids inhibit Ca^{2+} uptake and enhance Ca^{2+} efflux measured in vesicles derived from the sarcoplasmic reticulum of skeletal muscle. These effects of the fatty acids are impaired by the Ca^{2+} -ATPase ligands Mg^{2+} , Ca^{2+} and K^{+} , and by

drugs that block the leakage of Ca^{2+} through the Ca^{2+} -ATPase such as Ruthenium Red, spermine [de Meis (1991) *J. Biol. Chem.* **266**, 5736–5742] and thapsigargin [de Meis and Inesi (1992) *FEBS Lett.* **299**, 33–35].

INTRODUCTION

Vesicles derived from the light fraction of the sarcoplasmic reticulum of rabbit skeletal muscle retain a membrane-bound Ca^{2+} -ATPase (EC 3.6.1.38) [1]. This protein is able to pump Ca^{2+} into the vesicles at the expense of ATP hydrolysis. The pump can be reversed and Ca^{2+} can be released from the vesicles in a process coupled with the synthesis of ATP from ADP and P_i [1–4]. The reversal of the Ca^{2+} pump is initiated by phosphorylation of the enzyme by P_i , which forms an acylphosphate residue at the catalytic site of the enzyme [4,5]. This reaction is highly dependent on the Mg^{2+} concentration in the medium and is inhibited by Ca^{2+} ($K_a = 10^{-6}$ M), K^{+} ($K_a = 10^{-4}$ M), Ruthenium Red, the polyamines spermidine and spermine [5–9], and thapsigargin, a highly specific inhibitor of Ca^{2+} -ATPase [10–12].

The ATPase is asymmetrically embedded in the membrane with two domains, an extramembranous catalytic site facing the cytoplasm and a transmembrane helical domain responsible for Ca^{2+} translocation [13,14]. Coupling between catalysis and Ca^{2+} transport is not well understood at present. Measurements of Ca^{2+} efflux from Ca^{2+} -loaded vesicles showed that, in the absence of other enzyme ligands, the portion of the ATPase molecule where the catalytic site is located is no longer able to control the part of the protein involved in Ca^{2+} translocation. As a result, Ca^{2+} leaks through the ATPase at a rate similar to that observed during the reversal of the pump [15]. This leakage is greatly increased by hydrophobic drugs such as phenothiazines, β -adrenergic blocking agents and anaesthetics [9,12,16]. The Ca^{2+} leakage through the pump measured with and without hydrophobic drugs is abolished by the ATPase ligands P_i , Mg^{2+} , K^{+} and Ca^{2+} and by the same drugs that impair the reversal of the Ca^{2+} pump, i.e. thapsigargin, Ruthenium Red and polyamines. Experimental evidence indicates that the effect of these drugs on the rate of Ca^{2+} efflux is related to their binding to the enzyme and not to alterations of the physical state of the membrane lipids [9,12].

Fatty acids, particularly arachidonic acid, are known to regulate several physiological events in different cell types [17–23]. Fatty acids are also known to produce a variety of effects on sarcoplasmic-reticulum vesicles; these depend on concentration, acyl chain length, degree of saturation, and experimental conditions used [24–30]. The effects observed seem to be promoted by interaction of the fatty acids added with the membrane phospho-

lipids surrounding the ATPase [29,31]. In this report we show that, similarly to the phenothiazines, the saturated fatty acids (C_{16} , C_{18} and C_{20}) and arachidonic acid ($\text{C}_{20:4}$) inhibit Ca^{2+} accumulation and stimulate Ca^{2+} efflux from the vesicles. The effects of these fatty acids are antagonized by the same substances that impair the effects of the phenothiazines, suggesting a common mechanism of action.

MATERIALS AND METHODS

Sarcoplasmic-reticulum vesicles were prepared from rabbit skeletal muscle as described by Eletr and Inesi [32]. In this preparation the rate of Ca^{2+} efflux is not altered by ryanodine concentrations ranging from 0.4 to 10 μM [9], and gel electrophoresis reveals only minimal traces of the ryanodine-sensitive Ca^{2+} -channel protein [33].

ATP hydrolysis was determined by measuring the release of [^{32}P] P_i from [^{32}P]ATP [34].

Ca^{2+} uptake and efflux were measured by a filtration method using Millipore filters [35] and ^{45}Ca . After filtration, the filters were washed with 3 $\times$ 5 ml of 3 mM $\text{La}(\text{NO}_3)_3$, and the radioactivity remaining on the filters was counted in a liquid-scintillation counter.

For the efflux experiment, the vesicles were preloaded in a medium with 50 mM Mops/Tris, pH 7.0, 10 mM MgCl_2 , 20 mM P_i , 0.3 mM $^{45}\text{CaCl}_2$, 2 mM ATP and 0.06 mg/ml sarcoplasmic-reticulum vesicles. After 30 min at 35 $^{\circ}\text{C}$, most of the Ca^{2+} present in the medium was taken up by the vesicles. The loaded vesicles were sedimented by centrifugation at 30000 *g* for 20 min, the supernatant was discarded, and the walls of the tubes were blotted to minimize contamination by the residual loading medium. The pellet was kept on ice and resuspended in water immediately before use. The efflux medium contained 40 mM Mops/Tris, pH 7.0, 2 mM EGTA, 0.1 mM P_i and 0.1 mM MgCl_2 . For the efflux measurements, the vesicle concentration was 0.01 mg of protein/ml. Other additions were as indicated in the legends of the Figures. All experiments were performed at pH 7.0 and at 35 $^{\circ}\text{C}$.

Fatty acids were dissolved in ethanol. The amounts used were such that the ethanol concentration in the reaction mixture was never higher than 0.5 % (v/v). At this concentration, ethanol had no effect on either Ca^{2+} uptake or Ca^{2+} efflux. Unsaturated fatty acids were kept under N_2 to avoid spontaneous oxidation. All the

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Table 1 Effects of Mg^{2+} , K^+ , Na^+ and Li^+ on the inhibition of Ca^{2+} uptake induced by fatty acids

The reaction-medium composition was 50 mM Mops/Tris buffer (pH 7.0), 2 mM ATP, 20 mM P_i , 0.1 mM $CaCl_2$ and the additions shown below. Control experiments were performed with 1 mM $MgCl_2$. The reaction time was 20 min. Values are means $\pm$ S.E.M. of 3–7 experiments.

Additions	Inhibition of Ca^{2+} uptake (%)			
	$C_{16:0}$ (50 μ M)	$C_{18:0}$ (16 μ M)	$C_{20:0}$ (16 μ M)	$C_{20:4}$ (8 μ M)
Control	63.3 $\pm$ 3.8	73.3 $\pm$ 6.7	66.9 $\pm$ 2.8	95.0 $\pm$ 1.0
10 mM $MgCl_2$	22.5 $\pm$ 1.5	11.2 $\pm$ 4.1	10.2 $\pm$ 3.1	54.2 $\pm$ 5.1
100 mM KCl	18.3 $\pm$ 2.4	13.6 $\pm$ 1.8	13.6 $\pm$ 2.2	87.2 $\pm$ 1.2
100 mM NaCl	—	—	9.8 $\pm$ 3.7	74.3 $\pm$ 7.6
100 mM LiCl	—	—	8.9 $\pm$ 2.0	85.0 $\pm$ 9.2

Figure 1 Effects of arachidic and arachidonic acids on Ca^{2+} uptake (a) and ATPase activity (b)

The reaction-medium composition was 40 mM Mops/Tris buffer (pH 7.0), 2 mM ATP, 20 mM P_i , 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. Reaction was started by the addition of vesicles to a final concentration of 0.01 mg/ml. The reaction time was 10 min. ○, Arachidic acid ($C_{20:0}$); △, arachidonic acid ($C_{20:4}$).

Figure 2 Effect of Mg^{2+}

The reaction medium composition was 50 mM Mops/Tris buffer (pH 7.0), 2 mM ATP, 20 mM P_i , 0.1 mM $CaCl_2$ and 0.01 mg/ml vesicles. The $MgCl_2$ concentrations used were 1 mM in (a) and (c) and 10 mM in (b) and (d). ○, Control; △, 16 μ M stearic acid ($C_{16:0}$) (a and b) or 16 μ M arachidonic acid ($C_{20:4}$) (c and d).

Figure 3 Effect of Ruthenium Red and spermine on the inhibition of Ca^{2+} uptake induced by arachidonic acid

The reaction medium was the same as in Figure 1. The reaction time was 20 min. ○, No addition; △, 16 μ M arachidonic acid ($C_{20:4}$).

fatty acid solutions were freshly prepared. Fatty acids were obtained from Sigma.

The data shown in this paper are representative of experiments that were repeated with at least three preparations.

RESULTS

Effects of saturated and unsaturated fatty acids on Ca^{2+} uptake and ATPase activity

Ca^{2+} uptake was inhibited by the different fatty acids tested (Table 1). In agreement with Katz et al. [29], we found that unsaturated fatty acids were more effective inhibitors than were the saturated acids. This was measured by using arachidonic acid ($C_{20:4}$) and arachidic acid ($C_{20:0}$) (Figure 1a). The ATPase activity was not impaired by these acids (Figure 1b); on the contrary, it was activated by arachidonic acid ($C_{20:4}$). Thus the inhibition of Ca^{2+} uptake seems to be related to a decrease in the rate of pumping, but rather to leakage of Ca^{2+} accumulated by the vesicles [1].

The inhibition of Ca^{2+} uptake promoted by the different fatty acids decreased when the $MgCl_2$ concentration in the medium was raised from 1.0 to 10.0 mM (Table 1 and Figure 2). Mg^{2+} was more effective in antagonizing the effect of saturated than of unsaturated fatty acids (Table 1).

The univalent cations K^+ , Na^+ and Li^+ were also able to decrease the inhibition promoted by the saturated fatty acid ($C_{20:0}$) but, as observed for Mg^{2+} , had only a minimal effect when arachidonic acid ($C_{20:4}$) was used (Table 1).

The organic compounds Ruthenium Red and spermine antagonized the effects of unsaturated fatty acids (Figure 3). In control experiments without fatty acids, these organic compounds had no effect on the rate of Ca^{2+} uptake.

Effect of fatty acids on Ca^{2+} efflux

Both saturated and unsaturated fatty acids were found to increase the rate of Ca^{2+} efflux (Figure 4). This was observed when vesicles preloaded with calcium phosphate were diluted in a medium containing EGTA and low concentrations of P_i and Mg^{2+} . Ca^{2+} inhibited the efflux induced by saturated fatty acids, but had little or no effect on the efflux induced by arachidonic acid (Figure 5a and Table 2). The Ca^{2+} concentration in the medium needed to antagonize the effect of saturated fatty acids was in the same range as that needed to saturate the high-affinity Ca^{2+} binding site of the ATPase [36]. The effects of both saturated and unsaturated fatty acids on the rate of Ca^{2+} efflux were

Figure 4 Effect of fatty acids on the rate of Ca²⁺ efflux

The reaction-medium composition was 50 mM Mops/Tris buffer (pH 7.0), 0.1 mM P_i, 0.1 mM MgCl₂, 2 mM EGTA and 0.01 mg/ml vesicles previously loaded with ⁴⁵Ca. (a) ○, no addition; △, 16 μM stearic acid (C_{18:0}); □, 32 μM stearic acid (C_{18:0}); (b) ○, no addition; △, 8 μM arachidonic acid (C_{20:4}); □, 16 μM arachidonic acid (C_{20:4}). The symbols show the ⁴⁵Ca remaining in the vesicles after incubation in the efflux medium for the times shown on the abscissae.

Figure 5 Effects of Ca²⁺ (a) and thapsigargin (b) on the increased rate of Ca²⁺ efflux induced by fatty acid

In (a) the reaction medium and experimental conditions were as described in Figure 4. The efflux time was 1 min. ●, No addition; △, 16 μM stearic acid (C_{18:0}). The free Ca²⁺ concentration was calculated by using the apparent association constants for Ca/EGTA provided by Schwartzbach and Flaschka [37] using a computer program as described by Fabiato and Fabiato [38]. In (b), the efflux medium composition was: ●, 4 mM P_i, 10 mM MgCl₂ and no fatty acid; △, ▲, 50 mM Mops/Tris buffer (pH 7.0), 0.1 mM P_i, 0.1 mM MgCl₂, 2 mM EGTA and either (△) 8 μM or (▲) 16 μM arachidonic acid (C_{20:4}). The concentration of loaded vesicles was 0.01 mg/ml and the efflux time was 2 min.

Table 2 Ca²⁺ efflux

The reaction was started by addition of vesicles (0.010 mg of protein/ml) loaded with 2.0–2.3 μmol of Ca²⁺/mg of protein. The reaction mixture contained 50 mM Mops/Tris buffer (pH 7.0), 2 mM EGTA, 0.1 mM P_i, 0.1 mM MgCl₂ and the additions shown below. The Ca²⁺ efflux rates are the difference between the ⁴⁵Ca in the vesicles before and after 1 min incubation at 35 °C. Values are means ± S.E.M. of 3–7 experiments.

Additions	Rate of Ca ²⁺ efflux (μmol/min per mg of protein)		
	Control	C _{20:0} (16 μM)	C _{20:4} (16 μM)
None	0.23 ± 0.04	—	—
Fatty acid	—	1.42 ± 0.08	2.23 ± 0.02
MgCl ₂ (10 mM)	0.01 ± 0.01	0.18 ± 0.03	0.01 ± 0.08
KCl (100 mM)	0.04 ± 0.02	0.06 ± 0.09	0.61 ± 0.14
CaCl ₂ (0.1 mM)	0.01 ± 0.02	0.16 ± 0.08	1.96 ± 0.05
Ruthenium Red (0.1 mM)	0.01 ± 0.01	0.01 ± 0.01	0.16 ± 0.02
Spermine (1 mM)	0.02 ± 0.01	0.09 ± 0.03	1.31 ± 0.06
Thapsigargin (1 μM)	0.02 ± 0.01	0.85 ± 0.03	1.02 ± 0.03

Figure 6 Effect of Ruthenium Red and spermine on the increased rate of Ca²⁺ efflux induced by saturated (a) and unsaturated (b) fatty acids

The reaction medium and experimental conditions were as described in Figure 4. Time of efflux was 1 min. ●, No addition; ○, 100 μM Ruthenium Red; △, 2 mM spermine.

impaired by addition of Mg²⁺, K⁺, Ruthenium Red, spermine or thapsigargin (Figures 5b and 6, and Table 2). The effect of arachidonic acid on the Ca²⁺ efflux (Table 2) was antagonized more effectively by K⁺ than in Ca²⁺-uptake measurements (Table 1). The ability of thapsigargin to antagonize the effect of arachidonic acid varied, depending on the concentration of the fatty acid used (Figure 5b). In the conditions of Table 2, a half-maximal effect was obtained with 1 μM thapsigargin.

DISCUSSION

Among the different fatty acids tested, arachidonic acid was found to be the most active, not only as an inhibitor of Ca²⁺ uptake but also as an agent for increasing the rate of Ca²⁺ efflux. At present we do not know why the response observed with Ca²⁺ and K⁺ varies depending on whether a saturated or an unsaturated fatty acid is used (Figure 5a, Tables 1 and 2). Two hypotheses could be raised: (1) saturated and unsaturated fatty acids have different sites of action; (2) they are acting on the same site, but the affinity of the saturated fatty acid for the site is lower than the affinity of the unsaturated fatty acid, and therefore the former is more easily displaced. In a previous study [9] it was shown that phenothiazines uncouple the Ca²⁺ pump of the sarcoplasmic reticulum and greatly increase the leakage of Ca²⁺ through the ATPase. The finding that the effects of both phenothiazines and fatty acids are antagonized by the transport ATPase ligands Ca²⁺, Mg²⁺ and K⁺, as well as by Ruthenium Red, spermine and thapsigargin, suggests that phenothiazines and fatty acids uncouple the pump by the same mechanism. The mechanism by which Ca²⁺, Mg²⁺, K⁺, Ruthenium Red and spermine increase coupling of the pump has been discussed in detail in previous reports dealing with effects of phenothiazines [9,12,16]. Thapsigargin is a specific inhibitor of the Ca²⁺-ATPase. It stabilizes the ATPase in the E₂ conformation, blocking both the phosphorylation of the E₂ form by P_i and the E₂–E₁ interconversion of the enzyme. Thus the Ca²⁺ pump is locked and Ca²⁺ ions can no longer be translocated in either direction, to or from the vesicles [10–12]. The increment in efflux promoted by 8 μM arachidonic acid is completely antagonized by thapsigargin. However, the increment in efflux promoted by higher arachidonic acid concentrations is only partially impaired by thapsigargin. This raises the possibility that fatty acids may interact with two different regions of the membrane. At low concentrations they would bind only to the enzyme, and the resulting increment in Ca²⁺ efflux would be arrested by thapsigargin. At higher concentrations the fatty acids would bind to the ATPase and, in addition, alter the interactions of the ATPase with the membrane

phospholipids [29]. In this case Ca^{2+} would leave the vesicles through a second route which would not be affected by thapsigargin.

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REFERENCES

- de Meis, L. (1981) in *The Sarcoplasmic Reticulum: Transport and Energy Transduction* (Bittar, E., ed.), vol. 2, John Wiley and Sons, New York
- Tanford, C. (1984) *CRC Crit. Rev. Biochem.* **17**, 123–151
- de Meis, L. (1989) *Biochim. Biophys. Acta* **973**, 333–349
- de Meis, L. and Vianna, A. (1979) *Annu. Rev. Biochem.* **48**, 275–292
- Masuda, H. and de Meis, L. (1973) *Biochemistry* **12**, 4581–4585
- Beil, F. U., Chak, D. and Hasselbach, W. (1977) *Eur. J. Biochem.* **81**, 151–164
- Chaloub, R. M. and de Meis, L. (1979) *J. Biol. Chem.* **255**, 6168–6172
- Alves, E. W. and de Meis, L. (1986) *J. Biol. Chem.* **261**, 16854–16859
- de Meis, L. (1991) *J. Biol. Chem.* **266**, 5736–5742
- Witcome, M., Henderson, I., Lee, A. G. and East, J. M. (1992) *Biochem. J.* **283**, 525–529
- Sagara, Y., Fernandez-Belda, F., de Meis, L. and Inesi, G. (1992) *J. Biol. Chem.* **267**, 12606–12613
- de Meis, L. and Inesi, G. (1992) *FEBS Lett.* **299**, 33–35
- MacLennan, D. H., Brandl, C. J., Korczak, B. and Green, N. M. (1985) *Nature (London)* **316**, 696–700
- Clarke, D. M., Loo, T. W. and MacLennan, D. H. (1990) *J. Biol. Chem.* **265**, 6262–6267
- de Meis, L., Suzano, V. A. and Inesi, G. (1990) *J. Biol. Chem.* **265**, 18848–18851
- Wolosker, H., Pacheco, A. G. F. and de Meis, L. (1992) *J. Biol. Chem.* **267**, 5785–5789
- Randriamampita, C. and Trautmann, A. (1990) *J. Biol. Chem.* **265**, 18059–18062
- Hoffman, T., Lizzio, E. F., Ting, A., Marshall, L. A., Bonvini, E. and Jennings, M. K. (1987) *Clin. Immunol. Immunopathol.* **44**, 82–92
- Ordway, R. W., Walsh, J. V. and Singer, J. J. (1989) *Science* **244**, 1176–1178
- Giaume, C., Randriamampita, C. and Trautmann, A. (1989) *Pflügers Arch.* **413**, 273–279
- Piomelli, D., Volterra, A., Dale, N., Siegelbaum, S. A., Kandel, E. R., Schwartz, J. H. and Belardetti, F. (1987) *Nature (London)* **328**, 38–43
- Halenda, S. P., Banga, H. S., Zavoico, G. B., Lau, L. F. and Feinstein, M. B. (1989) *Biochemistry* **28**, 7356–7363
- Zietler, P. and Handwerker, S. (1985) *Mol. Pharmacol.* **28**, 549–554
- Hasselbach, W. and Makinose, M. (1962) *Biochem. Biophys. Res. Commun.* **7**, 132–136
- Sarzala, M. G. and Drabikowski, W. (1969) *Life Sci.* **8**, 477–483
- Messineo, F., Pinto, P. and Katz, A. M. (1980) *J. Mol. Cell. Cardiol.* **12**, 725–732
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974) *Biochemistry* **13**, 5501–5507
- Cheah, A. (1981) *Biochim. Biophys. Acta* **648**, 113–119
- Katz, A. M., Nash-Adler, P., Watras, J., Messineo, F., Takenaka, H. and Louis, C. F. (1982) *Biochim. Biophys. Acta* **687**, 17–26
- Herbette, L., Messineo, F. C. and Katz, A. M. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22**, 413–434
- Michelangelo, F., Grimes, E. A., East, J. M. and Lee, A. G. (1991) *Biochemistry* **30**, 342–351
- Eletr, S. and Inesi, G. (1972) *Biochim. Biophys. Acta* **282**, 174–179
- Inesi, G. and de Meis, L. (1989) *J. Biol. Chem.* **264**, 5929–5936
- Grubmeyer, C. and Penefsky, H. S. (1981) *J. Biol. Chem.* **256**, 3718–3727
- Chiesi, M. and Inesi, G. (1979) *J. Biol. Chem.* **254**, 10370–10377
- Inesi, G., Kurzmack, M., Coan, C. and Lewis, D. E. (1980) *J. Biol. Chem.* **255**, 3025–3031
- Schwartzbach, G. and Flaschka, H. (1965) *Die komplexometrische titration*, Euke Verlag, Stuttgart
- Fabiato, A. and Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505