Inactivation of sarcoplasmic-reticulum Ca^{2+} -ATPase in low-frequency-stimulated muscle results from a modification of the active site

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Molecular changes underlying the partial inactivation of the sarcoplasmic-reticulum (SR) Ca^{2+} -ATPase in low-frequency-stimulated fast-twitch muscle were investigated in the present study. The specific Ca^{2+} -ATPase activity, as well as the ATP- and acetyl phosphate-driven Ca^{2+} uptakes by the SR, were reduced by approx. 30 % in 4-day-stimulated muscle. Phosphoprotein formation of the enzyme in the presence of ATP or P_i was also decreased to the same extent. Measurements of ATP binding revealed a 30 % decrease in binding to the enzyme. These changes were accompanied by similar decreases in the ligand-induced (ATP, ADP, P_i) intrinsic tryptophan fluorescence. A decreased binding of fluorescein isothiocyanate (FITC) corresponded to the lower ATP binding and phosphorylation of the enzyme. Moreover, P_i -induced changes in fluorescence of the FITC-labelled enzyme did not differ between SR from stimulated and contralateral muscles, indicating that Ca^{2+} -ATPase molecules which did not bind FITC were responsible for the decreased P_i -dependent phosphorylation, and therefore represented the inactive form of the enzyme. No differences existed between the Ca^{2+} -induced changes in the intrinsic fluorescence of SR from stimulated and contralateral muscles which fit their similar Ca^{2+} -binding characteristics. Taking the proposed architecture of the Ca^{2+} -ATPase into consideration, our results suggest that the inactivation relates to a circumscribed structural alteration of the enzyme in sections of the active site consisting of the nucleotide-binding and phosphorylation domains.

INTRODUCTION

The Ca^{2+} -ATPase of the sarcoplasmic reticulum (SR), a Ca^{2+} -transporting enzyme, plays an essential role in muscle relaxation (Gillis, 1985). Previous work from our laboratory has shown that increased contractile activity as imposed by chronic low-frequency stimulation leads to a partial inhibition of the SR Ca^{2+} -ATPase in fast-twitch muscles of rat and rabbit (Heilmann & Pette, 1979; Leberer *et al.*, 1987; Simoneau *et al.*, 1989; Dux *et al.*, 1990; Matsushita *et al.*, 1991). The decreased Ca^{2+} -ATPase activity resulted from a decrease in the specific activity of the enzyme, because the immunochemically determined amount of the Ca^{2+} -ATPase protein was shown to be unaltered (Leberer *et al.*, 1987). Moreover, the Ca^{2+} -ATPase from stimulated muscle displayed decreases in formation of the phosphorylated intermediate and in the binding of fluorescein isothiocyanate (FITC) (Leberer *et al.*, 1987). In addition, the enzyme was characterized by an increased resistance towards trypsin at Arg-505, the first tryptic cleavage site (Dux *et al.*, 1990). These observations were interpreted as resulting from a structural modification of the enzyme in the region of its nucleotide-binding site, causally related to the inactivation of the enzyme.

The impaired function of the Ca^{2+} -ATPase in low-frequency-stimulated muscle was accompanied by a prolonged muscle relaxation and a decreased uptake of Ca^{2+} by the SR (Heilmann & Pette, 1979; Leberer *et al.*, 1987; Simoneau *et al.*, 1989). Prolonged relaxation and decreased Ca^{2+} uptake by the SR were also observed in muscles exercising at high intensity and have been related to the phenomenon of muscle fatigue (Byrd *et al.*, 1989; Gollnick *et al.*, 1991). In our view, the changes in the SR Ca^{2+} pump in low-frequency-stimulated muscle relate to the same functional impairment as in exercising muscle, and thus are also

related to muscle fatigue. Therefore the study of molecular changes underlying the partial inactivation of the SR Ca^{2+} -ATPase in stimulated muscle could be of general interest with regard to elucidating basic mechanisms of muscle fatigue.

In the present study, we explored Ca^{2+} -transporting activity, phosphoprotein formation, changes in intrinsic fluorescence by various ligands and ligand-binding capacity of the SR Ca^{2+} -ATPase from low-frequency-stimulated and contralateral muscles. Our results suggest that the inactivation of the Ca^{2+} -ATPase in low-frequency-stimulated fast-twitch muscles results from a modification of the enzyme at its active site.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade. Triton X-100, dodecyl octaethylene glycol monoether (C_{12}E_8) and 3-*O*-methylfluorescein phosphate were obtained from Sigma (Deisenhofen, Germany). Pyruvate kinase and lactate dehydrogenase were purchased from Boehringer (Mannheim, Germany). Fluorescein isothiocyanate (FITC) was from Aldrich (Steinheim, Germany). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5000 Ci/mmol), ^{45}Ca (10–40 mCi/mg of Ca) and D-[6- ^3H]-glucose (20–40 Ci/mmol) were obtained from Amersham (Braunschweig, Germany). $[\text{P}_i\text{-}^{32}\text{P}]\text{P}_i$ (9000 Ci/mmol) was from New England Nuclear (Du Pont de Nemours, Dreieich, Germany).

Animals and chronic stimulation

Adult male New Zealand White rabbits were subjected to low-frequency stimulation (10 Hz, 12 h/day) by the same methods and stimulation protocol as previously described (Schwarz *et al.*, 1983). Animals were killed after 4 days of stimulation, and both left (stimulated) and right (unstimulated control) extensor digi-

Abbreviations used: C_{12}E_8 , dodecyl octaethylene glycol monoether; Ca^{2+} -ATPase, Ca^{2+} -dependent Mg^{2+} -ATPase from sarcoplasmic reticulum (EC 3.6.1.38); DMSO, dimethyl sulphoxide; FITC, fluorescein isothiocyanate; 3-*O*-MFPase, 3-*O*-methylfluorescein phosphatase; SDH, succinate dehydrogenase (EC 1.3.99.1); SR, sarcoplasmic reticulum.

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torus longus and tibialis anterior muscles were excised and processed for measuring Ca^{2+} -ATPase activity in muscle homogenate and for isolation of SR.

Isolation of SR and purification of the Ca^{2+} -ATPase

SR was isolated from both contralateral muscles and stimulated muscles as described by Nakamura *et al.* (1976). Crude SR obtained in this way was dissolved in 0.3 M-sucrose/10 mM-Tris/maleate buffer, pH 7.0, at a protein concentration of 10 mg/ml and incubated overnight at 4 °C. Thereafter, the SR was washed twice by centrifugation for 30 min at 105 000 *g* and 4 °C. The final pellet was homogenized in the same buffer at a protein concentration of 25–30 mg/ml and stored at –70 °C (washed SR). Overnight incubation and successive washing steps were performed to remove contaminating phosphorylase, which was particularly enriched in the microsomal fraction from stimulated muscles. Consequently, the specific activity of the Ca^{2+} -ATPase was slightly increased, especially in the SR from stimulated muscles. Partially purified Ca^{2+} -ATPase was prepared from the washed SR vesicles as described by Meissner *et al.* (1973).

Assay for enzyme activity

The Ca^{2+} -ATPase activity was determined in both muscle homogenates and SR vesicles by using the coupled optical test as described by Simonides & van Hardeveld (1990) and Leberer *et al.* (1987) at pH 7.0 and 25 °C in the presence of 0.005 % Triton X-100 (homogenates) or in the absence and in the presence of 1 μg of calcium ionophore A23187/ml (SR). Ca^{2+} -dependent ATPase activity was defined as the difference between activity of total Ca^{2+} -/ Mg^{2+} -dependent ATPase activity and Mg^{2+} -ATPase activity. When the activity was measured after solubilization, 0.5 mg of C_{12}E_8 /ml was added to the assay mixture. Ca^{2+} -dependent 3-*O*-methylfluorescein phosphatase (3-*O*-MFPase) activity was measured in both muscle homogenates and SR at 25 °C as described by Everts *et al.* (1989). This activity was reported to represent phosphatase activity of the SR Ca^{2+} -ATPase (Everts *et al.*, 1989). The activity of succinate dehydrogenase (SDH) was determined photometrically at 30 °C (Reichmann *et al.*, 1985).

Assay for Ca^{2+} uptake

Ca^{2+} uptake by the SR was assayed at 25 °C with a Ca^{2+} -sensitive electrode in the presence of 20 mM-potassium oxalate as described by Leberer *et al.* (1987) with ATP (5 mM) or acetyl phosphate (10 mM) as substrates. Both initial and total Ca^{2+} uptakes were calculated after back-titration with 10 mM- CaCl_2 solution.

Fluorescence measurements

Fluorescence measurements on SR Ca^{2+} -ATPase were performed by using either the intrinsic tryptophan fluorescence or FITC-labelled Ca^{2+} -ATPase. Fluorescence-signal changes were measured at 20 °C (tryptophan fluorescence) or 30 °C (FITC fluorescence) with a Perkin-Elmer LS-5B or MPF 44A spectrofluorimeter with a continuously stirred cell. Excitation and emission wavelengths were 295 nm and 330 nm respectively for tryptophan fluorescence, or 495 nm and 525 nm respectively for FITC fluorescence. In each measurement 50–75 μg of SR vesicle protein was diluted into 2.5 ml of the appropriate solutions (see the Figure legends and Tables), and different substrates were added with a Hamilton syringe. Changes in fluorescence intensities were evaluated by using a digital integrator, taking into account the effects of dilution and the molar absorption of nucleotides at 295 nm. Changes were expressed as increases or decreases in fluorescence relative to the initial values. For FITC fluorescence

measurement at pH 7.0, the samples were preincubated for 30 min at 30 °C before addition of substrates. This preincubation was effective to give stable and reproducible fluorescence intensities, as suggested previously (Froud & Lee, 1986).

Solubilization of Ca^{2+} -ATPase

SR vesicles were solubilized with C_{12}E_8 in 10 mM-Mops/Tris (pH 7.0)/100 mM-KCl to give final concentrations of protein and detergent of 2 and 4 mg/ml respectively. The insoluble residue was removed by centrifugation for 30 min at 105 000 *g*. The supernatant was used for measurement of the Ca^{2+} -ATPase activity and intrinsic fluorescence studies.

Phosphoprotein formation

SR (0.8 mg/ml) was phosphorylated with either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\text{P}^{32}]\text{P}_i$, essentially as described by Barrabin *et al.* (1984). Phosphorylation by ATP was carried out at 25 °C for 10 s in a reaction medium containing 50 mM-Mops/Tris buffer (pH 7.0), 100 mM-KCl, 5 mM- MgCl_2 , 10 mM- CaCl_2 and 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. In some experiments, higher concentrations of ATP up to 5 mM were used. Phosphorylation by P_i was performed in a reaction medium containing 50 mM-Mes/Tris buffer (pH 6.0), 20 mM- MgCl_2 , 2 mM-EGTA, with or without 40 % (v/v) dimethyl sulphoxide (DMSO) and 2 mM- $[\text{P}^{32}]\text{P}_i$ for 10 min at 25 °C. The reaction was initiated by addition of either ATP or P_i and quenched with ice-cold 0.125 M- HClO_4 and 2 mM- P_i (final concentrations). The quenched protein was washed four times by centrifugation with the same solution, and the final sediment was dissolved in a medium containing 0.1 M-NaOH, 2 % (w/v) Na_2CO_3 , 2 % (w/v) SDS and 5 mM P_i . Samples were taken for protein determination and liquid-scintillation counting.

ATP and Ca^{2+} binding

Measurement of ATP and Ca^{2+} binding were carried out by filtration on Millipore filters (HAWP; 0.45 μm pore size) as described by Champeil & Guillain (1986) and Watanabe *et al.* (1981) respectively. The wet volume of the filters was determined from the $[\text{H}]\text{glucose}$ in the medium. For ATP binding, 200 μg of SR protein was first layered on to the filter in a medium consisting of 150 mM-Mops/Tris (pH 7.0), 5 mM- MgCl_2 and 2 mM-EGTA. After loading, the filter was perfused with the same medium containing the radioactive ATP (different concentrations) and 10 mM- $[\text{H}]\text{glucose}$. The filter was then counted for radioactivity in a scintillation mixture. For Ca^{2+} binding, SR vesicles (0.5 mg/ml) were first incubated at 25 °C for 1 min in a medium containing 50 mM-Mops/Tris (pH 7.0), 100 mM-KCl, 5 mM- MgCl_2 , 10 mM- $[\text{H}]\text{glucose}$, 50 μM - CaCl_2 and various concentrations of EGTA to give the desired concentrations of free calcium. A 200 μg probe was placed on a filter and the radioactivity was measured in a scintillation mixture.

FITC labelling

The SR preparations were suspended in 25 mM-Tris/HCl (pH 8.0)/100 mM-KCl/5 mM- MgCl_2 , at a protein concentration of 1 mg/ml. FITC was added to the suspension from a stock solution freshly prepared in dimethyl formamide. The final concentration of dimethyl formamide did not exceed 0.5 % (v/v). The labelling was carried out at 25 °C for 60 min with FITC concentrations of 1, 2, 3, 4, 5 and 10 μM , and was stopped by centrifugation through a Sephadex G-25 column (Baba *et al.*, 1986). The amount of bound FITC was determined photometrically in 1 % (w/v) SDS and 0.1 M-NaOH by using a molar absorption coefficient of 80 000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 495 nm (Pick & Karlsh, 1980).

Miscellaneous

SDS/PAGE was performed as described by Laemmli (1970).

Gels were stained with Coomassie Brilliant Blue, destained and scanned on a LKB 2202 Ultrascan laser densitometer. Protein concentration was determined as described by Lowry *et al.* (1951), with BSA as a standard. Free Ca^{2+} concentrations were estimated by using the computer program described by Bulos & Sacktor (1979).

RESULTS

Purity of isolated microsomes

Contamination with non-SR membranes has been reported to be increased in microsomal preparations isolated from low-frequency-stimulated fast-twitch muscles (Klug *et al.*, 1983; Leberer *et al.*, 1987). SR vesicles from short-term-stimulated muscles are also contaminated with glycogen particles (Ramirez & Pette, 1974). Indeed, we observed by SDS/PAGE in the SR from stimulated muscle a prominent 94 kDa band, presumably phosphorylase, migrating just below the 102 kDa Ca^{2+} -ATPase (Fig. 1, lane B). By using the washing procedure described in the Materials and methods section, we could remove most of this 94 kDa protein (Fig. 1, lane D). Densitometric analysis of the washed SR preparations showed that the Ca^{2+} -ATPase of contralateral and stimulated muscles amounts to 78 % and 75 % of the total protein respectively, agreeing with previous results (Leberer *et al.*, 1987). With deoxycholate treatment, proteins other than the Ca^{2+} -ATPase were largely extracted, so that the Ca^{2+} -ATPase bands of the SR from contralateral and stimulated muscles amounted to 95 % and 94 % of the total proteins. The amount of the proteins extracted by deoxycholate was 243 $\mu\text{g}/\text{mg}$ of total protein in the contralateral washed SR and 254 $\mu\text{g}/\text{mg}$ in the stimulated washed SR. In addition, SDH activity and Mg^{2+} -

dependent ATPase activity in the washed SR preparations from contralateral and stimulated muscles were similar (respectively 18.0 and 20.0 nmol/min per mg for SDH, 0.35 and 0.33 $\mu\text{mol}/\text{min}$ per mg for Mg^{2+} -ATPase). These values indicated that no significant differences existed between washed SR preparations from stimulated and contralateral muscles with regard to contamination with non-SR membranes. The relative amount of protein of the Ca^{2+} -ATPase was estimated to differ by less than 5 % in SR from stimulated and contralateral muscles. Therefore, washed SR preparations were used for all further studies.

Decreased Ca^{2+} -ATPase and Ca^{2+} -uptake activities in homogenates and SR preparations from stimulated muscles

Measurements on muscle homogenates showed a 24 % decrease in SR Ca^{2+} -ATPase activity of 4-day-stimulated muscle compared with the contralateral value. 3-*O*-MFPase activity was decreased to a similar degree in stimulated muscles (Table 1). Taking into account our previous observation that the amount of Ca^{2+} -ATPase protein did not change up to 28 days of stimulation (Leberer *et al.*, 1987), these findings were interpreted as partial inactivation of the SR Ca^{2+} -ATPase in the stimulated muscle. This notion was supported by activity measurements on washed SR preparations, showing an approx. 30 % decrease in activity for stimulated muscle in both the absence and the presence of ionophore. A similar decrease was observed by the 3-*O*-MFPase activity measurement (Table 1). The decrease in ATPase activity was also observed in deoxycholate-treated SR (results not shown), and thus could not have resulted from contamination with non-SR membranes. Moreover, both initial rate and total capacity of ATP-dependent Ca^{2+} -uptake were decreased in SR preparations from stimulated muscle to the same extent as Ca^{2+} -ATPase activity. A similar decrease in Ca^{2+} uptake was observed in stimulated SR when acetyl phosphate was used as a substrate (Table 1). It is remarkable that not only initial Ca^{2+} -uptake rate but also total Ca^{2+} -uptake capacity were decreased in stimulated SR. This finding is in agreement with the identification of an inactive SR vesicle fraction rich in non-phosphorylating Ca^{2+} -ATPase molecules (Matsushita *et al.*, 1991).

Intrinsic fluorescence

Conformational changes in the Ca^{2+} -ATPase were studied fluorimetrically by using changes in the intrinsic tryptophan fluorescence in washed SR preparations from both stimulated and contralateral muscles. Addition of ATP in the absence of Ca^{2+} enhanced the fluorescence signal. This increase, which is considered to derive from a conformational change produced by ATP binding to the enzyme (Dupont *et al.*, 1985), was smaller in the sample from the stimulated muscle (Table 2). The difference between stimulated and contralateral samples was observed at pH 7, and also at pH 6, where Mg^{2+} does not cause any change in fluorescence (Lacapère *et al.*, 1990). Concentrations of ATP up to 1 mM did not abolish the difference between fluorescence of stimulated and contralateral samples. A lower fluorescence of the stimulated than of the contralateral sample was also observed in the presence of ADP. Similarly, P_i -induced phosphorylation of the enzyme in the absence of Ca^{2+} (Masuda & de Meis, 1973) led to a smaller increase in fluorescence in the stimulated than in the contralateral sample (Table 2). On the contrary, the fluorescence changes induced by EGTA (sufficient for chelating free Ca^{2+} below 10 nM) and CaCl_2 (sufficient for keeping free Ca^{2+} above 0.1 mM) did not show any difference between contralateral and stimulated SR preparations (Table 2). These changes are supposed to reflect Ca^{2+} removal and binding at the high-affinity Ca^{2+} -binding site of the enzyme, which initiate the $\text{E}_1\text{-E}_2$ conformational transition (Dupont *et al.*,

Fig. 1. SDS/PAGE of crude and washed SR from contralateral and stimulated muscles

SDS/PAGE (10 % gel) was carried out as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue. Lanes: A and B, crude SR; C and D, washed SR; A and C, from contralateral unstimulated rabbit fast-twitch muscle; B and D, from 4-day-stimulated rabbit fast-twitch muscle. CaS and GP-53 denote calsequestrin and 53 kDa glycoprotein. Numbers on the left indicate molecular masses of standards in kDa.

Table 1. Catalytic activities and Ca^{2+} -uptake capacity of homogenates and isolated SR from contralateral and stimulated muscles

Muscle homogenates and washed SR preparations were obtained from contralateral and 4-day-stimulated extensor digitorum longus and tibialis anterior muscles. Values are given as means $\pm$ S.E.M. of 3–4 measurements and as ratios of stimulated to contralateral muscles.

	Contralateral	Stimulated	Ratio
Homogenate			
ATPase (nmol/min per mg)	112.7 $\pm$ 4.3	85.7 $\pm$ 3.2	0.76
3-O-MFPase (nmol/min per mg)	2.87 $\pm$ 0.11	2.21 $\pm$ 0.11	0.77
SR			
ATPase ($\mu\text{mol}/\text{min}$ per mg)			
Mg^{2+} -dependent	0.35 $\pm$ 0.02	0.33 $\pm$ 0.03	0.94
Ca^{2+} -dependent	1.35 $\pm$ 0.06	0.96 $\pm$ 0.05	0.71
With ionophore	5.91 $\pm$ 0.23	4.14 $\pm$ 0.18	0.70
3-O-MFPase (nmol/min per mg)	23.3 $\pm$ 1.0	16.7 $\pm$ 0.8	0.72
Ca^{2+} uptake (ATP-driven)			
Initial uptake (μmol of $\text{Ca}^{2+}/\text{min}$ per mg)	9.47 $\pm$ 0.31	6.63 $\pm$ 0.58	0.70
Total uptake (μmol of Ca^{2+}/mg)	20.24 $\pm$ 1.82	16.01 $\pm$ 0.88	0.79
Ca^{2+} uptake (acetyl phosphate-driven)			
Initial uptake (μmol of $\text{Ca}^{2+}/\text{min}$ per mg)	2.16 $\pm$ 0.20	1.56 $\pm$ 0.08	0.72
Total uptake (μmol of Ca^{2+}/mg)	5.44 $\pm$ 0.36	4.16 $\pm$ 0.24	0.76

Table 2. Ligand-induced changes in the intrinsic fluorescence of the SR Ca^{2+} -ATPase

Changes in fluorescence signal were recorded in the following solutions: 150 mM-Mops/Tris (pH 7.0)/5 mM- MgCl_2 /2 mM-EGTA for ATP- or ADP-induced changes at pH 7.0; 150 mM-Mes/Tris (pH 6.0)/5 mM- MgCl_2 /2 mM-EGTA for ATP-induced changes at pH 6.0; 150 mM-Mes/Tris (pH 6.0)/20 mM- MgCl_2 /2 mM-EGTA with or without 20% (v/v) DMSO for P_i -induced changes; 50 mM-Mops/Tris (pH 7.0)/100 mM-KCl/5 mM- MgCl_2 /0.1 mM- CaCl_2 for EGTA- and Ca^{2+} -induced changes. Ligands were added at the final concentrations indicated. For EGTA- and Ca^{2+} -induced changes, 0.5 mM-EGTA and 0.7 mM- CaCl_2 were added successively to the medium. Values are expressed as percentage changes (means $\pm$ S.E.M., $n = 3$ –4) and as ratios of stimulated to contralateral muscles.

Ligand	Concn. (mM)	pH	Change (%)		
			Contralateral	Stimulated	Ratio
ATP	0.1	7.0	4.19 $\pm$ 0.09	2.93 $\pm$ 0.12	0.70
	0.5	7.0	4.32 $\pm$ 0.12	3.20 $\pm$ 0.08	0.74
	1.0	7.0	4.33 $\pm$ 0.22	2.99 $\pm$ 0.25	0.69
	0.1	6.0	4.62 $\pm$ 0.11	3.14 $\pm$ 0.18	0.68
ADP	0.2	7.0	3.78 $\pm$ 0.03	2.46 $\pm$ 0.00	0.65
P_i	10 (No DMSO)	6.0	3.05 $\pm$ 0.01	2.07 $\pm$ 0.08	0.68
	2 (+DMSO)	6.0	3.17 $\pm$ 0.18	2.16 $\pm$ 0.07	0.68
EGTA		7.0	-5.79 $\pm$ 0.26	-5.50 $\pm$ 0.21	0.95
CaCl_2		7.0	5.63 $\pm$ 0.02	5.40 $\pm$ 0.12	0.96

1985). The observed differences in the fluorescence signal changes between SR preparations from stimulated and contralateral muscles by ATP, ADP or P_i matched the difference in Ca^{2+} -ATPase activity (Tables 1 and 2). This finding suggested the presence of inactive enzyme molecules in the SR from stimulated muscle which do not react with ATP and ADP or with phosphate, but are capable of undergoing the E_1 – E_2 conformational transition.

Changes in intrinsic fluorescence were studied also in SR Ca^{2+} -ATPase preparations solubilized with C_{12}E_8 , a non-ionic detergent, previously shown to achieve monomerization of the enzyme without affecting its catalytic activity (Dean & Tanford, 1978; Le Maire *et al.*, 1978). Maximum Ca^{2+} -ATPase activity of the solubilized enzyme amounted to 7.01 $\mu\text{mol}/\text{min}$ per mg for

Fig. 2. Intrinsic fluorescence change of solubilized SR Ca^{2+} -ATPase from (a) contralateral and (b) stimulated muscles induced by ATP

Fluorescence conditions are described in the Materials and methods section. ATP (20 μM) was added to C_{12}E_8 -solubilized Ca^{2+} -ATPase (25 $\mu\text{g}/\text{ml}$) in a medium containing 50 mM-Mops/Tris (pH 7.0), 100 mM-KCl, 5 mM- MgCl_2 , 50 μM - CaCl_2 and 0.5% C_{12}E_8 as indicated by the arrows. The effects of dilution and the molar absorption of ATP at 295 nm were negligible in these measurements.

the SR from contralateral and 5.04 $\mu\text{mol}/\text{min}$ per mg for the SR from stimulated muscle, providing an activity ratio of stimulated to contralateral of 0.72. The fluorescence response associated with addition of ATP to the C_{12}E_8 -solubilized enzyme in the presence of 50 μM - Ca^{2+} consisted of a rapid decrease in fluorescence, indicating the phosphorylation-induced conformational changes of the enzyme (Andersen *et al.*, 1985). This change was smaller in the stimulated (Fig. 2b) than in the contralateral sample (Fig. 2a). Most likely the difference was due to the presence of non-phosphorylatable enzyme molecules in the SR from stimulated muscle. Thus, as previously suggested (Dux *et al.*, 1990), it is unlikely that the inactivation results from a protein–protein interaction in the SR Ca^{2+} -ATPase from stimulated muscle.

Phosphoprotein formation and measurements of Ca^{2+} and ATP binding

The altered properties of the inactive Ca^{2+} ATPase was further examined by measuring phosphoprotein formation and ligand-

Table 3. Phosphoprotein formation of the SR Ca²⁺-ATPase from contralateral and stimulated muscles

Phosphoenzyme formation from ATP or P_i was determined as described in the Materials and methods section. Values are given as means ± S.E.M. (n = 3–4) and as ratios of stimulated to contralateral muscles.

	Phosphoenzyme formation (nmol/mg)		Ratio
	Contralateral	Stimulated	
ATP	3.97 ± 0.25	2.82 ± 0.23	0.71
P _i (No DMSO)	1.90 ± 0.04	1.24 ± 0.28	0.65
(+DMSO)	4.41 ± 0.03	3.00 ± 0.17	0.68

Fig. 3. Binding of ATP to SR from contralateral (○) and stimulated (●) muscles

ATP binding was measured as described in the Materials and methods section.

Fig. 4. Binding of Ca²⁺ of the SR from contralateral (○) and stimulated (●) muscles

SR (0.5 mg/ml of protein) was incubated in a medium with defined free ⁴⁵Ca²⁺ concentrations. Thereafter, 200 μg samples were applied to Millipore filters and the amount of bound Ca²⁺ was determined in a scintillation counter.

binding capacity with radioactive substances (Table 3). Confirming our previous observation (Leberer *et al.*, 1987), the amount of the ATP-dependent phosphorylated intermediate was decreased to 70 % in the SR from stimulated muscle as compared with contralateral SR. This decrease was observed with ATP concentrations between 0.1 and 5 mM. The amount of phosphorylated intermediate was similarly decreased when the phosphorylation was performed in the reverse direction with P_i (Table 3).

Measurements of ATP binding demonstrated that the amount of bound ATP was lower over a wide range of ATP concentrations in stimulated SR (Fig. 3). The decrease in ATP binding

Fig. 5. Inhibition of the Ca²⁺-ATPase activity by FITC

FITC binding to the SR from contralateral (△) and stimulated (▲) muscles was performed as described in the Materials and methods section.

Table 4. Ligand-induced changes in the fluorescence of FITC-labelled SR Ca²⁺-ATPase from contralateral and stimulated muscles

Fluorescence changes were measured at 30 °C under the conditions described in the Materials and methods section. Samples containing 60 μg of protein were suspended in 2.5 ml of either 150 mM-Mes/Tris (pH 6.0)/20 mM-MgCl₂/2 mM-EGTA or 50 mM-Mops/Tris (pH 7.0)/100 mM-KCl/5 mM-MgCl₂/0.2 mM-EGTA. P_i (10 mM) was added to the first medium. CaCl₂ (0.3 mM) and EGTA (0.7 mM) were added successively to the second medium. Values are given as means ± S.E.M. (n = 3–4) and as ratios of stimulated to contralateral muscles.

	Change (%)		Ratio
	Contralateral	Stimulated	
P _i	−2.62 ± 0.03	−2.71 ± 0.01	1.03
CaCl ₂	−6.32 ± 0.06	−6.07 ± 0.11	0.96
EGTA	6.08 ± 0.12	6.28 ± 0.28	1.03

demonstrated by direct measurement was in agreement with the lower fluorescence signal evoked by ATP (Table 2). Moreover, the altered ATP-binding capacity explained the lower amount of phosphorylated intermediate in stimulated muscle than in contralateral muscle (Table 3).

Contrary to the decreased ATP binding, low-frequency stimulation had no effect on the Ca²⁺ binding characteristic of Ca²⁺-ATPase (Fig. 4). This finding was in agreement with the results obtained by fluorescence measurement (Table 2) and indicated that the high-affinity Ca²⁺-binding site was unaltered in the inactive non-phosphorylating enzyme molecules in the stimulated sample. The molar ratio of phosphoenzyme to bound Ca²⁺ at 100 μM-ATP and 10 μM-Ca²⁺ was 0.513 and 0.375 for SR preparations from contralateral and stimulated muscles respectively.

FITC labelling and conformational changes

The results from activity measurement and phosphoprotein formation suggested that the inactive Ca²⁺-ATPase was not phosphorylated by either ATP or P_i, but that the phosphorylated enzyme molecules were catalytically active. In order to confirm this assumption, we performed studies on FITC-labelled SR samples. FITC binds to Ca²⁺-ATPase in a manner competitive with ATP (Pick & Karlsh, 1980). As shown in Fig. 5, Ca²⁺-ATPase activity of the SR from stimulated muscle was inhibited 100 % by 3.4 nmol of FITC/mg of protein, whereas inhibition was complete at 5.0 nmol of FITC/mg of protein in the SR from contralateral muscle. The 30 % higher value for the contralateral

sample suggested that the enzyme molecules which bound FITC represented the active Ca^{2+} -ATPase. This agrees with the finding that FITC-labelled SR from stimulated and contralateral muscles displayed equal fluorescence responses to addition of P_i (Table 4). Thus Ca^{2+} -ATPase molecules which did not bind FITC were responsible for the decreased phosphorylation by P_i .

DISCUSSION

In our previous studies we demonstrated that the SR Ca^{2+} -ATPase in low-frequency-stimulated rabbit muscle displays a decreased binding of FITC and a low susceptibility to trypsin at its first cleavage site (T_1) (Leberer *et al.*, 1987; Dux *et al.*, 1990). Considering the locations of the FITC-binding site (Lys-515) and the T_1 cleavage site (Arg-505), a modification of the nucleotide-binding site was suggested as a possible cause of the Ca^{2+} -ATPase inactivation. However, Champeil *et al.* (1988) have shown that FITC does not exclude binding of ATP at higher (millimolar) concentrations. Moreover, replacement of Lys-515 by site-directed mutagenesis did not abolish ATP-dependent phosphoenzyme formation (Maruyama *et al.*, 1989). These results indicate that FITC-binding and nucleotide-binding sites are not identical. Therefore it was decided to investigate directly the nucleotide-binding capacity of the partially inactivated Ca^{2+} -ATPase from low-frequency-stimulated muscle.

Our results show that the ATP- or ADP-induced change in the intrinsic fluorescence of the SR Ca^{2+} -ATPase is decreased in the stimulated muscle to the same extent as the catalytic activity of the enzyme. This change depends on the amount of bound nucleotide and relates to a conformational alteration of the enzyme (Lacapère *et al.*, 1990). Therefore our findings suggest the presence of Ca^{2+} -ATPase molecules that do not bind ATP or ADP. This interpretation is supported by the results obtained from direct measurement of ATP binding.

In addition to the decreased binding of ATP and ATP-dependent formation of the phosphorylated intermediate, we found a decreased P_i -dependent formation of the phosphorylated intermediate, the latter being in agreement with a similarly decreased change in P_i -induced fluorescence. However, P_i induces similar fluorescence changes of the FITC-labelled enzyme in both stimulated and contralateral SR preparations. This indicates that the lower P_i -induced intrinsic fluorescence changes observed in stimulated SR relate to the presence of inactive enzyme molecules that do not bind FITC. Hence we conclude that the inactive Ca^{2+} -ATPase is altered with regard to phosphorylation by both ATP and P_i . On the contrary, Ca^{2+} binding to the high-affinity site appears unaffected, since no differences were found with regard to ^{45}Ca binding, or to Ca^{2+} -induced fluorescence changes between SR preparations from stimulated and contralateral muscles.

The Ca^{2+} -ATPase has been shown to have an extramembrane domain composed of a large pear-shaped lobe on the cytoplasmic surface of the SR, connected to the membrane by a narrow stalk (Taylor *et al.*, 1986; Stokes & Green, 1990). According to the model proposed by MacLennan and colleagues, the extramembrane portion consists of three major domains, namely the nucleotide-binding domain where ATP binds, the phosphorylation domain which contains the phosphorylating Asp-351, and the transduction domain which links phosphorylation to Ca^{2+} transport (MacLennan *et al.*, 1985; Brandl *et al.*, 1986). The two high-affinity Ca^{2+} -binding sites are located in the transmembrane domain (Clarke *et al.*, 1989), some distance from the ATP-binding site, which is supposed to be exposed into the cytoplasmic region (Scott, 1985; Gutierrez-Merino *et al.*, 1987). Recent studies with monoclonal antibodies also suggest that the nucleotide-binding domain is located on the uppermost cyto-

plasmic surface of the ATPase (Tunwell *et al.*, 1991). On the other hand, the ATP-binding site on the nucleotide-binding domain and Asp-351 on the phosphorylation domain have been postulated to be in close proximity. Segments of these domains form the active site of the enzyme (Serrano, 1989; Taylor & Green, 1989). Based on these structural analyses, we interpret the results of our study as pointing to a preferential modification of the active site on the cytoplasmic membrane surface, resulting in an impaired phosphorylation by both ATP and P_i .

Contrary to the steric hindrance of nucleotide-binding and phosphorylation of the enzyme by FITC, which can be partially overcome by high ATP concentrations (Champeil *et al.*, 1988), elevated ATP concentrations do not have a similar effect on the inactive Ca^{2+} -ATPase from low-frequency-stimulated muscle. Moreover, Ca^{2+} -uptake measurements in the presence of acetyl phosphate do not provide evidence that this substrate is able to counteract the impaired function of the SR Ca^{2+} -ATPase from stimulated muscle, whereas acetyl phosphate can act as a phosphorylating substrate of the FITC-labelled enzyme (Pick & Karlsh, 1980). These results suggest that the restriction of ATP binding and phosphoprotein formation in the inactive Ca^{2+} -ATPase cannot be explained by a spacial interference of ligands binding to the enzyme, as in the case of FITC. This notion is supported by the impaired phosphorylation of the inactive enzyme by P_i in the reverse direction. It is therefore assumed that the inactivation of SR Ca^{2+} -ATPase in low-frequency-stimulated muscle results from an essential structural change of the enzyme at its active site which affects the co-ordinated functions of both nucleotide-binding and phosphorylation domains. Further studies are necessary in order to define the molecular nature of this modification, as well as to elucidate the intracellular events causally related to this phenomenon.

In conclusion, chronic low-frequency stimulation induces inactivation of the SR Ca^{2+} -ATPase in rabbit fast-twitch muscle in a manner which leaves the Ca^{2+} -binding capacity intact, but affects the phosphorylation by both ATP and P_i . A limited structural alteration of the enzyme at its active site is suggested as a possible cause of the inactivation.

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