The dimeric form of Ca\textsuperscript{2+}-ATPase is involved in Ca\textsuperscript{2+} transport in the sarcoplasmic reticulum

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INTRODUCTION

The possibility that an interaction between SR (sarcoplasmic reticulum) Ca\textsuperscript{2+}-ATPase molecules is required for Ca\textsuperscript{2+} transport is an important issue that has been under investigation since the 1970s [1,2]. A three-dimensional structure of the SR Ca\textsuperscript{2+}-ATPase has been deduced from X-ray diffraction analysis of crystals of solubilized Ca\textsuperscript{2+}-ATPase [3]. However, the details of this structure do not address this fundamental issue, because detergent-solubilized Ca\textsuperscript{2+}-ATPase monomers do not interact stably. Studies on the interaction of ATPase monomers have based their conclusions variously on analysis of the stoichometry of the interaction between Ca\textsuperscript{2+}-ATPase and a bound ligand [4–6], the unusual kinetic behaviour of the protein [7] and visualization of Ca\textsuperscript{2+}-ATPase on membranes by electron microscopy [8,9]. However, none of these methods has provided a way to correlate Ca\textsuperscript{2+} transport with an interaction between Ca\textsuperscript{2+}-ATPase monomers.

The ATPase activity of Ca\textsuperscript{2+}-ATPase does not depend on a physical interaction between Ca\textsuperscript{2+}-ATPase monomers. This was established using detergent-solubilized monomeric Ca\textsuperscript{2+}-ATPase, which was shown to hydrolyse ATP via the formation of a phosphoenzyme intermediate [10,11]. This phosphoenzyme intermediate was found to have an affinity for Ca\textsuperscript{2+} equivalent to that of the membrane-bound Ca\textsuperscript{2+}-ATPase [11]. It was previously shown that Ca\textsuperscript{2+}-ATPase on Xenopus oocyte membranes was as active as a monomer for hydrolysis of ATP [12]. Although these reports indicate that ATPase activity is independent of a molecular interaction between Ca\textsuperscript{2+}-ATPase monomers, they do not establish whether such an interaction is necessary for Ca\textsuperscript{2+} transport.

Analysis of the activity of membrane-bound Ca\textsuperscript{2+}-ATPase revealed a strong correlation between Ca\textsuperscript{2+} transport and the molecular interaction of Ca\textsuperscript{2+}-ATPase monomers. Nagata et al. [13] reported that Ca\textsuperscript{2+} transport by native rabbit SR Ca\textsuperscript{2+}-ATPase is inhibited in reconstituted vesicles by the presence of heat-denatured scallop SR Ca\textsuperscript{2+}-ATPase. Birmachu and Thomas [14] used time-resolved phosphorescence anisotropy and fluorescence energy transfer measurements at physiological temperatures to show that membrane-bound Ca\textsuperscript{2+}-ATPase is stable as an oligomer. Thus these results show that Ca\textsuperscript{2+} transport by the SR Ca\textsuperscript{2+}-ATPase requires an interaction between Ca\textsuperscript{2+}-ATPase monomers.

In the present paper, we elucidate the functional unit of SR Ca\textsuperscript{2+}-ATPase activity by demonstrating that a heterodimer comprising an active Ca\textsuperscript{2+}-ATPase monomer and a FITC-inactivated Ca\textsuperscript{2+}-ATPase monomer neither transports Ca\textsuperscript{2+} nor hydrolyses ATP. Our results indicate that both Ca\textsuperscript{2+} transport and ATP hydrolysis by Ca\textsuperscript{2+}-ATPase require an interaction between individual functional monomers during at least one stage of the catalytic cycle. This finding suggests that the physiologically functional unit of Ca\textsuperscript{2+}-ATPase is a dimer.

EXPERIMENTAL

Reagents

FITC was purchased from Sigma–Aldrich. PEG [poly(ethylene glycol)] 1540 was obtained from Nacali Tesque (Kyoto, Japan). All other reagents were of analytical grade.

Preparation of SR microsomes

SR microsomes were prepared from rabbit skeletal muscle according to the method of Yamada et al. [15]. SR microsomes were suspended in a solution containing 0.1 M sucrose, 0.1 M KCl and 10 mM Hepes/Na (pH 7.0), and were stored at −80°C. Protein concentrations were measured by the method of Lowry et al. [16] using BSA as a protein standard.

FITC labelling of SR Ca\textsuperscript{2+}-ATPase

SR Ca\textsuperscript{2+}-ATPase was labelled with FITC using the methods of Papp et al. [17]. Briefly, 2 mg/ml of SR microsomes were incubated at 30°C with 60 μM FITC in a solution containing 0.3 M sucrose, 5 mM MgCl\textsubscript{2}, 0.1 mM Tris/EGTA and 50 mM Tris/HCl (pH 8.0). After incubation for 10 min at 30°C, the mixture was exhibited neither Ca\textsuperscript{2+} transport nor ATP hydrolysis, suggesting that Ca\textsuperscript{2+} transport by the Ca\textsuperscript{2+}-ATPase requires an interaction between functional Ca\textsuperscript{2+}-ATPase monomers. This finding implies that the functional unit of the Ca\textsuperscript{2+}-ATPase is a dimer.

Key words: Ca\textsuperscript{2+}-ATPase, dimer, FITC, functional unit, membrane fusion, sarcoplasmic reticulum (SR).
ultracentrifuged at 100 000 rev./min (Hitachi S100AT5 rotor) for 10 min to precipitate the FITC-labelled vesicles. The precipitate was washed twice with solution I [0.1 M sucrose, 0.1 M KCl and 10 mM Hepes/Tris (pH 7.0)]. The concentration of residual FITC was less than 1 μM; at this concentration, unbound FITC did not inhibit the activity of Ca²⁺-ATPase. The final precipitate was suspended in solution I. The ATP hydrolysis and Ca²⁺ transport activities of FITC-labelled Ca²⁺-ATPase were 17% and < 1% respectively compared with the activities of native Ca²⁺-ATPase.

Fusion of SR microsomes

The fusion of vesicles containing FITC-labelled and intact Ca²⁺-ATPase was performed using PEG 1540 by the method of Rahamimoff et al. [18] with a slight modification. Briefly, vesicles were incubated for 10 min at 30 ºC in a solution of 25% (v/v) PEG 1540 containing 20% (v/v) DMSO, 150 mM KCl and 20 mM Hepes/Na (pH 7.0). The mixture was the diluted with 7 vol. of 10 mM Hepes/Na (pH 7.0) and allowed to stand for 1 h at room temperature (approx. 23 ºC). The diluted mixture was ultracentrifuged at 100 000 rev./min (Hitachi S100AT5 rotor) for 10 min to precipitate the fused vesicles. The precipitate was suspended in 100 μl of solution I. The Ca²⁺ transport and Ca²⁺-dependent ATPase activities of PEG-treated vesicles were 94.6 ± 15.9% and 31.5 ± 13.5% (means ± S.E.M., n = 7) respectively compared with the activities of the native (PEG-untreated) SR microsomes.

To confirm that Ca²⁺-ATPase molecules from separate vesicles co-existed on the same vesicle after fusion, we measured FRET (fluorescence resonance energy transfer) between FITC- and EITC (eosin 5′-isothiocyanate)-labelled Ca²⁺-ATPase molecules. Samples of SR microsomes were treated separately with 60 μM FITC or 30 μM EITC. The FITC-treated and EITC-treated vesicles were mixed at a 1:1 ratio of protein concentration and then fused using PEG 1540 as described above. As a control, a mixture of FITC- and EITC-labelled microsomes at the same ratio of protein as in the fusion vesicles was prepared immediately prior to FRET measurement. The vesicles were excited at 492 nm. The emission spectrum for the control vesicles had one peak at 515 nm and a shoulder at approx. 550 nm (Figure 1); the peak at 515 nm was derived from the emission of FITC, whereas the slight peak at approx. 550 nm was derived from the emission of EITC (results not shown). The spectrogram of the fusion vesicles revealed peaks at 515 and 546 nm. The intensity of the peak at 515 nm was about half that of the controls, whereas the intensity at 546 nm was approx. 20% higher than that of the controls (Figure 1). The decrease in fluorescence intensity at 515 nm and the increase at 546 nm were attributable to FRET from FITC to EITC. These results indicated that the Ca²⁺-ATPase molecules were present in close proximity on the PEG-treated vesicles.

ATPase activity determination

Fusion vesicles were incubated with 1 mM ATP for 10–20 min at 30 ºC in the presence of 2 μM A23187 in an assay solution [0.1 M sucrose, 0.1 M KCl, 5 mM MgCl₂ and 10 mM Hepes/Na (pH 7.0)] with either 0.1 μM CaCl₂ or 1 mM EGTA. Ca²⁺-dependent ATPase activity was estimated by subtracting the amount of P liberated in the presence of 1 mM EGTA from that liberated in the presence of 0.1 mM CaCl₂. The amount of P, was determined according to the method of Fiske and SubbaRow [19].

Assay of Ca²⁺ transport

Ca²⁺ transport was assayed by continuously monitoring the concentration of Ca²⁺ in the assay solution. Fusion vesicles were suspended in an assay solution containing 0.2 mM antipyrylazo III (Ca²⁺ indicator) and 5 mM sodium oxalate to precipitate Ca²⁺. The Ca²⁺ concentration was calculated photometrically by measuring the absorbance at 700 nm (A₇₀₀) of antipyrylazo III. CaCl₂ (50 μM) and 0.2 mM ATP were added sequentially after the initiation of absorbance measurement at 700 nm. The Ca²⁺ transport activity was determined by monitoring the change in absorbance for 50 or 100 s after ATP addition.

pNPP (p-nitrophenyl phosphate) hydrolysis activity assay

Fusion vesicles were incubated with 5 mM pNPP for 15 min at 30 ºC in an assay solution [0.1 M sucrose, 0.1 M KCl, 5 mM MgCl₂ and 10 mM Hepes/Na (pH 7.0)] containing 2 μM A23187 with either 0.1 μM CaCl₂ or 1 mM EGTA. The Ca²⁺-dependent ATPase activity was estimated by subtracting the amount of p-nitrophenol formed in the presence of 1 mM EGTA from that formed in the presence of 0.1 mM CaCl₂. The amount of pNPP was determined by monitoring the absorbance at 420 nm (A₄₂₀).

RESULTS

To test the possibility that transport of Ca²⁺ by the SR Ca²⁺-ATPase requires an intermolecular interaction between enzyme monomers, we measured the activity of a FN dimer (heterodimers comprising native and FITC-labelled Ca²⁺-ATPase molecules). We hypothesized that if transport of Ca²⁺ by Ca²⁺-ATPase requires an interaction between Ca²⁺-ATPase molecules (i.e., if the functional unit is a monomer), then the FN dimer should transport Ca²⁺. Conversely, if a molecular interaction is unnecessary (i.e., if the functional unit is a monomer), then the FN dimer should transport Ca²⁺.

To obtain FN dimers on single vesicles, we prepared fusion vesicles (FN vesicles) containing native and FITC-labelled Ca²⁺-ATPase molecules. We hypothesized that if transport of Ca²⁺ by Ca²⁺-ATPase requires an interaction between Ca²⁺-ATPase monomers (i.e., if the functional unit is a dimer), then the FN dimer should not transport Ca²⁺. Conversely, if a molecular interaction is unnecessary (i.e., if the functional unit is a monomer), then the FN dimer should transport Ca²⁺.

To obtain FN dimers on single vesicles, we prepared fusion vesicles (FN vesicles) containing native and FITC-labelled Ca²⁺-ATPase molecules. We hypothesized that if transport of Ca²⁺ by Ca²⁺-ATPase requires an interaction between Ca²⁺-ATPase monomers (i.e., if the functional unit is a dimer), then the FN dimer should not transport Ca²⁺. Conversely, if a molecular interaction is unnecessary (i.e., if the functional unit is a monomer), then the FN dimer should transport Ca²⁺.
The ATP-hydrolysis (grey bar) and Ca\(^{2+}\)-transport (white bar) activities of the native fusion vesicles were 5.19 ± 1.2 μmol/mg per min and 0.27 ± 0.2 μmol/mg per min (means ± S.E.M.; n = 7) respectively. Under the same assay conditions, the ATP-hydrolysis and Ca\(^{2+}\)-transport activities of PEG-untreated SR microsomes were 5.63 ± 1.74 μmol/mg per min and 0.79 ± 0.29 μmol/mg per min (means ± S.E.M.; n = 7) respectively (results not shown). The activities of the fusion vesicles containing native and FITC-labelled Ca\(^{2+}\)-ATPase at a molar ratio of 1:1 (Native/FITC) were approx. 25% of the respective activities of the native fusion vesicles. Vesicles containing only FITC-labelled Ca\(^{2+}\)-ATPase (FITC) were analysed as a control.

We measured the initial velocity of Ca\(^{2+}\) transport and ATP hydrolysis in FN vesicles containing native and FITC-labelled Ca\(^{2+}\)-ATPases at a molar ratio of 1:1. We postulated that if the FN dimers could neither transport Ca\(^{2+}\) nor hydrolyse ATP, then the residual activity of the FN vesicles would be 25% that of the NN vesicles. Even if the efficiency of vesicle fusion was not 100%, the Ca\(^{2+}\) transport activity of the FN vesicles would be less than 50% of that of the NN vesicles. By contrast, if the FN dimers were active, then the residual activity of the FN vesicles would be 50% that of the NN vesicles. We found that Ca\(^{2+}\) transport and ATP hydrolysis by the FN vesicles were 32% and 24% respectively compared with the activities of the NN vesicles (Figure 2). The FF vesicles showed 0.8% and 0% of the ATP hydrolysis and Ca\(^{2+}\) transport activities respectively compared with the NN vesicles (Figure 2). The Ca\(^{2+}\) transport and ATP hydrolysis activities of the FN vesicles were equivalent to 25% of those of the NN vesicles. The residual activities of the FN vesicles suggest that these activities could be attributed to the NN dimers (which represented 25% of the total number of Ca\(^{2+}\)-ATPase molecules on the vesicle membrane). Thus the FN dimers neither transported Ca\(^{2+}\) nor hydrolysed ATP. This finding indicates that the Ca\(^{2+}\) transport activity of Ca\(^{2+}\)-ATPase in the dimeric state requires collaboration between active monomers. This may imply the existence of intermolecular interactions between functional Ca\(^{2+}\)-ATPase monomers.

To confirm that the FN dimers could neither transport Ca\(^{2+}\) nor hydrolyse ATP, we measured the Ca\(^{2+}\)-transport activity of fusion vesicles loaded with native and FITC-labelled Ca\(^{2+}\)-ATPase at various ratios. We expected that if the FN dimers did not transport Ca\(^{2+}\), then the residual activity should be proportional to the amount of the FN dimer. We therefore screened a series of native and FITC-labelled Ca\(^{2+}\)-ATPase molecules mixed at various ratios (0:4, 1:3, 2:2, 3:1 and 4:0). The ratios in these mixes resulted in estimated amounts of NN dimer formation of 0, 6.3, 25.0, 56.3 and 100% respectively of the total amount of Ca\(^{2+}\)-ATPase (Figure 3). We found that the initial velocity of Ca\(^{2+}\) trans-

![Figure 2](Image)  
**Figure 2** Ca\(^{2+}\)-dependent ATP hydrolysis and Ca\(^{2+}\)-transport activities of fusion vesicles

![Figure 3](Image)  
**Figure 3** Ca\(^{2+}\)-transport activity of fusion vesicles with various ratios of native and FITC-labelled Ca\(^{2+}\)-ATPases

Ca\(^{2+}\)-transport activity of fusion vesicles was measured photometrically (see the Experimental section). Results are presented as the ratio of the activity of the fusion vesicles to that of the vesicles containing only native Ca\(^{2+}\)-ATPase (1:1). Ca\(^{2+}\)-transport activity of NN dimer vesicles was 4.70 μmol/mg per min. Broken and solid lines represent the estimated percentages of native Ca\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPases that form the NN dimer in fusion vesicles respectively.

It is well known that FITC-labelled Ca\(^{2+}\)-ATPase retains pNPPase (pNPP hydrolase) activity [21]. Therefore Ca\(^{2+}\)-dependent pNPPase activity of fusion vesicles was measured (Figure 4). The FN and FF vesicles retained pNPPase activity equivalent to that of NN vesicles (approx. 75% and 70% of that of the NN vesicles respectively), whereas the ATPase activity of the FF vesicles used in this assay was 30.0% and 0.01% respectively of the activity of the NN vesicles (Figure 4). The equivalent pNPPase activities of the FN and FF vesicles were similar to that observed with solubilized
interactions between Ca\(^{2+}\) ATPase molecules on the membrane. Their results showed that partners randomly within the plane of the membrane. A TPase, the dimer formation is favoured and dimers exchange of the SR microsomes and the NN fusion vesicles. The pNPP hydrolysis activities of the FN and FF fusion vesicles were comparable with those with native Ca\(^{2+}\) ATPase. The dimer hypothesis. In terms of Ca\(^{2+}\) transport, however, we could not determine whether the results of Andersen et al. [10] fitted a linear or squared function, because there were too few results.

Contrary to our finding that Ca\(^{2+}\) ATPase in the heterodimers failed to hydrolyse ATP, Takeda and Kawamura [12] suggested that Ca\(^{2+}\) ATPase could hydrolyse ATP irrespective of the presence of inactivated Ca\(^{2+}\) ATPase on the membrane. These authors co-expressed wild-type SR Ca\(^{2+}\) ATPase on Xenopus oocyte membranes along with an inactive mutant protein in which Glu\(^{309}\) had been replaced by an alanine residue. The wild-type Ca\(^{2+}\)-ATPase retained ATP hydrolysis activity, which was independent of the amount of mutant Ca\(^{2+}\)-ATPase. Although the E309A mutant had completely lost the capacity to transport Ca\(^{2+}\), it retained the ability to form a phosphoenzyme intermediate with ATP. [24]. Therefore the discrepancy between their results and ours may be reconciled by assuming that the interaction necessary for Ca\(^{2+}\) transport occurs at a step before formation of the phosphoenzyme.

The step that requires an interaction between Ca\(^{2+}\)-ATPase monomers may be indicated by our pNPP hydrolysis experiments. The FN heterodimer retained pNPP hydrolysis activity, but had lost ATP hydrolysis activity (Figure 4). In FITC-labelled Ca\(^{2+}\)-ATPase, the ATP hydrolysis reaction is inhibited after initiation of the reaction, but before ATP binding; namely, E\(_2\) (Ca\(^{2+}\)-bound form of Ca\(^{2+}\)-ATPase) to E\(_1\) (Ca\(^{2+}\)-unbound form of Ca\(^{2+}\)-ATPase). Therefore the discrepancy between their results and ours may be reconciled by assuming that the interaction necessary for Ca\(^{2+}\) transport occurs at a step before formation of the phosphoenzyme.
between monomers necessary for Ca\(^{2+}\) transport driven by ATP hydrolysis.

The importance of the ATP-binding step for the interaction between Ca\(^{2+}\)-ATPase monomers has been suggested by experiments using detergent-solubilized Ca\(^{2+}\)-ATPase. Andersen et al. [10,11] showed that solubilized Ca\(^{2+}\)-ATPase in the monomeric state could hydrolyse ATP and that the phosphoenzyme intermediate of the solubilized Ca\(^{2+}\)-ATPase could bind Ca\(^{2+}\) with an affinity equivalent to that exhibited by the membrane-bound enzyme. These results are the principal evidence in favour of the monomer hypothesis. However, the Hill coefficient of ATP binding during ATP hydrolysis in the aforementioned experiments was inconsistent with that of membrane-bound Ca\(^{2+}\)-ATPase [25]. Because of this inconsistency in the Hill coefficient, Møller et al. [25] suggested that formation of the dimer (or ‘associated form’ in their terminology) was necessary for ATP regulation. Their results also indicated the possibility that dimer formation takes place at the stage of ATP binding, namely, the E\(_2\) to E\(_1\)-ATP partial reaction.

The issue of what comprises the functional unit of Ca\(^{2+}\)-ATPase should be considered in terms of the mode of its physiological action rather than the intrinsic attributes of the molecule. The aforementioned inconsistency between the Hill coefficient of ATP binding for ATP hydrolysis between solubilized and membrane-bound Ca\(^{2+}\)-ATPase [25] suggests that ATP hydrolysis by solubilized Ca\(^{2+}\)-ATPase reflects the intrinsic attributes of the molecule, but not the physiological properties of membrane-bound Ca\(^{2+}\)-ATPase. This discrepancy is reminiscent of that for oxygen transport by haemoglobin: thus the tetrameric form of haemoglobin, which is characterized by positive co-operation for oxygen binding, is the functional unit for the physiological actions of haemoglobin, regardless of whether each subunit possesses the intrinsic ability to bind oxygen. In this context, the functional unit of Ca\(^{2+}\)-ATPase that underlies the physiological processes of Ca\(^{2+}\) transport and ATP hydrolysis should be investigated using membrane-bound Ca\(^{2+}\)-ATPase rather than solubilized Ca\(^{2+}\)-ATPase.

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SUPPLEMENTARY ONLINE DATA

The dimeric form of Ca\textsuperscript{2+}-ATPase is involved in Ca\textsuperscript{2+} transport in the sarcoplasmic reticulum

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Figure S1  Re-calculation is overlaid on to the original plot by Andersen et al. [1].

REFERENCE


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