[Original]

Monomeric Sarcoplasmic Reticulum Ca^{2+} -ATPase is Functionally Active

Kazuo TAKEDA and Masaru KAWAMURA

Department of Biology, University of Occupational and Environmental Health, Japan. Yahatanishi-ku, Kitakyushu 807-8555, Japan

Abstract: cRNAs for wild type and a functionless mutant of sarcoplasmic reticulum (SR) Ca^{2+} -ATPase were co-injected into *Xenopus* oocytes in various molar ratios. SR Ca^{2+} -ATPase activity of the microsomal fraction prepared from the injected oocytes increased linearly with increasing amounts of wild type cRNA. This result suggests that monomers are functional units of membrane-bound SR Ca^{2+} -ATPase.

Key words: sarcoplasmic reticulum Ca^{2+} -ATPase, functional unit, *Xenopus* oocyte.

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Introduction

Sarcoplasmic reticulum (SR) Ca^{2+} -ATPase consists of a single polypeptide chain and belongs to the P-type ion transporting ATPase family, which forms phosphorylated intermediates during the catalytic cycle. Ca^{2+} -ATPases have a tendency to associate into a dimeric structure both in the solubilized [1] and membrane-bound forms [2]. It has been claimed that this dimeric protein-protein interaction is necessary for regulating the enzyme's kinetic mechanism [3] and a functional dimer has been proposed [4], although fully functional monomeric Ca^{2+} -ATPase, i.e., where the monomer constitutes the minimum functional unit, has been described [5, 6].

The aim of this study is to examine the oligomeric state of SR Ca^{2+} -ATPase in the membrane using the *Xenopus* oocyte expression system as was done for the Na^+ , K^+ -ATPase [7]. Combinations of wild type and functionless mutant cRNAs for Ca^{2+} -ATPase were injected into *Xenopus* oocytes, and Ca^{2+} -ATPase activities were assayed. If a protein-protein interaction *per se* is indispensable for the enzymatic function, heterogenous oligomers consisting of active wild type monomer and the inactive mutant should show little activity. If random association of wild type and mutant occurs, formation of homogeneous wild type oligomer will be proportional to p^n , where n is the number of monomers associated and p is the fraction of wild type monomer in mixtures of wild type and mutant monomers. Ca^{2+} -ATPase activity increases with increasing p , and this increase in activity depends on n . If enzyme activity is

proportional to p , Ca^{2+} -ATPase should be a functional monomer throughout the catalytic cycle. If enzyme activity is proportional to p^2 , Ca^{2+} -ATPase should be a functional dimer at any particular step or throughout the catalytic cycle. Theoretical curves indicating the relationship between Ca^{2+} -ATPase activity and p^n are shown in Fig. 1 of ref. [7]. Our findings indicate that the sarcoplasmic reticulum Ca^{2+} -ATPase is a functional monomer.

Materials and Methods

Construction of mutant cDNA

A functionless mutant (E309A) was constructed from cDNA of rabbit skeletal muscle SR Ca^{2+} -ATPase, in which glutamate 309 was substituted by alanine using a site-directed mutagenesis kit (Stratagene, La Jolla, USA). This glutamate is located in the 4th transmembrane segment and is conservative for all Ca^{2+} -ATPases. The fact that mutants of this residue are expressed at similar levels as wild type in COS-1 cells and have shown Ca^{2+} -dependent phosphorylation from P_i [8] suggests that this substitution will not introduce significant conformational changes.

Expression in Xenopus oocytes

cRNAs for wild type and E309A were synthesized using an in vitro transcription kit (Ambion, Austin, USA), mixed by changing the molar ratios (0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25, and 1:0), while keeping the total cRNA at 16 ng, and injected into Stage V or VI oocytes. Oocytes were incubated at 19°C for 3 days in modified Barth's medium [7].

Ca^{2+} -ATPase activity

About 80 oocytes for each preparation were homogenized and the microsomal fraction was recovered from the homogenate by centrifugation at 213000 $\times g$ for 20 min. Microsomes were further treated with 0.47 M NaSCN for 30 min at 20°C. Ca^{2+} -ATPase activity was measured at 37°C in a reaction mixture (20 μl) containing 20 mM HEPES (pH 7.2), 10 μM CaCl_2 , 100 mM KCl, 3 mM MgCl_2 , 5 mM NaN_3 , 100 μM ouabain, 0.6 mM ATP and microsomes (about 10 μg of protein). The released inorganic phosphate was assayed using the malachite green method [9]. The thapsigargin-sensitive Ca^{2+} -ATPase activity, defined as the SR Ca^{2+} -ATPase activity, was calculated as the difference between the activities in the presence and absence of 400 nM thapsigargin.

Quantification of expressed Ca^{2+} -ATPase

To measure the amount of expressed Ca^{2+} -ATPase, SDS-polyacrylamide gel electrophoresis followed by western blotting was performed. Microsomes (15 μg of protein) were incubated in a sample buffer containing 2% SDS, 2% β -mercaptoethanol, 10% glycerol and 10 mM Tris-HCl (pH 6.8) at 37°C for 10 min, and applied to an SDS-polyacrylamide gel. Sepa-

Fig. 1. Expression of cRNA in oocytes. (A): fifteen micrograms of each microsomal protein prepared from oocytes injected with cRNA for wild type alone (lane 10–12), cRNA for E309A mutant alone (lane 2–4), or cRNAs for wild type and E309 with changing molar ratios, holding the total amount of cRNAs constant at 16 ng per oocyte (lane 5–9), or un-injected (lane 1) were separated on an SDS-polyacrylamide gel, blotted on polyvinylidene difluoride membrane and Ca²⁺-ATPase was detected by anti-Ca²⁺-ATPase antisera. (B): expression of cRNA for wild type alone (●), E309A alone (△), and co-expression of cRNAs for wild type and E309A (◇) were quantified and plotted. Values were the mean of two different experiments.

Fig. 2. SR Ca^{2+} -ATPase activity of cRNA-injected oocytes. Oocytes were injected with cRNA for wild type alone (●) or cRNAs for wild type and E309A together, holding the total amount of cRNAs constant at 16 ng (△). The abscissa shows the amount of cRNA for wild type. Normalized activities from two different experiments were averaged.

rated proteins were transferred to polyvinylidene difluoride membrane, and Ca^{2+} -ATPase was detected by anti- Ca^{2+} -ATPase antisera (a kind gift of Dr. H. Suzuki of Asahikawa Medical College), followed by secondary antibody and chemiluminescence (Roche Molecular Biochemicals, Mannheim, Germany). Quantification of Ca^{2+} -ATPase bands was performed with a digital imaging system (Fluor Chem, Alpha Innoteck, San Leandro, USA).

Results and Discussion

The quantitative relationship between the amount of cRNA injected and expressed SR Ca^{2+} -ATPase is shown in Fig. 1. Both in the case of wild type and E309A mutant, the amount of synthesized protein depended on the amount of injected cRNA, and no significant difference between wild type and E309A was observed. In the series of co-expression experiments with wild type and E309A in different molar ratios, keeping the total injected cRNA constant, the amounts of Ca^{2+} -ATPases expressed were almost at the same levels irrespective of the amounts of each cRNA injected. These results indicate that the synthesis of wild type and E309A are independent of each other and are proportional to the amount of each cRNA

injected.

Thapsigargin-sensitive Ca²⁺-ATPase activities increased linearly with increasing amounts of cRNA for wild type both in the microsomes prepared from oocytes injected with wild type cRNA alone and those injected with wild type and E309A cRNAs together (Fig. 2). An increase of enzyme activity proportional to the fraction of wild type in the co-injections suggests that *n*, the number of associated monomers, is one, that is, the SR Ca²⁺-ATPase is a functional monomer. Dominant-negative effects expected for monomer-monomer interactions were not observed.

Our data suggest that the minimum functional unit of the Ca²⁺-ATPase is a monomer in microsomes obtained from *Xenopus* oocyte driven to express the enzyme by injection of cRNA, as is the case for Na, K-ATPase [7], in which the minimum functional unit is suggested as an $\alpha\beta$ -protomer.

The glutamate-309 is invariant residue in the highly conserved PEGGL motif located in the 4th transmembrane segment of all Ca²⁺-ATPases and plays a key role in the trapping of Ca²⁺ ion. The mutants carrying the substitution of this glutamate were expressed at similar levels as wild type in COS-1 cells and have shown Ca²⁺-dependent phosphorylation from Pi [8] suggesting that this substitution will not result in the significant conformational changes. When the same substitution was introduced to the plasma membrane Ca²⁺-ATPase, which shared overall structural features with SR Ca²⁺-ATPase including the distribution of secondary structures and the number and location of the transmembrane domains, the mutant was correctly transported to the plasma membrane indicating that the mutation did not affect the proper folding that was a requirement for the targeting to the plasma membrane [10]. These observations rule out the possibility that wild type was unable to associate with E309A because of the mutation leading to malconformation.

The differences in density of Ca²⁺-ATPase in the membrane may determine whether the SR Ca²⁺-ATPase is a functional monomer or dimer (or oligomer). The density of Ca²⁺-ATPase in SR is estimated to be 1000 times more than that in oocytes injected with cRNA for Ca²⁺-ATPase. A monomer-rich situation like that found in SR membrane purified from skeletal muscle provides a preferable environment for the self-association of monomers and the resulting protein-protein interaction. On the other hand, in the case of low density of Ca²⁺-ATPase in the membrane, provided by the *Xenopus* oocyte expression system or by reconstituted vesicles with an excess of phospholipid, SR Ca²⁺-ATPase functions in the monomeric state. When SR Ca²⁺-ATPase was reconstituted with excess phospholipid, leading to lipid-rich vesicles with sufficient Ca²⁺-transporting capacity, the predominant portion of Ca²⁺-ATPase is found in the monomeric form in the reconstituted vesicles [6].

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筋小胞体 Ca²⁺-ATPase は単量体で活性をもつ

竹田 和夫, 川村 越

産業医科大学医学部 分子細胞生物学教室

要 旨: 筋小胞体 Ca²⁺-ATPase の野生型酵素および活性をもたない変異体酵素の cRNA を調製し, それらの合計量を一定に保ちながら, 量比を変えてアフリカツメガエル卵母細胞に注射し発現させた. 卵母細胞より調製したミクロソーム画分の筋小胞体 Ca²⁺-ATPase 活性は, 野生型酵素の cRNA 量の増加に比例して直線的に増加した. この結果は筋小胞体 Ca²⁺-ATPase の最小機能単位が単量体であることを示唆する.

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