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IONOPHOROUS PROPERTIES OF THE 13000-Da FRAGMENT FROM SARCOPLASMIC RETICULUM (Ca^{2+} + Mg^{2+})-ATPase

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The 25000-Da tryptic fragment from rabbit muscle sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase was subjected to cyanogen bromide digestion, and the four fragments isolated. Only the 13000-Da fragment induced ionophorous activity in planar thin lipid membranes made with 5:1 (w/w) phosphatidylcholine/cholesterol in decane. The membranes became cation selective, with a selectivity sequence among divalent of Mn^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}. This is different from that of the 25000-Da fragment (A.E. Shamoo, 1978, J. Memb. Biol. 43, 227–242), it's 'parent' 55000-Da fragment, and the intact enzyme, all of which have the same selectivity sequence. The inhibitory effects of Hg^{2+}, Cd^{2+} and Zn^{2+} were also examined. All were inhibitory, with Zn^{2+} being the most effective of these. The heavy-metal-induced inhibition of Ca^{2+} conductance could be reversed by selective chelation of the heavy metals by EDTA. From changes in the selectivity as well as changes in heavy-metal-induced inhibition behavior, we conclude that the ion transport site of the 13000-Da fragment may not be the same site as that of the parent fragment. It is either a different site altogether or has been physically modified by peptide cleavage.

Introduction

The (Ca^{2+} + Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum is the primary ion pump protein for sequestration of Ca^{2+} by that membrane system. The single polypeptide of molecular weight 119000 [1] is recognized to contain the entire Ca^{2+}-pump mechanism. Some time ago our laboratory looked for and found an ion-translocating site now called the Ca^{2+} ionophorous site in the (Ca^{2+} + Mg^{2+})-ATPase [2]. It was also shown that tryptic digestion of the enzyme results first in a cleavage to 55000 Da (A) and 45000 Da (B) fragments, followed by cleavage of the A fragment into 30000 Da (A_1) and 25000 Da (A_2) fragments [3]. The ionophorous site has been shown to reside in fragments A and A_2. [4,5].

Because the fragments are held together by strong hydrophobic interactions, only harsh detergents such as SDS are useful in separating them, mainly by preparative SDS-polyacrylamide gel electrophoresis. It has been shown that the ionophorous properties of these fragments are not due to residual detergent (SDS or cholate) bound to the protein [5,6].

Digestion of the A_2 fragment with cyanogen bromide cleaves it at the methionine residues, of which three are internal and one is N-terminal. The resulting fragments have molecular weights 13000, 7500, 3000 and less than 1000 [7].

In this paper we continue 'narrowing down' the location of the Ca^{2+} ionophorous site. We confirm

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our earlier report [8] that only the 13000-Da fragment induces ionophorous activity in the planar bilayer membrane assay, and characterize this activity with respect to selectivity sequence and heavy metal inhibition.

Materials and Methods

Preparations. Sarcoplasmic reticulum membranes were isolated from rabbit white muscle [9] and exposed to trypsin [3] as described. ATPase fragments were separated on Bio-Gel A 1.5 m and Bio-Gel HTP columns in the presence of SDS [3]. The 25000-Da fragment subjected to CNBr digestion and separated on Sephadex G-100 and hydroxyapatite columns as previously described [7]. SDS was removed by prolonged dialysis against 8 M urea. Residual SDS was assayed by the method of Hayashi [10], and protein by the method of Lowry et al. [14]. Egg phosphatidylcholine and cholesterol were obtained from Supelco.

Planar bilayer membrane (BLM) measurements. Planar lipid films were formed on a 1 mm hole from egg phosphatidylcholine/cholesterol, 5 : 1 mg/mg as a 10 mg/ml solution in *n*-decane. Conductance, capacitance and ionic selectivities were measured according to published methods (see Shamoo and Goldstein [11] for a review). Calomel electrodes were used here. Measurements of P_{Ca}/P_{Cl} were done with a starting bath composition of 5 mM $CaCl_2$/10 mM Hepes-Tris, pH 7.2 on both sides of the membrane. After introduction of ionophore, the initial open-circuit potential (as an *I-V* curve intercept) was determined whereupon concentrated $CaCl_2$ was added to one side. Open-circuit potentials were then determined for concentration ratios of 2 : 1, 5 : 1 and sometimes 10 : 1. If the initial open-circuit potential was larger than 3 mV, the electrodes were shorted and placed in heated 100 mM KCl until symmetry was restored, or they were replaced. Biionic potentials (for selectivity sequence determination) were measured with 5 mM $CaCl_2$ on one side and 5 mM divalent metal chloride on the other, both salts being introduced after membrane formation.

Results

Estimation of residual SDS. After dialysis, the amount of SDS bound to the 13000-Da fragment was below the detection threshold of the assay. A calculation gave an upper limit of < 0.05 μg SDS bound/μg protein. Usually less than 10 μg/ml protein was used in the bathing solution, giving an upper limit of 0.5 μg/ml or 1.7 μM. This is just on the threshold of SDS effects on a phosphatidylcholine/cholesterol BLM [6]. However, since this is a generous upper limit, the actual [SDS] is much less, especially when less protein is used. Assays for SDS in preparations of the other fragments were low, as well, with the exception of the 7500-Da fragment. In one case here we measured 0.023 μg SDS/μg protein. This preparation exhibited almost no ionophorous activity, however, even at 17 μg protein/ml bathing solution, corresponding to [SDS] ≈ 1.3 μM.

Comparison of the fragments. Fig. 1 shows representative Ca^{2+} conductance time courses of BLMs formed in the presence of each of the four CNBr fragments. The 13000-Da fragment is the only one inducing significant conductance, even though the other fragments were added at much higher concentrations (see figure legend). Further characterization of ionophorous activity was there-

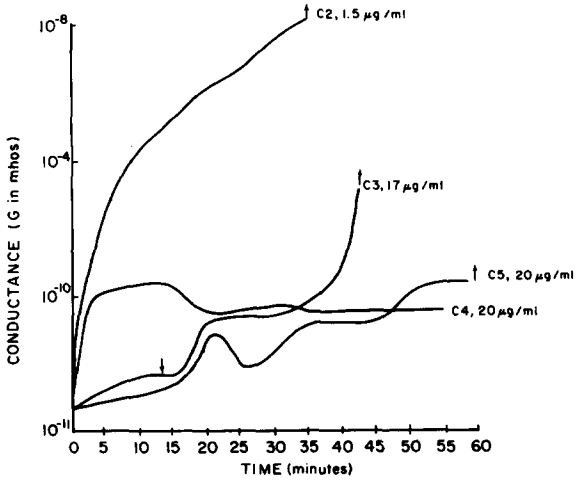

Fig. 1. The development of conductance in planar lipid films formed in the presence of CNBr fragments of the 25000-Da tryptic fragment of $(Ca^{2+} + Mg^{2+})$-ATPase. The bathing solution on both sides of the membranes was 5 mM $CaCl_2$/10 mM Hepes-Tris, pH 7.2. Membranes were formed at time zero, and upward arrows indicate membrane breakage. The concentrations of protein in the bathing solutions were: 13000-Da, 1.5 μg/ml; 7500-Da, 17 μg/ml; 3000-Da, 20 μg/ml; and 1000-Da, 20 μg/ml.

fore directed at the 13000-Da fragment.

Relative permeability of ions. We obtained the results for biionic potential measurements with $CaCl_2$ on one side and selected divalent metal chlorides on the other side of 13000-Da fragment-doped BLMs. To obtain values for the permeability ratios P_{Ca}/P_M (M = divalent metal), it is first necessary to measure P_{Ca}/P_{Cl} [11]. This was done using $CaCl_2$ gradients. The calculated P_{Ca}/P_{Cl} value decreases with increasing gradients from 1.8; 1.2; and 0.9 for 2:1; 5:1 and 10:1, respectively. This is probably because the Ca^{2+} permeability is limited by the amount of protein in the BLM, whereas the Cl^- permeability is not so dependent on protein incorporation ('bare' BLMs are known to have a much higher permeability to Cl^- than to divalent ions). Thus, the Ca^{2+} permeability saturates faster than Cl^- permeability with increasing concentration, and this is reflected in decreasing P_{Ca}/P_{Cl} values. We will use the value obtained for a 2:1 gradient, since this is closest to the conditions for biionic potential measurements.

The selectivity sequence for those divalent ions tested is Mn^{2+} (0.90 ± 0.2; $n = 13$) > Ca^{2+} (1.0) > Ba^{2+} (1.37 ± 0.06; $n = 4$) $\geq$ Sr^{2+} (1.5 ± 0.4; $n = 8$) > Mg^{2+} (2.3 ± 0.7; $n = 7$).

Properties of Ca^{2+} conductance inhibitor ions. We examined the effect of Hg^{2+}, Cd^{2+} and Zn^{2+} on the 13000-Da fragment-induced BLM Ca^{2+} conductance in the presence of 5 mM $CaCl_2$ and various concentrations of inhibitor ions. As shown in Fig. 2, each of these metals inhibits the development of Ca^{2+} conductance. The inhibition could be reversed by chelating the inhibitor ion with EDTA. The K_d values of EDTA for Hg^{2+}, Cd^{2+} and Zn^{2+} are much greater than that for Ca^{2+}, so the inhibitor ions are selectively removed (log K_1 = 10.6 for Ca^{2+}, 16.5 for Cd^{2+}, 21.8 for Hg^{2+} and 16.2 for Zn^{2+} [12]).

We obtained measurements for the relative permeabilities of Cd^{2+} and Hg^{2+} vs. Ca^{2+}. Assuming $P_{Ca}/P_{Cd} = 0.52 \pm 0.05$ ($n = 4$). We had sufficient protein for only one measurement with Hg^{2+} and obtained $P_{Ca}/P_{Hg} \approx 50$. Zn^{2+} was too inhibitory to permit a measurement of P_{Ca}/P_{Zn}.

The effect of small amounts of Zn on the value of P_{Ca}/P_{Cl} was also studied. In four experiments for measurement of P_{Ca}/P_{Cl}, 0.5 or 1.0 mM $ZnCl_2$ was added after the final voltage intercept was measured (three times with a 10:1 gradient and once with a 5:1 gradient). In every case the side with lower [Ca^{2+}] became more negative, indicating a decrease in P_{Ca}/P_{Cl}. Before addition of $ZnCl_2$, average P_{Ca}/P_{Cl} with a 10:1 gradient was 0.9; after $ZnCl_2$ addition it was 0.49. This indicates a selective decrease in Ca^{2+} conductance versus Cl^- conductance.

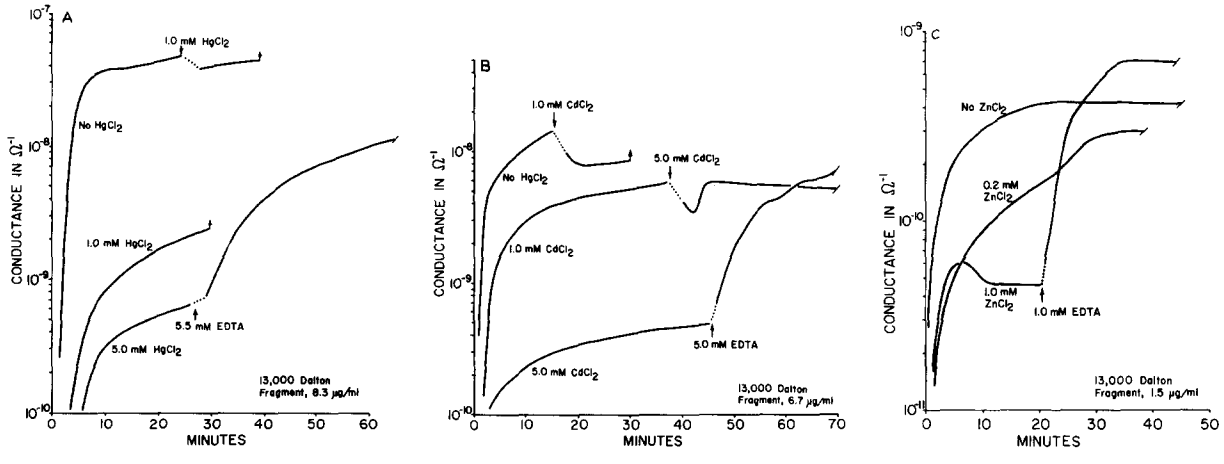

Fig. 2. Effect of heavy metals on the development of 13000-Da fragment induced conductance of planar lipid membranes. All bathing solutions contained 5 mM $CaCl_2$/10 mM Hepes-Tris, pH 7.2, and 1.5 to 8.0 µg/ml 13000-Da protein. The horizontal axis indicates time from membrane formation. Additions shown were made to both sides of the membrane. Heavy metals used were (A) $HgCl_2$, (B) $CdCl_2$ and (C) $ZnCl_2$.

Discussion

Previous studies on ionophores derived from skeletal muscle ($Ca^{2+} + Mg^{2+}$)-ATPase localized the Ca^{2+}-selective ionophorous activity in, successively, the intact solubilized enzyme, the A fragment (55 000-Da), and the A2 fragment (25 000-Da). These fragments are located at the N-terminus of the enzyme [8]. The selectivity sequence for all of these fragments in oxidized cholesterol BLMs is $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$ [4]. For the A_2 fragment treated with cholate to remove SDS, the selectivity is modified in phosphatidylcholine/cholesterol membranes: $P_{Mn} > P_{Mg}$.

The 13 000-Da fragment characterized in this paper is also situated at the N-terminus of the enzyme, although it is missing the N-terminal methionine [7]. The selectivity sequence in phosphatidylcholine/cholesterol BLMs is $Mn^{2+} > Ca^{2+} > Ba^{2+} \geq Sr^{2+} > Mg^{2+}$, distinctly different from that of the parent fragments. The sensitivity to heavy metal ion inhibitors is qualitatively different as well. The 25 000-Da fragment is more sensitive to Cd^{2+} than to Zn^{2+}, retaining significant Ca^{2+}-ionophorous activity in the presence of up to 3 mM Zn^{2+}. In contrast, the 13 000-Da fragment is marked by more sensitivity to Zn^{2+} than to Cd^{2+}, as seen in Fig. 2B and C. (Hg^{2+} was not examined for the 25 000-Da fragment.)

We consider it unlikely that SDS contamination is responsible for the observed ionophoric activity. It has been previously shown that in 8 M urea removes most of the SDS. Furthermore, the ionophorous activity of SDS in planar lipid films exhibits a selectivity sequence among divalent cations of Ba $\geqslant$ Mn > Sr > Sr > Mg, which is clearly different from that we observe for the 13 000-Da fragment [6].

The protein moiety inducing ion conductance in the BLM may be in the same peptide region as that of the fragments, or it may not. If it is, then this region has apparently been modified by CNBr cleavage. If it is not, then the original site has been destroyed by CNBr cleavage and the ionophorous activity is due to another site which has somehow been unmasked. The change in the selectivity sequence, described in terms of those predicted by Sherry [13] for 'closely spaced' anionic sites, is from sequence II for the 25 000-Da fragment to sequence III for the 13 000-Da fragment. This is excluding Mn^{2+}, which is left out of Sherry's analysis since he only considered alkaline earth ions. These sequences are close to each other as a function of anionic field strength and it is conceivable that even a small alteration in the peptide site could shift the sequence from II to III.

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