Ca^{2+} UPTAKE IN RECONSTITUTED SARCOPLASMIC RETICULUM VESICLES

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SUMMARY

The reconstitution of functional sarcoplasmic reticulum vesicles capable of Ca^{2+} transport has been achieved. Sarcoplasmic reticulum vesicles are first solubilized with deoxycholate and then reassembled into membranous vesicles by removal of the detergent using dialysis. The Ca^{2+} pump protein can, by itself, be reconstituted to form membranous vesicles capable of energized Ca^{2+} binding and uptake. The lipid content of the reconstituted vesicles is about the same as that of the original sarcoplasmic reticulum vesicles. The reconstituted vesicles have an elevated ATPase activity. Ca^{2+} binding and uptake in the presence of ATP are restored to about 25% and 50%, respectively.

Sarcoplasmic reticulum of skeletal muscle is a highly differentiated membrane with apparently one function, the release and uptake of Ca^{2+} during the contraction-relaxation cycle of muscle (for reviews, see refs. 1 and 2). The singleness of purpose together with a relatively simple protein and lipid composition make sarcoplasmic reticulum a good system for studying membrane function and reconstitution. Only three major proteins are consistently observed by polyacrylamide gel electrophoresis (3). One of these, the Ca^{2+} pump protein accounts for two-thirds of the total sarcoplasmic reticulum protein and has been purified using deoxycholate (3,4) or Triton X-100 (5). The Ca^{2+} pump protein, which binds phospholipid, forms membranes upon removal of bile acids (3,6), and has many of the characteristic properties of sarcoplasmic reticulum. It catalyzes the Ca^{2+} dependent hydrolysis of ATP via formation of a phosphoenzyme intermediate (1,2). Also, it contains one specific ATP and two specific Ca^{2+} binding sites per phosphorylation site (7) and is capable of transporting Ca^{2+} when incorporated into liposomes (8).

This communication describes the reconstitution of energized Ca^{2+} binding and uptake by sarcoplasmic reticulum vesicles which were solubilized with deoxycholate and then allowed to reform into membranous vesicles by removal of

the detergent using dialysis. We could show, further, that the purified and reconstituted Ca^{2+} pump protein by itself is capable of energized Ca^{2+} binding and uptake.

METHODS

Sarcoplasmic reticulum vesicles were prepared by zonal centrifugation essentially as previously described (3). Following the second zonal centrifugation the preparation was treated on ice overnight in the presence of 15% sucrose with 0.6 M KCl to remove small amounts of extraneous muscle proteins.

Energized Ca^{2+} binding and uptake were measured as previously described (3) using $^{45}Ca^{2+}$ and the Millipore filtration technique of Martonosi and Feretos (9). The Ca^{2+} uptake reaction was carried out for 10 min at 23° C in a medium containing 10-20 µg sarcoplasmic reticulum protein/ml, 0.1 M KCl, 5 mM Mg^{2+}, 5 mM ATP, 100 µM Ca^{2+}, 65 µM EGTA, 7.5 mM oxalate and 10 mM HEPES (pH 7.1). Ca^{2+} binding, i.e. Ca^{2+} accumulation in the presence of ATP and in the absence of oxalate, was measured at 23° C in a medium containing 100-200 µg sarcoplasmic reticulum/ml, 0.1 M KCl, 5 mM Mg^{2+}, 5 mM ATP, 45-55 µM Ca^{2+}, 30 µM EGTA, and 10 mM HEPES(pH 7.1). The reaction was started by the addition of ATP and was terminated after 30 sec and 2 min. In the binding assay total Ca^{2+} concentrations were determined by atomic absorption spectroscopy (3).

Ca^{2+} stimulated ATPase activity was determined at 23° C as the difference between total ATPase and "basic" ATPase activity. Total ATPase activity was measured in the medium used for Ca^{2+} binding. "Basic" ATPase was estimated in a medium containing 1 mM EGTA (ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid) and no added Ca^{2+}. Inorganic phosphate was determined by the procedure of Fiske and SubbaRow (10) using Elon as a reducing agent.

Protein was determined by the procedure of Lowry et al. (11) using bovine serum albumin as a standard. Total phosphorus was measured as an estimate of lipid phosphorus (12).

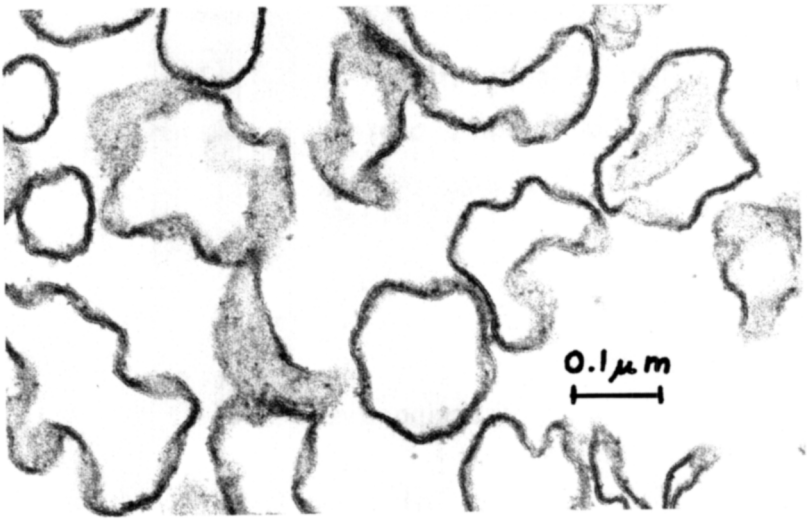

Fig. 1. Electron micrograph of reconstituted sarcoplasmic reticulum vesicles

RESULTS

Sarcoplasmic reticulum vesicles were solubilized with deoxycholate under conditions similar to those used previously (3). Solubilized sarcoplasmic reticulum is no longer membranous as observed by electron microscopy (not shown, cf. also ref. 3). Membranous vesicles are then reassembled by removal of the detergent by dialysis (Fig. 1). The conditions of reconstitution, i.e., dialysis for 22 hours at 22° C, result in removal of most of the detergent (Table 1). Energized Ca^{2+} uptake is restored and Ca^{2+} stimulated ATPase activity is reduced in the reconstituted vesicles as compared with the solubilized preparation. The protein profile of reconstituted sarcoplasmic reticulum vesicles is similar to the one of the original sarcoplasmic reticulum vesicles in that the Ca^{2+} pump protein is the major protein component. (Gels 1 and 2 in Fig. 2). It differs in that two other major proteins of sarcoplasmic reticulum, the Ca^{2+} binding and M_{55} proteins (3), are only in part rebound by the membrane.

The characteristic properties of original and reconstituted sarcoplasmic reticulum vesicles are compared in Table 2. Accordingly, the reconstituted sarcoplasmic reticulum vesicles contain approximately the same amount of phos-

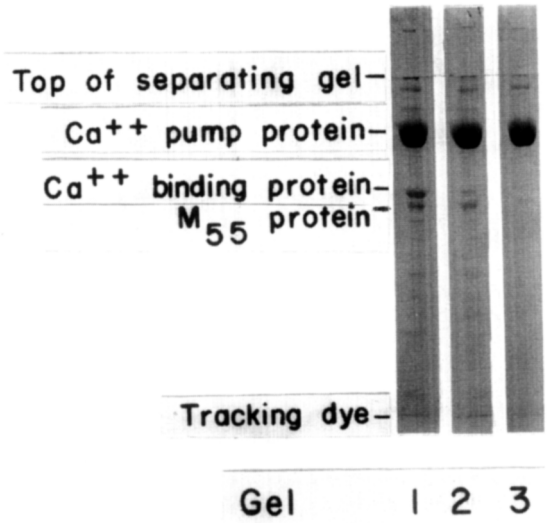

Fig. 2. Separation of sarcoplasmic reticulum proteins by gel electrophoresis
Sodium dodecyl sulfate gels, containing 10% and 5% acrylamide in the separating and stacking gel, respectively, were prepared and run as described by Laemmli (13). Samples (40 μg protein per gel) were reduced with 2.5% 2-mercaptoethanol by heating for 4 min at 100° C prior to gel electrophoresis. Gels were stained with 1% Amido Schwartz (3). Gel 1, original sarcoplasmic reticulum; Gel 2, reconstituted sarcoplasmic reticulum (cf. Table 2); Gel 3, reconstituted Ca^{2+} pump protein (cf. Table 3).

pholipid (expressed as μmoles P/mg protein) as the original vesicles. Both preparations are also capable of forming about the same amount of phosphoenzyme indicating that practically none of the Ca^{2+} pump protein activity is lost during solubilization and dialysis. Ca^{2+} stimulated ATPase activity is increased several-fold, while Ca^{2+} binding and uptake are restored to approximately 25% and 50% of their original values, respectively. In both preparations the two latter activities are dependent on the presence of ATP and are inhibited by the ionophore X537A. X537A has been shown, recently, to cause the rapid release of Ca^{2+} from sarcoplasmic reticulum (14).

The Ca^{2+} pump protein alone can be reconstituted to form membranous vesicles capable of energized Ca^{2+} binding and uptake. The purified Ca^{2+} pump protein is solubilized and then reassembled by dialysis in a similar way as used for the reconstitution of total sarcoplasmic reticulum membranes (Table 3). Ca^{2+} uptake of the reconstituted vesicles is somewhat increased

TABLE 1

RECONSTITUTION OF Ca^{2+} UPTAKE BY SARCOPLASMIC RETICULUM

	Sarcoplasmic Reticulum	
	solubilized	reconstituted
mg deoxycholate/ml	3.4	0.07
Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)	< 0.1	3.2
Ca^{2+} stimulated ATPase (μmoles P_i/mg protein per min)	3.1	2.1

Sarcoplasmic reticulum vesicles (7.0 mg protein/ml) were solubilized at 0° C with ^{14}C-deoxycholate (3.5 mg/ml, 10^3 cpm per mg) in a 10 mM Tris buffer (pH 7.9) containing 0.4 M KCl, 0.4 M sucrose, 1 mM EDTA, 1.5 mM Mg^{2+} and 0.1 mM Ca^{2+}. Insoluble material (10-25% of the protein) was removed by centrifugation in a Spinco 65 rotor at 50,000 rpm for 75 min. The solubilized sample(1 ml aliquot) was then reconstituted by dialysis for 22 hours at 22° C against 500 ml of a 8 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer, pH 7.25, containing 0.4 M KCl, 0.25 M sucrose, 1 mM EDTA, 1.5 mM Mg^{2+} and 0.1 mM Ca^{2+}. It should be noted that the solubilized and reconstituted samples were diluted about 500-fold when assayed for Ca^{2+} uptake and ATPase activity.

TABLE 2

PROPERTIES OF ORIGINAL AND RECONSTITUTED SARCOPLASMIC RETICULUM VESICLES

	Sarcoplasmic Reticulum Vesicles	
	Original	Reconstituted
Bound phosphorus (μmoles P/mg protein)	0.78	0.95
^{32}P-phosphoenzyme (nmoles ^{32}P/mg protein)	6.4	7.0
ATPase (μmoles P_i/mg protein per min)		
with 50 μM Ca^{2+}-30 μM EGTA	0.35	1.9
with 1 mM EGTA	0.1	0.1
Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)		
complete system	6.0	3.6
minus ATP	< 0.1	< 0.1
plus X 537A (20 μg/ml)	< 0.1	< 0.1
Ca^{2+} binding (nmoles Ca^{2+}/mg protein)		
complete system	160	44
minus ATP	14	12
plus X 537A (25 μg/ml)	20	9

Sarcoplasmic reticulum vesicles were solubilized and reconstituted as described in the legend of Table 1. One ml aliquots of the dialyzed samples were then diluted with 8 ml of dialysis buffer, centrifuged in a Spinco 65 rotor at 45,000 rpm for 60 min, and resuspended in 1 ml of 0.3 M sucrose - 1 mM HEPES (pH 7.4).

when additional sarcoplasmic reticulum phospholipid in form of an aqueous microdispersion (15,16) is added to the sample before its solubilization with deoxycholate. Under these conditions vesicles are reformed which contain about twice the amount of phospholipid of original sarcoplasmic reticulum vesicles. The reconstituted Ca^{2+} pump protein is approx. 90% pure and essentially devoid of Ca^{2+} binding and M_{55} proteins (Gel 3 in Fig. 2).

DISCUSSION

In the present study, we have described the reconstitution of membranous vesicles capable of energized Ca^{2+} binding and uptake. Sarcoplasmic reticulum vesicles or the Ca^{2+} pump protein, by itself, have been solubilized by deoxycholate and then reassembled by dialysis to remove the detergent. Martonosi (17) was the first to describe attempts at reconstituting functional sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles suspensions were clarified by the use of deoxycholate and by sonication. Removal of detergent by Sephadex chromatography or simple dilution resulted in restoration of part of the original Ca^{2+} uptake activity. In our hands, rapid dilution was not sufficient to restore Ca^{2+} uptake in our solubilized sample (cf. Table 1). In this respect Martonosi (1) has recently commented on his earlier studies: "The partial restoration of Ca^{2+} transport after treatment with deoxycholate may represent reassociation of large membrane fragments which were not disrupted by the detergents, instead of a reassembly of functionally competent membranes from molecularly dispersed constituents". We were careful to remove all membrane fragments by high-speed centrifugation. The absence of membranous fragments was further ascertained by electron microscopy.

The reconstituted sarcoplasmic reticulum vesicles contain only part of the Ca^{2+} binding and M_{55} proteins originally present. The reconstituted Ca^{2+} pump protein was essentially devoid of these two major proteins of sarcoplasmic reticulum. Thus, vesicles composed mainly of the Ca^{2+} pump protein and amounts of phospholipid normally present in sarcoplasmic reticulum vesicles

TABLE 3

PROPERTIES OF RECONSTITUTED MEMBRANOUS VESICLES CONSISTING OF Ca^{2+} PUMP PROTEIN AND PHOSPHOLIPID

PREPARATION	PROPERTIES OF RECONSTITUTED VESICLES			
	Bound phosphorus (μmoles P/ mg protein)	Ca^{2+} uptake (μmoles Ca^{2+} mg protein)	Ca^{2+} binding (nmoles Ca^{2+}/ mg protein)	
			-	+ X537A
Ca^{2+} pump protein	0.6	1.7	27	10
Ca^{2+} pump protein + SR phospholipid	1.45	2.5	34	9
Ca^{2+} pump protein + deoxycholate extract	1.0	2.3	28	9
Control (Reconstituted SR vesicles)	0.9	3.5	42	7

Sarcoplasmic reticulum (SR) vesicles (11 mg/ml) were treated with deoxycholate (3.4 mg/ml) in a 10 mM Tris buffer (pH 8.0) containing 0.4 M sucrose, 0.4 M KCl, 1 mM EDTA, 1.5 mM Mg^{2+} and 0.1 mM Ca^{2+}. A soluble and membranous fraction were obtained by centrifugation for 60 min at 49,000 rpm in a Spinco 65 rotor. The membranous fraction was once washed by resuspending the pellet in the above Tris buffer and recentrifugation. The resulting pellet (purified Ca^{2+} pump protein) was taken up in one-third of the original volume of the above Tris buffer to give a protein concentration of approx. 25 mg/ml and then divided into three equal aliquots. One aliquot was diluted with 1/3 volume of the above deoxycholate extract, a second aliquot was diluted with a corresponding volume of the above Tris buffer, and to the third aliquot a corresponding volume of the above Tris buffer containing an aqueous microdispersion of sarcoplasmic reticulum phospholipid (4.2 μmoles P/ml)(15,16) was added. Clarification was obtained by adding 7% deoxycholate to a final concentration of 3.9, 1.4 and 3.4 mg/ml, respectively. After centrifugation to remove insoluble material, the solubilized samples were dialyzed and washed as described in the legends of Tables 1 and 2. Reconstituted sarcoplasmic reticulum vesicles prepared as described in the legend of Table 2 served as the control.

possess two of the important characteristics of sarcoplasmic reticulum, i.e., they are capable of energized Ca^{2+} uptake and of maintaining a permeability barrier for Ca^{2+}. Our reconstituted vesicles are somewhat inferior to the original sarcoplasmic reticulum vesicles in that they have an elevated ATPase activity and accumulate lower amounts of Ca^{2+} in the presence of ATP. The absence of the Ca^{2+} binding and M_{55} proteins may account for some of these differences.

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