Effect of Ca^{2+} on the Dimeric Structure of Scallop Sarcoplasmic Reticulum

Loriana Castellani,* Peter M. D. Hardwicke,‡ and Clara Franzini-Armstrong§

*Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254-9110; ‡Department of Medical Biochemistry and Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901-4409; and §Department of Anatomy, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018

Abstract. Scallop sarcoplasmic reticulum (SR), visualized in situ by freeze-fracture and deep-etching, is characterized by long tubes displaying crystalline arrays of Ca^{2+}-ATPase dimer ribbons, resembling those observed in isolated SR vesicles. The orderly arrangement of the Ca^{2+}-ATPase molecules is well preserved in muscle bundles permeabilized with saponin. Treatment with saponin, however, is not needed to isolate SR vesicles displaying a crystalline surface structure. Omission of ATP from the isolation procedure of SR vesicles does not alter the dimeric organization of the Ca^{2+}-ATPase, although the overall appearance of the tubes seems to be affected: the edges of the vesicles are scalloped and the individual Ca^{2+}-ATPase molecules are not clearly defined. The effect of Ca^{2+} on isolated scallop SR vesicles was investigated by correlating the enzymatic activity and calcium-binding properties of the Ca^{2+}-ATPase with the surface structure of the vesicles, as revealed by electron microscopy. The dimeric organization of the membrane is preserved at Ca^{2+} concentrations where the Ca^{2+} binds to the high affinity sites (half-maximum saturation at pCa ~7.0 with a Hill coefficient of 2.1) and the Ca^{2+}-ATPase is activated (half-maximum activation at pCa ~6.8 with a Hill coefficient of 1.84). Higher Ca^{2+} concentrations disrupt the crystalline surface array of the SR tubes, both in the presence and absence of ATP. We discuss here whether the Ca^{2+}-ATPase dimer identified as a structural unit of the SR membrane represents the Ca^{2+} pump in the membrane.

FRAGMENTED sarcoplasmic reticulum (FSR)[1] from the striated adductor muscle of the deep sea scallop *Placopecten magellanicus* has been used in electron microscope investigations to determine the organization of the calcium pump protein (Ca^{2+}-ATPase) in the membrane. Although scallop FSR shares many important similarities with the well-studied rabbit skeletal muscle FSR, scallop FSR differs in that extensive dimeric arrays of Ca^{2+}-ATPase subunits are observed in isolated preparations without exposure to vanadate or phosphate (Castellani and Hardwicke, 1983; Castellani et al., 1985; Ferguson et al., 1985). Studies of the scallop sarcoplasmic reticulum (SR) in situ by examination of fixed tissue sections show that it is located between the sarcolemma and the single myofibril of the adductor muscle cells (Sanger, 1971; Nunzi and Franzini-Armstrong, 1981; Sanger and Sanger, 1985; Castellani et al., 1985). The scallop SR forms a system of tubes and cisternae, the latter often being associated with surface couplings to the sarcolemma that resemble the triad structures in vertebrate cross-striated muscle (Nunzi and Franzini-Armstrong, 1981; Sanger and Sanger, 1985). Presumably the tubular structures in scallop FSR preparations represent relatively intact pieces of the native SR.

Exposure of rabbit FSR to vanadate induces formation of a dimeric arrangement of Ca^{2+}-ATPase molecules in the membrane very similar to that seen in scallop SR (Dux and Martonosi, 1983*a*; Buhle et al., 1983; Taylor et al., 1984, 1986). The effect of different ligands on the rabbit system was analyzed and Ca^{2+} was found to disrupt the dimeric array produced by vanadate (Dux and Martonosi, 1983*b*). Further studies showed that Ca^{2+} and lanthanide ions produce an alternative monomeric arrangement of Ca^{2+}-ATPase molecules in rabbit SR (Dux et al., 1985). Recently, microcrystals of solubilized rabbit Ca^{2+}-ATPase have been grown in the presence of 20 mM Ca^{2+} (Dux et al., 1987; Pikula et al., 1988; Taylor et al., 1988).

We have visualized the scallop SR in situ by freeze-fracture and deep-etching and have shown that the Ca^{2+}-ATPase molecules are arrayed into orderly dimeric ribbons, confirming the membrane structure observed in isolated SR vesicles. We have also investigated by electron microscopy the effect of Ca^{2+} on isolated scallop SR vesicles using negatively stained and freeze-dried, rotary-shadowed preparations. A close

Portions of this work have been published previously in preliminary form (Franzini-Armstrong, C., D. G. Ferguson, L. Castellani, and L. J. Kenney. 1986. *Ann. NY Acad. Sci.* 483:44–56).

1. *Abbreviations used in this paper*: FSR, fragmented sarcoplasmic reticulum; SR, sarcoplasmic reticulum; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid.

correspondence was found between the dimeric organization of the Ca^{2+}-ATPase molecules in the membrane and the enzymatic activity of the Ca^{2+}-ATPase in solution. Loss of dimeric organization was observed to occur together with the inhibition of the Ca^{2+}-ATPase activity and the binding of Ca^{2+} to low affinity sites.

Materials and Methods

Preparation of FSR

Live sea scallops (*Placopecten magellanicus*) were obtained from the Marine Biological Laboratory, Woods Hole, MA. SR vesicles were prepared from saponin-treated striated adductor muscle essentially according to Castellani and Hardwicke (1983). An alternative isolation procedure, which involved the same purification steps but omitted treatment with saponin and ATP, was also used. The last step of the purification procedure involving a discontinuous sucrose gradient was omitted when vesicles were to be used for electron microscopy. Protein concentrations were determined by the modified Lowry procedure of Bensadoun and Weinstein (1976).

Ca^{2+} Binding Studies

Studies of Ca^{2+} binding to scallop FSR were performed essentially according to Inesi et al. (1980) and Scofano et al. (1985), using the method of Hummel and Dreyer (1962). 8 × 0.7-cm columns of Sephadex G-50 (fine) were equilibrated at 22°C with solutions of 40 μM [^{45}Ca]Cl_2, 80 mM KCl, 5 mM $MgCl_2$, 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES), pH 7.5, containing varying amounts of EGTA. Samples of scallop FSR (20 mg/ml) in 0.32 M sucrose, 0.1 M KCl, 0.1 mM $CaCl_2$, 20 mM TES, pH 7.5, were loaded onto the columns and the excess Ca^{2+} over baseline which eluted with the FSR fractions was taken as being bound.

Ca^{2+}-ATPase Activity

Ca^{2+}-ATPase activities were determined by a coupled assay (Warren et al., 1974) in a medium of 100 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 1 mM EGTA, 0.26 mM β-NADH, 10 μg ml^{-1} oligomycin, 100 mM TES, pH 7.5, 10 U/ml of pyruvate kinase, and 20 U/ml of lactate dehydrogenase at 25°C with $CaCl_2$ added to vary the pCa.

Calcium Determination

Standard $CaCl_2$ solutions for Ca^{2+}-binding measurements were prepared by drying $CaCO_3$ (Aldrich Chemical Co., Milwaukee, WI) overnight in an electric oven at 107°C, storing over NaOH pellets in a desiccator, and dissolving to a known volume in HCl. Total calcium in buffers was determined by the method of standard addition with an atomic absorption spectrophotometer (model 475; Varian Associates, Inc., Palo Alto, CA) used in the atomic emission mode. A stock Ca-acetate aqueous solution was used for electron microscopy. The free calcium and magnesium ion concentrations were calculated using a program kindly supplied by Dr. P. D. Chantler (Department of Anatomy, The Medical College of Pennsylvania, Philadelphia, PA), which uses a reiterative procedure (Chantler and Szent-Györgyi, 1980). In some samples for electron microscopy, the free-calcium concentration was kindly measured by Dr. Yale Goldman (Department of Physiology, University of Pennsylvania, Philadelphia, PA), using a calibrated Ca^{2+}-sensitive electrode.

Electron Microscopy

Whole Muscle. For freeze-fracture, small bundles of muscle fibers, either fresh or after skinning with 0.1% saponin in relaxing solution (100 mM NaCl, 10 mM EGTA, 8 mM $MgSO_4$, 5 mM ATP, 1 mM DTT, 20 mM TES, pH 7.0), were fixed in 2% glutaraldehyde in artificial sea water or relaxing solution. The fixed bundles were infiltrated with 30% glycerol for a minimum of 15 min, frozen in freon, fractured at −110°C, and unidirectionally shadowed with platinum at 45°. Other bundles were infiltrated in 30% methanol, frozen, and fractured as described above, but deep-etched for 15 min at −100°C and rotary-shadowed at 25°.

Isolated Vesicles. SR vesicles were diluted (~1:30) in 100 mM Na-acetate, 1 mM Mg-acetate, 0.2 mM EGTA, 10 mM TES, pH 7.0, with or without the addition of 1 mM Mg-ATP. A drop of suspension was placed on a 400-mesh carbon-coated grid for negative staining or on a freshly cleaved sheet of mica for freeze-drying/rotary-shadowing. The vesicles absorbed onto these supports were washed for 1 min with the acetate buffer described above containing increasing amounts of Ca^{2+}-acetate. Longer incubations were carried out in solution.

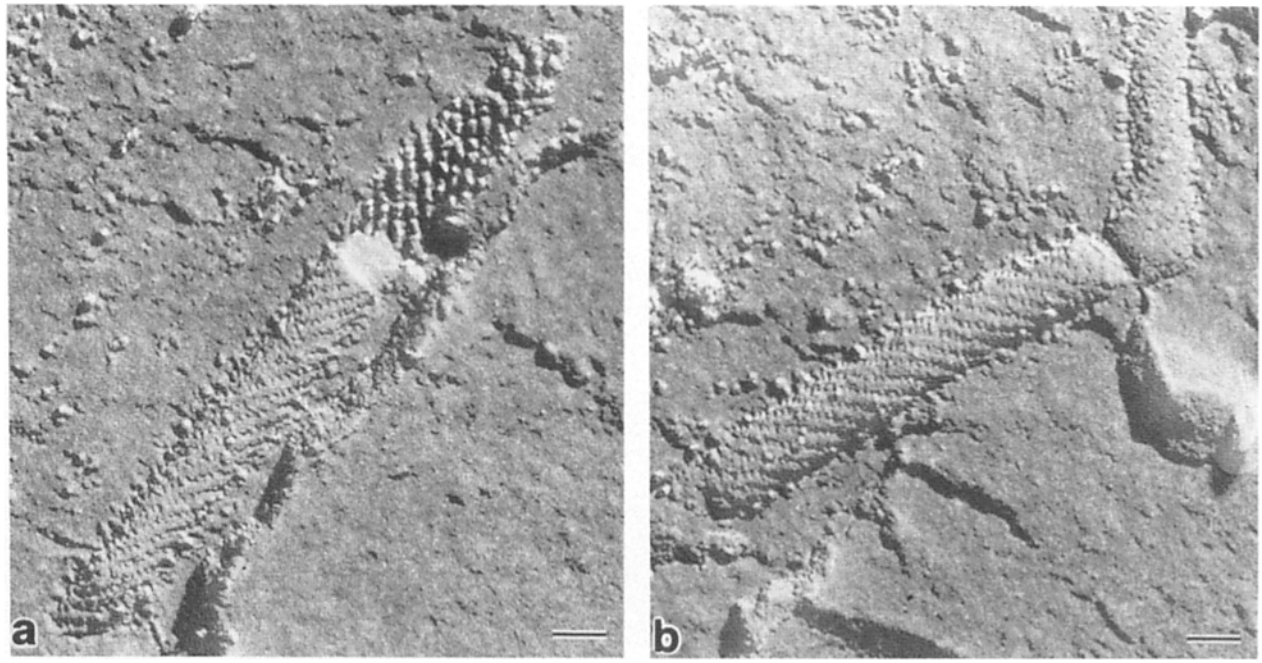

Figure 1. Electron micrographs of freeze-fractured, unidirectionally shadowed, scallop striated muscle showing SR tubes. The tube in *a* displays both cytoplasmic and luminal leaflets and the one in *b* only the luminal leaflet. The cytoplasmic leaflet is characterized by left-handed helical rows of particles (*a*), whereas the luminal leaflet shows the complementary pits, ordered within equally spaced right-handed grooves (*a* and *b*). The micrographs are shown in reverse contrast. Direction of shadowing from the top of the figure down. Bars, 35 nm.

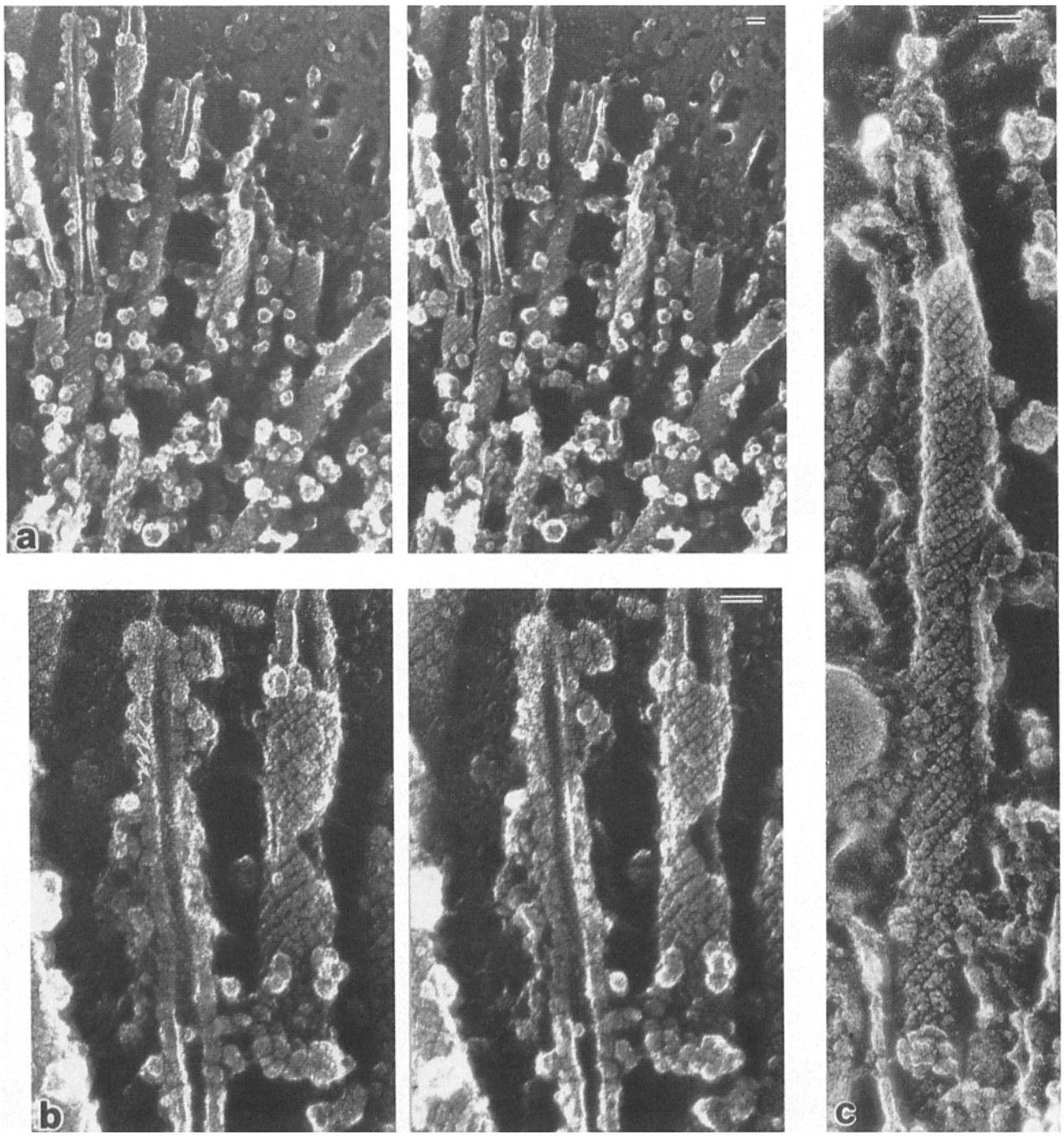

Figure 2. Deep-etched, rotary-shadowed SR tubes in scallop striated muscle. (*a*) Stereomicrographs showing SR tubes longitudinally oriented and characterized by a regular array of Ca^{2+}-ATPase dimer ribbons on the cytoplasmic surface. (*b*) Detail of *a* in stereo showing an SR tube split in half; rows of very small projections can be seen on the luminal surface (*arrows*). (*c*) SR tube at higher magnification; the dimeric organization of the Ca^{2+}-ATPase molecules can be seen on the cytoplasmic surface. Micrographs are shown in reverse contrast. Bars, 35 nm.

The vesicles on the grids were stained with three drops of 1% aqueous uranyl acetate solution. The vesicles on the mica were treated with 2% aqueous uranyl acetate solution for 30 s and rinsed with water. The final wash was dried to a thin film and the mica sheets were frozen in liquid nitrogen and loaded in a double replica holder. Rotary-shadowing with platinum was carried out at 25°. Electron micrographs were recorded on a Philips 301, 420, or 410, and on a Joel 100B electron microscope. The 301, 420, and 410 electron microscopes were fitted with an anticontamination device and calibrated using tropomyosin Mg^{2+}-paracrystals or catalase crystals.

Results

SR in Intact Muscle Fibers

In the intact muscle fibers, the SR occupies a thin layer of cytoplasm immediately beneath the plasmalemma. It is mostly in the form of uniform-size tubes, with a preferentially longitudinal orientation. In freeze-fracture preparations of

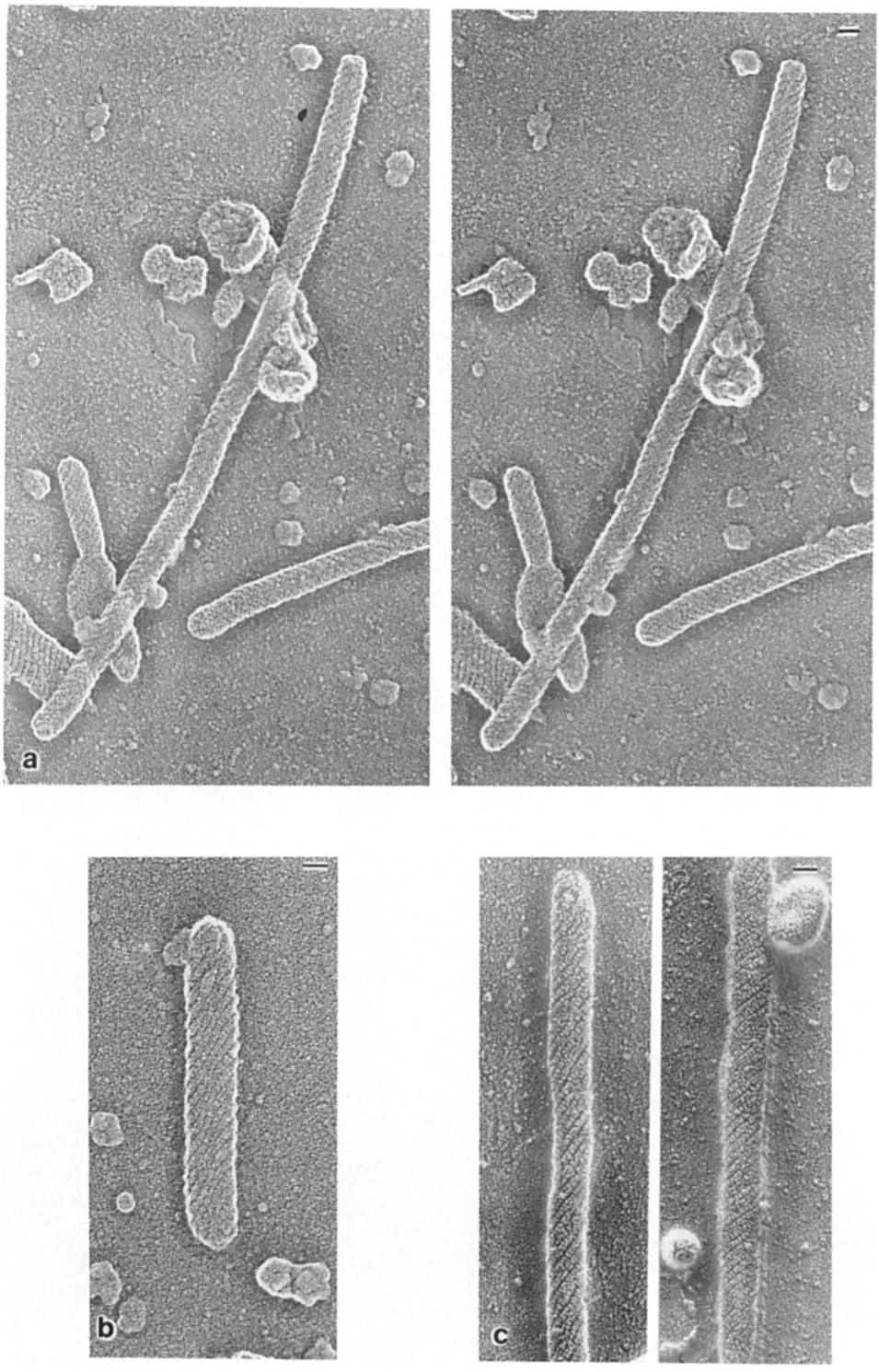

Figure 3. Comparison of freeze-dried, rotary-shadowed SR tubes isolated in EGTA-containing solution in the absence (*a* and *b*) and presence (*c*) of ATP. (*a*) Stereomicrographs of SR tubes isolated from intact scallop striated muscle; these tubes are characterized by a regular surface array of Ca^{2+}-ATPase dimer ribbons and scalloped edges. The tube in *b*, isolated in the absence of ATP, shows pronounced grooves between the dimer ribbons, but the individual Ca^{2+}-ATPase molecules are not clearly identified. The tubes in *c*, isolated from saponin-skinned scallop muscle in the presence of ATP, display straight edges and the individual Ca^{2+}-ATPase molecules are clearly seen. Micrographs printed in reverse contrast. Bars, 35 nm.

saponin-skinned scallop fiber bundles, the cytoplasmic and luminal leaflets of the split SR membrane show helically arranged rows of particles and complementary pits, respectively (Fig. 1). The spacing of the grooves on the luminal leaflet of the fractured membrane corresponds to the center-to-center spacing between the dimeric rows on the cytoplasmic surface. The fine elongated pits within the grooves correspond to the interdimer distance along the rows. The intramembranous particles observed on the cytoplasmic leaflet encompass the width of the dimeric row and each particle covers the area occupied by up to two dimers. The disposition and size of these particles, however, are seldom regular over large distances, suggesting that distortion of the preparation occurs during fracturing.

In deep-etched preparations, the exposed cytoplasmic surface of the SR tubes reveals the typical dimeric coupling of

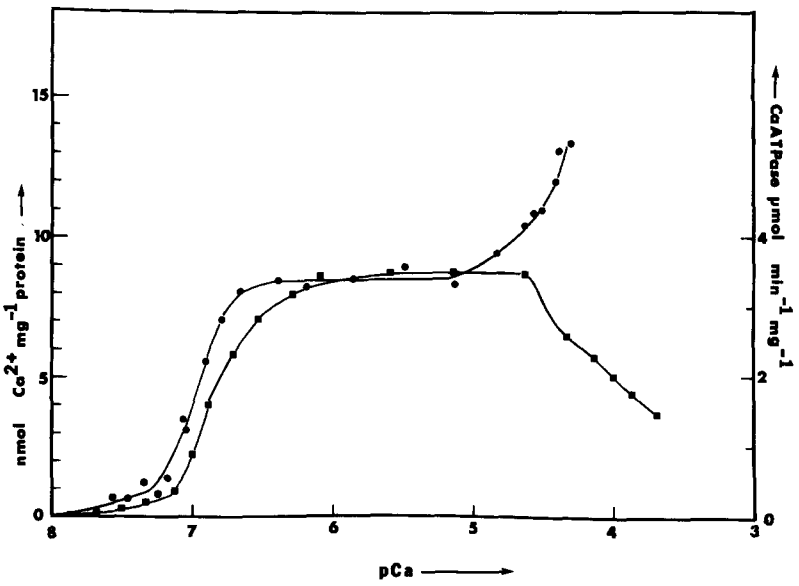

Figure 4. Ca^{2+}-ATPase enzymatic activity (■) and Ca^{2+} binding property (●) of isolated scallop SR vesicles measured at various free Ca^{2+} concentrations. Binding of Ca^{2+} to high affinity sites increases Ca^{2+}-ATPase activity. The ATPase activity is inhibited when the weak Ca^{2+} binding sites are filled.

the ATPase molecules, first described in the isolated vesicles (Fig. 2). Occasionally, when the tubes are split in half, rows of small projections running diagonally across the tubes can be seen on the luminal surface (Fig. 2 *b*).

Effect of ATP on Isolation of SR Vesicles

Preparations of FSR isolated from scallop are made up of a mixture of round and tubular vesicles characterized by a regular surface array of ribbons of Ca^{2+}-ATPase dimers (cf. Castellani and Hardwicke, 1983). Although the standard isolation procedure of scallop FSR vesicles is done from saponin-treated muscle strips with solutions containing ATP and EGTA (muscle in the relaxed state), vesicles isolated from intact fiber bundles in the absence of ATP also reveal the ordered surface array of Ca^{2+}-ATPase dimers (Fig. 3). The treatment of the muscle strips with saponin only affects the yield of SR vesicles obtained. The absence of ATP, however, appears to affect the overall appearance of the tubes. These tubes show scalloped edges and pronounced grooves between the ribbons of dimers (Fig. 3, *a* and *b*), whereas tubes isolated in the presence of ATP show straight edges, less pronounced separation beween the dimeric rows, and clear definition of the individual Ca^{2+}-ATPase molecules (Fig. 3 *c*). These differences suggest that in the absence of ATP the Ca^{2+}-ATPase molecules may undergo a conformational change so that the rows appear to be raised on the membrane surface. Exposure of these vesicles to ATP-containing solutions restores the typical appearance of control preparations. It should be noted that preparations without ATP are unstable upon storage unless trace amounts of Ca^{2+} are added.

Ca^{2+}-ATPase Activity and Ca^{2+} Binding

Standard FSR preparations were used to measure Ca^{2+}-ATPase enzymatic activity and Ca^{2+} binding. Half-maximal activation of the FSR ATPase occurs at 0.14 μM free Ca^{2+} (pCa ~6.8) (Fig. 4). A Hill plot of the data gives a value of 1.84 for the Hill coefficient, similar to that seen with FSR from rabbit skeletal muscle. At pCa values <5.0, the Ca^{2+}-ATPase is progressively inhibited by Ca^{2+}. Thus, there are three sections to the curve: a region where increasing Ca^{2+} concentration increases the activity (between pCa 8.0 and 6.0), a plateau region, and, at pCa <5.0, a region where increasing Ca^{2+} concentration inhibits the ATPase activity.

The Ca^{2+} binding studies correlate well with the ATPase activity curve (Fig. 4). The high affinity Ca^{2+}-specific binding sites show a half-maximal saturation at 0.1 μM free Ca^{2+} (pCa ~7.0). The Hill plot for this region of the binding curve gives a Hill coefficient of 2.1. At higher calcium ion concentrations (pCa values <5.0) the Ca^{2+} binding curve indicates the presence of weak Ca^{2+} binding sites.

Effect of Ca^{2+} on SR Vesicles

Incubation of SR vesicles with various concentrations of Ca^{2+} (pCa ≥6.4) in the presence of ATP for 1 min does not affect the dimeric arrangement of the Ca^{2+}-ATPase molecules on the surface of SR tubes (Fig. 5, *a–c* and *g–i*). At pCa ~6.0 some tubes begin to show partial disruption of the ordered surface array (Fig. 5, *d* and *j*). The fully flattened tubes, usually observed at lower free Ca^{2+} concentrations (cf. Fig. 5, *a–c* and *g–i*), appear to be largely replaced by a population of partially flattened tubes (Fig. 5, *d–f*, *j*, and *k*). In negative stained preparations they are characterized by a uniform stain distribution, as are the flattened tubes in the absence of Ca^{2+}, but show a fringe of projections embedded in a pool of stain along the edges, reminiscent of the one observed in stain-filled tubes (cf. Castellani and Hardwicke, 1983). At pCa ~5.4 or below, the disrupting effect of Ca^{2+} becomes more evident: the ribbons of dimers are clearly seen only in limited areas, and local "melting" of the membrane produces kinks along the tubes (cf. Fig. 5 *k*). Occasionally, in small regions of the tube surface, single rows of ATPase molecules are observed. Given the size and the limted order of these regions, however, it is difficult to ascertain whether they represent true monomeric arrays. Increasing the time of incubation with buffer at pCa ~5.4 (Fig. 6, *a–d*) or the free Ca^{2+} concentration (up to pCa ~4.0) increases the disorder of the membrane surface array. More-

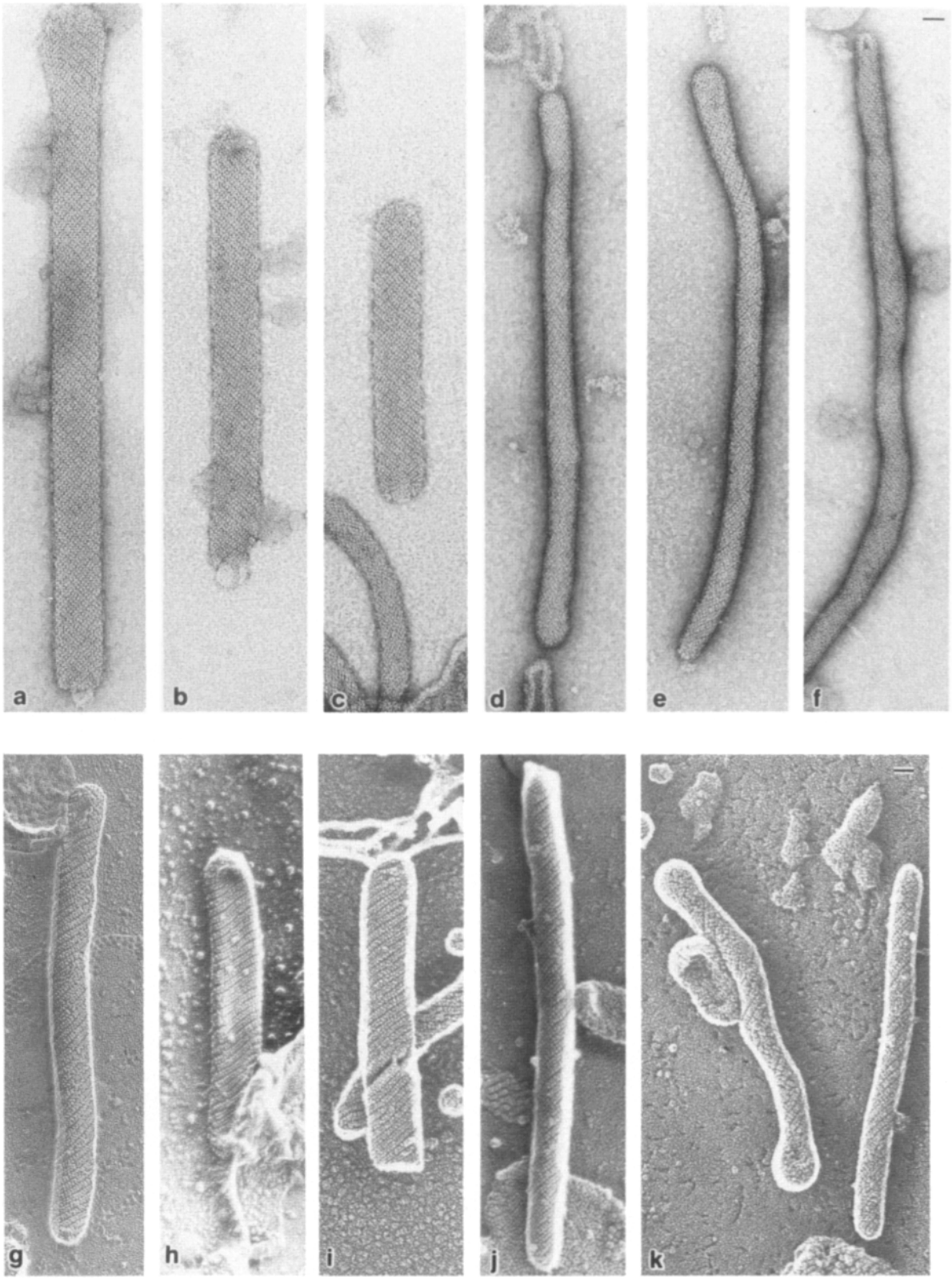

Figure 5. Negatively stained (*a–f*) and freeze-dried, rotary-shadowed (*g–k*), isolated scallop SR tubes exposed to solutions containing ATP and various free Ca^{2+} concentrations. Tubes in *a–f* were incubated for 1 min at pCa >8.0 (*a*), ~6.9 (*b*), ~6.4 (*c*), ~6.0 (*d*), ~5.4 (*e*), and ~4.0 (*f*). Tubes in *g–k* were incubated for varying times at selected pCas: >8.0 (*g*), ~6.4 for 1 min (*h*) and 24 h (*i*), ~6.0 for 22 h (*j*), and ~5.4 for 1 min (*k*). Note that at pCa ~6.0 and below tubes appear less flattened and show a fringe of projections (clearly visible in negatively stained tubes [*e* and *f*]). The ordered surface array of dimer ribbons is disrupted. Micrographs in *g–k* are shown in reverse contrast. Bars, 35 nm.

over, many tubes appear fragmented and an increasing number of round vesicles is observed.

The reversibility of the Ca^{2+} effect was analyzed by incubation of the SR suspension at pCa ~5.4 with ATP for 1 h followed by overnight dialysis vs. EGTA-containing solution (Fig. 6). Under these conditions, the dimer ribbons, as well as the typical appearance of control preparations, are completely regained (compare Fig. 6, *a* and *e*). Washing the sample on the grid with EGTA-containing buffer for up to 5 min restores only in part the crystalline appearance, probably due to slow removal of Ca^{2+} from the lumen of the tubes.

The effect of Ca^{2+} on SR vesicles was also tested in the absence of ATP (Fig. 7). Exposure of vesicles to solutions at pCa ~6.0 or above for 1 min does not alter the dimeric array of Ca^{2+}-ATPase molecules (Fig. 7, *a–d*). Although the ribbons of dimers are pronounced, the individual Ca^{2+}-ATPase molecules do not appear as clearly defined as in the presence of ATP (see Fig. 5 for comparison). The tubes begin to show loss of the crystalline array at pCa ~6.0 and below, in a manner similar to those washed with ATP-containing solutions. As the free calcium concentration increases (up to pCa ~4.0) (Fig. 7, *e* and *f*), the surface of the tubes becomes more disordered, although rows of Ca^{2+}-ATPase molecules are still visible in limited areas. Longer times of incubation did not essentially change the range of sensitivity to free Ca^{2+} of the Ca^{2+}-ATPase dimers.

Discussion

The SR in scallop striated muscle in situ is characterized by longitudinal tubes with an ordered surface array of Ca^{2+}-ATPase. This crystalline appearance of the scallop SR resembles that observed in isolated scallop SR vesicles (Castellani and Hardwicke, 1983; Castellani et al., 1985; Ferguson et al., 1985) and in vanadate-treated SR vesicles from rabbit striated muscle (Dux and Martonosi, 1983*a*; Taylor et al., 1984). In whole muscle the orderly arrangement of Ca^{2+}-ATPase molecules is better preserved when the fiber bundles are fixed after permeabilization with saponin. Since saponin is known to perforate the surface but not the internal membranes of various cell types (Endo and Iino, 1980), it is likely that the skinning procedure allows a more rapid penetration of glutaraldehyde while the presence of EGTA ensures buffering of any calcium that may escape the SR. The orderly arrangement of the Ca^{2+}-ATPase is not dependent on treatment with saponin since crystalline SR vesicles can be isolated from scallop muscle strips which have not been exposed to detergent. In addition, skinning of vertebrate muscle fibers under the same conditions used for scallop muscle does not result in ordering of the SR Ca^{2+}-ATPase (Ferguson et al., 1985).

The appearance of scallop SR in situ is quite different from that of SR in vertebrate muscles, where the tubules have a variable shape and the Ca^{2+}-ATPase is irregularly disposed on the surface of the membrane (Peachey and Franzini-Armstrong, 1983). Freeze-fractured preparations of scallop SR show a regular arrangement of intramembranous particles and corresponding pits. The apparent size of these particles suggests that they represent up to four Ca^{2+}-ATPase molecules, in contrast with the dimeric surface array of the membrane. The regular spacing of the pits, however, is consistent with a dimeric grouping of the Ca^{2+}-ATPase molecules, suggesting that the size of the intramembranous particles may represent an artifact of the preparation. Corresponding images of SR from vertebrates reveal a very irregular arrangement of particles showing barely visible pits on the luminal leaflet (cf. Franzini-Armstrong and Ferguson, 1985). Controversial interpretations of the size of these particles have been reported in the literature (cf. Scales and Inesi, 1976; Napolitano et al., 1983). In contrast, isolated rabbit

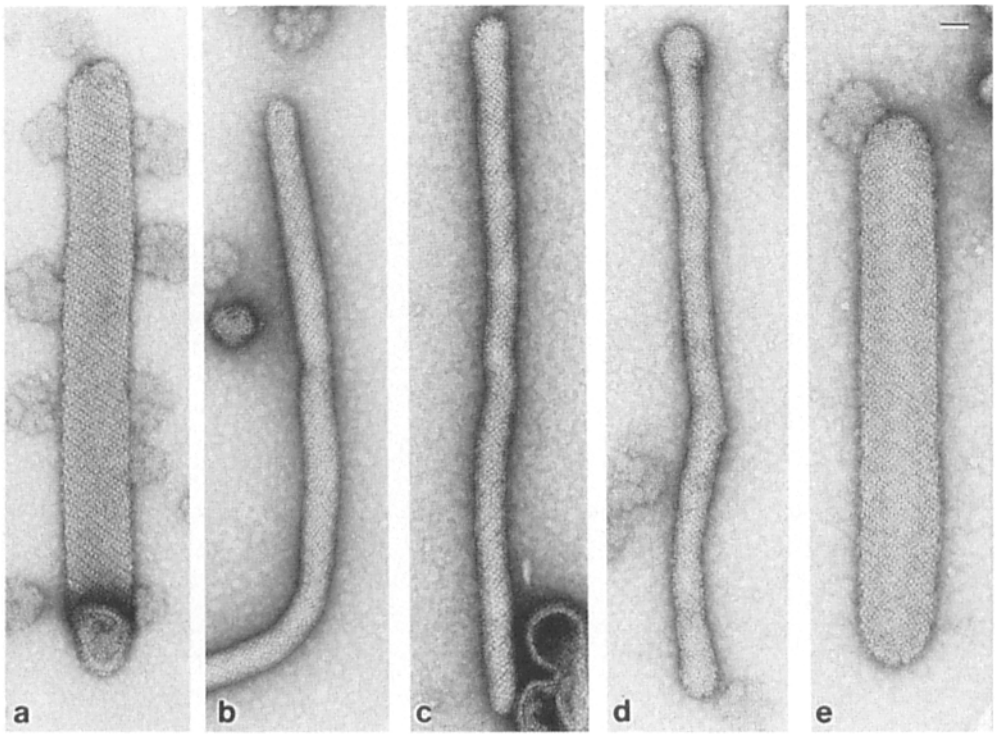

Figure 6. Negatively stained SR tubes illustrating the effect of exposure to pCa ~5.4 in the presence of ATP for increasing lengths of time and the effect of reversal: tube in EGTA (pCa >8.0) as control (*a*), selected tubes at pCa ~5.4 for 15 min (*b*), 1 h (*c*), overnight (*d*), and for 1 h followed by overnight dialysis vs. EGTA-containing solution (*e*). Note that the disrupting effect of Ca^{2+} on the surface array of the SR tubes (*b–d*) can be reversed by removal of Ca^{2+} (*e*). Bar, 35 nm.

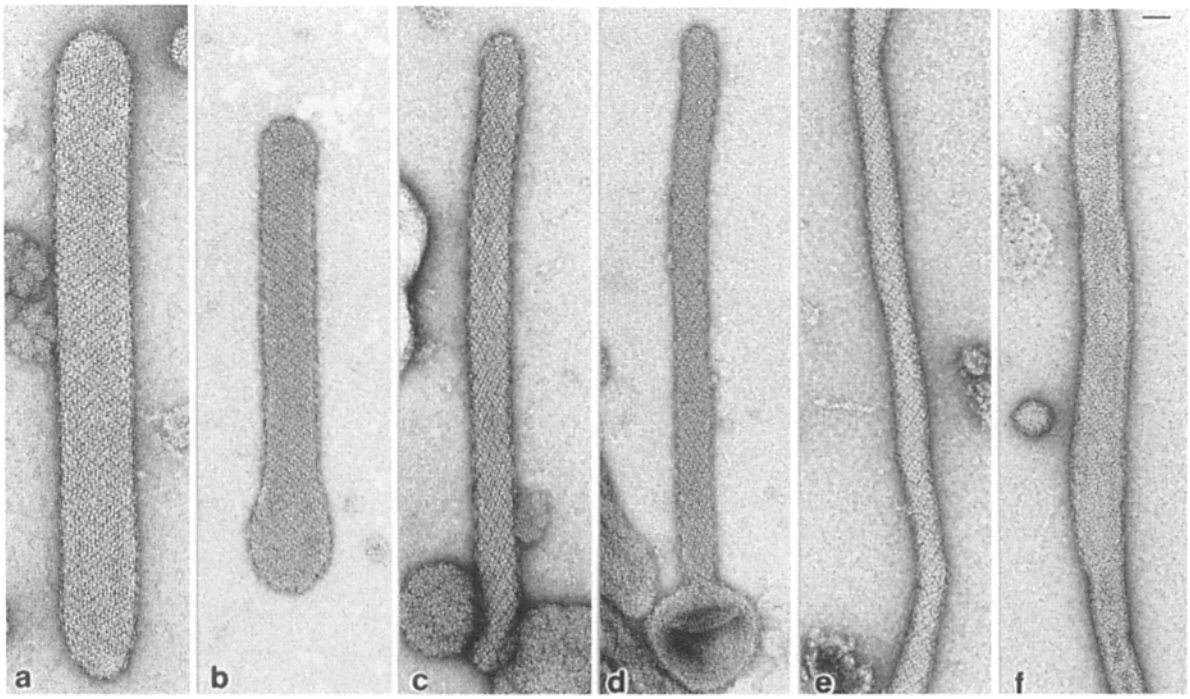

Figure 7. Gallery of negatively stained SR tubes exposed for 1 min to solutions without ATP at various free Ca^{2+} concentrations: pCa >8.0 (*a*), ~6.9 (*b*), ~6.4 (*c*), ~6.0 (*d*), ~5.4 (*e*), and ~4.0 (*f*). Note that the disrupting effect of Ca^{2+} begins in patches at pCa ~6.0 and becomes more evident at lower pCas. Bar, 35 nm.

SR, in which the Ca^{2+}-ATPase is polymerized by exposure to vanadate, has freeze-fracture profiles similar to scallop SR in situ (Peracchia et al., 1984).

Deep-etching of scallop muscle reveals regular arrays of ribbons of Ca^{2+}-ATPase dimers on the cytoplasmic surface of the SR tubes and rows of very small projections on the luminal surface. The precise relationship between the dimer ribbons and the rows of small projections observed on the luminal surface (see Fig. 2) is difficult to establish, given the differential amount of metal deposited on the two surfaces. These images, however, suggest that the Ca^{2+}-ATPase molecules span the lipid bilayer emerging into the luminal surface but do not project very far into the lumen. Three-dimensional reconstructions calculated from negatively stained isolated SR tubes do not show the ATPase molecules protruding into the lumen, although the exact position of the lipid bilayer cannot be clearly defined (Castellani et al., 1985; Taylor et al., 1986).

The effect of Ca^{2+} on the scallop FSR was analyzed by correlating the enzymatic activity and Ca^{2+} binding properties of the Ca^{2+}-ATPase with the surface structure of the vesicles at various Ca^{2+} concentrations. The Ca^{2+} activation curve for the Ca^{2+}-ATPase activity follows the Ca^{2+} binding curve closely at pCa values above 5.0, and both processes exhibit a Hill coefficient close to 2. This behavior is very similar to that of rabbit skeletal muscle FSR (Inesi et al., 1980; Hasselbach, 1983; Martonosi, 1983; and for review see Inesi, 1985) and implies that Ca^{2+} is bound with positive cooperativity to the outside of the vesicles.

The inhibition of the Ca^{2+}-ATPase activity at pCa ~5.0 and below indicates the presence of low affinity inhibitory sites on a catalytic intermediate form of the scallop Ca^{2+}-ATPase. In the Ca^{2+}-ATPase from rabbit SR, however, the low affinity, Ca^{2+} binding sites appear on the E_2P form of the enzyme (Andersen et al., 1985) and do not coexist with the high affinity sites on the same Ca^{2+}-ATPase molecule. Indeed, the transformation of high affinity, Ca^{2+} binding sites on the E_1 form of the enzyme to low affinity sites in the E_2P form is the basis of several models of Ca^{2+} translocation across the SR membrane (e.g., Tanford et al., 1987). Thus, the low affinity sites detected in addition to the high affinity sites by $^{45}Ca^{2+}$ binding to scallop SR do not represent the specific low affinity luminal sites of the Ca^{2+}-ATPase protein as such, but are accounted for by the binding of Ca^{2+} to the phospholipid component of the membrane. A similar picture has been suggested for the low affinity binding of Ca^{2+} observed in addition to the high affinity binding with rabbit SR (Kalbitzer et al., 1978).

Electron microscopy of isolated FSR, visualized by negative staining or by rotary-shadowing, reveals that, at calcium concentrations where Ca^{2+} binds to the high affinity sites and the Ca^{2+}-ATPase is activated, the regular array of dimer ribbons is preserved. The overall appearance of the tubes, however, seems to be affected at pCa ~6.0 (presence of partially flattened tubes) suggesting possible rearrangement of the Ca^{2+}-ATPase dimers within the membrane. A detailed structural analysis will be required to establish the exact nature of these changes. Monomeric Ca^{2+}-ATPase has been found to be enzymatically active (Martin et al., 1984; Andersen et al., 1985). Radiation-inactivation analysis of SR vesicles has been interpreted to indicate that the Ca^{2+}-ATPase is associated into dimers during the pumping cycle (Chamberlain et al., 1983), although this conclusion is still subject to uncertainty. The high degree of cooperativity observed in measurements of Ca^{2+} binding at different pHs also suggest that the Ca^{2+} pump is a dimer (Inesi et al., 1980; Hill and

Inesi, 1982), in keeping with the electron microscopic structural studies presented here. More recently, studies on the Ca^{2+}-ATPase catalytic cycle have suggested that, although conformational changes of the pump protein are involved in the binding and dissociation of Ca^{2+} and Pi, the occurrence of dimeric arrays induced by vanadate reflects a basic property of the Ca^{2+}-ATPase molecules rather than depicting an intermediate state of the cycle (Jorges-Garcia et al., 1988). The role of the Ca^{2+}-ATPase dimers observed in scallop SR when the Ca^{2+} pump is activated may therefore be that of providing an additional level of control to the functionally active monomer, possibly through an increase of cooperativity of Ca^{2+} binding.

The loss of crystallinity in the isolated scallop FSR tubes that begins in patches at pCa ~6.0 and becomes evident when the pCa falls below 5 seems to occur independently of ATP. These structural changes appear to correlate with the onset of low affinity, Ca^{2+} binding sites measured in solution. Monomeric arrays of Ca^{2+}-ATPase are observed in rabbit SR at similar Ca^{2+} concentrations, although only in a limited portion of the vesicles population and at slightly alkaline pH (Dux et al., 1985). The apparent differences between the observations described by Dux et al. (1985) and the ones presented here may possibly be attributed to the different pHs at which the experiments were carried out. Further studies are, however, required to explain these observations. It should also be noted that many investigations of liposome model systems provide evidence for interactions between Ca^{2+} and phosphatidylserine (e.g., Hauser et al., 1976; Feigenson, 1986). Relatively high concentrations of Ca^{2+} have been reported to be necessary for significant effects on model membranes (e.g., Silvius and Gagne, 1984). Thus, it is likely that Ca^{2+} binding to the phospholipid component of the SR, rather than to Ca^{2+} binding sites on the Ca^{2+}-ATPase, is involved in some of the structural effects described.

We thank Drs. Carolyn Cohen and Peter Vibert for continuous support during the development of this project and comments on the manuscript, Dr. Donald Ferguson for earlier work, Judith Black for photography, and Louise Seidel and Beth Finkelstein for typing the manuscript.

This work was supported by grants from the National Institutes of Health, AR35829 (to L. Castellani), AR17346 (to C. Cohen), and HL-15835 to the Pennsylvania Muscle Institute; from the National Science Foundation, DCB85-09699 (to P. Hardwicke) and DMB85-02233 (to C. Cohen and P. Vibert); and from the Muscular Dystrophy Association (to the H. H. Watts Research Center and to C. Cohen). Funds to purchase and maintain the Philips Electronics Instruments EM420T transmission electron microscope were obtained from a Shared Instrumentation Grant 1-S10-RR02464-01 awarded to C. Cohen by the National Institutes of Health.

Received for publication 8 August 1988, and in revised form 24 October 1988.

References

Andersen, J. P., K. Lassen, and J. V. Moller. 1985. Changes in Ca^{2+} affinity related to conformational transitions in the phosphorylated state of soluble monomeric Ca^{2+}-ATPase from sarcoplasmic reticulum. *J. Biol. Chem.* 260:371–380.

Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70:241–250.

Buhle, E. L., B. E. Knox, E. Serpersu, and U. Aebi. 1983. Structural analysis of crystalline Ca^{2+} transport ATPase vesicles. *J. Ultrastruct. Res.* 85:186–203.

Castellani, L., and P. M. D. Hardwicke. 1983. Crystalline structure of sarcoplasmic reticulum from scallop. *J. Cell Biol.* 97:557–561.

Castellani, L., P. M. D. Hardwicke, and P. Vibert. 1985. Dimer ribbons in the three-dimensional structure of sarcoplasmic reticulum. *J. Mol. Biol.* 185:579–594.

Chamberlain, B. K., C. J. Berenski, C. Y. Jung, and S. Fleischer. 1983. Determination of the oligomeric structure of the Ca^{2+} pump protein in canine cardiac sarcoplasmic reticulum membranes using radiation inactivation analysis. *J. Biol. Chem.* 258:11997–12001.

Chantler, P. D., and A. G. Szent-Györgyi. 1980. Regulatory light chains and scallop myosin: full dissociation, reversibility and co-operative effect. *J. Mol. Biol.* 138:473–492.

Dux, L., and A. Martonosi. 1983*a*. Two-dimensional arrays of proteins in sarcoplasmic reticulum and purified Ca^{2+}-ATPase vesicles treated with vanadate. *J. Biol. Chem.* 258:2599–2603.

Dux, L., and A. Martonosi. 1983*b*. The regulation of ATPase–ATPase interactions in sarcoplasmic reticulum membrane. *J. Biol. Chem.* 258:11896–11902.

Dux, L., S. Pikula, N. Mullner, and A. Martonosi. 1987. Crystallization of Ca^{2+}-ATPase in detergent-solubilized sarcoplasmic reticulum. *J. Biol. Chem.* 262:6439–6442.

Dux, L., K. A. Taylor, H. P. Ting-Beall, and A. Martonosi. 1985. Crystallization of the Ca^{2+}-ATPase of sarcoplasmic reticulum by calcium and lanthanide ions. *J. Biol. Chem.* 260:11730–11743.

Endo, M., and M. Iino. 1980. Specific preparation of muscle cell membranes with preserved SR functions by saponin treatment. *J. Muscle Res. Cell Motil.* 1:89–100.

Feigenson, G. W. 1986. On the nature of calcium ion binding between phosphatidylserine lamellae. *Biochemistry.* 25:5819–5825.

Ferguson, D. G., C. Franzini-Armstrong, L. Castellani, P. M. D. Hardwicke, and L. J. Kenney. 1985. Ordered arrays of Ca^{2+}-ATPase on the cytoplasmic surface of isolated sarcoplasmic reticulum. *Biophys. J.* 48:597–605.

Franzini-Armstrong, C., and D. G. Ferguson. 1985. Density and disposition of Ca^{2+}-ATPase in sarcoplasmic reticulum membrane as determined by shadowing techniques. *Biophys. J.* 48:607–615.

Franzini-Armstrong, C., D. G. Ferguson, L. Castellani, and L. J. Kenney. 1986. The density and disposition of Ca-ATPase in situ and isolated sarcoplasmic reticulum. Recent advances in electron and light optical imaging in biology and medicine. *Ann. NY Acad. Sci.* 483:44–56.

Hasselbach, W., and H. Oetliker. 1983. Energetics and electrogenicity of the sarcoplasmic reticulum calcium pump. *Annu. Rev. Physiol.* 45:325–329.

Hauser, H., A. Darke, and M. C. Phillips. 1976. Ion-binding to phospholipids. *Eur. J. Biochem.* 62:335–344.

Hill, T. L., and G. Inesi. 1982. Equilibrium cooperative binding of calcium and protons by sarcoplasmic reticulum ATPase. *Proc. Natl. Acad. Sci. USA.* 79:3978–3982.

Hummel, J. P., and W. J. Dreyer. 1962. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta.* 63:530–532.

Inesi, G. 1985. Mechanism of calcium transport. *Annu. Rev. Physiol.* 47:573–601.

Inesi, G., M. Kurzmack, L. Coan, and D. E. Lewis. 1980. Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 255:3025–3031.

Jorge-Garcia, I., D. J. Bigelow, G. Inesi, and J. B. Wade. 1988. Effect of urea on the partial reactions and crystallization pattern of sarcoplasmic reticulum adenosine triphosphate. *Arch. Biochem. Biophys.* 265:82–90.

Kalbitzer, H. R., D. Stehlik, and W. Hasselbach. 1978. The binding of calcium and magnesium to sarcoplasmic reticulum vesicles as studied by manganese electron paramagnetic resonance. *Eur. J. Biochem.* 82:245–255.

Martin, D. W., C. Tanford, and J. A. Reynolds. 1984. Monomeric solubilized sarcoplasmic reticulum Ca pump protein: demonstration of Ca binding and dissociation coupled to ATP hydrolysis. *Proc. Natl. Acad. Sci. USA.* 81: 6623–6626.

Martonosi, A. N. 1983. The regulation of cytoplasmic Ca^{2+} concentration in muscle and non-muscle cells. *In* Muscle and Non-muscle Motility. A. Stracher, editor. Vol. 1. Academic Press, New York. 233–257.

Napolitano, C. A., P. Cooke, K. Segalman, and L. Herbette. 1983. Organizaton of calcium pump protein dimers in the isolated sarcoplasmic reticulum membrane. *Biophys. J.* 42:119–125.

Nunzi, M. G., and C. Franzini-Armstrong. 1981. The structure of smooth and striated portions of the adductor muscle of the valves in scallop. *J. Ultrastruct. Res.* 76:134–148.

Peachey, L. D., and C. Franzini-Armstrong. 1983. Structure and function of membrane systems of skeletal muscles. *In* Handbook of Physiology, Skeletal Muscle. Chapt. 2. 23–71.

Peracchia, C., L. Dux, and A. N. Martonosi. 1984. Crystallization of intramembrane particles in rabbit sarcoplasmic reticulum vesicles by vanadate. *J. Muscle Res. Cell Motil.* 5:431–442.

Pikula, S., N. Mullner, L. Dux, and A. Martonosi. 1988. Stabilization and crystallization of Ca^{2+}-ATPase in detergent-solubilized sarcoplasmic reticulum. *J. Biol. Chem.* 263:5277–5286.

Sanger, J. W. 1971. Sarcoplasmic reticulum in the cross-striated adductor muscle of the bay scallop *Aequipecten irradians*. *Z. Zellforsch. Mikrosk. Anat.* 118:156–161.

Sanger, J. W., and J. M. Sanger. 1985. Sarcoplasmic reticulum in the adductor muscles of a Bermuda scallop: comparison of smooth versus cross-striated

portions. *Biol. Bull. (Woods Hole).* 168:447–460.
Scales, D., and G. Inesi. 1976. Assembly of ATPase protein in sarcoplasmic reticulum membranes. *Biophys. J.* 16:735–751.
Scofano, H., H. Barrabin, G. Inesi, and J. A. Cohen. 1985. Stoichiometric and electrostatic characterization of calcium binding to native and lipid-substituted adenosine triphosphatase of sarcoplasmic reticulum. *Biochim. Biophys. Acta.* 819:93–104.
Silvius, J. R., and G. Gagne. 1984. Lipid phase behavior and calcium-induced fusion of phosphotidyl-thenolamine–phosphatidyl-serine vesicles: colorimetric and fusion studies. *Biochemistry.* 23:3232–3240.
Tanford, C., J. A. Reynolds, and E. A. Johnson. 1987. Sarcoplasmic reticulum calcium pump: a model for Ca^{2+} binding and Ca^{2+}-coupled phosphorylation. *Proc. Natl. Acad. Sci. USA.* 84:7094–7098.
Taylor, K. A., L. Dux, and A. Martonosi. 1984. Structure of the vanadate-induced crystals of sarcoplasmic reticulum Ca^{2+}-ATPase. *J. Mol. Biol.* 174: 193–204.
Taylor, K. A., L. Dux, and A. Martonosi. 1986. Three-dimensional reconstruction of negatively stained crystals of the Ca^{2+}-ATPase from muscle sarcoplasmic reticulum. *J. Mol. Biol.* 187:417–427.
Taylor, K. A., N. Mullner, S. Pikula, L. Dux, C. Peracchia, S. Varga, and A. Martonosi. 1988. Electron microscope observations on Ca^{2+}-ATPase microcrystals in detergent-solubilized sarcoplasmic reticulum. *J. Biol. Chem.* 263:5287–5294.
Warren, G. R., P. A. Toon, N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe. 1974. Reconstruction of a calcium pump using defined membrane components. *Proc. Natl. Acad. Sci. USA.* 71:622–626.