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Effect of Na_3VO_4 and Membrane Potential on the Structure of Sarcoplasmic Reticulum Membrane

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Summary. Two-dimensional crystalline arrays of Ca^{2+}-ATPase molecules develop after treatment of sarcoplasmic reticulum vesicles with Na_3VO_4 in a Ca^{2+}-free medium. The influence of membrane potential upon the rate of crystallization was studied by ion substitution using oxonol VI and 3,3′-diethyl-2,2′-thiadicarbocyanine (Di−S−C_2(5)) to monitor inside positive or inside negative membrane potentials, respectively. Positive transmembrane potential accelerates the rate of crystallization of Ca^{2+}-ATPase, while negative potential disrupts preformed Ca^{2+}-ATPase crystals, suggesting an influence of transmembrane potential upon the conformation of Ca^{2+}-ATPase.

Key Words Ca^{2+}, Mg^{2+}-ATPase · Ca^{2+} transport · membrane potential · vanadate · crystals · sarcoplasmic reticulum

Introduction

Two-dimensional crystalline arrays of the Ca^{2+} transport ATPase develop after treatment of sarcoplasmic reticulum vesicles with Na_3VO_4 in a Ca^{2+}-free medium (Dux & Martonosi, 1983*a*). In order to form crystals the Ca^{2+}-ATPase molecules must assume the E_2 conformation, which is stabilized by vanadate. Ca^{2+} in concentration sufficient to saturate the high affinity Ca^{2+} binding site of the Ca^{2+}-ATPase prevents the formation of ATPase crystals and disrupts the crystals that were formed previously (Dux & Martonosi, 1983*a*). These effects of Ca^{2+} can be attributed to the changes in the conformation of Ca^{2+}-ATPase associated with the binding of Ca^{2+}. Based on these observations the crystallization of Ca^{2+}-ATPase may serve as an indicator of the conformational requirements of ATPase-ATPase interactions in sarcoplasmic reticulum membranes.

Artificially imposed inside positive membrane diffusion potential strongly accelerated the rate of formation of Ca^{2+}-ATPase crystals (Dux & Martonosi, 1983*b*). Inside negative potential interfered with the crystallization, and disrupted preformed Ca^{2+}-ATPase crystals. It is assumed that the membrane potential alters the conformational equilibrium of Ca^{2+}-ATPase in a manner that either promotes or hinders the interaction between ATPase molecules, depending upon the direction of the potential (Dux & Martonosi, 1983*b*). These observations may have physiological significance in light of the membrane potential changes in sarcoplasmic reticulum that accompany the uptake and release of Ca^{2+} *in vivo* (Bezanilla & Horowicz, 1975; Vergara & Bezanilla, 1981) and *in vitro* (Beeler, 1980; Beeler, Farmen & Martonosi, 1981).

Incorporation of the Ca^{2+} transport ATPase into phospholipid vesicles increases the permeability of the membrane by several orders of magnitude (Jilka, Martonosi & Tillack, 1975; Martonosi, 1975; Jilka & Martonosi, 1977). Therefore, in addition to its role in mediating ATP-dependent active Ca^{2+} transport, the Ca^{2+}-ATPase also contributes to the passive permeability of the membrane to ions and other small molecules. Considering the effect of membrane potential on ATPase-ATPase interactions (Dux & Martonosi, 1983*b*) and the potential changes associated with the uptake and release of Ca^{2+} by sarcoplasmic reticulum (Beeler et al., 1981; Vergara & Bezanilla, 1981) it is tempting to speculate that changes in the conformational equilibrium of Ca^{2+}-ATPase under the influence of membrane potential may contribute to the changes in the Ca^{2+} permeability of sarcoplasmic reticulum membrane by altering the monomer-polymer equilibrium of Ca^{2+}-ATPase (Martonosi et al., 1977; Vanderkooi et al., 1977)

As a first step to test this hypothesis we analyzed the effect of Na_3VO_4 upon the magnitude and duration of membrane potential generated by ion substitutions in rabbit sarcoplasmic reticulum vesicles, using the voltage sensitive dyes diethylthiadicarbocyanine and oxonol VI, and compared the changes in membrane potential with the rate of crystallization of Ca^{2+}-ATPase.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit skeletal muscle, as described earlier (Nakamura et al., 1976), with slight modifications (Beeler et al., 1981).

Diffusion potential across the sarcoplasmic reticulum membrane was generated by ion substitution, as described in detail in the text and in the Figure legends. The difference absorbances of oxonol VI (Molecular Probes, Inc., Junction City, Oregon) and 3,3′diethyl-2,2′-thiadicarbocyanine (Di−S−C_2(5)) (Eastman Kodak, Rochester, New York) were recorded on an Aminco DW-2 spectrophotometer. The absorbance changes of oxonol VI and Di−S−C_2(5) served to evaluate the changes in inside-positive and inside-negative membrane potential, respectively (Beeler et al., 1981). Oxonol VI contains delocalized negative charges and accumulates into compartments under the influence of inside positive potential, resulting in absorbance and fluorescence changes. Di−S−C_2(5), a dye with delocalized positive charges, responds in an analogous manner to inside negative potential.

For electron microscopy, the vesicle suspensions ($\simeq$1 mg protein/ml) were placed on carbon-coated parlodion films and negatively stained with freshly prepared 1% uranyl acetate (pH 4.3). The specimens were viewed with a Siemens Elmiskop 102 electron microscope at 60 kV accelerating voltage. For magnification calibration catalase crystals negatively stained with 1% uranyl acetate were used.

Results

Ca^{2+}-ATPase Membrane Crystals in Sarcoplasmic Reticulum

Crystalline arrays of Ca^{2+}-ATPase develop upon exposure of sarcoplasmic reticulum vesicles to 5 mM Na_3VO_4 in a Ca^{2+}-free medium, at 2 °C (Fig. 1). The Ca^{2+}-ATPase crystals are usually observed on the surface of elongated tubules which are approximately 600 to 800 Å in diameter. The crystal lattice consists of chains of ATPase dimers which are in register with neighboring chains, and surround the tubules in a right-handed helix (Taylor, Dux & Martonosi, 1983a; Peracchia et al.[1]). The diagonal lattice arises from superimposition of the front and rear images of the collapsed cylinders. The lattice constants are: $a=56.34$ Å, $b=104.78$ Å and $\gamma=72.72°$ (Taylor et al., 1983). Under the conditions described in Fig. 1, crystallization of about 2/3 of the vesicles requires 1 to 2 days.

Very much faster crystallization of the Ca^{2+}-ATPase was observed after dilution of the vesicles preincubated in 0.15 M choline chloride medium into a medium containing 0.15 M K-glutamate and 5 mM Na_3VO_4 (Fig. 2). Under these conditions outward movement of Cl^- and inward movement of K^+ generate an inside positive diffusion potential across the vesicle membrane. Within 15 sec after dilution, Ca^{2+}-ATPase crystals were observed on the surface of about half of the vesicles. Control preparations which were pre-equilibrated in K-glutamate medium, showed essentially no crystallization 15 sec after transfer into K-glutamate medium containing 5 mM Na_3VO_4 (Fig. 3). Under these conditions no potential is expected to develop, and significant crystallization requires several hours of incubation with vanadate.

These observations, together with earlier findings (Dux & Martonosi, 1983*b*), establish that conditions which are expected to generate positive membrane potential facilitate the association of ATPase molecules into extended crystalline arrays.

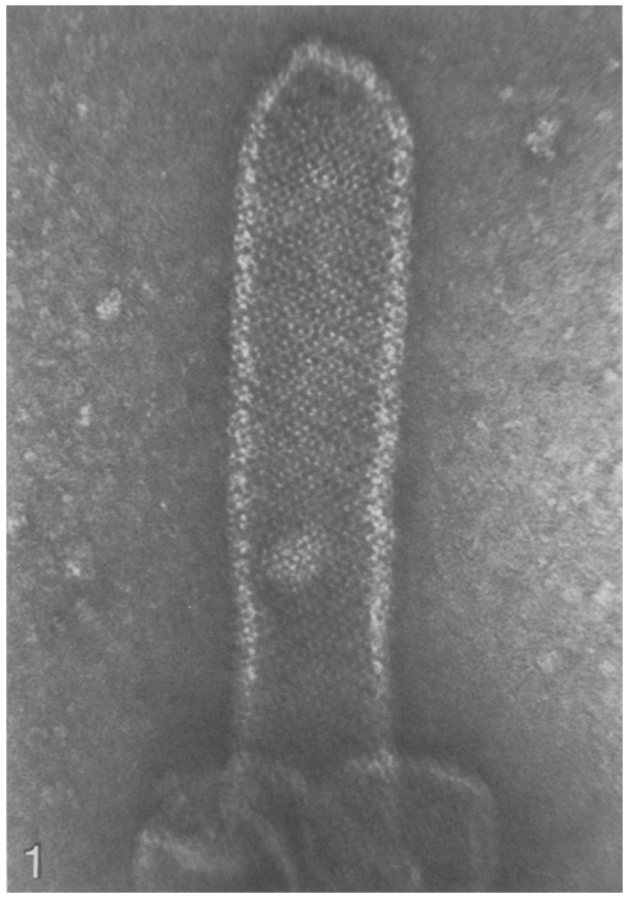

Fig. 1. Crystalline arrays of Ca-ATPase molecules in rabbit sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were incubated in 0.1 M KCl, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM Na_3VO_4 at 2 °C and samples were taken for negative staining after 4 days. Magnification: 239,519×

[1] C. Peracchia, L. Dux & A. Martonosi. Crystallization of intramembrane particles in rabbit sarcoplasmic reticulum vesicles by vanadate. (*unpublished*)

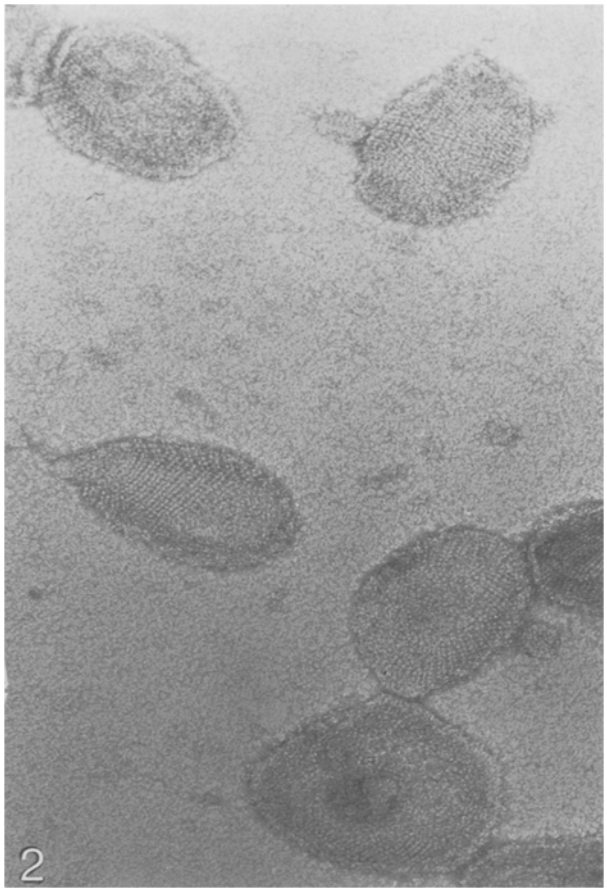

Fig. 2. Rapid crystallization of Ca-ATPase molecules under the influence of inside positive potential. Sarcoplasmic reticulum vesicles (10 mg/ml) were preincubated in 0.15 M choline chloride, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$ and 0.5 mM EGTA for 16 hr at 2 °C, and then diluted 10-fold in 0.15 M K-glutamate, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM Na_3VO_4 containing medium. Negative staining was carried out 15 sec after dilution. Magnification: 102,651 ×

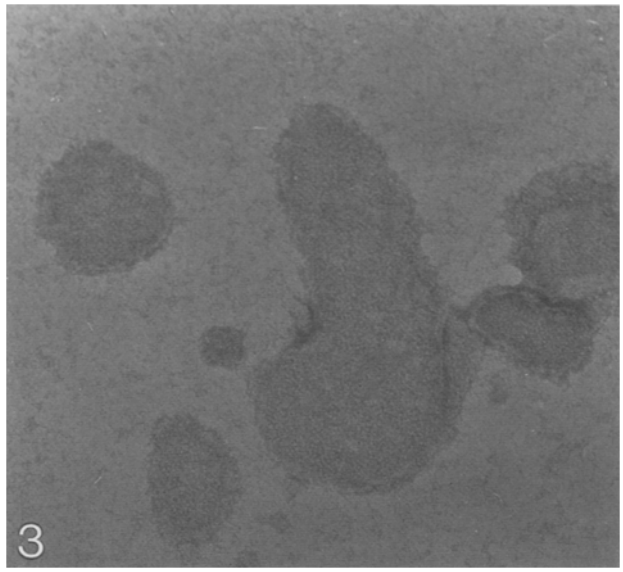

Fig. 3. Surface structure of vesicles after dilution into medium of identical composition. Sarcoplasmic reticulum vesicles (10 mg/ml) were pre-incubated in 0.15 M K-glutamate, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, and 0.5 mM EGTA medium for 16 hr, then diluted 10-fold in the same medium containing 5 mM Na_3VO_4. Negative staining was completed 15 sec after dilution. Magnification: 102,651 ×

Oxonol VI Signals in Vanadate-Treated Sarcoplasmic Reticulum Vesicles after Transfer from Choline Chloride into K-Glutamate Medium

The time course of the potential change generated by transfer of vesicles from choline chloride into K-glutamate medium was followed by measuring the difference absorbance of the voltage sensitive dye oxonol VI at 625 to 586 nm (Fig. 4).

Dilution of control vesicles from K-glutamate into K-glutamate media containing 50 μM Ca (Fig. 4*A*, sample 1′) increases the absorbance by about 0.14 to 0.15 units; the absorbance change of control samples containing 5 mM vanadate (sample 2′) is significantly smaller. The absorbance change in control samples rapidly achieves a steady

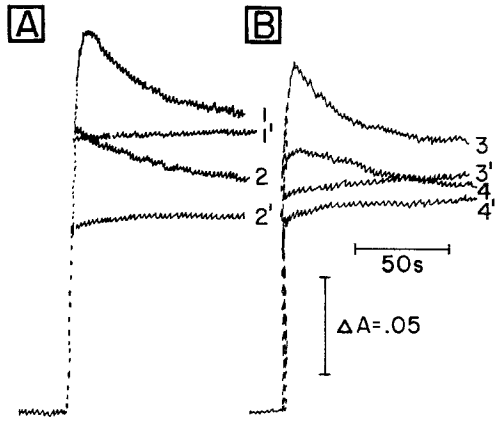

Fig. 4. Absorbance changes of oxonol VI upon dilution of vesicles pre-equilibrated in choline chloride into K-glutamate medium. Sarcoplasmic reticulum vesicles (30 mg protein/ml) were equilibrated overnight at 2 °C in medium containing 0.15 M choline chloride, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$, and 50 μM $CaCl_2$. Inside positive membrane potentials were generated by diluting 4 μl aliquots of this suspension into 2 ml of medium containing 0.15 M K-glutamate, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$, 10 μg/ml oxonol VI and: 50 μM $CaCl_2$ (trace 1); 50 μM $CaCl_2$, 5 mM Na_3VO_4 (trace 2); 1 mM EGTA (trace 3); or 1 mM EGTA, 5 mM Na_3VO_4 (trace 4). The difference absorbance of oxonol VI at 625 to 586 nm was measured in an Aminco DW-2 spectrophotometer at 10 °C. For controls, the sarcoplasmic reticulum vesicles were equilibrated in K-glutamate instead of choline chloride medium and diluted as described above (traces 1′–4′)

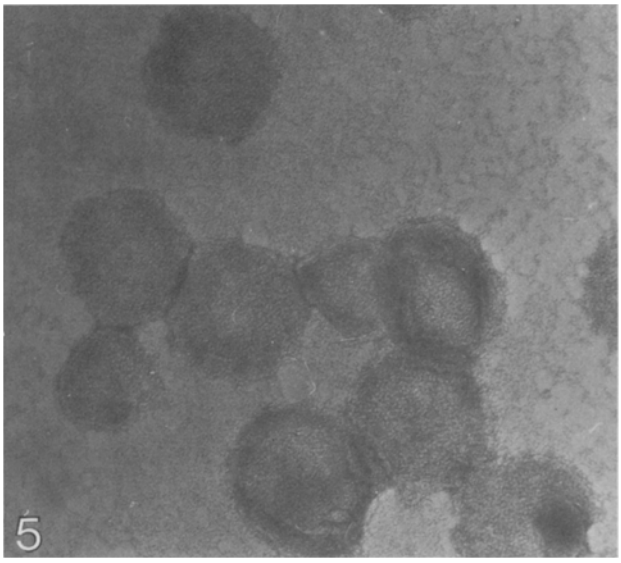

Fig. 5. Disruption of Ca^{2+}-ATPase membrane crystals under the influence of inside negative potential. Sarcoplasmic reticulum vesicles (10 mg/ml) were precrystallized in 0.15 M K-glutamate, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM Na_3VO_4 medium at 2 °C for 16 hr. Under this condition 65 to 70% of the vesicles show crystalline arrays (Dux & Martonosi, 1983*b*). Negative potential was generated by 10-fold dilution of the samples in a 0.15 M choline chloride containing medium of otherwise identical composition. Magnification: 102,651 ×

level that presumably reflects the binding of oxonol VI to the sarcoplasmic reticulum vesicles in the absence of potential.

Dilution of vesicles from choline chloride into K-glutamate medium containing 50 μM Ca^{2+} (Fig. 4*A*, sample 1), causes a much greater absorbance change ($\Delta A \sim 0.20$) due to the generation of inside positive potential; this is followed by a rapid decline of the optical response as the potential is dissipated. Dilution into K-glutamate medium containing 50 μM Ca and 5 mM Na_3VO_4 (Fig. 4*A*, sample 2) gave an initial absorbance change of 0.151 which declined to a level intermediate between that of samples 1′ and 2′. The smaller absorbance change observed in the presence of vanadate (sample 2) may be due to at least two causes:

1. An effect of Na_3VO_4 on the absorbance of oxonol VI, as also reflected by the difference between the absorbance response of the two control samples (samples 1′ and 2′).

2. Na_3VO_4 decreases the potential generated by ion substitution presumably because it changes the permeability of the membrane to ions.

The difference between samples 1 and 2 cannot be attributed to crystallization of the Ca^{2+}-ATPase since 50 μM Ca^{2+} completely inhibits the formation of Ca^{2+}-ATPase crystals.

In Fig. 4*B* a similar experiment is described except that the dilution medium contained 1 mM EGTA to lower free [Ca^{2+}] below 10^{-8} M. The absorbance change observed upon transfer of vesicles from choline chloride into K-glutamate medium containing EGTA is much greater in the absence (Fig. 4*B*, sample 3) than in the presence of 5 mM Na_3VO_4 (Fig. 4*B*, sample 4); in both samples the difference absorption rapidly decreases and in the presence of Na_3VO_4 (sample 4) within 1 to 2 min approaches the level of the corresponding control (sample 4′) without preimposed potential. In the absence of vanadate (sample 3) the potential signal reaches a relatively steady level 2 min after dilution, that is significantly greater than in the control (sample 3′) without pre-imposed potential. These observations imply that the signal of oxonol VI to inside positive potential generated by ion substitution is small and short-lived in the presence of EGTA and Na_3VO_4, and within 2 min the potential is largely dissipated. Therefore conditions that promote the crystallization of Ca^{2+}-ATPase decrease the potential response of oxonol VI to ion substitution, presumably by changing the ion permeability of sarcoplasmic reticulum.

Influence of Inside Negative Potential upon Preformed Ca^{2+}-ATPase Crystals

Ca^{2+}-ATPase crystals were formed by incubation of sarcoplasmic reticulum vesicles in a medium of 0.15 M K-glutamate, 10 mM imidazole pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA and 5 mM Na_3VO_4 at 2 °C for 16 hr. About 2/3 of the vesicles showed crystalline arrays similar to those in Fig. 1 on at least a portion of their surface. Dilution of this suspension into a similar medium in which K-glutamate was replaced with 0.15 M choline chloride caused the complete disappearance of the crystal lattice within 15 to 30 sec (Fig. 5). Substitution of K-glutamate with choline chloride is expected to produce inside negative potential and it is assumed that the conformational change of the Ca^{2+}-ATPase under the influence of this potential is responsible for the disruption and disappearance of the Ca^{2+}-ATPase crystals.

The time course and magnitude of the potential were measured using the voltage sensitive dye Di–S–C_2(5) as indicator. Dilution of sarcoplasmic reticulum vesicles equilibrated in a K-glutamate medium into a medium containing choline chloride causes a decrease in the difference absorbance of Di–S–C_2(5) at 660 to 700 nm in response to negative potential; this is followed by a slow return of absorbance to near starting levels as the poten-

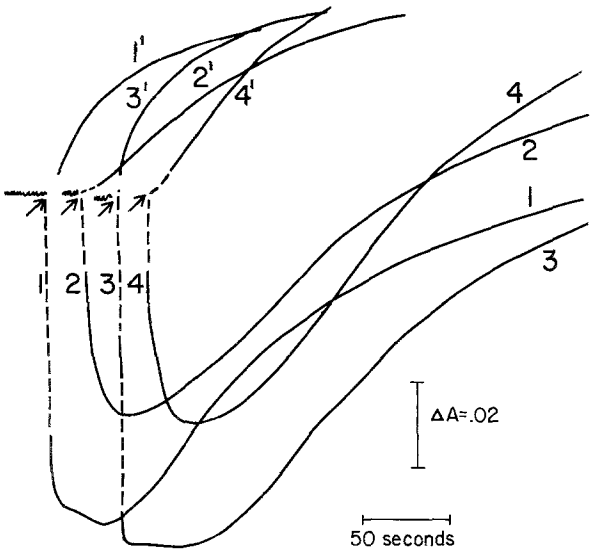

Fig. 6. Changes in the absorbance of Di−S−C_2(5) after dilution of sarcoplasmic reticulum vesicles from K-glutamate into choline chloride medium. Sarcoplasmic reticulum vesicles (30 mg protein/ml) were equilibrated overnight at 2 °C in medium containing 0.15 M K-glutamate, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$ and 50 μM $CaCl_2$. Inside negative potentials were generated by diluting 4 μl aliquots of this suspension into 2 ml of medium containing 0.15 M choline chloride, 10 mM histidine (pH 6.8), 5 mM $MgSO_4$, 10 μg/ml Di−S−C_2(5), 1 μM valinomycin and: 50 μM $CaCl_2$ (trace 1); 50 μM $CaCl_2$, 5 mM Na_3VO_4 (trace 2); 1 mM EGTA (trace 3); 1 mM EGTA, 5 mM Na_3VO_4 (trace 4). The difference absorbance of Di−S−C_2(5) at 660 to 700 nm was measured in an Aminco DW-2 spectrophotometer at 10 °C. For controls, the sarcoplasmic reticulum vesicles were equilibrated in choline chloride instead of K-glutamate and diluted as described above (traces 1′–4′)

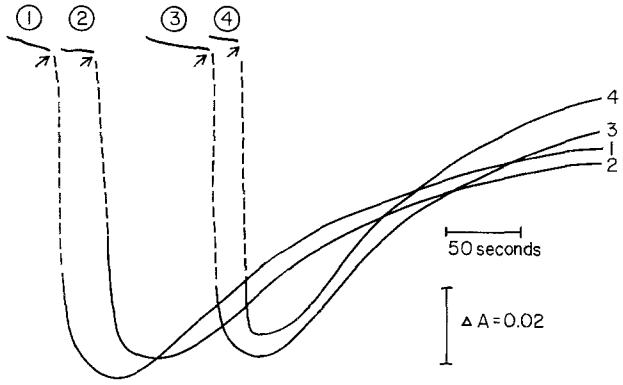

Fig. 7. Changes in absorbance of Di−S−C_2(5) after dilution of sarcoplasmic reticulum vesicles from K-glutamate into choline chloride medium. Sarcoplasmic reticulum vesicles (30 mg protein/ml) were equilibrated overnight in a medium containing 0.15 M K-glutamate, 10 mM histidine pH 6.8, 5 mM $MgSO_4$, and either 50 μM $CaCl_2$ (samples 1 and 2) or 1 mM EGTA (samples 3 and 4). Samples 2 and 4 contained 5 mM Na_3VO_4. During incubation in the presence of EGTA and Na_3VO_4 (sample 4) Ca^{2+}-ATPase crystals develop while samples 1–3 serve as crystal-free controls. After overnight incubation the samples were diluted into media containing 0.15 M choline chloride, 10 mM histidine pH 6.8, 5 mM $MgSO_4$, 50 μM $CaCl_2$ and 10 μg/ml Di−S−C_2(5). The difference absorbance of Di−S−C_2(5) was measured at the wavelength pair of 660 to 700 nm

tial is dissipated (Fig. 6, samples 1–4). Control samples diluted from choline-Cl into choline-Cl (Fig. 6, samples 1′–4′) are not expected to generate potential and show only a slow increase in difference absorbance that is probably attributable to slow penetration of the dye into the vesicles. The rate of this change is much slower than the change observed under the influence of potential, and is not likely to affect significantly the conclusions. The magnitude of the absorbance change is greater in the absence (samples 1 and 3) than in the presence (samples 2 and 4) of vanadate whether the dilution medium contains 50 μM Ca (samples 1 and 2) or 1 mM EGTA (samples 3 and 4).

These observations suggest that Na_3VO_4 decreases the magnitude and duration of the potential change induced by ion substitution, presumably due to a change in the permeability of the membranes for ions.

There is no significant difference in the amplitude of the optical response between samples preincubated in the presence of 50 μM $CaCl_2$, that interferes with crystallization of the Ca^{2+}-ATPase (Fig. 7, samples 1, 2), and samples pre-incubated with 1 mM EGTA (Fig. 7, samples 3–4), that promotes crystallization of Ca^{2+}-ATPase in the presence of Na_3VO_4 (Fig. 7, sample 4). Therefore preexisting crystalline arrays of Ca^{2+}-ATPase do not affect the magnitude of potential change after transfer into choline chloride medium. The rate of disappearance of the optical response is slightly faster in samples containing 1 mM EGTA (Fig. 7, samples 3 and 4) in agreement with earlier observations (Duggan & Martonosi, 1970) that EGTA increases the permeability of sarcoplasmic reticulum membrane. This effect of EGTA is neutralized by Ca^{2+}.

Effect of Other Ion Substitutions

Transfer of vesicles from a Na-methanesulfonate solution into a medium containing K-methanesulfonate and 1 μM valinomycin also generates positive potential and promotes the crystallization of Ca^{2+}-ATPase (Dux & Martonosi, 1983*b*), while transfer from K-methanesulfonate solution into a

medium of Na-methanesulfonate + 1 μM valinomycin disrupts Ca^{2+}-ATPase crystals. Therefore the effects described in Figs. 1 through 6 are indeed attributable to changes in diffusion potential across the sarcoplasmic reticulum rather than to specific ion effects.

Discussion

Two effects of ion substitutions on the rate of crystallization of Ca^{2+}-ATPase were observed.

1. Transfer of vesicles from choline chloride into K-glutamate medium generates an inside positive potential across the vesicle membrane as indicated by a change in the absorbance of oxonol VI. The positive transmembrane potential favors the interaction between ATPase molecules with the formation of crystalline arrays of Ca^{2+}-ATPase in the presence of Na_3VO_4, presumably by changing the disposition of Ca^{2+}-ATPase in the membrane. Imposition of positive potential in the absence of Na_3VO_4 did not cause significant crystallization of Ca^{2+}-ATPase, although it inhibited the ATPase and Ca^{2+} transport activities of sarcoplasmic reticulum (Beeler et al., 1981). Therefore even in the absence of vanadate, transmembrane potential affects the conformation of the Ca^{2+}-ATPase in a functionally important manner.

The absorbance change of oxonol VI caused by ion substitution is generally smaller under conditions that promote the crystallization of Ca^{2+}-ATPase, raising the possibility that the vanadate-induced formation of Ca^{2+}-ATPase crystals increases the ion permeability of the membrane and facilitates the dissipation of transmembrane potential. A direct effect of vanadate upon the optical response of oxonol VI is observed even in the presence of Ca^{2+}, where crystallization cannot take place. The mechanism of this effect is not clear.

The crystalline arrays formed under the influence of positive potential usually occur on spherical vesicles in contrast to the elongated tubules that form after several days of crystallization. This may imply that the formation of tubular structures is a secondary, relatively slow process that follows the rapid crystallization.

2. Preformed crystalline arrays of Ca^{2+}-ATPase are disrupted when the microsome suspension is diluted from K-glutamate into choline chloride medium. Under these conditions an inside negative potential develops, as indicated by the absorbance change of $Di-S-C_2(5)$. The magnitude of the optical response is smaller in the presence than in the absence of vanadate in the dilution medium. However this difference cannot be attributed to crystallization of Ca^{2+}-ATPase since crystallization did not occur in media containing 50 μM Ca^{2+} and all crystals were destroyed upon dilution of vesicles from K-glutamate into choline chloride media. Na_3VO_4 may directly interfere with the optical response of $Di-S-C_2(5)$ or could produce other changes in the membrane not related to crystallization.

The disruption of Ca^{2+}-ATPase crystals by negative membrane potential is probably attributable to an effect on the conformation of the Ca^{2+}-ATPase that destabilizes ATPase-ATPase interactions. Since such interactions are presumed to take place in the E_2 conformation of the enzyme (Dux & Martonosi, 1983*a*), positive potential presumably favors the E_2 conformation, while negative potential would have the opposite effect.

Although active Ca^{2+} transport generates an inside positive potential (Beeler, 1980; Beeler et al., 1981), and a transient negative potential may arise during activated Ca^{2+} release (Beeler et al., 1981; Vergara & Bezanilla, 1981), there is no indication so far that these processes would be accompanied by changes in the monomer-polymer equilibrium of the Ca^{2+}-ATPase.

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