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Decavanadate Is Responsible for Vanadate-Induced Two-Dimensional Crystals in Sarcoplasmic Reticulum[1,2]

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Abstract

Two-dimensional protein crystals of the calcium pump protein of sarcoplasmic reticulum (SR) from fast skeletal muscle were induced using Na_3VO_3 as first described by Dux and Martonosi. These crystals exhibit repeat rows ~11 nm apart which contain discrete units with ~7 nm repeats. Four different methods of sample preparation for electron microscopy, i.e., negative staining, freeze-drying, freeze-fracturing, and thin-sectioning electron microscopy, each give complimentary repeat units. The SR-membrane crystals exhibit surface structure by the freeze-drying technique and row-like structures on the normally smooth outer face of normal SR. The formation of the membrane crystals is dependent on the pH and concentration of the vanadate. Only conditions favoring the presence of decavanadate yield crystals. At low concentrations and neutral pH, decavanadate is unstable and with time converts to smaller oligomers and the monomer. The presence of membrane crystals was correlated with the life span of the decavanadate. Membrane crystals were obtained in the SR membrane from fast twitch muscle from light and heavy SR, referable to longitudinal and terminal cisternae as well as from reconstituted SR. Canine cardiac SR did not crystallize under these conditions.

Key Words: Decavanadate; vanadate; two-dimensional crystals; sarcoplasmic reticulum; calcium pump protein; freeze-fracture; freeze-drying; electron microscopy.

Introduction

The sarcoplasmic reticulum membrane (SR) from fast skeletal muscle is a highly specialized membrane that consists mainly (~90%) of calcium pump

[1]Dedicated to the memory of Prof. David E. Green, friend, mentor, and colleague.

[2]Abbreviations: Tris, (tris[hydroxymethyl])aminomethane; TES, (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 2-(2-hydroxy-1-bis[hydroxymethyl]ethyl)aminoethanesulfonic acid; SR, sarcoplasmic reticulum; CPP, calcium pump protein.

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protein (CPP), also referred to as the Ca-ATPase (Tada *et al.*, 1978; Meissner *et al.*, 1973). Freeze-fracture studies of normal and reconstituted SR reveal that the content of CPP is proportional to the intramembrane particles observed in the P face by freeze-fracture (Saito *et al.*, 1978; Wang *et al.*, 1979). The CPP is transmembrane with a major portion extending out from the cytoplasmic surface (Blasie *et al.*, 1982; Saito *et al.*, 1978). It is unidirectionally oriented in normal SR, but bidirectional after reconstitution (Wang *et al.*, 1979). The CPP was found to be a dimer in the membrane in three states of the pumping cycle (Hymel *et al.*, 1984). Two-dimensional crystals of the CPP in the SR membrane from skeletal muscle have been obtained by incubation with vanadate (Dux and Martonosi, 1983). The present study describes conditions which favor its formation and characterizes the two-dimensional crystals by electron microscopy using four different methods of sample preparation.

Materials and Methods

Sodium vanadate was obtained from Fisher, and electrophoresis reagents were from Bio-Rad. Deoxycholic acid (sodium salt) was obtained from Sigma (St. Louis, Missouri) and twice recrystallized before use (Meissner *et al.*, 1973). All other reagents were of analytical grade.

The subcellular fractions from fast skeletal muscle were prepared from New Zealand white rabbits. These included SR isolated as described by Meissner *et al.* (1973), and heavy and light SR isolated according to Meissner (1975). The terminal cisternae fraction, isolated according to Saito *et al.* (1984), was a gift from Dr. Alice Chu. Each of these fractions were stored in the frozen state in storage medium containing 0.3 M sucrose, 0.1 M KCl, 10 mM HEPES, pH 7.4, with the exception of terminal cisternae; in this case, KCl was omitted from the storage medium. Plasma membranes, prepared according to Seiler and Fleischer (1982), were provided by Dr. Brian Costello. The triad fraction (pyrophosphate variant), prepared according to Mitchell *et al.* (1983), was a gift of Dr. Pompeo Volpe. Purified cardiac SR, isolated from dog heart (Chamberlain *et al.*, 1983a), was a gift from Dr. Brian Chamberlain.

Reconstituted skeletal SR was carried out according to Meissner *et al.* (1974) with different lipid-to-protein ratios (Wang *et al.*, 1979). Enrichment of the CPP was achieved by incubating SR membranes (6 mg/ml protein) in 1 or 2 mg deoxycholate/mg protein at 0°C for 10 min, followed by centrifugation for 75 min at 40,000 rpm in a 75 Ti rotor (Beckman, Spinco Division, Palo Alto, California). The supernatant was discarded and the pellet was resuspended in storage buffer. SR sheets were prepared by incubating purified SR

membranes (4.8 mg protein/ml) with 2.3 mg deoxycholate/mg protein at 0°C. "Giant" SR vesicles were prepared by lyophilizing normal SR in 10 mM TES (pH 7.3) in the absence of sucrose and resuspending to a protein concentration of 10 mg/ml in 10 mM TES, pH 7.3 (Crowe *et al.*, 1983).

Protein was measured according to Lowry *et al.* (1951) using bovine serum albumin as a standard. Phosphorus was determined by the procedure of Chen *et al.* (1956) as described in Rouser and Fleischer (1967).

Formation of Two-Dimensional Crystals

A vanadate stock solution was prepared by dissolving Na_3VO_4 in water to a concentration of 100 mM; the pH was 12. A crystallization medium was prepared containing different concentrations of vanadate and 6.25 mM $MgCl_2$, 125 mM KCl, 12.5 mM imidazole, and 0.63 mM EGTA. The pH was adjusted with HCl to 7.4. Four parts of this medium were diluted with one part of the membrane fraction. When the concentration of Na_3VO_4 exceeded 1–2 mM, a yellowish color developed at pH lower than ~8.5. This yellowish color is indicative of the presence of the decavanadate oligomers (Pope and Dale, 1968; Rubinson, 1981) which can be correlated with its absorption at around 375–400 nm (see Fig. 4). For crystallization of SR, these solutions were used promptly after pH adjustment, since the vanadate oligomers, especially decavanadate, are unstable (Pope and Dale, 1968), converting to smaller oligovanadates and vanadate monomer and losing its yellow color. Absorption spectra from 200 to 600 nm of solutions containing Na_3VO_4 at different concentrations and conditions were taken using an HP 8450 A UV/VIS spectrophotometer.

Crystallization was carried out in a medium containing 1 mg protein/ml, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, and 10 mM imidazole, pH 7.4. The Na_3VO_4 concentration was varied from 10 μM to 10 mM. The samples were either processed immediately for electron microscopy or kept at 2–6°C or room temperature for varying periods of time. In some experiments, detergents (Triton X-100 and deoxycholate) were included as indicated.

Electron Microscopy and Sample Preparation

For negative staining, the samples were absorbed onto carbon films, pretreated by glow discharge to make them positive, washed twice with distilled water, and subsequently stained with 1% uranyl acetate. For freeze-drying, the samples were absorbed onto mica that was freshly cleaved to obtain a clean and positively charged surface (Nermuth, 1982), washed with distilled water, and quick-frozen in slush nitrogen. Freeze drying was

performed as described by Maurer and Mühlethaler (1981). Freeze-fracture was carried out according to Moor (1969) on glutaraldehyde-fixed (2% v/v) pellets that were suspended in 30% (v/v) glycerol. Thin sections were prepared from pellets fixed with OsO_4 and poststained with uranyl acetate and lead as described in Saito *et al.* (1978). The specimens were examined in a JEOL 100S electron microscope at 80 kV acceleration voltage at magnifications of 32,000 and 51,000. Calibrations were made with a cross grating grid with 2160 lines/mm (Balzers, Nashua, New Hampshire). All prints were made directly from the negatives. For freeze-fracturing and freeze-drying the shadowing direction is indicated with an arrowhead in a white circle.

Results

Incubation of skeletal sarcoplasmic reticulum in 5 mM Na_3VO_4 induces formation of two-dimensional crystalline protein arrays of the calcium pump protein (Fig. 1) (Dux and Martonosi, 1983). This is achieved in a crystallization medium containing Mg^{2+} and phosphate and which is devoid of Ca^{2+} by using EGTA as chelator. Electron micrographs of negatively stained samples show lattice lines that run diagonally (~60°) to the perimeter of the vesicles (see Fig. 1A). The giant vesicles of SR are originally round shaped but become cylindrical in shape with the ordering of the calcium pump protein in the membrane. The vesicles that contain more ordered crystalline arrays have a cylindrical shape with a diameter of 50–80 nm and variable length. Rounder-shaped crystalline arrays were obtained when normal SR was dialyzed against 5 mM Na_3VO_4 in the same crystallization medium. The two-dimensional arrays in these round-shaped vesicles appear to have more distorted lattice order. The center-to-center distance of the repeat units within the row averages ~7 nm apart, and the repeat distance between rows is ~11 nm. The crystal lattice is ordered only over a limited range, i.e., 4 or 5 repeat units.

The surface of sarcoplasmic reticulum was visualized with the freeze-drying technique (Nermuth, 1982). Normal SR, reconstituted SR, and "sheets" of SR obtained by limited detergent treatment exhibit a smooth outer surface (Figs. 2A and 2B). After crystallization, two-dimensional order can be seen at the surface of the vesicles (Fig. 2C). Noncrystalline portions of the vesicle devoid of defined surface structure are also present in the same vesicle containing ordered structures. By freeze-fracture, the particles asymmetrically distributed largely in the P face of normal SR (Deamer and Baskin, 1969; Saito *et al.*, 1978). However, freeze-fracture of the SR ordered into two-dimensional crystals reveals parallel rows on both P and E fracture faces (see Fig. 3A). Repeating rows can be visualized in the E fracture face while the P fracture face contains distinct particles within the rows as well. The

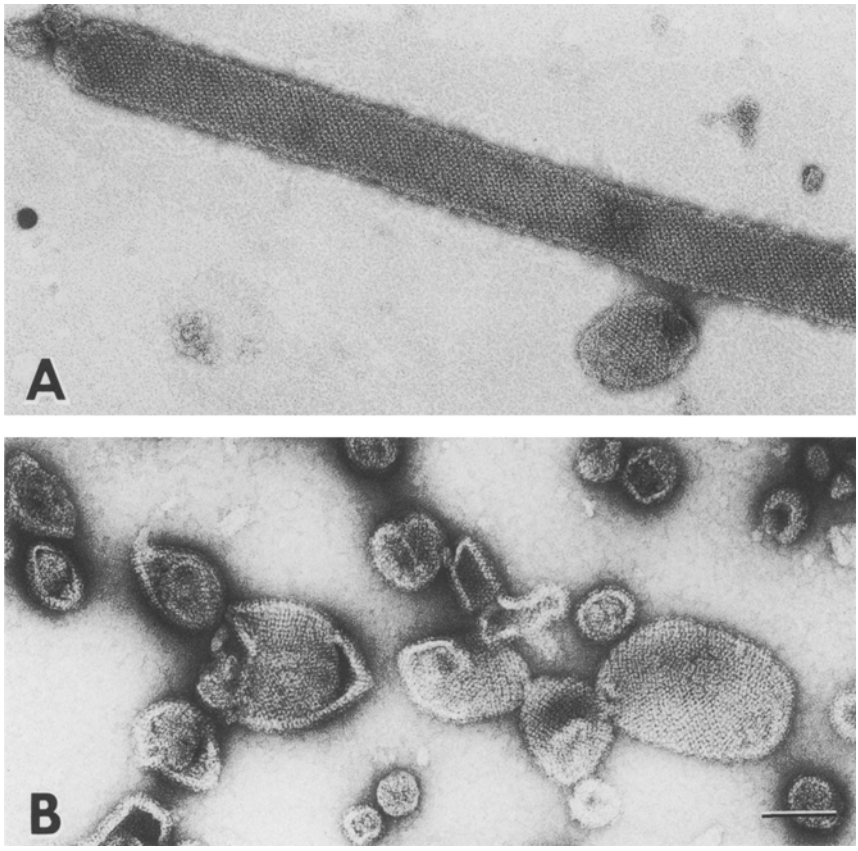
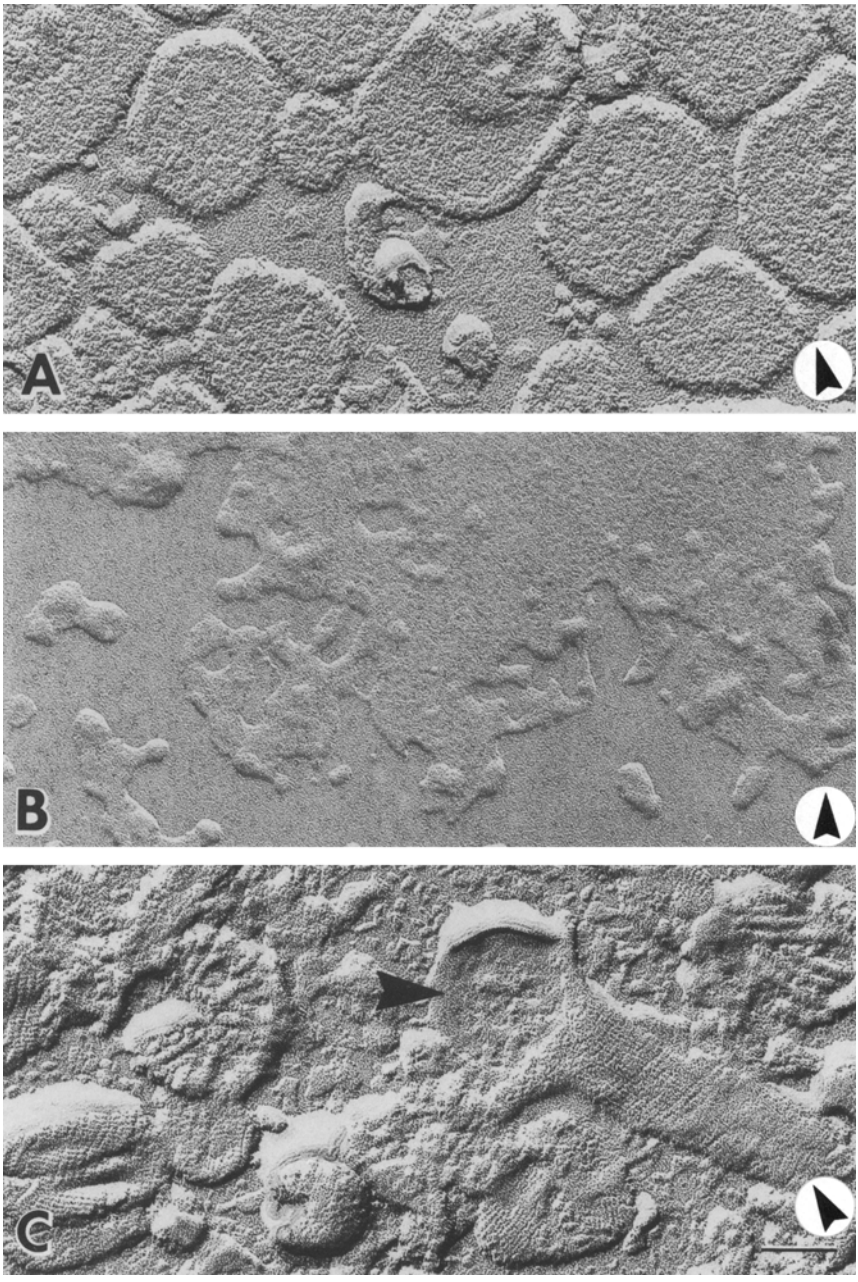


Fig. 1. Two-dimensional membrane crystals of SR induced by vanadate. The giant SR (A) has a cylindrical shape, while normal SR that was dialyzed against 5 mM Na_3VO_4 in the same crystallization buffer (B) shows mainly round vesicles. The samples were negatively stained with 1% (w/v) uranyl acetate. "Giant" SR (prepared according to Crowe *et al.*, 1983) at a protein concentration of 1 mg/ml was admixed in crystallization medium (100 mM KCl, 5 mM $MgCl_2$, and 0.5 mM EGTA) containing 5 mM Na_3VO_4 and 10 mM Imidazole, pH 7.4. The crystals exhibit repeat rows (11 nm) containing units with a center-to-center distance of ~7 nm. The crystals prepared by slower ordering by dialysis were even less ordered. Magnification 100,000 ×. (Bar represents 100 nm.)

row-like repeat distances on both fracture faces is ~11 nm and corresponds to repeat distances on the surface obtained by freeze-drying. Thin sections of samples fixed with glutaraldehyde and osmium tetroxide that were post-stained with lead citrate show cylindrically shaped vesicles containing repeat rows of electron-dense material (see Fig. 3B). Discrete particles cannot be discerned within the rows, although particle-like structures can clearly be observed at the outer edges of the membranes (see arrows).


A
B
C

The conditions which induce formation of two-dimensional crystals were investigated. Skeletal muscle SR was incubated in crystallization medium with different concentrations of freshly neutralized Na_3VO_4, pH 7.4. We found that vanadate at a concentration of 2.5 mM or higher induced crystal formation (Table I), while lower concentrations prepared in the usual manner by dilution of stock Na_3VO_3 (100 mM) into crystallization medium and then neutralization did not. This ability to induce ordered arrays was qualitatively correlated with the yellow color of the medium (Fig. 4) which is obtained with neutralization. The yellow color at low concentration and neutral pH is unstable. For a 5-mM solution, the loss of the yellow color occurs in 2 h at 40°C, overnight at 22°C, or two weeks at 2–6°C. The yellow color can be distinguished spectrophotometrically (Fig. 4) by its absorption at 375–400 nm and is indicative of decavanadate form (Pope and Dale, 1968; Rubinson, 1981). When the stock solution (100 mM) of Na_3VO_3 is first neutralized and then diluted to 1 mM into crystallization medium, some yellow color was observed and crystal formation was obtained. When decavanadate prepared from 5 mM Na_3VO_3 in crystallization medium was converted to smaller oligomers or the monomer by warming (Fig. 4B), this colorless solution no longer induced the two-dimensional crystals. Thus, it would appear that the ability to induce two-dimensional order in SR membranes is correlatable with the decavanadate form.

The time required for the formation of two-dimensional order was also studied. Membrane crystals were observed when negative staining was performed immediately after adding crystallization medium containing decavanadate at 5 mM Na_3VO_4. Membrane crystals were no longer observed when the yellow color was spontaneously lost with prolonged storage time, i.e., when the decavanadate was converted into monomers and smaller vanadate oligomers (not shown). The addition of fresh decavanadate to such samples did not restore the order. Incubation of SR first with monovanadate, followed by decavanadate, prevented the formation of crystals. Incubation of membranes with decavanadate followed by monovanadate did not lead to loss of crystals already formed. Membrane crystals were not obtained in crystallization medium in the absence of vanadate even after 4 weeks storage at 2–6°C,

Fig. 2. Surface structure of freeze-dried SR. Reconstituted SR (A), partially solubilized SR sheets (B), and reconstituted SR-membrane crystals (C) induced with 5 mM Na_3VO_4 in crystallization medium were visualized using the freeze-drying technique. The lipid-to-protein ratio for the reconstituted SR in (A) and (C) is 120:1, while the SR membrane sheet has a lipid-to-protein ratio of 60:1 (B). While noncrystalline reconstituted SR and SR membrane sheets [(A) and (B)] have smooth outer surfaces, the crystalline arrays in (C) can be observed at the surface of the cylindrical vesicles. Repeat rows containing repeat units of 7 × 11 nm can be observed. Note that the noncrystalline portion of the cylinder (C) has a smooth surface (see arrow). The arrowheads in the white circles indicate the shadowing direction. Magnification 100,000 ×. (Bar represents 100 nm.)

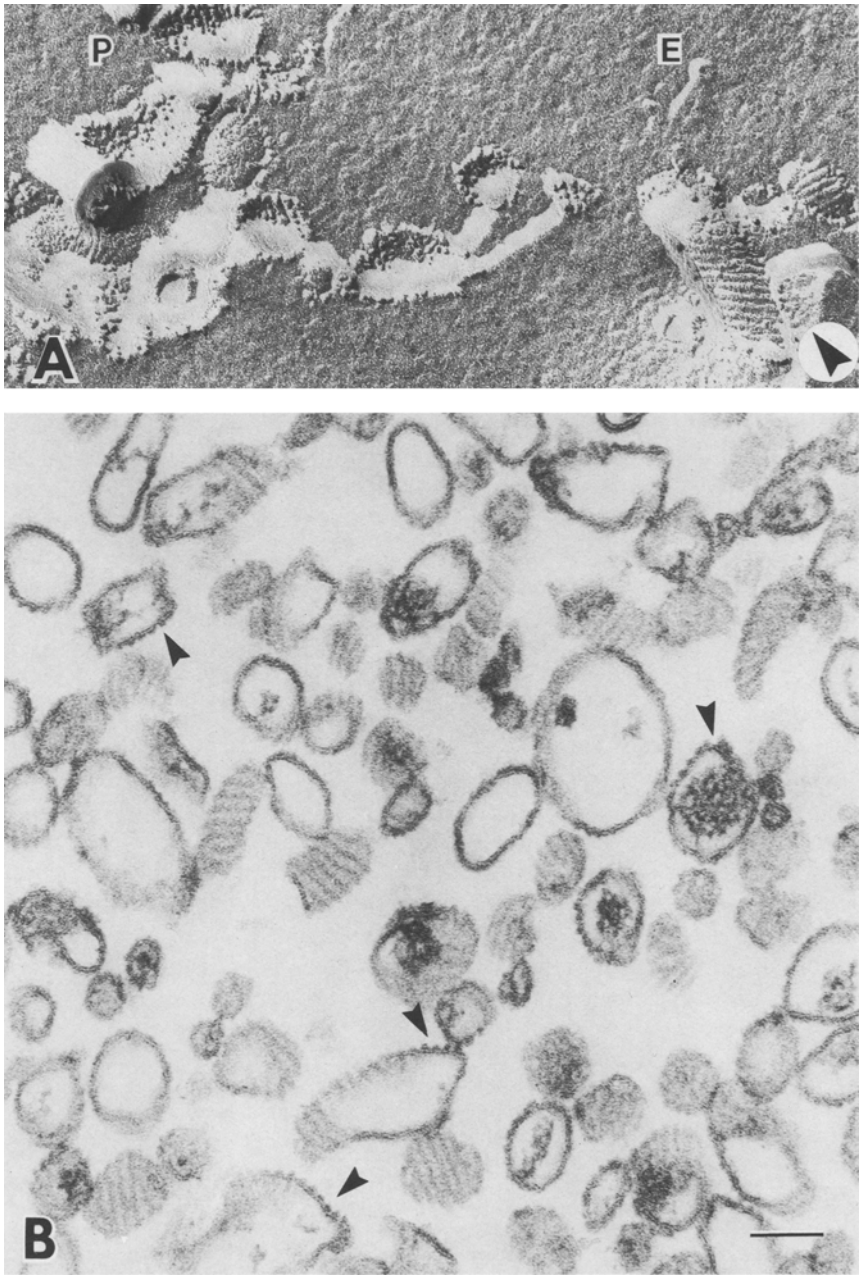

P
E
A
B

Table I. Influence of Vanadate Concentration on Formation of Two-Dimensional Arrays[a]

Vanadate concentration	Presence of crystals[c] in percentage of total vesicles	
	2 h	24 h
10 μM	—	—
100 μM	—	—
500 μM	—	—
1 mM	—	—
1 mM[b]	5–10	10–15
2.5 mM	10–15	10–15
5 mM	50–70	50–70
10 mM	50–70	50–70

[a]The protein concentration for all experiments was 1 mg/ml. Unless otherwise specified, the vanadate solutions were prepared by diluting the vanadate with crystallization medium (100 mM KCl, 10 mM imidazole, 5 mM $MgCl_2$, and 0.5 mM EGTA, pH 7.4) to the concentration indicated and then neutralized.

[b]100 mM Na_3VO_4 was neutralized to pH 7.4 and then diluted to 1 mM final concentration in crystallization medium. The solution was yellow, indicating the presence of decavanadate.

[c]The percentage of vesicles containing any ordered arrays was averaged for several preparations. A range is indicated to indicate the range of scatter of data because the SR vesicles did not spread evenly on carbon films in the absence of sucrose, resulting in some aggregates of vesicles which limited our confidence that we were observing representative samples. The dashes indicates that we did not observe ordered arrays in any of the vesicles.

or in several different buffers, i.e., in a buffer containing 100 mM KCl, 10 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$, or when the E_2P-conformation of the CPP was induced in the absence of vanadate by addition of 10 mM Tris-maleate, 10 mM potassium phosphate, pH 6.2, 10 mM $MgCl_2$, 5 mM EGTA, and 0.5 M sucrose (Inesi *et al.*, 1983), or 100 mM KCl, 5 mM PO_4, pH 7.5, 3 mM $MgCl_2$, and 0.5 mM EGTA.

Different types of membrane fractions from fast skeletal muscle were subjected to treatment in crystallization medium containing 5 mM vanadate. Each of the fractions containing SR formed two-dimensional crystals including light and heavy SR, terminal cisternae, and triads (Table II). Reconstituted SR at different lipid-to-protein molar ratios and normal SR, in the presence of detergents, were also studied, and the results are summarized in

Fig. 3. Freeze-fracture and thin-section electron micrographs of vanadate-induced membrane crystals of normal SR. Crystallization was induced by incubation of the SR in crystallization medium containing decavanadate at 5 mM Na_3VO_4. The membranes were fixed with 2% (v/v final concentration) glutaraldehyde and then prepared for freeze-fracture (A) or thin sections (B). Freeze-fracture replicas reveal rows of discrete particles on the P fracture face, and repeating rows can be discerned on the E fracture face of normal SR. Thin sections of these crystalline arrays show parallel rows of electron-dense material and particles at the edges of the membrane (see arrowheads). The repeat of the rows have the same center-to-center distance (11 nm) as observed with negative staining, freeze-drying, and freeze-fracture. Most of the vesicles exhibit a cylindrical shape. Magnification 100,000 ×. (Bar represents 100 nm.)

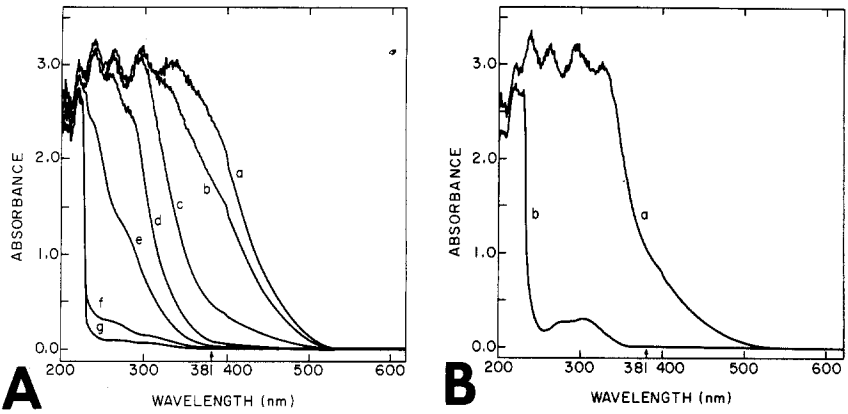


Fig. 4. Absorption spectra of vanadate solutions. The different concentrations of vanadate, 10 μM to 10 mM, are in crystallization medium. The pH was neutralized with concentrated HCl to 7.4. When the vanadate concentration was above 1 mM, the solution turned yellow when neutralized to pH 7.4. The intensity of the color at 375–400 nm (absorption maximum of 381 nm—see Lemerle *et al.*, 1980) is indicative of the decavanadate ion (A). Curve a corresponds to 5 mM, b to 10 mM, c to 2.5 mM, d to 1 mM, e to 500 μM, f to 100 μM, and g to 10 μM Na_3VO_4. The spectra were taken shortly after pH adjustment. In (B), 5 mM Na_3VO_4 was neutralized to form decavanadate (a) (Lemerle *et al.*, 1980) which was then converted to monovanadate (b) and smaller oligomers by warming the sample for 2 h to 40°C at which time the yellow color was no longer detected.

Table III. Reconstituted SR readily forms two-dimensional crystals. However, at low phospholipid-to-protein molar ratio (below 60) or in high detergent concentration (0.1% or higher), the formation of membrane crystals was prevented. The presence of glycerol or sucrose also prevented the formation of membrane crystals. It is noteworthy that canine cardiac SR did

Table II. Decavanadate-Induced Two-dimensional Order[a]

Membrane fraction	Presence of crystals
Skeletal muscle	
Normal SR (NSR)	+
Light SR	+
Heavy SR	+
Terminal cisternae	+
Triads	+
Plasma membrane	—
Dog heart	
SR	—

[a]All membrane fractions were admixed at 1 mg protein/ml in crystallization medium containing 5 mM vanadate, pH 7.4, in the cold. Negative staining was carried out using 1% uranyl acetate after 24, 48, and 96 h. The membrane fractions were prepared as described under Materials and Methods. The preparation was considered positive when ~30% of the observed vesicles were in crystalline form, as viewed by negative staining.

Table III. Factors Influencing Decavanadate-Induced Two-Dimensional Order in SR[a]

Condition	Presence of crystals[c]
Normal SR (NSR) L/P 115[b]	+
Reconstituted SR (RSR) L/P 50:1[b]	—
" " L/P 100:1[b]	+
" " L/P 120:1[b]	+
Normal SR, treated with 0.1% DOCA, then washed without DOCA	+
Normal SR, treated with 0.2% DOCA, then washed without DOCA	+
Normal SR, treated with 0.3% DOCA, then washed without DOCA	—
Normal SR + 0.25% DOCA	—
Normal SR + 0.05% Triton-X-100	+
Normal SR + 0.1% Triton-X-100	—
Normal SR + 0.2% Triton-X-100	—
Normal SR in 20% (w/v) glycerol	—
Normal SR in 0.3 M sucrose	—

[a] Samples at 1 mg protein/ml were placed in crystallization medium containing 100 mM KCl, 10 mM Imidazole, 5 mM $MgCl_2$, 0.5 mM EGTA, and 5 mM vanadate, pH 7.4. To some samples, detergents, sucrose, or glycerol were first added. Treatment with deoxycholate (DOCA) was performed as described in Materials and Methods.

[b] L/P refers to the mole ratio of phospholipid to calcium pump protein.

[c] In this study, the preparation was considered positive when ~30% of the observed vesicles were in crystalline form, as viewed by negative staining.

not form membrane crystals using the same conditions, nor did plasma membrane from skeletal muscle.

Discussion

Concentrations of vanadate (~2 mM and higher) induce the calcium pump protein of skeletal muscle SR to form two-dimensional crystalline arrays (Dux and Martonosi, 1983). The two-dimensional arrays have been observed by four different methods of sample preparations. The structure of the vesicle and the membrane changes dramatically with formation of the two-dimensional arrays. Repeat units can be observed in thin section (Fig. 3B), by freeze-drying (Fig. 2C), by freeze-fracture (Fig. 3A), as well as by negative staining (Fig. 1). Each of the methods shows rows with repeat distances of 11 nm. The outer surface of the vesicle as revealed by freeze-drying is normally smooth (Figs. 2A and 2B). In the ordered membrane, the rows as well as repeat units are clearly observed within the rows. In thin section the repeats units are clearly discerned at the surface of the membrane (Fig. 3B). The E face of normal SR normally appears smooth in freeze-fracture replicas. The ordered crystals exhibit repeat rows on both fracture faces, and unit repeats are observed only in the P face. Thus, each of the methods of sample preparation complement one another, reflecting the ordered arrays in the membrane. The same repeat unit, approximately 7 × 11

nm, is obtained by each of the methods. This value is close to that reported by Taylor *et al.* (1984) using low-dose electron microscopy and image reconstruction. The repeat units represent two calcium pump protein molecules (Taylor *et al.*, 1984; see also Hymel *et al.*, 1984). It is worth noting that scallop SR membranes are oriented *in situ* in similar two-dimensional arrays (Castellani and Hardwicke, 1983).

Vanadate exists in aqueous solutions in different isopolyanion forms depending upon pH and concentration (Pope and Dale, 1968). At pH 4 and higher and concentrations of 1 mM or less, the monomer is the predominant species. At concentrations greater than 1 mM, when the pH is lowered, other oligomeric forms including the dimer, trimer, tetramer, and, finally, decamers are formed. Higher concentration and neutral pH favor the formation of the decamer, called decavanadate. Lowering the vanadate concentrations to less than 1 mM at neutral pH favors the formation of the monomer and smaller oligomers. The decavanadate is not stable at neutral pH and 5 mM concentration and slowly relaxes to the monomer and smaller oligomeric forms (Pope and Dale, 1968). Decavanadate can also be readily distinguished from the other vanadate forms by its yellowish color absorbing at 375–400 nm (Lemerle *et al.*, 1980; Rubinson, 1981). This property has been useful in the correlations in this study (see Fig. 4).

Vanadate is a powerful inhibitor of ATP-driven pumps effective in the micromolar concentration range (Cantley *et al.*, 1978; Pick, 1982; Carafoli *et al.*, 1982; Goffeau *et al.*, 1982). It has been suggested that vanadate binds to a special region of the ATP-binding site of the calcium pump protein of SR (Pick, 1982) or of the Na,K pump (Skriver *et al.*, 1981). Jørgensen and his colleagues found that vanadate can be used to obtain two-dimensional crystals of the enriched Na,K pump membrane from hog kidney. More recently, Dux and Martonosi (1983) have applied the use of vanadate to order the calcium pump of skeletal muscle sarcoplasmic reticulum. In our studies, we correlate the ability to induce two-dimensional order in skeletal muscle SR with the yellow color which is indicative of the decavanadate polyanion. Decavanadate is clearly not the form which is inhibitory to ATP-driven pumps in the micromolar concentration range (Rubinson, 1981).

The decavanadate form is not stable even in the 5-mM concentration range. It is transiently formed by lowering the pH to 7, and appears to be limited kinetically in relaxing back to the monomer or smaller vanadate oligomers in a manner that is time and temperature dependent. This kinetic barrier would also explain why, on occasion, a higher absorption, indicative of decavanadate, is obtained using a lower concentration of sodium vanadate (cf. Fig. 4B; compare 5 and 10 mM).

The calcium pump protein of SR and the Na,K pump react differently to vanadate. The (Na^+, K^+) pump forms crystals very slowly at vanadate

concentrations that favor monovanadate (Hebert *et al.*, 1982). Two-dimensional crystals of the Na,K pump can also be induced by phosphate and Mg^{2+} in the absence of vanadate and appears to form crystals in both the E_1P and E_2P conformations, the latter form with vanadate. On the other hand, the calcium pump protein is oriented rapidly, and it is the decavanadate form which is effective. For the Ca^{2+} pump of SR, it would appear that the E_2P or E_2 vanadate conformations of the pump become ordered. The use of decavanadate is one of several methods to induce the crystallization of adenylate kinase, a soluble enzyme, to yield three-dimensional crystals (Pai *et al.*, 1977).

Vanadate under conditions which are effective in forming two-dimensional crystals in skeletal muscle SR did not induce such order in cardiac SR or skeletal muscle plasma membrane (Table III). Two-dimensional crystals could be obtained with reconstituted membranes of calcium pump protein of different phospholipid-to-protein ratios. However, when the L/P molar ratio was reduced to below 60/1, there was no ordering. Likewise, addition of detergent prevented crystal formation. These two observations, taken together, would suggest that the fluidity and integrity of the membrane must be maintained in order to obtain two-dimensional crystals. Saturation of the vanadate binding of SR sites with monovanadate followed by decavanadate addition did not lead to crystallization. This observation is consistent with a slow off-rate and suggests that both forms of vanadate compete for the same binding site, but only decavanadate is effective in crystal formation. The life span of the membrane crystals correlates with the presence of the yellow color of decavanadate. When the decavanadate is converted back into monomer or smaller oligovanadates, the crystalline arrays can no longer be detected.

When the skeletal muscle SR was treated with trypsin to cleave 90% of the calcium pump protein (Stewart *et al.*, 1976), two-dimensional crystals were obtained. Also, fragmentation of greater than 80% of the Ca^{2+} pump protein by radiation-induced cleavage (Hymel *et al.*, 1984) did not block formation of two-dimensional order.[4]

We could not obtain two-dimensional crystals by incubation of canine cardiac SR under conditions which were successful with skeletal muscle SR. Although the cardiac calcium pump protein is similar to skeletal muscle pump protein in a number of respects, including that it exists as a dimer in the membrane (Hymel *et al.*, 1984, and Chamberlain *et al.*, 1983b), the calcium pump protein in cardiac SR membrane differs in other aspects from the skeletal SR-membrane (Chamberlain *et al.*, 1983). Most notably, it is different immunologically (DeFoor *et al.*, 1980) and its function is modulated by cAMP and Ca^{2+}-calmodulin dependent protein kinases apparently by way

[4]Maurer, A., and Fleischer, S. (1983), unpublished results.

of a regulatory protein, designated phospholamban (Tada *et al.*, 1978). The possible association of cardiac Ca^{2+} pump protein with phospholamban and/or protein kinase might limit ordering. Also, the concentration of the calcium pump protein in heart SR is only about half that in skeletal muscle SR so that the pump molecule in the cardiac SR membrane is surrounded by more "contaminating" proteins which may limit its ability to orient into two-dimensional crystals.

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