Electron Microscopic Evidence for the Transmembrane Displacement of Calcium ATPase

Donald J. Scales * and Stefan R. Highsmith **

* Department of Physiology and ** Department of Biochemistry, University of the Pacific, School of Dentistry, 2155 Webster Street, San Francisco, California 94115

and

* Department of Biological Chemistry, University of Maryland, School of Medicine, Baltimore, Maryland 21201

Z. Naturforsch. **39 c,** 177–179 (1984);
received May 30/September 29, 1983

Sarcoplasmic Reticulum, Ca^{2+}-ATPase, Ion Transport, Vanadate

Incubation of the Ca^{2+}-ATPase in vanadate solutions leads to the formation of two-dimensional arrays in the sarcoplasmic reticulum membrane. Electron micrographic freeze fracture replicas show depressions on the inner leaflet for the first time. This indicates that the ATPase has moved perpendicular to the plane of the membrane. Our results also suggest that aggregation of the Ca^{2+}-ATPase into the two-dimensional arrays occurs before they move into the membrane. These phenomena were observed as soon as 15 minutes after vanadate was added. The effects of vanadate appear to be completely reversible. When SR was incubated in the vanadate solutions and was then diluted into a buffer containing Ca^{2+} and ATP, the ATPase activity was normal for up to several hours of incubation and only somewhat reduced after 3 days.

Introduction

The native sarcoplasmic reticulum (SR) membrane is assembled asymmetrically according to both electron microscopic (Hasselbach and Elfvin-Lars [1]) and X-ray (Dupont *et al.* [2]) studies. The outer, or cytoplasmic, leaflet consists predominantly of protein (Ca^{2+}-ATPase) and the inner leaflet consists predominantly of lipid (Scales and Inesi [3]). Recent studies have shown that in the absence of calcium, vanadate rapidly binds to low affinity phosphate binding sites and stabilizes the Ca^{2+}-ATPase in a vanadate-enzyme structure that appears to be an analog of one of the phosphate-enzyme intermediates (Inesi *et al.* [4]; Dupont and Bennett [5], Pick [6] and Pick and Karlish [7]). More recently, vanadate was shown, after many hours, to induce two-dimensional arrays of the Ca^{2+}-ATPase in negatively-stained SR membranes (Dux and Martonosi [8]).

Reprint requests to Dr. D. Scales

0341-0382/84/0100-0177 $ 01.30/0

Materials and Methods

SR vesicles were prepared as described in Scales and Inesi [3]. These were incubated for either 15 minutes or three hours at room temperature in 10 mM imidazole, pH 7.0, 0.1 mM KCl, 0.5 mM EGTA, 5 mM Na_3VO_4 (Sigma Chemical Company) at a protein concentration of 1 mg/ml. At all times, some vesicles treated with vanadate demonstrated arrays of surface projections when negatively stained with 1% uranyl acetate. At various times samples were also prepared for freeze fracturing as described in Scales and Inese [3]. Electron micrographs were taken with JEOL 100CX or Philips EM200 transmission electron microscopes.

ATPase activities were done under the conditions described in the text. Inorganic phosphate was determined by a phosphomolybdate assay [9]. Typical control activities were 5 μmol P_i mg^{-1} min^{-1} at 37 °C. Chemicals were reagent grade or better. Fresh solutions of Na_3VO_4 (pH 7.0) were made for each experiment.

Results and Discussion

Frozen specimens prepared without vanadate demonstrate randomly distributed intramembranous particles (IMP's) on the outer leaflet (concave fracture faces) and a featureless inner leaflet (convex faces). A typical freeze fracture replica of untreated SR is shown in Figure 1. Figure 2 shows an array that formed after a sample incubated for three hours in vanadate and was then negatively stained. In this example the vesicle is disrupted on the top end and the protein lattice has unraveled onto the carbon support film. Although lacking in long-range order, this lattice of subunits is clearly arranged as pairs of projections. Dux and Martonosi reported similar results [8].

Freeze fracture replicas show alignment of IMP's on many concave faces whether the vesicles are incubated at room temperature in 5 mM Na_3VO_4 for 15 minutes (Fig. 3) or 3 hours (Fig. 4). In addition, a new feature appears on formerly smooth convex faces. Many inner leaflets now show rows of depressions or pits which obviously correspond to the rows of IMP's on the concave faces. These rows of pits are

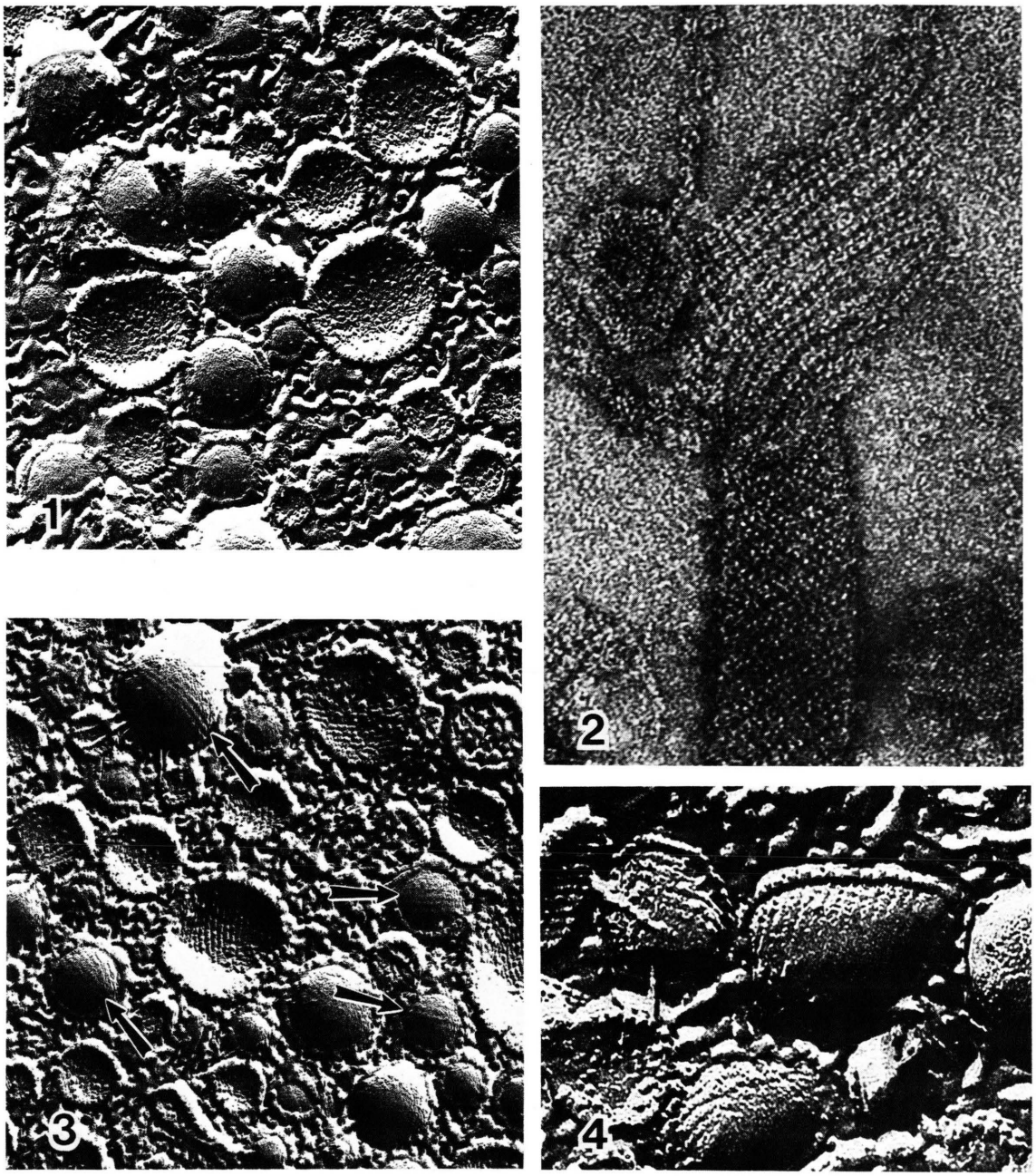

Fig. 1. Freeze fractured vesicles without vanadate: particles are distributed randomly on concave faces, whereas convex faces are smooth. ×83 400.

Fig. 2. SR vesicles incubated for 3 hours in vanadate and negatively stained with uranyl acetate: surface projections are aligned and are arranged as pairs, clearly seen at the top where the vesicle has broken open. ×325 000.

Fig. 3. Freeze fractured vesicles after 15 minutes in vanadate at room temperature: particles are aligned on many concave faces and convex faces show corresponding grooves (arrows), which are sometimes resolved as rows of pits. ×83 400.

Fig. 4. Freeze fractured vesicles after 3 hours in vanadate at room temperature: rows of pits can be seen on the convex, or inner, leaflet. ×181 000.

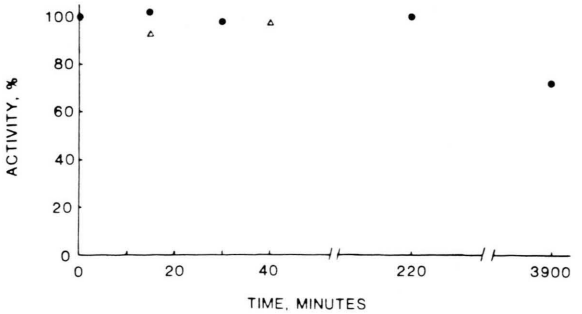

Fig. 5. Vanadate inhibition of ATPase activity: SR vesicles were incubated (at 1 mg/ml) in 5 mM vanadate, 100 mM KCl, 5 mM $MgCl_2$, 0.50 mM EGTA, 10 mM imidazole pH 7.0, at 25 °C for up to 40 minutes and then at 0 °C for up to 65 hours. Aliquots were taken and diluted into 68 mM KCl, 4.3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.83 mM EGTA, 43 mM MOPS (KOH, pH 7.0), 5 mM ATP, 0.01 mg/ml SR, 0.1 μg/ml A23187, 37 °C and P_i production was monitored. In some experiments 0.01 mg/ml A23187 was included in the incubation mixture (△).

never seen when vanadate is omitted, nor are randomly arranged pits seen on convex faces after vanadate treatment. These results suggest that the vanadate-treated ATPase moves in the transmembrane direction far enough to leave a depression in the inner-leaflet fracture face. Since there are no depressions found in a "noncrystalline" state, the vanadate-ATPase complex must move in the transmembrane direction after alignment within the plane of the membrane.

Parallel measurements of ATPase activities indicate that the effects of 5 mM vanadate are reversible. When SR is incubated in 5 mM vanadate solutions and then Ca^{2+}, ATP and A23187 are added to the incubation mixture, the activity is normal after 1 minute of incubation and drops to near zero after 15 minutes. The time course of this loss of activity seems to parallel array formation. If the [vanadate] is only 100 μM, no inhibition is seen after 15 minutes incubation. When aliquots of SR incubating in 5 mM vanadate are taken and diluted into an assay buffer so the [vanadate] is less than 100 μM, and assayed immediately, the activity remains high for up to 3 days of incubation (Fig. 5). These results indicate that the vanadate induced inhibition of ATPase activity is rapidly and completely reversed by dilution into a solution containing Ca^{2+} and ATP, even after times sufficient to have complete formation of the two dimensional arrays.

We conclude that vanadate induces a spatial redistribution of the enzyme perpendicular to as well as in the plane of the membrane. It seems that the redistribution in the plane occurs first. Since our conditions do not lead to irreversible inactivation of the ATPase (within 3 days), our results may be of biological significance since the vanadate-ATPase complex may correspond to one of the phosphoenzyme states that occurs during ATP hydrolysis.

Acknowledgements

We are grateful to Thomas Yasumura for making the freeze fracture replicas and Douglas Barker for excellent technical assistance. This work was supported by National Institutes of Health, Grants 1-PO-1-HL27867, AM25177 and AM00509 and a Grant from the Muscular Dystrophy Association.

[1] W. Hasselbach and G. Elfvin-Lars, J. Ultrastruct. Res. **17,** 598 (1967).
[2] Y. Dupont, S. C. Harrison, and W. Hasselbach, Nature **244,** 555 (1973).
[3] D. Scales and G. Inesi, Biophys. J. **16,** 735 (1976).
[4] G. Inesi, M. Kurzmack, R. Nakamoto, L. de Meis, and S. Bernhard, J. Biol. Chem. **255,** 6040 (1980).
[5] Y. Dupont and N. Bennett, FEBS Lettr. **139,** 237 (1982).
[6] U. Pick, J. Biol. Chem. **257,** 6111 (1982).
[7] U. Pick and S. D. Karlish, J. Biol. Chem. **257,** 6120 (1982).
[8] L. Dux and A. Martonosi, J. Biol. Chem. **258,** 2599 (1983).
[9] A. J. Murphy, J. Biol. Chem. **256,** 12046 (1981).