Crystallization of the Ca2+−ATPase of skeletal muscle sarcoplasmic reticulum Inhibition by myotoxin a

ARTICLE *in* FEBS LETTERS · DECEMBER 1987

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(87)80428-3 · Source: PubMed

3 AUTHORS, INCLUDING:

Anthony T. Tu
Colorado State University
236 PUBLICATIONS **4,200** CITATIONS
SEE PROFILE

Pompeo Volpe
University of Padova
144 PUBLICATIONS **5,792** CITATIONS
SEE PROFILE

Volume 224, number 1, 89–96 FEB 05262 November 1987

Crystallization of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum

Inhibition by myotoxin *a*

Andreas Maurer, Anthony T. Tu* and Pompeo Volpe°

*Department of Microbiology, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland, *Department of Biochemistry, Colorado State University, Fort Collins, CO 80253, USA* and *°Centro di Studio per la Biologia e la Fisiopatologia Muscolare del CNR, Istituto di Patologia Generale dell'Universita di Padova, Via Loredan 16, 35131 Padova Italy*

Received 25 August 1987

Decavanadate produces extensive ordered arrays of Ca^{2+}-ATPase molecules on sarcoplasmic reticulum (SR) vesicle surfaces [(1984) J. Bioenerg. Biomembranes 16, 491–505] and the basic unit of these crystalline structures seems to be a dimer of Ca^{2+}-ATPase [(1983) J. Ultrastruct. Res. 24, 454–464; (1984) J. Mol. Biol. 174, 193–204]. Myotoxin *a*, isolated from the venom of the prairie rattlesnake *Crotalus viridis viridis*, is a muscle-degenerating polypeptide and its primary site of interaction is the SR membrane, where it uncouples Ca^{2+}-translocation from Ca^{2+}-dependent ATP hydrolysis [(1986) Arch. Biochem. Biophys. 246, 90 97]. The effect of myotoxin *a* on decavanadate-induced two-dimensional Ca^{2+}-ATPase crystals of SR membranes has been investigated. The toxin inhibits the formation of two-dimensional SR-membrane crystals and disrupts previously formed crystals in a time- and concentration-dependent manner, which parallels the uncoupling of ATP hydrolysis from Ca^{2+} translocation. Two-dimensional crystalline arrays of the SR membrane have a typical diffraction pattern which, after myotoxin *a* treatment, displays a progressive loss of order. Decavanadate is an uncompetitive inhibitor of the Ca^{2+}-ATPase enzyme-myotoxin *a* complex. The present results suggest that a Ca^{2+}-ATPase dimer is required for coupling Ca^{2+} translocation to Ca^{2+}-dependent ATP hydrolysis.

Enzyme crystallization; Ca^{2+}-ATPase; Myotoxin *a*; Vanadate; Enzyme inhibition

1. INTRODUCTION

The SR of skeletal muscle is an endomembrane system that controls the myoplasmic Ca^{2+} concentration and, thus, regulates contraction and relaxation. Active transport into the SR lumen, i.e. muscle relaxation, is mediated by an ATP-dependent Ca^{2+}-ATPase (Ca^{2+} pump), an intrinsic membrane protein with an M_r of about 115000 [5].

Correspondence (present) address: A. Maurer, F. Hoffmann-La Roche & Co. AG, CH-4002 Basel, Switzerland

Transition between two configurational states of the Ca^{2+}-ATPase (EP_1 and EP_2) is involved in the Ca^{2+} transport cycle [6]. Important yet unsettled issues are whether the Ca^{2+}-ATPase is present in the membrane as an oligomer both in the resting state and during the Ca^{2+}-transport cycle, and whether the oligomeric state changes during such a cycle. A related question is whether the oligomeric state of the Ca^{2+}-ATPase influences the coupling of Ca^{2+} uptake to Ca^{2+}-dependent ATP hydrolysis. Hymel et al. [7] reported that the Ca^{2+}-ATPase is a constant dimer during the catalytic cycle, whereas Dux et al. [8] and Dux and Mar-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

tonosi [9] suggested that the EP_1-EP_2 transition involves a shift in the monomer-oligomer equilibrium of the Ca^{2+}-ATPase. Based on the cDNA-deduced sequence of the Ca^{2+}-ATPase [5] a mechanistic model for Ca^{2+} translocation has also been proposed [10] in which an ATPase monomer seems to be sufficient for Ca^{2+} translocation. However, the degree of Ca^{2+}-binding cooperativity [11,12] would require interaction of four binding domains corresponding to two Ca^{2+}-ATPase molecules and this implies that ATPase dimers permit full reactivity of the sites of each monomer. Therefore, it is not yet clear whether a dimer or a monomer of the Ca^{2+} pump is required for Ca^{2+} translocation.

If the polyanion decavanadate is added to purified SR vesicles, two-dimensional crystals are formed [1]. The Ca^{2+}-ATPase, locked in the EP_2 configuration, is present as a dimer in these membrane crystals [2,3]. Decavanadate induces substantial immobilization or aggregation of the Ca^{2+}-ATPase molecules in native membranes [13] and seems to act as a nonfunctional substrate analogue [14]. In scallop SR two-dimensional membrane crystals are found without the addition of vanadate and the crystalline Ca^{2+}-ATPase is present as a dimer [15,16].

The snake venom myotoxin *a*, a polypeptide of 43 amino acid residues isolated from the prairie rattlesnake *Crotalus viridis viridis* [17] causes muscle degeneration [18]. Microscopic studies have shown that myotoxin *a* binds to the myoplasmic face of the SR [19] and causes SR vacuolization and later degeneration of myofibrils [20]. We have recently shown that myotoxin *a* inhibits Ca^{2+} loading and stimulates Ca^{2+}-dependent ATPase activity of isolated SR vesicles [4]. Since the membrane integrity of the SR vesicles and unidirectional Ca^{2+} efflux are not affected, myotoxin *a* seems to be a true uncoupler of the Ca^{2+} pump. We report here the effects of myotoxin *a* on decavanadate-induced two-dimensional membrane crystals and show that the destruction of the crystals by the toxin is, like the inhibition of Ca^{2+} loading and the stimulation of the Ca^{2+}-dependent ATPase, time- and concentration-dependent. We also provide additional evidence that myotoxin *a* interacts with the Ca^{2+}-ATPase and not with SR phospholipids. The excellent correlation between uncoupling of Ca^{2+} transport [4] and effects on decavanadate-induced two-dimensional membrane crystals leads to the suggestion that Ca^{2+}-ATPase dimers are needed to have a coupled Ca^{2+} transport cycle.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Sodium vanadate was obtained from Aldrich. Deoxycholic acid (sodium salt) was from Sigma (St Louis, MO) and recrystallized twice before use. Pyruvate kinase, phospho*enol*pyruvate, lactic dehydrogenase, phosphatidylcholine, and phosphatidylethanolamine (from egg yolk) were also from Sigma. All other reagents were of analytical grade.

2.2. *Isolation of myotoxin* a

Myotoxin *a* was isolated and purified from the venom of *Crotalus viridis viridis* [18]. Homogeneity of each preparation was established by disc gel electrophoresis and by analytical isotachophoresis.

2.3. *Isolation of SR vesicles*

SR vesicles were isolated and purified from rabbit fast twitch skeletal muscle by differential and sucrose density gradient centrifugation [21]. The fraction referred to as R2 (mainly derived from longitudinal SR) was suspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, and stored at −70°C until used. Protein concentrations were estimated according to Lowry et al. [22]. Phosphorus was determined according to Rouser and Fleischer [23].

2.4. *Biochemical assays*

(Mg^{2+}-Ca^{2+})-ATPase was measured by a spectrophotometric enzyme-coupled assay in the presence of 0.15 mM NADH, 0.5 mM phospho*enol*pyruvate, 5 U pyruvate kinase and 5 U lactate dehydrogenase, as described [4,24]. The Ca^{2+}-dependent ATPase activity is defined as the difference between the total ATPase activity in the presence of 0.2 mM $CaCl_2$ and basal ATPase activity in the presence of 0.2 mM EGTA. Ca^{2+}-dependent ATPase activity was also determined by measuring the inorganic phosphate production [25]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [26].

2.5. *Preparation of SR-limit membranes*

SR vesicles (6 mg protein/ml) were incubated with deoxycholate (1 mg/ml) at 0°C for 10 min and then centrifuged for 25 min in a Beckman airfuge A-100 rotor at 22 lb/inch^2. The pellet (SR-limit membranes) was resuspended in 0.3 M sucrose, 0.1 M KCl, 10 mM K^+-Hepes, pH 7.4 [1]. Ca^{2+}-ATPase represented more than 95% of the total protein as indicated by densitometry of Coomassie blue stained gels.

2.6. *Preparation of liposomes*

Phosphatidylcholine and phosphatidylethanolamine (1:1) were suspended in chloroform. The organic solvent was evaporated under a stream of nitrogen and the lipids were further dried under vacuum. After addition of 20 mM Tris and 1 mM EDTA, pH 8.1, the mixture was vortexed and then sonicated for 10 min. The liposomes were stored under nitrogen at 4°C until used.

2.7. *Preparation of decavanadate-induced two-dimensional crystals of the Ca^{2+}-ATPase and electron microscopy*

Sodium vanadate and sodium decavanadate were prepared as in [1]. Decavanadate-induced crystals were prepared as described in [1,4]. Myotoxin *a* was either added prior to the decavanadate or after the addition of the decavanadate. The specimens were prepared and examined as described in [4]. The negatives were examined in a light-optical diffractometer [27]. The observed diffraction patterns were then recorded on 35 mm film and their positions on the micrographs were marked with a circle.

3. RESULTS AND DISCUSSION

Myotoxin *a* has been used in the present study as a tool to investigate the oligomeric state of the Ca^{2+}-ATPase during Ca^{2+} translocation. The polyanion decavanadate induces the formation of two-dimensional membrane crystals of SR vesicles [1] which are observed by negative stain electron microscopy. Fig.1a shows decavanadate-induced membrane crystals of control vesicles and fig.1b a sample of decavanadate-induced crystals incubated for 60 min at room temperature with myotoxin *a*. The crystals are no longer visible after myotoxin *a* treatment but the vesicles seem to be

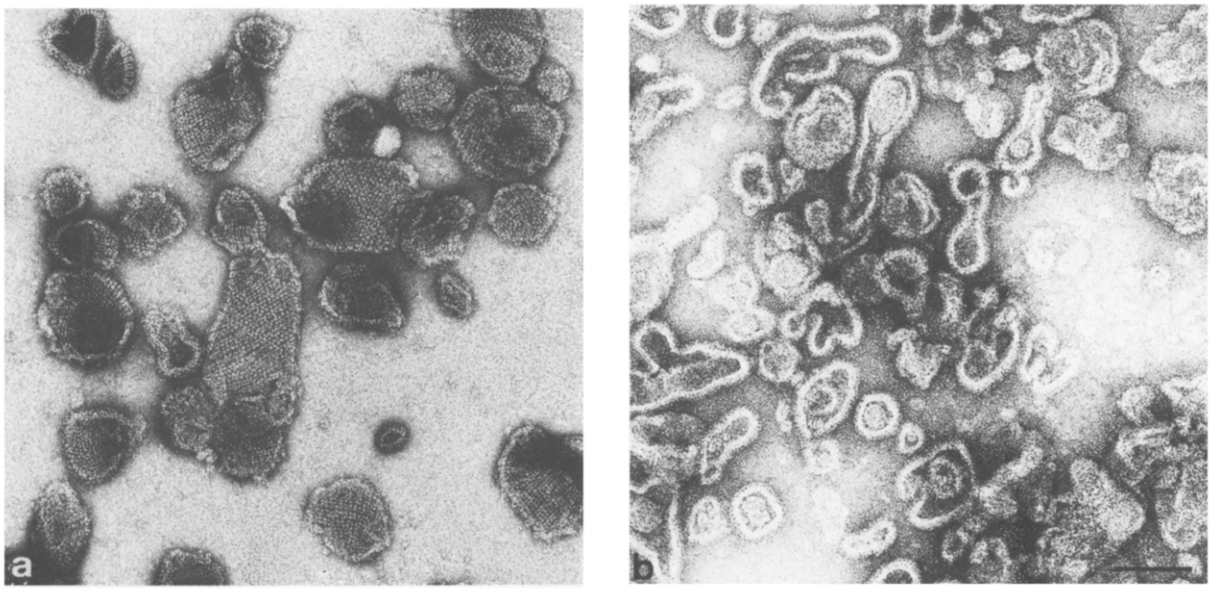


Fig.1. Decavanadate-induced two-dimensional crystals of SR vesicles. For crystallization, SR vesicles (1 mg/ml) were incubated in 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM Na_3VO_4 and 10 mM imidazole, pH 7.4, at room temperature for 60 min. Negative staining electron microscopy was carried out as described in section 2. The control (a) shows large crystalline arrays. (b) The crystals were incubated with 0.8 mg myotoxin *a* per mg of SR protein. Bar indicates 100 nm.

intact (see also [4]). Many SR vesicles are still rod-shaped, a characteristic feature of decavanadate-induced two-dimensional crystals [9].

Table 1 presents the time course of the myotoxin *a* action on SR membrane crystals. With increasing incubation time, the amount of crystals remaining decreases. This decrease is concomitant with the decrease of energized Ca^{2+} uptake by SR vesicles [4]. Almost all crystals are destroyed after 40 min of preincubation. However, short incubation times (less than 10 min) leave most of the crystals intact.

Table 2 shows the concentration dependence of myotoxin *a* effect on two-dimensional SR crystals after 60 min preincubation at room temperature. With increasing toxin concentrations fewer crystals remain intact. The disappearance of the decavanadate-induced crystals parallels the decrease of Ca^{2+} loading by SR vesicles [4]. Many membrane crystals still remain intact at 0.05–0.1 mg myotoxin *a* per mg SR protein. However, higher toxin concentrations lead to rapid crystal destruction and, at 0.6 mg toxin per mg SR protein, most crystals are destroyed. Table 3 shows that SR vesicles preincubated at room temperature for 60 min with myotoxin *a* concentrations of 0.5 mg or 1.0 mg per mg of SR protein, are no longer capable of forming decavanadate-induced two-dimensional crystals. At low myotoxin *a* concentrations, some crystals are still visible.

The quality of the crystals can be assessed by means of diffraction studies performed on an optical bench. Fig.2 shows the crystals and their diffraction patterns after incubation with different concentrations of myotoxin *a*. The control (panel a) exhibits 8 strong first-order reflections. The diffraction pattern consists of spots from the bottom and from the top of the flattened crystal. With increasing myotoxin *a* concentrations, the reflections

Table 1

Time course of myotoxin *a* action on decavanadate-induced two-dimensional SR membrane crystals

Incubation time (min)	Amount of crystalline SR (% of total vesicles)[a]
0 (control)	50–70
5	50–70
10	20–40
20	5–20
40	no crystals visible
60	no crystals visible

[a] The percentage of crystalline vesicles is averaged from counting several vesicles (50–80) for each incubation time of two different SR preparations and 3 crystallization experiments for each SR preparation. Since SR vesicles do not spread very evenly on carbon films in the absence of sucrose, a range is indicated for the percentage of crystalline vesicles. Control values are comparable to those previously reported [1]

SR membrane crystals were obtained as detailed in section 2 and were incubated at room temperature with 0.5 mg myotoxin *a* per mg SR protein

Table 2

Influence of myotoxin *a* concentration upon decavanadate-induced two-dimensional SR membrane crystals

[Myotoxin *a*] (mg per mg SR protein)	Amount of crystalline SR (% of total vesicles)[a]
Control	50–70
0.05	50–70
0.10	35–50
0.20	10–30
0.40	5–20
0.60	<5
0.80	no crystals visible
1.00	no crystals visible

[a] See footnote to table 1

Crystals were incubated at room temperature for 60 min with myotoxin *a*

Table 3

Preincubation of SR vesicles with myotoxin prevents decavanadate-induced SR membrane crystal formation

[Myotoxin *a*] (mg per mg SR protein)	Amount of crystalline SR (% of total vesicles)[a]
Control	50–70
0.10	20–40
0.50	no crystals visible
1.00	no crystals visible

[a] See footnote to table 1

Preincubation was carried out for 60 min at room temperature. Membrane crystals were obtained as detailed in section 2. A single SR preparation was tested

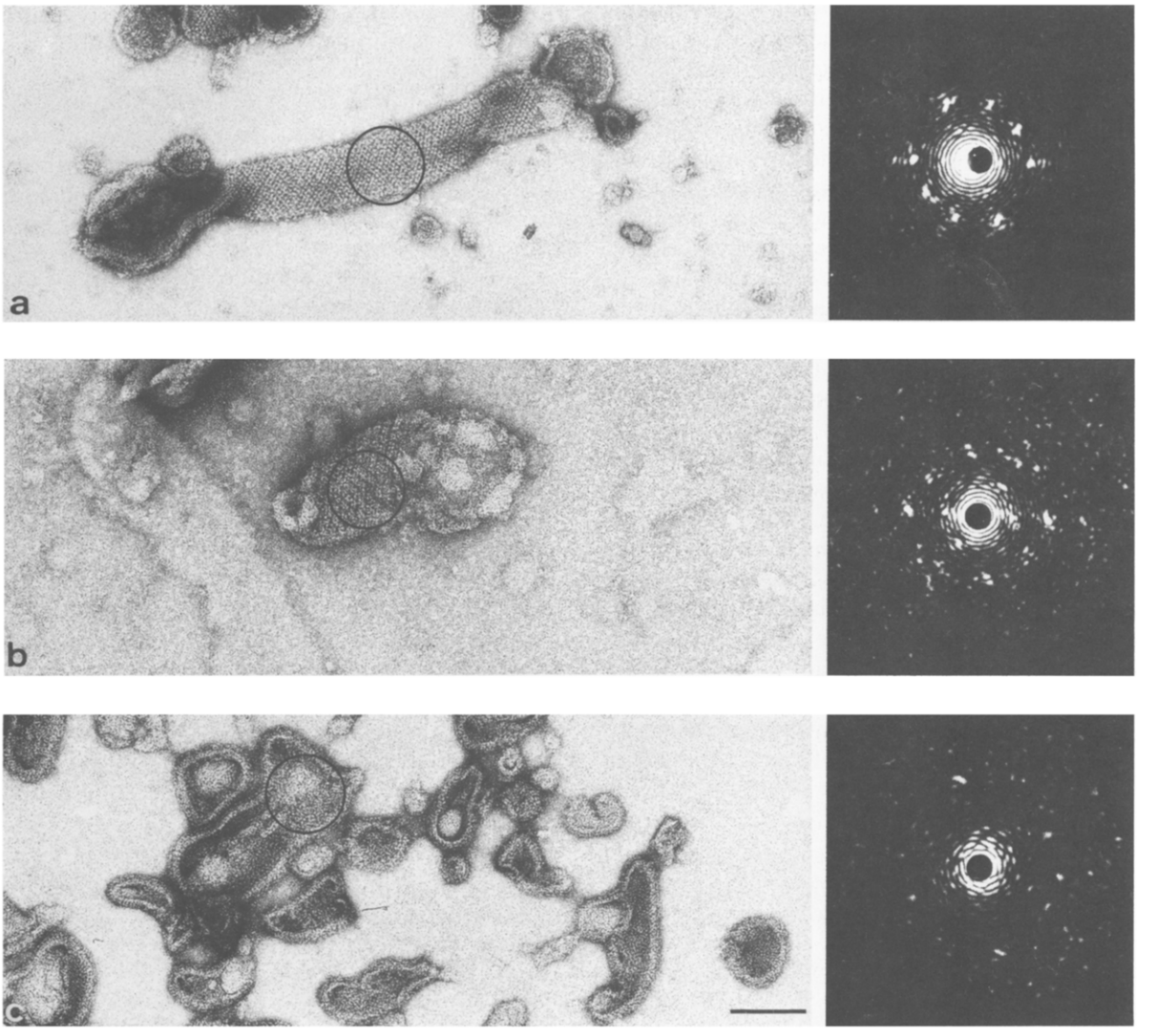


Fig.2. Diffraction patterns of decavanadate-induced two-dimensional SR membrane crystals after treatment with myotoxin *a*. On the left side negative staining electron microscopy of decavanadate-treated SR vesicles in the absence (a) and presence (b,c) of myotoxin *a* (0.4 and 0.8 mg of myotoxin *a* per mg of SR). Crystallization was carried out as described in the legend to fig.1. Optical diffraction patterns obtained from the images of each micrograph in the area enclosed by the circle are shown on the right side. The untreated sample (control, a) exhibits 8 distinct diffraction spots. After treatment with myotoxin *a* the spots become weaker, and start to disappear. Bar indicates 100 nm.

become weaker and finally disappear. In panel b the diffraction pattern no longer exhibits the 4 spots in the middle of the pattern and only shows 4 reflections farther out. The reflections disappear altogether (panel c) at 0.8 mg myotoxin *a* per mg of SR. Since the number and the intensity of the reflections increase with the quality of the crystal, it can be concluded that the order of the crystals decreases with increasing toxin concentrations. The quaternary structure of the Ca^{2+}- ATPase of membrane crystals is a dimer [2,3].

We have shown that binding of specific anti-(SR Ca^{2+}-ATPase) antibodies was partially blocked by myotoxin *a* [4]. In order to assess whether the

Ca^{2+}-ATPase represents the predominant binding site of myotoxin *a*, a 'limit membrane' of the SR was formed by selective extraction with deoxycholate [1]. This membrane is practically devoid of proteins other than the Ca^{2+}-ATPase (see section 2) and the lipid-to-protein ratio decreases from 24 to 18 μg phosphorus/mg protein. This latter value corresponds to 87 mol of phospholipids per mol Ca^{2+}-ATPase compared to 115 mol of phospholipids per mol Ca^{2+}-ATPase for native SR membranes. As shown in fig.3a control limit membranes still formed two-dimensional membranes. The treatment of such crystals with myotoxin *a* leads again to their destruction (fig.3b).

The addition of liposomes (17.3 μg phospholipids per mg protein) to the limit-membrane, i.e. the equivalent amount of phospholipid present in 1 mg of SR limit-membrane, does not inhibit crystal destruction by myotoxin *a* (not shown). The Ca^{2+}-ATPase appears to be the target site of myotoxin *a*. The toxin may induce a conformational change by binding to the polar region of the Ca^{2+} pump [4] and in turn lead to the disruption of the membrane crystals.

Decavanadate either binds with high affinity to the phosphate receptor site of the Ca^{2+}-ATPase [28] or interacts with both the phosphorylation site and the substrate site [14]. Myotoxin *a* might displace decavanadate from its site either by direct competition or by a shift in equilibrium in favor of the EP_1 conformation. In order to address this question, vanadate and decavanadate were added in various concentrations both to SR and to SR preincubated for 60 min at room temperature with myotoxin *a*. Fig.4a shows that vanadate and decavanadate still inhibit the Ca^{2+}-dependent ATPase activity in the presence of myotoxin *a*. A Dixon plot of the same data (fig.4b and c) indicates that different amounts of myotoxin *a* do not change the rate of inhibition by vanadate and decavanadate and that both are uncompetitive inhibitors of the Ca^{2+}-ATPase enzyme-myotoxin *a* complex. (Deca)vanadate and myotoxin *a* do not share the same binding site and crystal disruption is not simply caused by replacement of the (deca)vanadate from its binding site. Other vanadate-sensitive ATPases from insect brush border membranes and from dog kidney outer medulla are not affected by myotoxin *a* (Maurer, A., unpublished) and this indirectly supports our findings that myotoxin *a* does not share binding sites with (deca)vanadate.

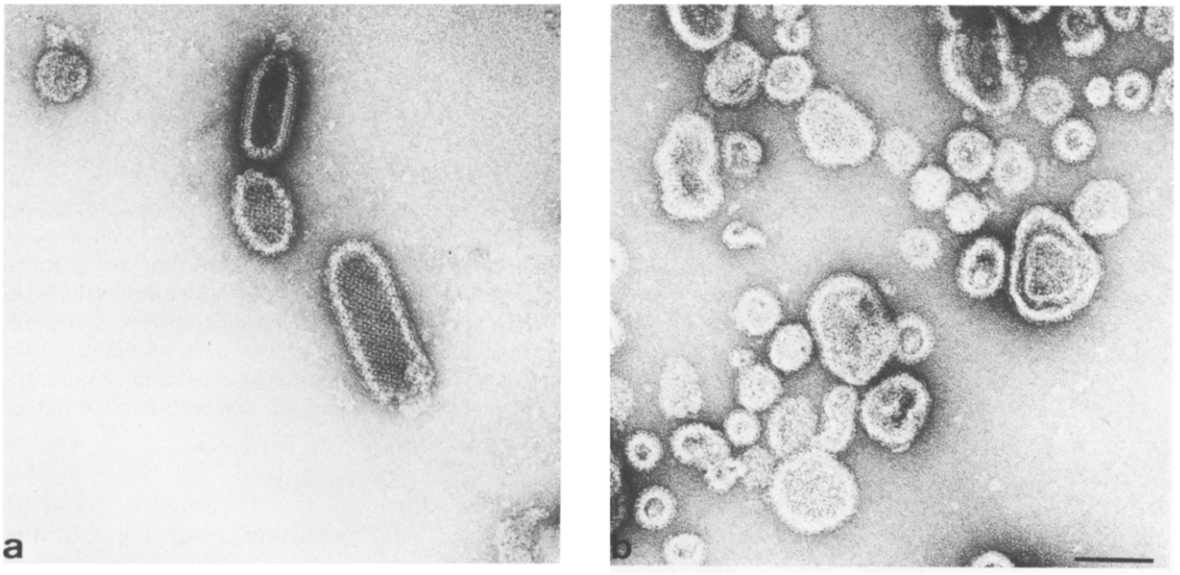
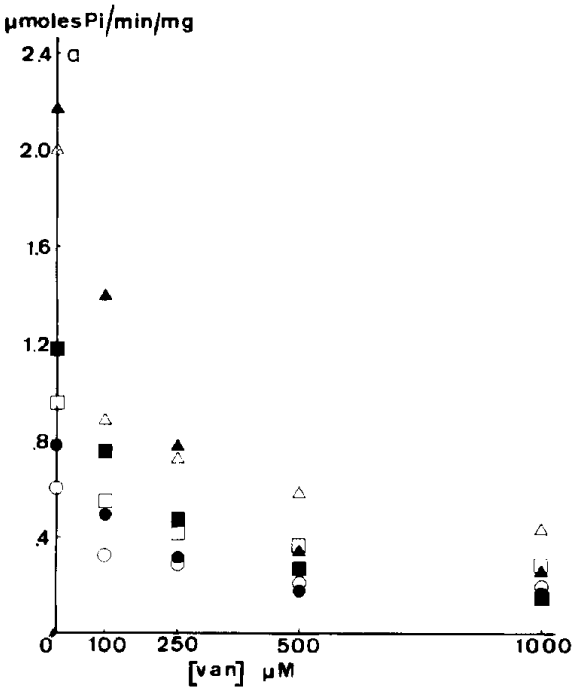
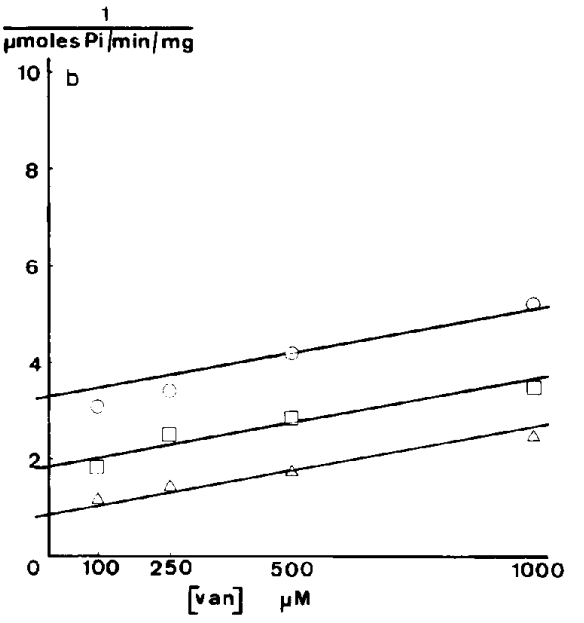


Fig.3. Influence of myotoxin *a* on decavanadate-induced two-dimensional crystals of limit SR membranes. SR membranes were delipidated with deoxycholate to form limit membranes. Negative staining was carried out as described in section 2. 30–45% of the control vesicles (a) showed decavanadate-induced crystals. After treatment with 0.8 mg myotoxin *a* per mg SR at 22°C for 60 min, no more crystals were found (b). Bar indicates 100 nm.





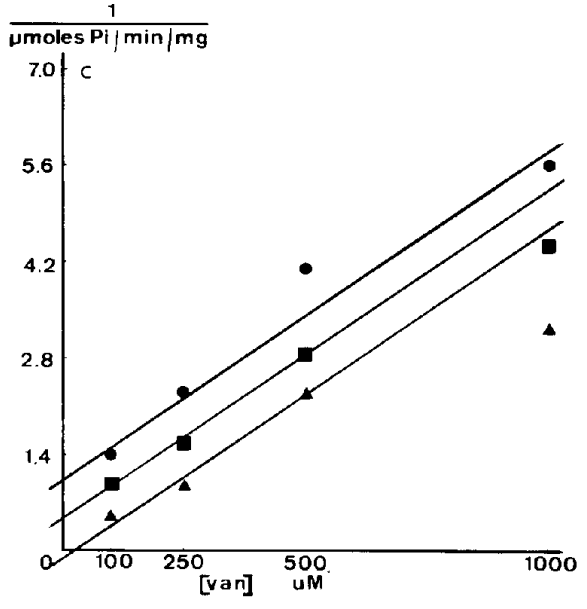


Fig.4. Effect of vanadate and decavanadate on Ca^{2+}-dependent ATPase activity. SR was incubated with increasing amounts of vanadate (open symbols) or decavanadate (closed symbols) and the Ca^{2+}-ATPase activity was determined as described in section 2. (a) The controls are represented by open and closed circles and the myotoxin *a*-treated samples (0.4 mg and 1.0 mg per mg SR) by squares and triangles, respectively. The vesicles were preincubated for 60 min at 22°C with or without myotoxin *a* in 0.3 M sucrose and 5 mM imidazole, pH 7.4. In panels b and c the same data are presented in the form of a Dixon plot. Panel b shows the inhibition with vanadate and panel c shows the inhibition with decavanadate.

In this study we show that the toxin disrupts decavanadate-induced two-dimensional membrane crystals and that the action of myotoxin *a* on these crystals is time- and concentration-dependent as was the inhibition of Ca^{2+}-uptake and the stimulation of Ca^{2+}-dependent ATPase [4]. The crystals remaining after myotoxin *a* treatment exhibit a progressive loss of order that is suggestive of a conformational change of the Ca^{2+}-ATPase. Thus, myotoxin *a* alters the dimeric association of the Ca^{2+}-ATPase molecules.

The uncoupling of the Ca^{2+}-transport cycle and

the disruption of the decavanadate-induced SR crystals by myotoxin *a* might be due to the same mechanism. Assuming that decavanadate-induced crystalline arrays reflect physiological states of the Ca^{2+}-ATPase, a plausible interpretation of the present data is that myotoxin *a* interferes with the dimeric association of the Ca^{2+}-ATPase and causes increase of Ca^{2+}-dependent ATP hydrolysis and inhibition of Ca^{2+}-uptake. Our results imply that a Ca^{2+}-ATPase dimer is needed to have coupled Ca^{2+}-transport cycle. Additional insight into the molecular mechanism of myotoxin *a* action can be obtained by studying structural and configurational changes of the Ca^{2+}-ATPase.

ACKNOWLEDGEMENTS

This work was supported by institutional funds from the Consiglio Nazionale delle Ricerche of Italy (P.V.) and grant no. 5R01GM15591 from the NIH (A.T.T.). The authors thank Dr Brian Costello (Vanderbilt University, Nashville, TN) for critical reading of the manuscript.

REFERENCES

[1] Maurer, A. and Fleischer, S. (1984) J. Bioenerg. Biomembr. 16, 491–505.
[2] Buhle, E.L., Knox, B.E., Serpersu, E. and Aebi, U. (1983) J. Ultrastr. Res. 24, 454–464.
[3] Taylor, K., Dux, L. and Martonosi, A. (1984) J. Mol. Biol. 174, 193–204.
[4] Volpe, P., Damiani, E., Maurer, A. and Tu, A.T. (1986) Arch. Biochem. Biophys. 246, 90–97.
[5] MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Nature 316, 696–700.
[6] Martonosi, A.N. and Beeler, T.J. (1983) in: Handbook of Physiology – Skeletal Muscle (Peachey, L. and Adrian, R.H. eds) pp.417–485, American Physiological Society, Bethesda.
[7] Hymel, L., Maurer, A., Berenski, C., Jung, C.Y. and Fleischer, S. (1984) J. Biol. Chem. 259, 4890–4895.
[8] Dux, L., Taylor, K.A., Ting-Beall, H.P. and Martonosi, A. (1985) J. Biol. Chem. 260, 11730–11743.
[9] Dux, L. and Martonosi, A. (1983) J. Biol. Chem. 258, 2599–2603.
[10] Brandl, C.J., Green, N.M., Korczak, B. and MacLennan, D.H. (1986) Cell 44, 597–607.
[11] Hill, T. and Inesi, G. (1982) Proc. Natl. Acad. Sci. USA 79, 3978–3982.
[12] Verjosky-Almeida, S. and Silva, J.L. (1981) J. Biol. Chem. 256, 2940–2944.
[13] Lewis, S.M. and Thomas, D.D. (1986) Biochemistry 25, 4615–4621.
[14] Coan, C., Scales, D.J. and Murphy, A.T. (1986) J. Biol. Chem. 261, 10394–10403.
[15] Castellani, L. and Hardwicke, P.M.D. (1983) J. Cell Biol. 97, 557–561.
[16] Castellani, L., Hardwicke, P.M.D. and Vibert, P. (1985) J. Mol. Biol. 185, 579–594.
[17] Fox, J.W., Elzinga, M. and Tu, A.T. (1979) Biochemistry 18, 678–684.
[18] Cameron, D.L. and Tu, A.T. (1977) Biochemistry 16, 2546–2553.
[19] Tu, A.T. and Morita, M. (1983) Br. J. Exp. Pathol. 64, 633–637.
[20] Ownby, C.L., Cameron, D.L. and Tu, A.T. (1976) Am. J. Pathol. 85, 149–158.
[21] Saito, A., Seiler, S., Chu, A. and Fleischer, S. (1984) J. Cell Biol. 99, 875–885.
[22] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[23] Rouser, G. and Fleischer, S. (1967) Methods Enzymol. 10, 385–406.
[24] Volpe, P., Damiani, E., Salviati, G. and Margreth, A. (1982) J. Muscle Res. Cell Mot. 3, 213–230.
[25] Baginski, E.S., Foa, P.P. and Zak, B. (1967) Clin. Chim. Acta 15, 155–158.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Klug, A. and Berger, J.E. (1964) J. Mol. Biol. 10, 565–569.
[28] Varga, S., Csermely, P. and Martonosi, A. (1985) Eur. J. Biochem. 148, 119–126.