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High-affinity and low-affinity vanadate binding to sarcoplasmic reticulum Ca^{2+}-ATPase labeled with fluorescein isothiocyanate

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Conditions were found that allowed both the fluorescence detection of vanadate binding to the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum and the vanadate-induced formation of two-dimensional arrays of the enzyme. The fluorescence intensity of fluorescein isothiocyanate-labeled Ca^{2+}-ATPase increased with high-affinity vanadate binding ($K_a = 10^6\ M^{-1}$) as reported by Pick and Karlish (Pick, U. and Karlish, S.D. (1982) J. Biol. Chem. 257, 6120–6126). The Ca^{2+} and Mg^{2+} dependencies for high-affinity vanadate binding were similar but not identical to those for orthophosphate. In addition, it was found that there is low-affinity ($K_a = 380\ M^{-1}$) vanadate binding, which causes a 25% decrease in fluorescence. The Ca^{2+} and Mg^{2+} dependencies of the low-affinity vanadate binding were different from those of orthophosphate or high-affinity vanadate binding. The covalent attachment of fluorescein isothiocyanate (FITC) in the ATP site of the Ca^{2+}-ATPase did not affect the formation of two-dimensional arrays, as detected by negatively stained electron micrographs. Vanadate concentrations high enough to saturate the low-affinity binding caused two-dimensional arrays as reported by Dux and Martonosi (Dux, L. and Martonosi, A. (1983) J. Biol. Chem. 258, 2599–2603). In addition, freeze-fracture replicas of quick-frozen specimens showed rows of indentations in the inner leaflet of the bilayer that corresponds to the arrays seen on the outer leaflet. This appearance of indentations suggests that low-affinity vanadate binding causes a transmembrane movement of the Ca^{2+}-ATPase. By contrast, high-affinity vanadate binding was shown to cause neither array formation nor the appearance of indentations.

Introduction

In muscle sarcoplasmic reticulum, one of the crucial steps in the sequence of events that tranduces the chemical energy of ATP binding and hydrolysis into the osmotic energy of a Ca^{2+} gradient, is the conformational change of the Ca^{2+}-ATPase from a structure with high-affinity Ca^{2+}-binding sites on the outside of the membrane to one with low-affinity Ca^{2+}-binding sites on the inside [1]. During Ca^{2+} pumping, this step is one of many that occur while ATP is hydrolyzed to form a phosphoenzyme complex and the products released. As a result, it is difficult to isolate and investigate this conformational change from high to low-affinity Ca^{2+}-binding sites during Ca^{2+} uptake. In the absence of ATP, equilibrium measure-

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Abbreviations: FITC, fluorescein isothiocyanate; EM, electron micrograph; Mops, 4-morpholinepropanesulfonic acid; SDS, sodium dodecyl sulfate.

ments of Ca^{2+} binding to the high-affinity sites have been made; but, under those conditions, the low-affinity Ca^{2+} binding form is inherently inaccessible in the presence of $[Ca^{2+}]$ above 10^{-6} M. However, observations of Ca^{2+}-ATPase catalysis of oxygen exchange between orthophosphate and water [2] led to the demonstration, that in the absence of ATP and Ca^{2+}, the Ca^{2+}-ATPase could be phosphorylated by orthophosphate directly [3]. It is generally thought that the phosphoenzyme produced is one of the intermediates of the kinetic scheme for Ca^{2+} transport and that is has the empty Ca^{2+}-binding sites in the low-affinity internally oriented form [4]. Mg^{2+} is required for phosphoenzyme formation, and the binding of Mg^{2+} and phosphate to the Ca^{2+}-ATPase has been well-studied. The association constant for P_i is low, about 10^3 M^{-1}, and Ca^{2+} induces phosphoenzyme hydrolysis at 10^{-6} M.

A few years ago, a useful phosphoenzyme analog was developed. It was shown by Pick and his co-workers that the fluorescence intensity of vesicular Ca^{2+}-ATPase, specifically labeled in the ATP-binding site with fluorescein isothiocyanate (FITC), was sensitive to ligand binding [5]. This modified enzyme is useful because the fluorescence changes are due to conformational changes of the Ca^{2+}-ATPase specifically. In the absence of Ca^{2+}, vanadate binding caused a 10% fluorescence increase that was reversed by Ca^{2+} [6]. The vanadate association constant was estimated to be 10^6 M^{-1} and the dependencies of the vanadoenzyme complex formation on Mg^{2+}, Ca^{2+}, ATP and P_i all indicated the vanadate bound in the ATP-binding site produced on analog of the phosphoenzyme with enhanced stability [6,7].

More recently, vanadate was shown by Dux and Martonosi [8], to have a striking effect on the state of Ca^{2+}-ATPase aggregation in vesicles, as detected by negatively stained electron micrographs. At $5 \cdot 10^{-3}$ M, vanadates induces mass aggregation of the Ca^{2+}-ATPase into regular two-dimensional arrays [8–10]. This vanadoenzyme complex also may be an analog of a phosphoenzyme intermediate during Ca^{2+}-transport. Phosphate can induce similar aggregation [9,11] and freeze-fracture electron micrographs of the arrays of vanadoenzyme suggest that the Ca^{2+}-ATPase is more firmly anchored or even moves into the plane of the bilayer and toward the interior of the vesicles [10]. The latter phenomenon is easy to relate to exposure of the Ca^{2+}-binding sites to the interior of the sarcoplasmic reticulum during Ca^{2+} uptake.

Reported here are results from experiments aimed at correlating the FITC fluorescence detected conformational changes [5–7] and the electron micrograph (EM)-detected formation of two-dimensional arrays [8–11], both of which are induced by vanadate. Our purpose was to determine whether or not the vanadoenzyme analog of the phosphoenzyme causes the two-dimensional arrays. It is clear from the results that in addition to the high-affinity vanadate binding [5,12], there is reversible low-affinity vanadate binding that also can be detected by fluorescence. Labeling with FITC does not affect two-dimensional array formation. EM results indicate that it is vanadate binding to the low-affinity site(s) that induces the arrays of the Ca^{2+}-ATPase.

Experimental methods

Materials. Sarcoplasmic reticulum vesicles were isolated from hind-leg muscle of New Zealand rabbits by the method of Eletr and Inesi [13] and stored in 30% sucrose solution at 0°C for up to 3 days, until use. Vesicles prepared this way appear to have about 80% of the total protein appearing in the $M_r = 1.1 \cdot 10^5$ band as estimated from scanning the absorbance at 565 nm of Coomassie blue-stained polyacrylamide gels that had undergone electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS). The maximum amount of phosphoenzyme that can be made from ATP is 4–5 nmol phosphoenzyme/mg sarcoplasmic reticulum protein [14,26]. Two calcium ions are transported per phosphoenzyme produced under typical assay conditions. Protein concentrations were determined by the biuret method using isolated sarcoplasmic reticulum protein as a standard.

Chemicals were of the highest commercial grade and used without further purification. FITC was from Sigma and vanadate solutions were freshly prepared from Na_3VO_4 (99%).

Activities. ATPase activities typically were measured in 63 mM KCl, 4.2 mM $MgCl_2$, 0.83 mM EGTA, 0.2 μM ionophore A23187, 42 mM (pH

7.0 KOH) at 37°C with 0.01 mg sarcoplasmic reticulum protein/ml. Vanadate inhibition measurements were done using assay conditions given in the text. Phosphate production was measured by a phosphomolybdate method [15]. Free Ca^{2+} concentrations were calculated using an effective association constant for Ca^{2+} and EGTA of $4 \cdot 10^6$ M^{-1} at 25°C.

Spectroscopy. Absorption and fluorescence were measured using a Mac-Pherson Model EU-700 spectrophotometer and a Perkin-Elmer Model MPF-44B fluorospectrophotometer, respectively. Inner-filter effects of vanadate were eliminated using the expression:

$$F_{corr} = F_{obs} \cdot 10^{-(\epsilon_{em}+\epsilon_{ex})0.5cl}$$

where F_{corr} and F_{obs} are the correlated and observed fluorescence intensities, respectively, and ϵ_{em}, ϵ_{ex}, c and l are the extinction coefficients at the emission and excitation wavelengths, the concentration and the cuvette pathlength, respectively. Only corrections for vanadate absorbance were necessary. Control measurements of FITC fluorescence (no protein) as a function of [vanadate] matched the values predicted by the above equation with better than 1% accuracy for [vanadate] up to 20 mM.

Negative Staining. Droplets of each experimental suspension were placed directly on carbon-coated Formvar grids. None of the solutions were diluted and the protein concentrations were 0.8–1.0 mg/ml. These were negatively stained with 1% uranyl acetate and viewed at 80 kV with a Philips EM200 electron microscope [16].

Quick Freezing. Some samples were prepared by a rapid-freezing technique using the Gentleman Jim Quick Freezing Device (Quick Freezing Devices, Baltimore, MD). Sarcoplasmic reticulum vesicles were incubated for 15 min at room temperature in 10 mM imidazole (pH 7.0), 100 mM KCl, 0.5 mM EGTA and 5 mM Na_3VO_4 at a protein concentration of 1 mg/ml. The suspension was centrifuged at 18500 rpm for 1 h at 4°C. Samples of the pellet were pipetted on to cardboard discs, which were attached with silicone glue to aluminium planchets, designed to fit a Balzers freeze etch cold stage. The pellet samples were dropped onto a liquid-nitrogen-cooled, highly polished pure copper freezing bar [17,18]. No cryoprotectants or chemical fixatives were present. The frozen pellets were transferred to a Balzers 360M, one at a time and scraped at -105°C by carefully lowering the microtome knife. The surfaces were platinum-replicated immediately, cleaned with bleach and examined in a Philips EM200.

Results

High-affinity vanadate binding

Specifically labeled Ca^{2+}-ATPase was prepared freshly the day of an experiment by incubating sarcoplasmic reticulum vesicles and excess FITC for 30 min in 100 mM KCl, 5 mM $MgCl_2$, 10 mM Mops (pH 7.5, KOH) and 100 μM $CaCl_2$ at 25°C, and then removing excess FITC by size-exclusion chromatography as described [33,34]. 5.3 ± 0.4 nmol bound FITC/mg protein caused complete inhibition of Ca^{2+}-ATPase activity, corrected for basal activity. In agreement with others [19,20], all the FITC was on the Ca^{2+}-ATPase, and appeared on the 45 kDa fragment after trypsin treatment when analysed by gel electrophoresis in the presence of 1% SDS. FITC labels the ATP-binding site [5,19,28,34].

In the buffer most often used for the preparation of two-dimensional arrays of Ca^{2+}-ATPase [8], the binding of vanadate to a high-affinity binding site was detected by changes in FITC fluorescence. A typical trace of fluorescence versus time after additions of EGTA, vanadate and Ca^{2+}, in that order, is shown in Fig. 1A for 25 μM vanadate. Upon subsequent addition of 100 μM free Ca^{2+}, the decrease in fluorescence to a value lower than the starting level (endogeneous $Ca^{2+} \approx 1$ μM) was always observed (Fig. 1A). These data are consistent with the original observations using different buffers [5–7]. Adding more Ca^{2+} had no effect. When the [vanadate] was varied between 0 and 100 μM, the fluorescence increment peaked near [vanadate] = 15 μM and then declined. The association constant for vanadate binding to Ca^{2+}-ATPase was estimated to be $1 \cdot 10^6$ M^{-1} from the mid-point the increase (data not shown). A seen in Fig. 1B, Mg^{2+} was required; when vanadate was added after EDTA, a small, fast decrease was observed, instead of an increase. It is clear that the high-affinity vanadate binding re-

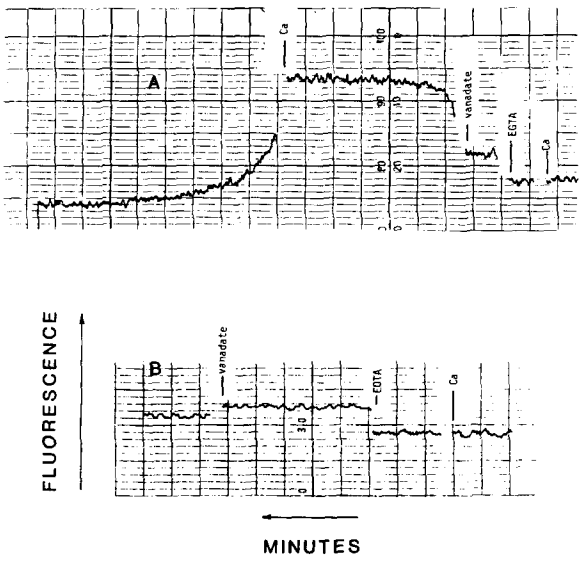


Fig. 1. High-affinity vanadate binding. Suspensions of 0.05 mg/ml FITC-labeled sarcoplasmic reticulum Ca^{2+}-ATPase in 10 mM imidazole (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, with about 1 μM endogenous Ca^{2+}, had 0.5 mM EGTA, 25 μM vanadate and 100 μM $CaCl_2$ added in that order (A). The fluorescence increase (λ_{ex} 490 nm, λ_{em} 520 nm) due to EGTA was too fast to detect (complete in less than 15 s). The increase due to vanadate and the decrease due to Ca^{2+} were completed within several minutes. If Mg^{2+} was not added and endogenous free Mg^{2+} and Ca^{2+} were removed with EDTA, adding vanadate caused a small but reproducible fast decrease instead of an increase (B). In all cases, the fresh stock solution of vanadate was 5 mM.

ported by Pick and Karlish [5], occurs in the buffer used to produce two-dimensional arrays of the Ca^{2+}-ATPase [8]. The dependence of the high-affinity vanadate binding on Mg^{2+} and Ca^{2+} is analogous to that of the Ca^{2+}-ATPase and orthophosphate.

Ca^{2+} antagonism to high-affinity vanadate binding

Having Ca^{2+} present in the solution alters the reaction of vanadate with the high-affinity binding sites on the Ca^{2+}-ATPase [6,12,19,23,24,36]. Increasing [Ca^{2+}] from 1 nM up to 1 μM showed the reaction rate for 25 μM vanadate by about 2-fold without having much of an effect on the extent of the reaction, as determined by the fluorescence increase (Table I). When [Ca^{2+}] was 10 μM or greater, the slow fluorescence increase did not occur; instead, there was a rapid small decrease. The decrease observed for 25 μM vanadate added

TABLE I

Ca^{2+} EFFECTS ON HIGH-AFFINITY VANADATE BINDING

Suspensions of 0.05 mg/ml of FITC-labeled sarcoplasmic reticulum in 100 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7, HCl), 0.5 mM EGTA and $CaCl_2$ added to give free [Ca^{2+}] as described in the text had 25 μM vanadate added, and the fluorescence at 520 nm was recorded over time.

[Ca^{2+}], free (μM)	Fluorescence increase (%)	Time to 50% (s)
0.001	7	46
0.005	6.5	62
1	6.25	100
11	−2	fast [a]
100	−2	fast

[a] The decreases in fluorescence, observed above 1 μM Ca^{2+}, were complete within 15 s.

to sarcoplasmic reticulum in 100 μM free Ca^{2+} (Table I) was close to the difference in fluorescence between the starting and final conditions in Fig. 1A, where 100 μM free Ca^{2+} was added to sarcoplasmic reticulum in 25 μM vanadate. Dilution or inner-filter effects are not the cause of this decrease in either case. These results suggest that vanadate (at 25 μM) may bind to the Ca^{2+}-ATPase when the high-affinity Ca^{2+}-binding sites are occupied.

The FITC-labeled Ca^{2+}-ATPase in the absence of vanadate appeared to bind Ca^{2+} identically to the native Ca^{2+}-ATPase. Using the FITC fluorescence to monitor Ca^{2+} binding (Fig. 2A), the association constant at 25°C in 100 mM KCl at pH 7.0 was $4 \pm 0.6 \cdot 10^6$ M^{-1} and the Hill coefficient was 1.9 ± 0.2 (plot not shown). These results are in good agreement with those obtained by others [19,21,22,35]. If the Ca^{2+} titration was done with 25 μM vanadate present, for additions of free [Ca^{2+}] less than 1 μM, the curve (Fig. 2B) was indistinguishable from the control. The fluorescence change induced in this low [Ca^{2+}] range was rapid and then stable for at least 10 min. Near 1 μM free Ca^{2+}, a fluorescence decrease similar to the one shown in Fig. 1A, but slower due to the lower free [Ca^{2+}], was triggered. The remainder of the titration curve was similar to the control (Fig. 2A). The slow fluorescence decrease that occurred during several minutes when the free Ca^{2+} reached

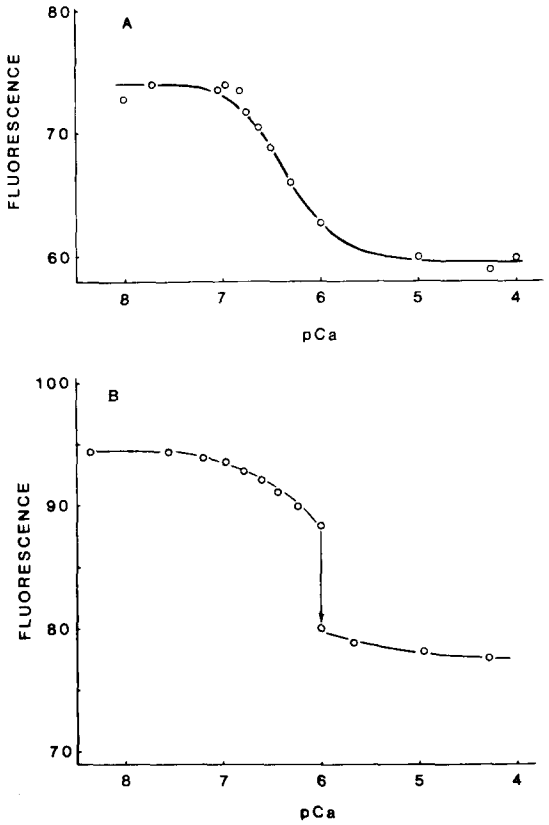


Fig. 2. Ca^{2+} binding to FITC-labeled Ca^{2+}-ATPase with and without vanadate present. Suspensions of 0.05 mg/ml of FITC labeled Ca^{2+}-ATPase in 100 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 0.5 mM EGTA and either 100 μM (A) or no (B) vanadate were titrated with $CaCl_2$ at 25°C. The FITC fluorescence decrements due to Ca^{2+} binding were all fast except the one that appears as a discontinuity in panel B. That decrease was similar to the decrease seen in Fig. 1A when Ca^{2+} was added in excess, but slower.

1 μM is shown as a downward pointing arrow in Fig. 2B. If irradiations were done by adding EGTA to Ca^{2+}-containing solutions, the curve was the same as that in Fig. 2B, except that the transition at the apparent discontinuity was upwards.

It has been suggested that the fluorescence decrease associated with the Ca^{2+}-induced change in vanadate binding occurs when only one Ca^{2+} is bound per vanadoenzyme complex [6]. The data in Fig. 2B are consistent with this hypothesis, in that the slow fluorescence decrease associated with the change in vanadate binding does not occur until after there has been a significant decrease in fluorescence due to Ca^{2+} binding. When the portion of the titration curve before the discontinuity is analyzed, the apparent association constant is $2 \pm 1 \cdot 10^6$ M^{-1} and the apparent Hill coefficient is 1.2 ± 0.4. These numbers are not precise and their calculation required the tacit assumption that the Ca^{2+} binding can be isolated in the curve shown in Fig. 2B. However, they are mentioned because for one Ca^{2+} per Ca^{2+}-ATPase to have an effect, the binding would have to be non-cooperative. Non-cooperative Ca^{2+} binding to sarcoplasmic reticulum has been observed under other conditions [21,22], and the data in fig. 2B suggest it may be occurring when Ca^{2+} binds the vanadoenzyme complex. In any case, the titration curves in Fig. 2 indicate that Ca^{2+} binding to the vanadoenzyme complex is reduced, at most, only a few-fold in comparison for Ca^{2+} binding to the vanadate-free enzyme.

Low-affinity vanadate binding

The 10% increase in FITC-labeled Ca^{2+}-ATPase fluorescence that occurs at low [vanadate] is followed by a 25% decrease when the vanadate concentration is increased to the mM range. The uncorrected fluorescence decrease due to added vanadate is shown in Fig. 3, and the data corrected for inner-filter effects due to the vanadate, (see Experimental methods) are shown in the inset. The pH of vanadate stock solution was 7.0 and the pH of the Ca^{2+}-ATPase solution after the titration was found to change by less than 0.1 unit. The apparent association constant for vanadate-binding, taken as the reciprocal of the added vanadate concentration at 50% of the total decrease, is 380 $\pm$ 47 M^{-1}. Low-affinity vanadate binding is reversible. As shown in Table II, the fluorescence of FITC-labeled sarcoplasmic reticulum in the presence of 5 mM vanadate is one-half that of a control, not containing vanadate. Dilution of both samples to reduce the [vanadate] to 0.25 mM caused the fluorescence intensities to be nearly equal, as expected if the low-affinity vanadate binding were reversible. Subsequent addition of 5 mM vanadate to the diluted sample caused the fluorescence intensity to decrease by 50%.

Unlike vanadate binding to the high-affinity binding site(s), vanadate binding to the presumed low-affinity binding site(s) was not sensitive to Ca^{2+} or Mg^{2+}. Varying the concentrations of free Ca^{2+} and Mg^{2+} between 10^{-8} and 10^{-3} M

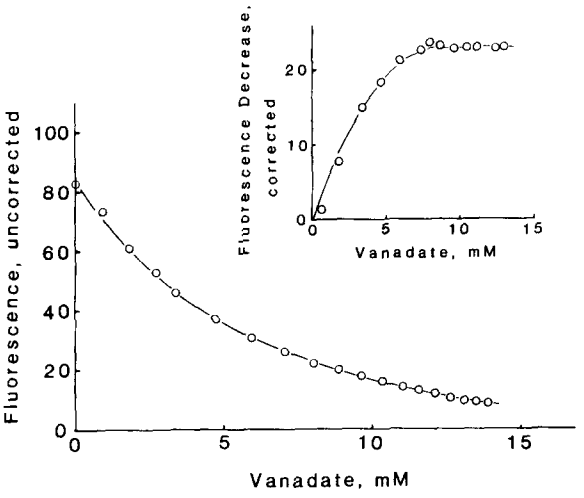


Fig. 3. Low-affinity vanadate binding. Suspensions of 0.05 mg/ml of FITC-labeled sarcoplasmic reticulum in 10 mM imidazole (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$ and 0.5 mM EGTA were titrated with vanadate (stock solution 50 mM) and the FITC fluorescence was monitored. The inset shows the fluorescence decrements corrected for dilution and vanadate inner-filter effects.

changed the 10 mM vanadate-induced uncorrected fluorescence decrease by less than 12% in all cases (data not shown). The fluorescence of free FITC was not effected by [vanadate] up to 10 mM, if the correction for inner-filter effects was made.

Vanadate-induced two-dimensional arrays

Vanadate is known to induce two-dimensional arrays for the Ca^{2+}-ATPase [8,9]. When vanadate is left out of the incubation medium, two-dimensional arrays are never seen in electron micrographs (Fig. 4A). When sarcoplasmic reticulum vesicles are incubated in the buffer used for the fluorescence measurements plus 5 mM vanadate for as few as 15 min at room temperature, centrifuged and then quick-frozen, freeze-fracture replicas show alignment of intramembranous particles on the concave fracture faces, as shown in Fig. 4B. Convex faces show parallel grooves, or depressions, that correspond in spacing and size to the particles on the outer leaflet. A preliminary report has been made [10] and Fig. 4B shows the improved resolution of the indentations obtained by quick-freezing. To the best of our knowledge, this is the first electron micrographic observation of the Ca^{2+}-ATPase being in the inner leaflet under any condition.

The labeling of sarcoplasmic reticulum with FITC did not alter its ability to form two-dimensional arrays or the corresponding rows of indentations in solutions containing high (5 mM) vanadate concentrations, as detected by freeze-fracture replicas or negatively stained electron micrographs. FITC-labeled and native sarcoplasmic reticulum vesicles behaved identically for all the

TABLE II

REVERSIBLE LOW-AFFINITY VANADATE BINDING

Suspensions of 0.05 mg/ml FITC-labeled sarcoplasmic reticulum in 100 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7, HCl), 0.5 mM EGTA and ±5 mM vanadate were irradiated at 490 nm and the fluorescence at 520 was monitored. Each was diluted with buffer (−vanadate) to give ±0.25 mM vanadate, and then 5 mM vanadate was added to one sample.

Perturbation	Fluorescence intensity	
	no vanadate	5 mM vanadate
None	80	40
20-fold dilution	4.1 [b]	4.2 [a]
5 mM vanadate added	–	2.2 [a]

[a] Corrected for increased amplification of signal to give larger scale reading.

TABLE III

VANADATE INHIBITION OF Ca^{2+}-ATPase ACTIVITY

Orthophosphate production was measured at 0.5-min intervals from 0 to 2.5 min after adding 5 mM ATP to 0.01 mg/ml sarcoplasmic reticulum protein at 25°C in 83 mM KCl, 4.2 mM $MgCl_2$, 8.3 mM imidazole (pH 7), 0.5 EGTA plus the ionophore A23187, $CaCl_2$, and Na_3VO_4 as described in the text. All samples were incubated 15 min before Ca^{2+} and ATP were added.

[A23187] (μg/ml)	$[Ca^{2+}]$ [a] (μM)	[Vanadate] (μM)	Activity (μmol P_i/min per mg)
1.0	1	0	6.21
1.0	1	100	0.45
0.1	130	0	3.10
0.1	130	100	3.35
1.0	130	0	3.43
1.0	130	100	0.87

[a] Free concentration using $0.4 \cdot 10^7$ M^{-1} as the association constant for Ca^{2+} and EGTA.

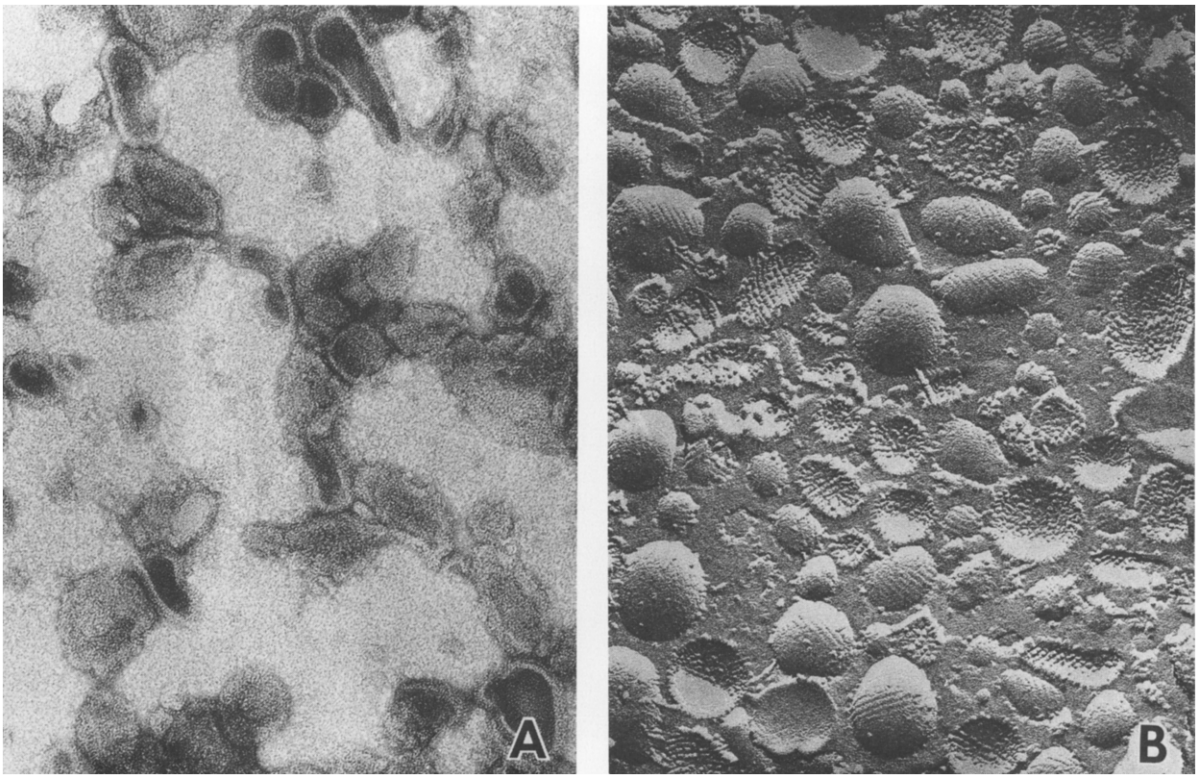


Fig. 4. Ultrastructure. (A) Control: sarcoplasmic reticulum vesicles negatively stained with 1% uranyl acetate×126000. (B) Freeze-fracture replica of quick-frozen sarcoplasmic reticulum that had been exposed to 5 mM vanadate. Particles are aligned on concave faces, and convex faces show corresponding grooves. We believe that the grooves are the result of increased penetration of the vanadoenzyme complex into the membrane×98500.

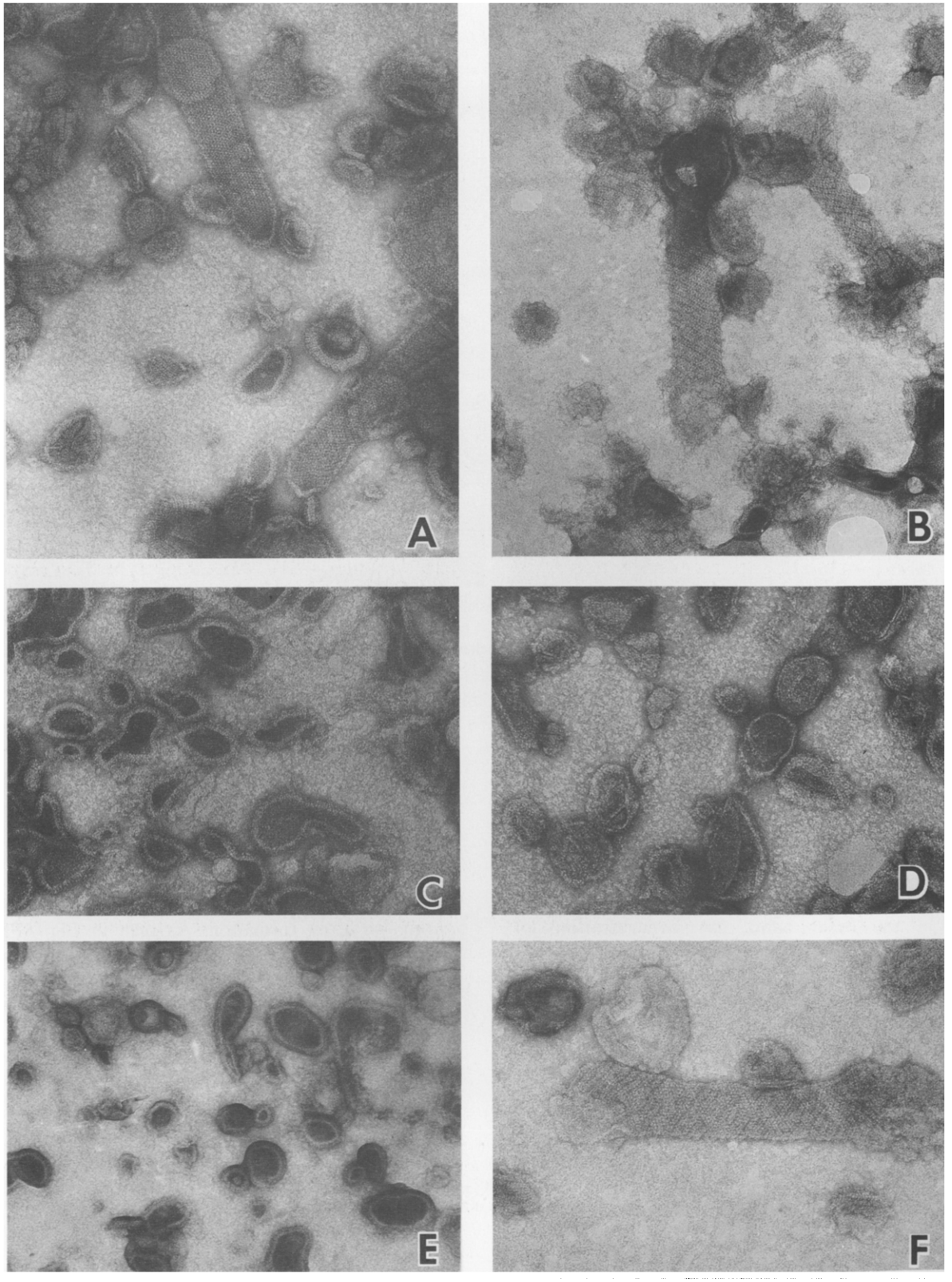
conditions used. Samples of FITC-labeled and unlabeled sarcoplasmic reticulum were incubated in 100 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7, KOH), 0.5 mM EGTA and 5 mM vanadate at 25°C for 18 h. The aggregated Ca^{2+}-ATPase arrays appeared to be identical, as shown in Figs. 5A and B, when negatively stained electron micrographs were made.

On the other hand, low concentrations of vanadate, that would saturate only the high-affinity binding sites, did not induce the formation of two-dimensional arrays of Ca^{2+}-ATPase, even after 1 week (Fig. 5C). Rarely, a vesicle with aggregated Ca^{2+}-ATPase was observed in both the native and FITC-labeled preparations in the presence of 25 μM vanadate; but it corresponded to a very small fraction of the vesicles and the array appeared to be different from those observed at high [vanadate]. These results eliminate high-affinity vanadate binding as the cause of array formation.

The effects of Ca^{2+} and Mg^{2+} on the formation of Ca^{2+}-ATPase arrays in the presence of 5 mM vanadate were investigated. Ca^{2+} greatly reduced the formation of two-dimensional arrays. Shown in Fig. 5D and E are micrographs obtained in the presence of 100 μM and 1 mM added $CaCl_2$, respectively. These results are in agreement with other recently reported data [9]. Mg^{2+} is not required for array formation. As seen in Fig. 5F, when Mg^{2+} is replaced by EDTA, the two-dimensional arrays are present and appear unaffected.

Inhibition of ATPase activity

Vanadate has been shown to inhibit sarcoplasmic reticulum Ca^{2+}-ATPase activity under some conditions. Complete inhibition of activity with 100 μM vanadate in the assay medium is well-documented [8,23,24]. On the other hand, full activity in the presence of 100 μM vanadate was observed when the reversibility of the effects of 5


A
B
C
D
E
F

mM vanadate was demonstrated [10]. This apparent contradiction prompted an investigation of the effects of the assay conditions on vanadate inhibition. It was found that higher concentrations of Ca^{2+} ionophore A23187 and lower concentrations of free Ca^{2+} enhanced vanadate inhibition (Table III, lines 1 and 2). On the other hand, lower [ionophore A23187] and higher [Ca^{2+}] made 100 μM vanadate a rather weak inhibitor (Table III, lines 3 and 4). When both Ca^{2+} and ionophore A23187 concentrations were high, vanadate was an effective inhibitor at 100 μM (Table III, lines 5 and 6). The Ca^{2+} and ionophore A23187 dependencies suggest that vanadate inhibits ATPase activity by binding the high-affinity binding site, which is antagonized by external Ca^{2+} and stabilized by internal Ca^{2+}. The optimal conditions for vanadate inhibition of Ca^{2+}-ATPase activity are high [ionophore A23187] and low external [Ca^{2+}].

Discussion

Solutions of vanadate are heterogenous under most conditions. At concentrations near 10^{-6} M, the major species at pH 7 is orthovanadate [27] and it seems likely that this is the vanadate species involved in binding to the high-affinity binding site, and that 25 μM vanadate saturates the site (Fig. 1). The results shown here for vanadate binding in this site support the conclusion that this is the same site that is phosphorylated by orthophosphate at pH 6 [20]. The Ca^{2+} and Mg^{2+} effects are analogous for phosphate and vanadate (Ref. 3; Fig. 1 and Tables I and III), and P_i and ATP inhibit high-affinity vanadate binding [5,20,34]. The inhibition of ATPase activity is probably due to binding to the same site, since the low-affinity vanadate binding site(s) would be less than 3% filled with 100 μM vanadate present.

With regard to Ca^{2+} binding, the vanadoenzyme differs from the phosphoenzyme in some ways. There is anatogonism between vanadate and Ca^{2+} binding, as seen in Table I where Ca^{2+} slows the vanadate reaction. But the strength of Ca^{2+} binding to the vanadoenzyme (Fig. 2) appears to be only slightly reduced, in comparison to the enzyme itself, rather than the 1000-fold reduction associated with the phosphoenzyme [4]. It appears that vanadate binds at the phosphorylation site at which phosphate binds, but has a different effect on the protein conformation and its Ca^{2+}-binding sites. The results in Fig. 2 suggest that Ca^{2+} binding causes the vanadoenzyme to be hydrolyzed rather than stabilizing the vanadate-free conformation.

The negatively stained electron micrographs shown in Figs. 5A and B indicate that labeling of the Ca^{2+}-ATPase with FITC does not affect the formation of arrays in 5 mM vanadate. Results shown in Fig. 5C clearly indicate that vanadate binding in the high-affinity binding site does not induce the aggregation of the Ca^{2+}-ATPase into the two-dimensional arrays first observed by Dux and Martonosi [8], who have recently reported that low [vanadate] induces little aggregation [9].

The higher concentrations of vanadate required to fill the presumed low-affinity binding site necessary for the Ca^{2+}-ATPase array formation (Figs. 3–5) contain oligomeric species of vanadate in addition to the orthovanadate that is prevalent at lower concentrations [27]. This is demonstrated by the large wavelength-dependent changes in the apparent extinction coefficient for Na_3VO_4 solutions at pH 7 when the concentration is varied (not shown). Thus, there is some uncertainty regarding the nature of the vanadate species that binds in the low-affinity binding site(s), and it is possible that the apparent low-affinity binding is due to polymeric vanadate species that appear only at high [vanadate] and bind the high-affinity sites. The conditions for obtaining two-dimensional arrays [8,9] were purposely copied. Nonetheless, the low-affinity vanadate binding does saturate (Fig. 3) and is rapidly reversible (Table II). The role or location of the low-affinity vanadate-binding site is not known.

The concentration dependence of the binding of vanadate to the low-affinity sites (Fig. 3) is similar

Fig. 5. Negatively stained electron micrographs. (A) Vesicles were exposed to 100 mM KCl, 10 mM imidazole (pH 7.0), 0.5 mM EGTA, 5 mM $MgCl_2$ and 5 mM Na_3VO_4 ×110400. (B) Same as (A) but enzyme was labeled with FITC×110400. (C) Same as (A) but 25 μM Na_3VO_4 ×126000. (D) Same as (A) but 0.1 mM $CaCl_2$ added instead of EGTA×124200. (E) Same as (A) but 1 mM $CaCl_2$ added instead of EGTA×126000. (F) Same as (A), but 1 mM EDTA added instead of $MgCl_2$ ×126000.

to that of array formation (Ref. 9 and Fig. 4), suggesting that the two phenomena are related. But the two do not seem to be identical. The fluorescence change is complete in less than 1 min, while the array formation seems to require at least 1 to be complete, and often longer, The conformational change induced by low-affinity vanadate binding and detected by FITC fluorescence (Fig. 3, Table II) appears to be a step that preceeds aggregation.

This assignment of the fluorescence-detected conformational change to a step before array formation is consistent with the difference of the effects of Mg^{2+} and Ca^{2+} on the low-affinite vanadate binding, which is not sensitive to [Mg^{2+}] and [Ca^{2+}], and on the formation of the arrays (Fig. 4) which is sensitive to [Ca^{2+}] but not [Mg^{2+}]. These cation dependencies are different in both cases from that of vanadate binding at the high-affinity binding site, and supports the conclusion that array formation is not due to high-affinity binding.

The electron micrographs of freeze-fracture replicas of rapidly frozen sarcoplasma reticulum specimens, which had no prior treatment, show clearly that vanadate-induced two-dimensional array formation also causes rows of indentations to appear on the inner-leaflet of the sarcoplasmic reticulum bilayer (Fig. 4). Identations can be seen for transmembrane proteins like the gap junction protein [29–31]. However, indentations have never been seen for sarcoplasmic reticulum vesicles that were not treated with vanadate. The most straightforward interpretation of this observation is that vanadate induces the Ca^{2+}-ATPase to move toward the interior of the membrane [10]. A reasonable speculation is that a movement of this type is involved in the ATP-driven Ca^{2+} transport that the Ca^{2+}-ATPase catalyzes.

Conclusions

There are two modes of vanadate binding to the Ca^{2+}-ATPase of sarcoplasmic reticulum. Both can be detected by fluorescence intensity changes of FITC covalently attached to the ATP-binding site, for conditions that also promote the formation of two-dimensional arrays of the Ca^{2+}-ATPase. The high-affinity binding mode of vanadate [5–7,12,20] is analogous to orthophosphate binding but clearly does not cause the formation of two-dimensional arrays. It also appears that the vanadoenzyme can bind Ca^{2+} at high-affinity sites, unlike the phosphoenzyme.

The newly observed low-affinity mode of vanadate binding is reversible with regard to fluorescence changes and inhibition of ATPase activity. The [vanadate] required to saturate this site is close to that required to obtain two-dimensional arrays, although the divalent cation dependencies are different. Currently, efforts are being made in other laboratories to determine the structure of the crystallized Ca^{2+}-ATPase. The tacit assumption has been that vanadate is acting like orthophosphate during Ca^{2+} transport. We have shown that that is not true, and that an additional mode of vanadate binding exists and seems to be the actual cause of array formation.

It appears that low-affinity vanadate binding induces a conformational change that is detectable by fluorescence whether or not Ca^{2+} is bound. Subsequence array formation occurs only if Ca^{2+} is removed.

The vanadoenzyme complex induced by low-affinity vanadate binding is a new species which may or may not be an analog of an intermediate on the kinetic pathway. The freeze-fracture results suggest this new phosphoenzyme analog may be moved in a transmembrane inward direction compared to the Ca^{2+}-ATPase which is not phosphorylated.

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