The effects of membrane potential and lanthanides on the conformation of the Ca^{2+} -transport ATPase in sarcoplasmic reticulum

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The effects of Ca^{2+} , lanthanide ions (Gd^{3+} , La^{3+} and Pr^{3+}) and membrane potential on the fluorescence of tryptophan and covalently bound fluorescein were analysed in native and fluorescein isothiocyanate (FITC)-labelled sarcoplasmic reticulum vesicles. The binding of Ca^{2+} and lanthanides to the Ca^{2+} -ATPase increases the fluorescence intensity of tryptophan and decreases the fluorescence intensity of FITC; the dependence of these effects on cation concentration is consistent with the involvement of the high-affinity Ca^{2+} -binding sites of the Ca^{2+} -ATPase in the cation-induced fluorescence changes. The fluorescence of FITC-labelled sarcoplasmic reticulum vesicles is also influenced by membrane potential changes induced by ion substitution. Inside positive potential increases, while inside negative potential decreases, the fluorescence of bound FITC. Smaller potential-dependent changes in tryptophan fluorescence were also observed. The effects of Ca^{2+} , lanthanides and membrane potential on the fluorescence of tryptophan and FITC are discussed in terms of the two major conformations of the Ca^{2+} -ATPase (E_1 and E_2), that are assumed to alternate during Ca^{2+} transport. The observations support the suggestion [Dux, Taylor, Ting-Beall & Martonosi (1985) *J. Biol. Chem.* **260**, 11730–11743] that the vanadate-induced crystals of Ca^{2+} -ATPase represent the E_2 , while the Ca^{2+} and lanthanide-induced crystals the E_1 , conformation of the enzyme.

INTRODUCTION

Vanadate and orthophosphate ions induce the crystallization of Ca^{2+} -ATPase in sarcoplasmic reticulum vesicles (Dux & Martonosi, 1983a–e, 1984; Taylor *et al.*, 1984a,b; Beeler *et al.*, 1984; Peracchia *et al.*, 1984). Vanadate is presumed to act by stabilizing the E_2 conformation of Ca^{2+} -ATPase as an analogue of inorganic orthophosphate (Dux & Martonosi, 1983a–c; Pick, 1982; Pick & Karlsh, 1982).

A different crystal form of the Ca^{2+} -ATPase develops in sarcoplasmic reticulum vesicles exposed to lanthanide ions or to Ca^{2+} (Dux *et al.*, 1984, 1985; Martonosi *et al.*, 1984). Lanthanides and Ca^{2+} probably promote the crystallization by interacting with the high-affinity Ca^{2+} -binding site of the Ca^{2+} -ATPase that is exposed in the E_1 conformation (Grisham, 1982).

If the two crystal forms indeed correspond to the E_2 and E_1 conformations of the enzyme, representing the beginning and the end of the Ca^{2+} -transport cycle, the difference between the two structures may give information about the physical basis of Ca^{2+} translocation. Presently both conformational assignments are tentative as they are based largely on kinetic (Martonosi & Beeler, 1983) rather than on structural evidence. Therefore we decided to analyse the effects of lanthanides and membrane potential on the conformation of the Ca^{2+} -ATPase by using the fluorescence of protein tryptophan (Dupont, 1976, 1978; Dupont & Leigh, 1978; Dupont *et al.*, 1982; Guillain *et al.*, 1980; Guillain &

Boyer, 1982) and of covalently bound FITC (Pick & Karlsh, 1980, 1982; Andersen *et al.*, 1982) as conformational indicators. Ca^{2+} increases the fluorescence of tryptophan (Dupont, 1976) and decreases the fluorescence of the covalently bound FITC (Pick & Karlsh, 1980). These fluorescence changes are presumed to indicate stabilization of the E_1 conformation of the Ca^{2+} -ATPase due to binding of Ca^{2+} to the high-affinity Ca^{2+} -binding sites (Pick & Karlsh, 1982; Andersen *et al.*, 1982; Dupont *et al.*, 1982).

Membrane potential changes induced by ion-substitution influence the rates of Ca^{2+} -activated ATP hydrolysis and Ca^{2+} transport in isolated sarcoplasmic reticulum vesicles (Zimniak & Racker, 1978; Beeler, 1980; Beeler *et al.*, 1981). The rate of Ca^{2+} transport increases under the influence of inside negative membrane potential; inside positive potential inhibits Ca^{2+} transport. Changes in membrane potential may accompany the release and uptake of calcium by sarcoplasmic reticulum *in vivo*, contributing an important element to the mechanism of excitation–contraction coupling (Beeler *et al.*, 1981; Oetliker, 1982; Martonosi & Beeler, 1983; Martonosi, 1984). The effect of membrane potential upon Ca^{2+} transport presumably involves a potential-dependent change in the conformation of the Ca^{2+} pump that alters the kinetics of a rate-limiting elementary step. So far neither the rate-limiting step nor the potential-dependent conformational state of the enzyme are precisely defined.

Inside positive membrane potential imposed by ion

Abbreviations used: FITC, fluorescein 5'-isothiocyanate; KG, potassium glutamate; CC, choline chloride.

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substitution promoted the formation of Ca^{2+} -ATPase crystals in sarcoplasmic reticulum vesicles exposed to Na_3VO_4 in a Ca^{2+} -free medium; inside negative potential inhibited the crystallization of Ca^{2+} -ATPase and disrupted preformed Ca^{2+} -ATPase crystals (Dux & Martonosi, 1983d; Beeler *et al.*, 1984). Based on kinetic and spectroscopic observations, it is plausible to assume that the vanadate-induced crystals represent the E_2 conformation of the Ca^{2+} -ATPase (Dux & Martonosi, 1983a-d). Therefore positive potential may promote the vanadate-induced crystallization of the Ca^{2+} -ATPase by stabilizing the E_2 conformation. By contrast, the crystallization of Ca^{2+} -ATPase induced by Ca^{2+} and lanthanides is promoted by inside negative potential (Dux *et al.*, 1985), suggesting that negative potential stabilizes the E_1 conformation of the Ca^{2+} -ATPase.

The effects of artificially imposed membrane potential, Ca^{2+} and lanthanide ions on the fluorescence of FITC-labelled sarcoplasmic reticulum vesicles, reported in the present paper, are consistent with this interpretation.

MATERIALS AND METHODS

Materials

The Ludox light-scattering standard (IBD 1019-69, 30.37% SiO_2 , average particle diameter, 15.4 nm) was a gift of E. I. Du Pont Nemours and Co. Industrial Chemicals Department, Wilmington, DE 19898, U.S.A. The sources of lanthanides were as follows: GdCl_3 , Aldrich Chemical Co., Milwaukee, WI 53201, U.S.A.; PrCl_3 , Alfa Products, Danvers, MA 01923, U.S.A. The Chelex chelating resin, FITC, Mops, LaCl_3 , Tris and potassium glutamate were obtained from Sigma Chemical Co., St. Louis, MO 63178, U.S.A. KCl and choline chloride were supplied by Fisher Scientific, Fairlawn, NJ 07410, U.S.A. CaCl_2 was provided by Baker Chemical Co., Phillipsburg, NJ 08865, U.S.A. All reagents were analytical grade.

Preparation of microsomes

Sarcoplasmic reticulum vesicles were isolated from the predominantly white muscles of rabbits according to the procedure of Nakamura *et al.* (1976). The animals were fasted for 24 h before death to reduce contamination of the preparations by glycogen and phosphorylase. The microsomes were used either immediately after preparation or were stored at -70°C in 0.3 M-sucrose/10 mM-Tris/maleate, pH 7.0, at a protein concentration of 25–30 mg/ml. Before use the microsomes were diluted to a protein concentration of 2 mg/ml in 0.1 M- KCl /30 mM-Tris/Mops, pH 7.0. For most experiments the microsomes were treated with Chelex-100 chelating resin (1 g of washed Chelex or 200 mg of dry resin/10 ml of solution) by stirring the suspensions with the chelating particles in a cold room for 10 h. The Chelex particles were removed by brief centrifugation in a clinical centrifuge; the centrifugation of the supernatant was repeated until all Chelex was removed. The purpose of the Chelex treatment was to reduce the contamination of microsomes by Ca^{2+} and other metals (Papp *et al.*, 1985).

Reaction of microsomes with FITC

Microsomes (2 mg of protein/ml) were reacted for 1 h at 25°C with 10 nmol of FITC/mg of protein in a medium of 0.3 M-sucrose/50 mM-Tris/maleate (pH 7.4)/5 mM- MgCl_2 /0.5 mM-EGTA. After centrifugation at 80801 g for 30 min, the microsomes were resuspended in the appropriate buffer solution, to a final protein concentration of 20 mg/ml. All preparations were used within 10 h after labelling with FITC.

The effects of Ca^{2+} and lanthanides were usually tested with microsomes suspended in 0.1 M- KCl /30 mM-Tris/Mops (pH 7.0)/5 mM- MgCl_2 . For the analysis of the effect of membrane potential the microsomes were suspended either in potassium glutamate or in choline chloride medium, as described below.

Generation of membrane potential

Membrane potential was generated in sarcoplasmic reticulum vesicles by ion substitution essentially as described earlier (Beeler *et al.*, 1981, 1984). Native or FITC-labelled microsomes (20–25 mg of protein/ml) were dialysed overnight (12–15 h) against two changes of 500 volumes each of one of the following media.

Potassium glutamate (KG) medium: 150 mM-potassium glutamate, 10 mM-potassium Mops, pH 7.0, containing 5 mM- MgCl_2 . The microsomes equilibrated with KG medium are designated as KG microsomes.

Choline chloride (CC) medium: 150 mM-choline chloride, 10 mM-Tris/Mops, pH 7.0, containing 5 mM- MgCl_2 . The microsomes equilibrated in CC medium are designated as CC microsomes.

The potentials were generated by 190-fold dilution of KG microsomes into CC medium (inside negative potential), or of CC microsomes into KG medium (inside positive potential). The final protein concentration after dilution was 0.1 mg/ml. The fluorescence changes obtained by dilution of KG microsomes into CC medium were corrected within the same experiment series for control data obtained by dilution of CC microsomes into CC medium or of KG microsomes into KG medium. Similarly the fluorescence changes obtained by dilution of CC microsomes into KG medium were corrected for CC into CC and KG into KG controls. Neither controls showed significant changes in fluorescence upon dilution and there was no indication of systematic differences between resting fluorescence intensities measured in either KG or CC media.

Steady state fluorescence measurements

Fluorescence measurements were carried out in an SLM 4800 fluorescence spectrometer at 10°C , except where otherwise stated. The tryptophan fluorescence of sarcoplasmic reticulum was measured by using an excitation wavelength of 295 nm and an emission wavelength of 330 nm, with slit widths of 4 and 16 nm, respectively. The fluorescence of fluorescein in FITC-labelled sarcoplasmic reticulum was measured by using light of 502 nm (2 nm slit width) for excitation and 517.5 nm (4 nm slit width) for emission. A diluted (1:10) Ludox light scattering standard suspension served as reference to correct for changes in the intensity of the light source; the final concentration of colloidal silica after dilution was 3% (w/v). The digital signal corrected for reference was stored on a computer at a sampling rate of 0.82 s for later analysis.

RESULTS

Effect of Ca^{2+} and La^{3+} on the tryptophan fluorescence of sarcoplasmic reticulum vesicles

In agreement with earlier observations of Dupont (1976) the intensity of tryptophan fluorescence of sarcoplasmic reticulum vesicles measured at 330 nm is enhanced by Ca^{2+} at micromolar concentrations that are expected to saturate the high-affinity Ca^{2+} binding sites (Fig. 1). The large decrease in fluorescence caused by EGTA (Fig. 1b) suggests the presence of endogenous calcium in the microsomes that contributes to the fluorescence intensity of the starting material.

Addition of 5–15 μM - LaCl_3 to untreated microsomes without added Ca^{2+} (Fig. 1c) or with 10 μM added Ca^{2+} (Fig. 1a), decreased the fluorescence intensity, while slight excess of La^{3+} added after EGTA (Fig. 1b) increased the fluorescence. Similar observations were made with 5–15 μM - GdCl_3 or $-\text{PrCl}_3$. We assume that the decrease in fluorescence intensity caused by lanthanides in the presence of 10–30 μM - Ca^{2+} is due to competition by lanthanides with Ca^{2+} at the high-affinity Ca^{2+} binding sites. The increase in fluorescence intensity upon addition of excess lanthanides to EGTA-containing test systems (Fig. 1b) may imply (a) displacement of Ca^{2+} from the Ca^{2+} -EGTA complex, or (b) a direct enhancement of tryptophan fluorescence, albeit at a lower quantum yield than that of Ca^{2+} , by the lanthanide bound to the high-affinity Ca^{2+} sites.

These possibilities were further analysed by using sarcoplasmic reticulum vesicles and test media that were depleted of endogenous Ca^{2+} by treatment with Chelex chelating resin (Figs 2 and 3). Chelex treatment decreases the Ca^{2+} content of sarcoplasmic reticulum from approx. 30 nmol/mg of protein to approx. 3 nmol/mg of protein or below.

In such Ca^{2+} -depleted systems, 5 μM - Gd^{3+} , $-\text{La}^{3+}$ or $-\text{Pr}^{3+}$ increase tryptophan fluorescence by 1–2% and the enhancement of fluorescence is reversed by EGTA (Fig. 2). The maximum increase in fluorescence by 10–100 μM - Ca^{2+} under these conditions was about 4% (Figs 2 and 3). Addition of 5 μM - Gd^{3+} to Chelex-treated microsome suspensions containing 10 μM added Ca^{2+} did not change significantly the fluorescence intensity (Fig. 3). A slight decrease in fluorescence, comparable with that described in Fig. 1, was produced by 5 μM - La^{3+} or $-\text{Pr}^{3+}$ in the presence of 10 μM - Ca^{2+} (Fig. 3). These observations are consistent with the interpretation that binding of Gd^{3+} , La^{3+} or Pr^{3+} to the Ca^{2+} -binding site of sarcoplasmic reticulum causes an increase in tryptophan fluorescence. The magnitude of this increase is less than that obtained upon saturation of the Ca^{2+} binding site by Ca^{2+} . Therefore, in systems containing 5–10 μM - Ca^{2+} , competition of lanthanides with Ca^{2+} at the high-affinity sites actually decreases the fluorescence intensity.

Relationship between tryptophan fluorescence and the concentration of Ca^{2+} , Gd^{3+} and Pr^{3+}

Chelex-treated microsomes were titrated with Ca^{2+} , Gd^{3+} or Pr^{3+} and the changes in tryptophan fluorescence were measured at 330 nm (Fig. 4). Saturation of the high-affinity Ca^{2+} sites of the Ca^{2+} -ATPase by Ca^{2+} increased the tryptophan fluorescence by about 3–4%, consistent with the proposition (Pick & Karlsh, 1980, 1982; Andersen *et al.*, 1982) that Ca^{2+} binding to the high-affinity sites stabilizes the E_1 conformation of the

Fig. 1. Effect of Ca , La , and EGTA on the fluorescence of tryptophan in sarcoplasmic reticulum vesicles without Chelex treatment

To untreated sarcoplasmic reticulum vesicles (0.1 mg of protein/ml) suspended in 0.1 M-KCl/30 mM-Tris/Mops (pH 7.0)/5 mM- MgCl_2 , CaCl_2 , EGTA and LaCl_3 were added in varying final concentrations in the order indicated: (a) CaCl_2 , 10 μM ; LaCl_3 , 5 μM ; EGTA , 100 μM . (b) EGTA , 10 μM ; LaCl_3 , 15 μM ; CaCl_2 , 100 μM . (c) LaCl_3 , 5 μM ; EGTA , 10 μM ; CaCl_2 , 100 μM . The fluorescence of tryptophan was measured at 330 nm using 295 nm light for excitation. The changes in fluorescence intensity (ΔF) are expressed as a percentage of the starting value. Temperature: 10 °C.

enzyme. Over the same concentration range, Gd^{3+} and Pr^{3+} increase the tryptophan fluorescence by about 1–2% (Fig. 4), in agreement with the observations in Figs. 1–3. Complete titration curve could not be obtained since at

Fig. 2. Effects of lanthanides on the fluorescence of tryptophan in sarcoplasmic reticulum vesicles after treatment with Chelex chelating resin

Sarcoplasmic reticulum vesicles (0.1 mg of protein/ml) were treated with Chelex for 10 h as described under 'Materials and methods'. To the Chelex-treated vesicles suspended in 0.1 M-KCl/30 mM-Tris/Mops (pH 7.0)/5 mM-MgCl₂, lanthanides (5 μ M), EGTA (10 μ M) and CaCl₂ (100 μ M) were added as indicated in the Figure. The fluorescence of tryptophan was measured at 330 nm using light of 295 nm for excitation. The changes in fluorescence intensity (ΔF) are expressed as a percentage of starting value. Temperature: 10 °C. (a) GdCl₃, (b) LaCl₃, (c) PrCl₃.

lanthanide concentrations above 10 μ M aggregation of microsomes interfered with fluorescence measurements. This is probably a form of isoelectric precipitation caused by the binding of lanthanides to several sites on the

Fig. 3. Effect of Ca²⁺, lanthanides and EGTA on the fluorescence of tryptophan in Chelex-treated sarcoplasmic reticulum

The experimental conditions were similar to those described in Fig. 2, except that the order of addition was changed to CaCl₂ (10 μ M), lanthanides (5 μ M) and EGTA (100 μ M). Temperature: 10 °C.

sarcoplasmic reticulum membrane (Itoh & Kawakita, 1984). The aggregation was reversed by slight excess of EGTA.

Effect of lanthanides on the fluorescence of FITC microsomes

Sarcoplasmic reticulum vesicles were covalently labelled with fluorescein isothiocyanate at a lysine residue near the T₁ tryptic cleavage site of the Ca²⁺-ATPase (Pick & Karlsh, 1980, 1982; Mitchinson *et al.*, 1982). The FITC-labelled ATPase loses its ATPase activity (Pick &

Fig. 4. Relationship between the change in tryptophan fluorescence and total concentration of Ca^{2+} , Gd^{3+} and Pr^{3+} in sarcoplasmic reticulum vesicles treated with Chelex resin

Sarcoplasmic reticulum vesicles were treated with Chelex chelating resin as described under 'Materials and methods'. The vesicles were suspended in 0.1 M-KCl/30 mM-Tris/Mops (pH 7.0)/5 mM-MgCl₂ to a final concentration of 0.1 mg/ml. From concentrated stock solution, CaCl_2 , GdCl_3 and PrCl_3 were added to a final concentration indicated on the abscissa and the changes in the tryptophan fluorescence were measured at 330 nm using 295 nm light for excitation. The fluorescence changes (ΔF), expressed as percentages of the starting value, were corrected for the dilution, which did not exceed 1.03%. ●, CaCl_2 ; ○, PrCl_3 ; △, GdCl_3 . Temperature: 10 °C.

Bassilian, 1981), but retains the ability to crystallize in the presence of Na_3VO_4 and EGTA (Csermely *et al.*, 1985), together with the characteristic conformational responses to Ca^{2+} and vanadate (Pick & Karlsh, 1982). The distinctive spectral properties of tryptophan and fluorescein permit the analysis of the responses of both fluorophores to Ca^{2+} in FITC-labelled sarcoplasmic reticulum preparations. The Ca^{2+} -induced conformational change is accompanied by an increase in the fluorescence of tryptophan and a decrease in the fluorescence of the covalently attached fluorescein (Pick & Karlsh, 1982; Andersen *et al.*, 1982). The Ca^{2+} -induced responses of both fluorophores are reversed by EGTA.

The Ca^{2+} -induced decrease of FITC fluorescence in FITC-labelled sarcoplasmic reticulum is mimicked by lanthanides. Gd^{3+} , La^{3+} and Pr^{3+} , at a total concentration of 5 μM , decrease the fluorescence intensity by approx. 1, 1.5 and 2% respectively, compared with a 4% decrease observed at saturating Ca^{2+} concentration (Fig. 5). Precipitation of microsomes prevented the use of higher lanthanide concentrations. The decrease in fluorescence caused by Ca^{2+} and lanthanides is essentially complete within the mixing time of about 5–10 s. The effects of Ca^{2+} and Gd^{3+} on the fluorescence of FITC are rapidly

reversed by excess EGTA; the reversal of the effects of La^{3+} and Pr^{3+} by EGTA was slower, suggesting that the release of La^{3+} and Pr^{3+} from the binding site or the subsequent change in conformation ($E_1 \rightarrow E_2$?) are relatively slow processes.

Effect of transmembrane potential on the fluorescence of FITC covalently bound to sarcoplasmic reticulum

Membrane potential generated by ion substitution causes large changes in the fluorescence of FITC in FITC-labelled sarcoplasmic reticulum vesicles. Dilution of vesicles equilibrated with CC medium into KG medium generates inside positive membrane potential (Beeler *et al.*, 1981, 1984), accompanied by a large initial increase in FITC fluorescence. The fluorescence change is complete within the mixing time of several seconds (Fig. 6a). The ion diffusion potential is slowly dissipated over a period of several minutes due to ion fluxes through the cation and anion channels of sarcoplasmic reticulum; with the decrease in membrane potential the fluorescence signal of FITC also returns to the resting level (Fig. 6a). Dilution of KG microsomes into KG medium does not generate ion diffusion potential and there is no significant change in the level of FITC fluorescence (results not shown).

Dilution of microsomes equilibrated in KG medium into CC medium generates inside negative membrane potential (Beeler *et al.*, 1981, 1984), accompanied by a rapid decrease in FITC fluorescence that is complete within the mixing time of a few seconds (Fig. 6b). With the dissipation of membrane potential the fluorescence rises to resting level over a period of 15–20 min (Fig. 6b). Dilution of CC microsomes into CC medium produced no significant fluorescence change and served as control. Inclusion of 0.5 mM-EGTA into the dilution medium (broken lines) produced a slight increase in the rate of decay of fluorescence response with potentials of either direction (Figs 6a and 6b). This effect is more pronounced in Fig. 6(b) and may indicate a modest increase in the permeability of ion channels to monovalent cations and anions upon chelation of divalent cations.

The decrease in FITC fluorescence under the influence of inside negative membrane potential (Fig. 6b) corresponds in direction to the decrease in FITC fluorescence observed upon the addition of Ca^{2+} and lanthanide ions to sarcoplasmic reticulum vesicles. Based on kinetic and spectroscopic observations, Ca^{2+} and lanthanides are assumed to stabilize the E_1 conformation of the Ca^{2+} -ATPase (Pick & Karlsh, 1982; the present paper). Therefore we propose that the conformation of Ca^{2+} -ATPase induced by inside negative potential may be similar to the E_1 conformation stabilized by Ca^{2+} and lanthanides.

By analogy, the increase in FITC fluorescence caused by inside positive potential (Fig. 6a) is comparable to the effects of EGTA and vanadate on FITC fluorescence (Pick & Karlsh, 1982) and may reflect the stabilization of an enzyme conformation analogous to or identical with the E_2 state of the Ca^{2+} -ATPase.

These propositions are consistent with the fact that positive potential promotes, while negative potential, Ca^{2+} and lanthanides disrupt, the vanadate-induced crystals of Ca^{2+} -ATPase that are presumed to reflect the E_2 conformation (Dux & Martonosi, 1983c,d).

Fig. 5. Effect of lanthanides on the fluorescence of FITC-labelled microsomes

FITC-labelled microsomes, prepared as described under 'Materials and methods', were treated with Chelex-100 for 16 h at 2 °C to decrease Ca^{2+} contamination. The microsomes were suspended in Chelex-treated 0.1 M-KCl/30 mM-Mops solution to which 5 mM- MgCl_2 was added just before the experiment. Protein concentration: 0.1 mg/ml. The fluorescence was measured using 500 nm light for excitation and 517.5 nm for emission (slits 2 and 4 nm, respectively). The following additions were made: (a) 10 μM - Ca^{2+} , 15 μM -EGTA; (b) 5 μM -EGTA, 15 μM - Ca^{2+} ; (c) 5 μM - Gd^{3+} , 10 μM -EGTA; (d) 5 μM -EGTA, 10 μM - Gd^{3+} ; (e) 5 μM - La^{3+} , 10 μM -EGTA; (f) 5 μM -EGTA, 10 μM - La^{3+} ; (g) 5 μM - Pr^{3+} , 10 μM -EGTA; (h) 5 μM -EGTA, 10 mM- Pr^{3+} . Temperature: 10 °C.

Changes in the tryptophan fluorescence of microsomes under the influence of potential generated by ion substitution

Inside positive potential generated by transfer of sarcoplasmic reticulum vesicles from CC medium into KG medium slightly increases the fluorescence of tryptophan (Figs. 7a and 7c), followed by a slower return to resting fluorescence as the potential is dissipated. The change in tryptophan fluorescence is usually of the order

of 1%, compared with the 8–10% change in FITC fluorescence under similar conditions.

A small decrease in tryptophan fluorescence was observed upon dilution of sarcoplasmic reticulum vesicles from KG medium into CC medium (Figs. 7b and 7d), followed again by slow return to resting levels.

Inclusion of 0.5 mM-EGTA into the dilution medium (Figs. 7c and 7d) did not alter significantly the time course or magnitude of the tryptophan fluorescence response, compared with dilution media that did not contain

Fig. 6. Effect of membrane potential on the fluorescence of FITC covalently bound to the Ca^{2+} -ATPase

Sarcoplasmic reticulum vesicles were labelled with FITC at a ratio of 10 nmol/mg of protein and dialysed overnight against two changes (500 volumes each) of either KG medium or CC medium as described under 'Materials and methods'. Protein concentration: 20 mg/ml. (a) Inside positive potential was generated by 190-fold dilution of CC microsomes into KG medium. Dilution of KG microsomes into KG medium served as control. (b) Inside negative potential was generated by 190-fold dilution of KG microsomes into CC medium. Dilution of CC microsomes into CC medium served as control. The changes in FITC fluorescence were measured using excitation and emission wavelengths of 500 and 517.5 nm, with slits of 2 and 4 nm, respectively. -----, dilution medium contained no EGTA; ----, dilution medium contained 0.5 mM-EGTA.

EGTA (Figs 7a and 7b). These observations imply that within the error of these experiments contaminating Ca^{2+} (approx. 10^{-5} M) did not influence significantly the tryptophan response of Ca^{2+} -ATPase to membrane potential.

The effect of membrane potential on the fluorescence of tryptophan in sarcoplasmic reticulum vesicles labelled with FITC (results not shown) is of similar magnitude and sign as in unlabelled control microsomes. Therefore blocking of the ATP binding site by FITC (Pick & Bassilian, 1981) has no significant effect on the conformational response of the Ca^{2+} -ATPase either to membrane potential or to the binding of Ca^{2+} and lanthanides to the Ca^{2+} -ATPase.

Fig. 7. Effect of membrane potential on tryptophan fluorescence

Sarcoplasmic reticulum vesicles equilibrated in CC medium were diluted into KG medium to generate inside positive potential (a and c). Inside negative potential was generated by dilution of KG vesicles into CC medium (b and d). The dilution media of (a) and (b) contained approx. 10^{-6} M free Ca^{2+} ; to the dilution media of (c) and (d) 0.5 mM-EGTA was added to decrease the free Ca^{2+} concentration to below 10^{-8} M. The curves in (a) and (b) represent averages of four separate experiments.

These observations give further credence to the reliability of FITC as a fluorescence probe of the conformational state of the Ca^{2+} -ATPase.

Fluorescence response of tryptophan to membrane potential in the presence of EGTA and vanadate

Preincubation of sarcoplasmic reticulum vesicles overnight in 0.5 mM-EGTA significantly alters their response to membrane potential changes induced by ion substitution (Fig. 8). The response of the vesicles to inside positive potential generated by dilution from CC medium into KG medium with or without 0.1 mM- Na_3VO_4 is essentially abolished (Figs. 8a and 8c). These observations can be rationalized as follows: removal of Ca^{2+} by EGTA, especially in the presence of vanadate, favours the E_2 conformation of the Ca^{2+} -ATPase. As the equilibrium in the presence of EGTA and vanadate is already in favour of the E_2 form, positive potential is not expected to have large additional effect on the $\text{E}_1 \rightarrow \text{E}_2$ equilibrium, hence no fluorescence signal is obtained. The influence of negative potential is still observed in the presence of EGTA, particularly with 0.1 mM- Na_3VO_4 in the dilution medium (Fig. 8d), suggesting that negative potential shifts the equilibrium from the E_2 to the E_1 conformation, with a decrease in fluorescence intensity. A slow re-equilibration occurs as the potential is dissipated.

Fig. 8. Effect of membrane potential on the tryptophan fluorescence of microsomes in the presence of EGTA and vanadate

Sarcoplasmic reticulum vesicles (protein concentration 22.5 mg/ml) were equilibrated in KG medium or CC medium containing 0.5 mM-EGTA by dialysis at 2 °C overnight. Positive potential was generated by dilution of CC vesicles into KG medium (*a* and *c*); dilution of KG vesicles into KG medium served as control. Negative potential was generated by dilution of KG vesicles into CC medium (*b* and *d*); dilution of CC vesicles into CC medium served as control. For (*a*) and (*b*) the dilution media contained 0.5 mM-EGTA; for (*c*) and (*d*) the dilution media contained 0.5 mM-EGTA and 0.1 mM- Na_3VO_4 . The Na_3VO_4 stock solutions (50 mM, pH 8.0) were boiled for 10 min before use to reduce decavanadate contribution. For other details, see the Materials and methods section.

DISCUSSION

We conclude that membrane potential generated in sarcoplasmic reticulum vesicles by ion substitution alters the conformation of the Ca^{2+} -ATPase. The potential dependent conformational change is reflected in the fluorescence of protein tryptophan and of fluorescein covalently bound to the Ca^{2+} -ATPase.

Inside positive potential generated by transfer of vesicles from CC medium into KG medium increases both tryptophan and FITC fluorescence. Transfer of vesicles from KG medium into CC medium generates negative potential and decreases the fluorescence of both tryptophan and FITC. The fluorescence transients caused by either type of ion substitution decline with the dissipation of membrane potential over a period of several minutes. To an estimated potential of 50–60 mV, FITC- Ca^{2+} -ATPase responds with a fluorescence change of 7–9%. The corresponding changes in tryptophan fluorescence are much smaller, usually of the order of 1%.

Therefore analysis of the tryptophan signals generally required averaging.

It is noteworthy that the fluorescence responses of FITC and tryptophan to membrane potential are of the same sign, while the two probes respond with changes of opposite sign to the binding of Ca^{2+} and lanthanides to the sarcoplasmic reticulum (Andersen *et al.*, 1982; Pick & Karlsh, 1982; the present paper). The disparate responses of tryptophan and of the covalently bound FITC to potential and to the binding of Ca^{2+} and lanthanides imply that one or both of these responses are significantly influenced by local changes in the environment of the probes. Local effects on tryptophan fluorescence would be expected if the binding site of Ca^{2+} and lanthanides is in the vicinity of the sampled tryptophan(s). Extensive control experiments excluded the possibility that the fluorescence changes induced by ion substitution arise from a direct influence of the dilution media on the quantum yield of the fluorescence, without involvement of transmembrane potential.

We proposed earlier (Dux & Martonosi, 1983*a–d*) that the Ca^{2+} -ATPase crystals induced by vanadate in Ca^{2+} -free media represent the E_2 conformation of the enzyme. The fluorescence responses of FITC to inside positive membrane potential, and to the interaction of vanadate with the Ca^{2+} -ATPase in the presence of EGTA, are consistent with the interpretation that the E_2 conformation of the Ca^{2+} -ATPase is similar to or equivalent with the 'high' fluorescence state of FITC-ATPase. Ca^{2+} , lanthanides and inside negative potential disrupt the vanadate-induced Ca^{2+} -ATPase crystals (Dux & Martonosi, 1983*c,d*) with a decrease in FITC fluorescence. Therefore the 'low' fluorescent state of FITC-ATPase is assumed to represent the E_1 conformation.

These observations, together with the similar effects of Ca^{2+} and lanthanides on the tryptic digestion profiles of the Ca^{2+} -ATPase (Dux *et al.*, 1985), support the conclusion that the Ca^{2+} -ATPase crystals induced by Ca^{2+} or lanthanides (Dux *et al.*, 1985) represent the E_1 conformation of the enzyme.

The identification of a particular state of fluorescence with the E_1 or E_2 conformations of the Ca^{2+} -ATPase is more difficult when the effects of Ca^{2+} , lanthanides, and membrane potential on tryptophan fluorescence are compared with their effects on the stability of Ca^{2+} -ATPase membrane crystals. The vanadate-induced crystals of Ca^{2+} -ATPase, presumed to represent the E_2 conformation, are disrupted by Ca^{2+} with an increase in tryptophan fluorescence, and by inside negative potential with a decrease in tryptophan fluorescence. Furthermore the formation of Ca^{2+} -ATPase crystals by vanadate is accelerated by EGTA with a decrease in tryptophan fluorescence, and by inside positive potential with an increase in tryptophan fluorescence. Therefore two distinct mechanisms, signified by the opposite changes in tryptophan fluorescence, appear to operate in the stabilization of the E_1 and E_2 crystals.

The changes in tryptophan fluorescence caused by Ca^{2+} or lanthanides may contain a large local contribution by the bound cation that is independent from the effects of the overall conformation of the Ca^{2+} -ATPase. This hypothesis is supported by the finding that the changes in tryptophan fluorescence caused by lanthanides are significantly smaller than those obtained with calcium. The co-ordination geometry of the bound Ca^{2+} ions in

complexes is frequently different from that of the bound lanthanides (Matthews & Weaver, 1974; Martin & Richardson, 1979). For Ca^{2+} 6-, 7-, and 8-fold, while for lanthanides 8- and 9-fold, co-ordination geometries are typical, and the effective ionic radii of Ca^{2+} and lanthanides increase with the co-ordination number. These structural differences in the active site occupied by Ca^{2+} or by lanthanides may provide sufficient explanation for the observed differences in tryptophan and FITC fluorescence.

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REFERENCES

- Andersen, J. P., Møller, J. V. & Jorgensen, P. L. (1982) *J. Biol. Chem.* **257**, 8300–8307
- Beeler, T. J. (1980) *J. Biol. Chem.* **255**, 9156–9161
- Beeler, T. J., Farnen, R. H. & Martonosi, A. (1981) *J. Membr. Biol.* **62**, 113–137
- Beeler, T. J., Dux, L. & Martonosi, A. (1984) *J. Membr. Biol.* **78**, 73–79
- Csermely, P., Varga, S. & Martonosi, A. (1985) *Eur. J. Biochem.* **150**, 455–460
- Dupont, Y. (1976) *Biochem. Biophys. Res. Commun.* **71**, 544–550
- Dupont, Y. (1978) *Biochem. Biophys. Res. Commun.* **82**, 893–900
- Dupont, Y., Bennett, N. & Lacapere, J. J. (1982) *Ann. N.Y. Acad. Sci.* **402**, 569–572
- Dupont, Y. & Leigh, J. B. (1978) *Nature (London)* **273**, 396–398
- Dux, L. & Martonosi, A. (1983a) *J. Biol. Chem.* **258**, 2599–2603
- Dux, L. & Martonosi, A. (1983b) *J. Biol. Chem.* **258**, 10111–10115
- Dux, L. & Martonosi, A. (1983c) *J. Biol. Chem.* **258**, 11896–11902
- Dux, L. & Martonosi, A. (1983d) *J. Biol. Chem.* **258**, 11903–11907
- Dux, L. & Martonosi, A. (1983e) *Muscle Nerve* **6**, 566–573
- Dux, L. & Martonosi, A. (1984) *Eur. J. Biochem.* **141**, 43–49
- Dux, L., Taylor, K. A. & Martonosi, A. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 1700
- Dux, L., Taylor, K. A., Ting-Beall, H. P. & Martonosi, A. (1985) *J. Biol. Chem.* **260**, 11730–11743
- Grisham, C. M. (1982) in *Membranes and Transport* (Martonosi, A., ed.), vol. 1, pp. 585–592, Plenum, New York
- Guillain, F. M. & Boyer, P. (1982) *Ann. N.Y. Acad. Sci.* **402**, 566–568
- Guillain, F. M., Gingold, P., Buschlen, S. & Champeil, P. (1980) *J. Biol. Chem.* **255**, 2072–2076
- Itoh, N. & Kawakita, M. (1984) *J. Biochem. (Tokyo)* **95**, 661–669
- Martin, R. B. & Richardson, F. S. (1979) *Q. Rev. Biophys.* **12**, 181–209
- Martonosi, A. (1984) *Physiol. Rev.* **64**, 1240–1320
- Martonosi, A. & Beeler, T. J. (1983) in *Handbook of Physiology: Skeletal Muscle* (Peachey, L. D. & Adrian, R. H., eds.), pp. 417–485, American Physiological Society, Bethesda
- Martonosi, A., Dux, L., Peracchia, C. & Taylor, K. A. (1984) in *Membrane Dynamics and Transport of Normal and Tumor Cells* (Tron, L., Damjanovich, S., Fonyo, A. & Somogyi, J., eds.), pp. 133–151, Akademiai Kiado, Budapest
- Matthews, B. W. & Weaver, L. H. (1974) *Biochemistry* **13**, 1719–1725
- Mitchinson, C., Wilderspin, A. F., Trinaman, B. J. & Green, N. M. (1982) *FEBS Lett.* **146**, 87–92
- Nakamura, H., Jilka, R. L., Boland, R. & Martonosi, A. N. (1976) *J. Biol. Chem.* **251**, 5414–5423
- Oetliker, H. (1982) *J. Muscle Res. Cell Motil.* **3**, 247–272
- Papp, S., Rutzke, M. & Martonosi, A. (1985) *Arch. Biochem. Biophys.* **243**, 254–263
- Peracchia, C., Dux, L. & Martonosi, A. (1984) *J. Muscle Res. Cell Motil.* **5**, 431–442
- Pick, U. (1982) *J. Biol. Chem.* **257**, 6111–6119
- Pick, U. & Bassilian, S. (1981) *FEBS Lett.* **123**, 127–130
- Pick, U. & Karlish, S. J. D. (1980) *Biochim. Biophys. Acta* **626**, 255–261
- Pick, U. & Karlish, S. J. D. (1982) *J. Biol. Chem.* **257**, 6120–6126
- Taylor, K. A., Dux, L. & Martonosi, A. (1984a) *J. Mol. Biol.* **174**, 193–204
- Taylor, K. A., Dux, L. & Martonosi, A. (1984b) *Biophys. J.* **45**, 145a
- Zimniak, P. & Racker, E. (1978) *J. Biol. Chem.* **253**, 4631–4637

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