Functional properties of a sarcoplasmic reticulum Ca^{2+} -ATPase with an altered Ca^{2+} -binding mechanism

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Treatment of sarcoplasmic reticulum vesicles with diethylpyrocarbonate in the presence of a large excess of reagent, at pH 6.2 and at room temperature, reveals both a fast- and a slow-reacting population of protein residues. The loss of the Ca^{2+} -ATPase activity is mainly associated with the fast-reacting population being partially sensitive to hydroxylamine. There is also an effect on the Ca^{2+} -binding mechanism. Shorter derivatization times (5 min) produce a loss of the positive cooperativity of Ca^{2+} binding. When the treatment was prolonged for 30 min there was an additional decrease in the overall Ca^{2+} affinity. Curve-fitting procedures applied to the non-cooperative binding isotherms provide the equilibrium constants for the two Ca^{2+} sites, although they cannot discriminate between interacting and independent site mechanisms. Prestationary

kinetics assays show 2 Ca^{2+} :1 ATP ratios, at any extent of Ca^{2+} saturation, indicating that the Ca^{2+} sites are not independent. The Ca^{2+} dissociation profile after derivatization shows a decrease in the dissociation constant for the release of the second Ca^{2+} , which is consistent with interacting sites. Isotopic exchange experiments show fast and slow components of equal amplitude even at subsaturating Ca^{2+} concentrations, which is incompatible with independent binding sites. The experimental data suggest a modification of the equilibrium binding constants making them more similar, but keeping the interacting character. The structural position of the external (cytoplasmic) and the internal (lumenal) Ca^{2+} sites remains unaltered in the absence of positive cooperativity.

INTRODUCTION

Ca^{2+} binding to the sarcoplasmic reticulum (SR) Ca^{2+} -ATPase protein is a prerequisite for the phosphoryl transfer from ATP to the enzyme (for reviews, see [1,2]). This fundamental step in the enzyme activation requires a long-range transmission of the Ca^{2+} signal from the transmembrane sector (transport sites) to the cytoplasmic head region (catalytic centre).

Ca^{2+} binding at equilibrium and in the absence of ATP presents a stoichiometry of 2 mol of Ca^{2+} per mol of phosphorylated enzyme [3] and the binding exhibits positive cooperativity that can be described by a sequential mechanism [3–6]. Site-directed mutagenesis [7,8], chemical modification [9], and chimeric constructs [10] indicate that the Ca^{2+} -binding sites are in the transmembrane domain of the protein. There is also some evidence that the Ca^{2+} ions are accommodated in a tube-like structure providing non-equivalent external accessibility [5,11,12].

A deeper knowledge of the Ca^{2+} -binding mechanism can be obtained by modification of the native binding properties and analysis of the functional characteristics of the altered enzyme. Diethylpyrocarbonate (DEPC), also termed diethyldicarbonate or ethoxyformic anhydride, is a relatively specific reagent for protein modification that under certain experimental conditions might derivatize histidine residues, although some caution may be needed when interpreting the results [13]. This acylating agent was found to be an effective inhibitor of different enzyme activities [14–19] including that of the Ca^{2+} -ATPase from SR [20–22].

Interestingly, the derivatized ATPase molecule binds Ca^{2+}

non-cooperatively. This fact was explained by the elimination of the cooperative character without alteration of the specific Ca^{2+} -binding affinities [22]. If this is true, the Ca^{2+} sites will become distinct and independent after the chemical treatment. Based on this observation, and considering the mechanistic implications that can be derived from such a situation, we exposed the SR vesicles to gradual DEPC derivatization. The present report analyses the absence of positive cooperativity of Ca^{2+} binding with respect to sequential binding models accounting for two interacting or two independent Ca^{2+} sites. These results, in conjunction with data derived from the functional properties of the enzyme, suggest the presence of interacting Ca^{2+} sites with similar dissociation constants after the chemical treatment. This study contributes to our understanding of the positive cooperativity mechanism of Ca^{2+} binding in the native enzyme.

EXPERIMENTAL

SR vesicles were obtained from rabbit leg fast-twitch muscle according to Eletr and Inesi [23]. The protein content was estimated by the method of Lowry et al. [24] using BSA as a standard.

Ca^{2+} -EGTA buffers were used to establish different free Ca^{2+} concentrations in the incubation/reaction media. The procedure was based on a computer program described by Fabiato and Fabiato [25]. The association constant for the Ca^{2+} -EGTA complex was taken from Schwartzbach et al. [26], and the

Abbreviations used: SR, sarcoplasmic reticulum; DEPC, diethylpyrocarbonate; pCa, negative logarithm of molar Ca^{2+} concentration; *h*, Hill coefficient.

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equilibrium constants for the EGTA protonation were from Blinks et al. [27].

DEPC (Sigma) was divided into aliquots under a nitrogen atmosphere and stored at 4 °C in amber glass vials sealed with rubber plugs. For each session, a sample taken through the rubber closure by microsyringe was diluted with ice-cold ethanol and kept on ice. The concentration of the diluted reagent was checked by measuring the absorbance change at 242 nm after reaction with 3 ml of 20 mM imidazole, pH 7.5 (ϵ_{242} 3.2 mM⁻¹·cm⁻¹, as described by Ovádi et al. [28]).

A typical modification reaction was performed by incubating SR vesicles (1 mg of protein/ml) at room temperature (22 °C) with 7 mM DEPC in the presence of 20 mM Mes, pH 6.2, and 80 mM KCl. The chemical reaction was stopped by an extensive dilution or by adding a medium containing 5 mM imidazole as specified in each case. In the experiments requiring the functional activity of the Ca²⁺-ATPase protein the modification reaction was restricted to 5 min. For time-resolved measurements of Ca²⁺ binding, dissociation, or exchange the reaction was allowed to proceed for 30 min. Hydroxylamine hydrochloride freshly prepared at pH 7.5 was used in a 30 min incubation to hydrolyse mono-*N*-carbethoxylated histidine [13]. The final concentration in the reaction medium was 0.5 M. The time course of the derivatization was followed by the absorbance increase at 242 nm or by difference U.V. spectra using 5-fold diluted samples (0.2 mg/ml) in the presence of 0.1 % SDS.

The Ca²⁺-ATPase activity of SR vesicles was monitored during the DEPC treatment. To this end, the chemical reaction of DEPC with the SR vesicles was arrested at different time intervals by a 50-fold dilution. The incubation medium after dilution contained 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.823 mM CaCl₂ (pCa_{free} 5.3), 0.02 mg/ml of DEPC-treated vesicles and 10 μ M A23187. The presence of ionophore in the reaction medium permits enzymic measurements at steady state. The rate of ATP hydrolysis was measured by a colorimetric procedure following the liberation of inorganic phosphate [29] after addition of 1 mM ATP. The Mg²⁺-ATPase activity (basal activity) was measured in the presence of 1 mM EGTA without any Ca²⁺ addition.

The dependence on Ca²⁺ of the ATPase activity was studied in SR vesicles treated for 5 min with 7 mM DEPC. After a 20-fold dilution in the presence of imidazole to stop the reaction, the mixture consisted of 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 1 mM EGTA, 0.05 mg/ml of modified vesicles and 5 mM potassium oxalate. Aliquots of 6 ml were supplemented with different Ca²⁺ concentrations. After addition of 1 mM ATP, samples of 1 ml were taken at serial time intervals to determine the ATPase activity [29].

The Ca²⁺ uptake capacity was measured in parallel experiments by including the ⁴⁵Ca²⁺ tracer (~ 10000 c.p.m./nmol) in the incubation medium. Samples of 1 ml were filtered (0.45 μ m pore size, HAWP Millipore filter), and rinsed with 3 ml of medium (50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂ and 5 mM LaCl₃) before counting the radioactivity retained by the filter.

Transient kinetics experiments of Ca²⁺ transport and ATP hydrolysis were performed as described previously [30]. SR vesicles were incubated with 7 mM DEPC for 5 min. After addition of the imidazole medium, the chemical composition of the mixture was 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 0.3 mg/ml of derivatized SR protein and 50 μ M ⁴⁵CaCl₂ (~ 10000 c.p.m./nmol) for the experiments of Ca²⁺ transport. Aliquots (0.6 ml) were preincubated with various concentrations of EGTA to yield different free Ca²⁺ concentrations. Then a volume of 0.6 ml, containing 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole,

0.1 mM EGTA and 1 mM ATP, was manually added under continual vortexing. Samples were filtered on HAWP Millipore filters within 10 s. The filters were rinsed once with 3 ml of medium containing 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole and 5 mM LaCl₃, and then subjected to liquid scintillation counting.

[³²P]P_i release was measured under the same experimental conditions, except that [γ -³²P]ATP (~ 15000 c.p.m./nmol) and CaCl₂ were now included in the mixing solution instead of ATP and ⁴⁵Ca²⁺. Samples were denatured by adding 1 ml of ice-cold solution containing perchloric acid and sodium phosphate to give final concentrations of 125 mM and 0.5 mM respectively. The reaction mixtures were centrifuged for 5 min in a benchtop centrifuge at 5000 rev./min and 4 °C, and the supernatants were used to quantify the radioactive phosphate [3].

Ca²⁺ binding at equilibrium was measured by a double labelling filtration technique [31] with the aid of radioactive ⁴⁵Ca²⁺ and tritiated glucose to evaluate the filter wet volume [12]. SR vesicles (1 mg/ml) were treated in the presence of 7 mM DEPC at room temperature (22 °C). The chemical reaction was arrested 5 or 30 min later by adding the imidazole medium. The mixture composition after dilution was 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 100 μ M ⁴⁵CaCl₂ (~ 10000 c.p.m./nmol), 1 mM [³H]glucose (~ 5000 c.p.m./nmol), 0.4 mg/ml DEPC-treated vesicles and 5 mM imidazole. Aliquots of 1 ml were supplemented with different concentrations of EGTA to obtain fractional saturation of the Ca²⁺ sites. After equilibration, samples of 0.25 ml (0.1 mg of protein) were filtered by hand to determine the ⁴⁵Ca²⁺ and ³H associated with the filter (0.45 μ m, HAWP Millipore). The Ca²⁺ bound to the protein was calculated by subtracting the free Ca²⁺ retained by the filter.

The kinetics of Ca²⁺ binding were measured after a 30 min treatment with 7 mM DEPC. The chemical modification was stopped by imidazole following a 10-fold dilution to give 50 mM Mops, pH 6.8, 5 mM MgCl₂, 80 mM KCl, 0.1 mg of modified SR protein/ml, 5 mM imidazole, 1 mM EGTA and 1 mM glucose. Aliquots of 1 ml were loaded on to Millipore filters (HAWP) and perfused at specific times with a medium containing 50 mM Mops, pH 6.8, 5 mM MgCl₂, 80 mM KCl, 5 mM imidazole, 1 mM [³H]glucose (~ 3500 c.p.m./nmol), 0.1 mM EGTA and 147.7 μ M ⁴⁵CaCl₂ (~ 6000 c.p.m./nmol) (pCa_{free} 4.3). An electronically controlled perfusion system from Bio-Logic Co. (Claix, France) was used for this task [5].

The Ca²⁺ dissociation kinetics were studied as previously described [12]. SR vesicles were initially treated with 7 mM DEPC for 30 min. The DEPC reaction was stopped by a 10-fold dilution in the presence of imidazole. The incubation medium after dilution consisted of 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 1 mM [³H]glucose (~ 4000 c.p.m./nmol), 0.1 mg of modified protein/ml, 100 μ M ⁴⁵CaCl₂ (~ 7000 c.p.m./nmol), 0.0512 mM EGTA (pCa_{free} 4.3) and 10 μ M A23187. The presence of ionophore ensures complete dissociation of bound Ca²⁺. Aliquots of 1 ml were applied to nitrocellulose filters (HAWP Millipore) and perfused with a medium consisting of 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 1 mM glucose and 1 mM EGTA in order to measure Ca²⁺ dissociation. The perfusion time in the millisecond time span was achieved by a rapid filtration apparatus (Bio-Logic Co.). The radioactive isotopes in the filters were evaluated by liquid-scintillation counting.

The ⁴⁵Ca²⁺-⁴⁰Ca²⁺ exchange in partially Ca²⁺-saturated ATPase [12] was also measured after stopping the 30 min reaction of SR vesicles (1 mg/ml) with 7 mM DEPC. The incubation medium for the isotopic exchange obtained after the imidazole dilution was 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂,

0.1 mg of DEPC-treated protein/ml, 5 mM imidazole, 1 mM [³H]glucose (~2000 c.p.m./nmol), 100 μ M ⁴⁵CaCl₂ (~10000 c.p.m./nmol) and 10 μ M ionophore A23187. The presence of 0.051, 0.167 or 0.274 mM EGTA concentrations allowed saturation of the Ca²⁺-binding sites to different extents. The experimental protocol is similar to that described for Ca²⁺ dissociation except that the perfusion medium consisted of 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 1 mM glucose and 1 mM ⁴⁰CaCl₂.

Selective labelling of the internal Ca²⁺ was achieved by partial isotopic exchange as previously published [11,32]. Native SR vesicles (0.1 mg/ml) were first equilibrated in a medium containing 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM [³H]glucose (~2000 c.p.m./nmol), 100 μ M ⁴⁵CaCl₂ (~10000 c.p.m./nmol), 0.0512 mM EGTA (pCa_{free} 4.3) and 10 μ M ionophore A23187. Aliquots of 1 ml were filtered (HAWP Millipore filter) and rinsed by hand with 0.25 ml of non-labelled medium containing 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM glucose and 1 mM CaCl₂. This allowed substitution at the external site of non-radioactive Ca²⁺. Within 2–3 s the kinetics of the Ca²⁺ exchange at the internal site were followed by perfusing the samples with the rinsing medium using the rapid filtration technique [33]. Note that, in the ordered sequential mechanism of Ca²⁺ binding, the internal site showing a slow rate of exchange corresponds to the first ion to bind whereas the external Ca²⁺ with a fast exchange rate is the second Ca²⁺ bound.

The labelling of the external Ca²⁺ was approached in a similar way. The incubation medium consisted of 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 100 μ M CaCl₂, 0.0512 mM EGTA, 0.1 mg/ml of native SR vesicles and 10 μ M ionophore A23187. Aliquots of 1 ml were added on to a Millipore filter (HAWP) and rinsed with 0.25 ml of radioactive medium containing 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM [³H]glucose, 100 μ M ⁴⁵CaCl₂ and 0.0512 mM EGTA in order to label the external Ca²⁺. Immediately thereafter, the samples were perfused in the millisecond time scale with rinsing medium devoid of the radioactive tracers. This allowed study of the Ca²⁺ exchange at the external site.

The experimental data shown in the Figures correspond to mean values of at least three independent assays. The standard errors were lower than $\pm 10\%$.

The absorbance increase due to the DEPC modification was fitted by a non-linear regression algorithm to the equation:

$$\Delta A = A_f[1 - \exp(-k_f t)] + A_s[1 - \exp(-k_s t)] \quad (1)$$

where A_f and A_s refer to the amplitude of the fast and slow components whereas k_f and k_s correspond respectively to the pseudo-first-order rate constants for the fast and slow components.

The equilibrium binding curves were fitted to the Hill equation expressed as a function of the $K_{0.5}$:

$$Y = N \times \text{Ca}^h / (K_{0.5}^h + \text{Ca}^h) \quad (2)$$

Y being the fraction of saturated binding sites, N the maximum Ca²⁺ binding, $K_{0.5}$ the Ca²⁺ concentration needed for half-saturation of the sites, and h the Hill coefficient expressing the interaction between the binding sites.

The fractional saturation of binding in a two distinct and interacting sites model can be expressed in terms of the intrinsic dissociation constants K'_{d1} and K'_{d2} as:

$$Y = (K'_{d2} \text{Ca} + \text{Ca}^2) / (K'_{d1} K'_{d2} + 2K'_{d2} \text{Ca} + \text{Ca}^2) \quad (3)$$

where K'_{d1} and K'_{d2} are related to the dissociation constants K_{d1} and K_{d2} by the corresponding statistical factor ($K_{d1} = \frac{1}{2} K'_{d1}$, and $K_{d2} = 2K'_{d2}$). K_{d1} corresponds to the dissociation in the first

calcium equilibrium: $E + \text{Ca} \rightleftharpoons \text{ECa}$, and K_{d2} is the dissociation constant in the second calcium equilibrium: $\text{ECa} + \text{Ca} \rightleftharpoons \text{ECa}_2$. The relative values of the intrinsic dissociation constants determine the cooperativity of the ligand binding (positive cooperativity when $K'_{d1} > K'_{d2}$).

In the case of two distinct and independent sites, the Ca²⁺ binding will be defined by the expression:

$$Y = N_1 \times \text{Ca} / (K_{d1} + \text{Ca}) + N_2 \times \text{Ca} / (K_{d2} + \text{Ca}) \quad (4)$$

that considers parameters related to the affinity (K_{d1} and K_{d2}) and the stoichiometry (N_1 and N_2) of the binding sites.

If the two sites are independent but they have the same K_d , the analytical expression will be:

$$Y = N \times \text{Ca} / (K_d + \text{Ca}) \quad (5)$$

Ca²⁺-binding isotherms were fitted by computer using the SigmaPlot software (Jandel Scientific, San Rafael, CA, U. S. A.).

RESULTS

The chemical reaction of DEPC with the SR vesicles can be monitored by an absorbance increase at 242 nm. The experiment presented in Figure 1 was performed with a large excess of reagent, therefore the modification reaction can be considered a pseudo-first-order process. The analysis of the experimental trace indicates the presence of two populations of reactive residues that can be characterized by the corresponding pseudo-first-order rate constants (1.18 min⁻¹ and 0.03 min⁻¹ respectively). We also show several absorbance spectra recorded during the time course of the reaction as they appear after subtraction of a proper blank (inset of Figure 1). Scan A was taken 5 min after the addition of DEPC, once the fast-reacting population has been modified, while scan C was taken at 30 min when a large proportion of the slow-reacting component has been completed. Some useful information can be derived by the subsequent addition of hydroxylamine (broken line spectra). The decrease in absorbance of scan B with respect to scan A can be considered

Figure 1 Time course of the SR vesicles derivatization by DEPC

SR vesicles (1 mg/ml) were incubated at room temperature in a medium containing 20 mM Mes, pH 6.2, 80 mM KCl and 7 mM DEPC. The extent of the reaction was monitored at different time intervals by a spectrophotometric technique. Samples of the reaction medium were 5-fold diluted and supplemented with 0.1% SDS before each measurement (○). Inset, difference absorbance spectra were recorded 5 min (scan A) or 30 min (scan C) after DEPC addition. Scans B and D correspond respectively to samples of scans A and C after a 30 min incubation in the presence of 0.5 M hydroxylamine at pH 7.5.

Figure 2 Dependence of the Ca^{2+} -ATPase activity on DEPC treatment

The modification reaction was performed at room temperature in a medium containing 20 mM Mes, pH 6.2, 80 mM KCl, 1 mg of SR protein/ml and 0.7 mM (○) or 7 mM (●) DEPC. Aliquots of the reaction medium were 50-fold diluted at different times and used to measure the rate of the ATP hydrolysis. The incubation medium after dilution was 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 0.823 mM CaCl_2 , 0.02 mg of modified protein/ml, and 10 μM A23187. The ATPase activity was assayed after addition of 1 mM ATP. Hydroxylamine at a final concentration of 0.5 M was added to the DEPC modification reaction after 30 min of treatment. The control value (100%) corresponds to 2.07 I.U./mg of protein; the basal ATPase activity (0.18 I.U./mg of protein) has been subtracted from each data point.

a demonstration that approximately half of the fast-reacting population is monocarboxylated histidine [13]. This is also the case for most of the slow-reacting residues (compare scans C and D; also B and D).

The Ca^{2+} -ATPase activity of SR vesicles during the modification reaction shows (Figure 2) that 7 mM DEPC induces a progressive decrease in activity, being completely abolished in approximately 20 min. The effect of DEPC is almost irreversible in a 30 min incubation since the hydroxylamine addition allowed only a small recovery of the Ca^{2+} -ATPase activity. The addition of 5 mM imidazole at 5 min protected from further inactivation, giving a residual activity of approximately 25% with respect to the control value (data not shown). These conditions were selected for the experiments related to the enzyme turnover. By using a DEPC concentration of 0.7 mM the inhibitory effect was more slowly developed. Likewise, the reactivation by hydroxylamine, at the reaction time checked, was more efficient.

The high-affinity Ca^{2+} binding to the enzyme in native vesicles (open circles in Figure 3) exhibits a stoichiometry of 7.4 nmol/mg of protein, the $K_{0.5}$ is 1.8 μM and $h = 1.64$. These parameters were deduced by fitting procedures to the Hill equation (eqn. 2). The incubation of SR vesicles with 7 mM DEPC alters these parameters. A 5 min treatment (Figure 3, closed triangles) mainly affects the cooperative character of the Ca^{2+} binding. Fitting of the experimental data to the Hill equation provides a $K_{0.5}$ of 1.1 μM , and the h is 0.91. When the reagent incubation was prolonged for 30 min (Figure 3, closed circles), the effect on cooperativity was more pronounced and the apparent binding affinity was lower. Under these conditions $K_{0.5}$ is 4.4 μM and h is 0.79.

The Ca^{2+} -binding isotherm in untreated SR (Figure 3, open circles) can be satisfactorily fitted with $K'_{a1} = 10.4 \mu\text{M}$ and $K'_{a2} = 0.3 \mu\text{M}$, assuming the two-interacting-sites model de-

Figure 3 Effect of DEPC on the ATPase Ca^{2+} binding at equilibrium

The chemical derivatization was performed in the presence of 7 mM DEPC. The reaction was stopped after 5 (▲) or 30 min (●) of treatment by imidazole dilution as described in the Experimental section. Aliquots of 0.25 ml were filtered to determine the radioactive isotopes retained by the filter. Open circles corresponding to the Ca^{2+} binding data of untreated vesicles were fitted to the two distinct and interacting sites model. The Ca^{2+} binding data of the 30 min DEPC treatment (●) were fitted to the two interacting (broken line) or two independent (solid line) sites equations. Since the traces were perfectly superimposed they were plotted slightly shifted to allow the visualization. The set of constants used to draw the curves were: 10.4 and 0.3 μM (untreated vesicles); 2.5 and 8.3 μM (interacting sites in treated vesicles); 1.34 and 16 μM (independent sites in treated vesicles).

scribed by eqn. (3). We also applied this analytical procedure to samples derivatized for 30 min with DEPC (Figure 3, closed circles) since the effect on Ca^{2+} binding was more evident. The fitting was now performed by considering the binding equation for interacting sites (eqn. 3) or that for independent sites (eqn. 4). As can be seen, the curves generated for both models can satisfy the equilibrium binding data with the same degree of confidence. When the experimental data are interpreted according to the mechanism of interacting sites (broken line), the dissociation constants are $K'_{a1} = 2.5 \mu\text{M}$ and $K'_{a2} = 8.3 \mu\text{M}$. Alternatively, the binding data can be consistent with independent sites of equal amplitude $N_1 = N_2 = 3.16$ nmol of Ca^{2+} /mg of protein, with $K_{a1} = 1.34 \mu\text{M}$ and $K_{a2} = 16 \mu\text{M}$ (solid line). Since this procedure does not provide a unique response we devised some additional experiments in order to discriminate between the two mechanisms.

Steady-state experiments on ATP hydrolysis and Ca^{2+} transport were unsuitable to provide information on the coupling phenomenon due to the Ca^{2+} leakage of the vesicles after the DEPC treatment (data not shown). This drawback can be overcome by performing pre-steady-state measurements, since the modified vesicles are still able to transport limited amounts of Ca^{2+} . The experimental approach consists of the simultaneous addition of ATP and EGTA to the enzyme, which has already been preincubated with Ca^{2+} . ATP causes phosphorylation of the enzyme, pushing the catalytic cycle in the forward direction, and EGTA chelates and eventually dissociates Ca^{2+} , preventing further turnovers. We initially selected conditions (1 mM ATP and 0.1 mM EGTA) in DEPC-modified vesicles leading to the transport of ~ 8 nmol of Ca^{2+} /mg of protein. The derivatized vesicles were then preincubated at subsaturating Ca^{2+} concentrations before the addition of ATP and EGTA. By using the

Figure 4 Pre-steady-state experiments of Ca²⁺ transport and ATP hydrolysis in DEPC-treated vesicles

(a) The modification reaction was performed for 5 min in the presence of 7 mM DEPC. Aliquots of this medium were supplemented with different concentrations of EGTA and used for the functional assays. The Ca²⁺ transport (●) was measured in the presence of ⁴⁵Ca²⁺ by adding an equal volume (0.6 ml) of medium containing EGTA and ATP. The ATP hydrolysis was followed by the release of [³²P]P_i (○) measured under the same experimental conditions although [³²P]ATP was now the radioactive tracer. See the Experimental section for complete details of the reaction media. (b) Plot of Ca²⁺ transport versus P_i release measured at different pCa to calculate the coupling factor.

proper tracer (⁴⁵Ca²⁺ or [³²P]ATP) we could study the effect of varying the Ca²⁺ concentration on Ca²⁺ transport or ATP hydrolysis (Figure 4a). The Ca²⁺ transport profile can be described by a K_{0.5} of 155 nM and *h* = 1.01 whereas the P_i release presents a K_{0.5} of 190 nM and *h* = 0.98. The stoichiometric ratio between Ca²⁺ transport and P_i release in the presence of different free Ca²⁺ concentrations can be obtained from Figure 4(b). The coupling between both processes is given by the slope of the straight line (1.93). These data clearly show that after the treatment to eliminate the cooperativity in Ca²⁺ binding, the Ca²⁺:ATP coupling remains unaltered (~2) within the Ca²⁺ concentration range used to saturate the binding sites.

Time resolution of Ca²⁺ binding was studied by a rapid filtration technique in the presence of 50 μM free Ca²⁺. The binding kinetics of native vesicles shows a typical saturation

Figure 5 Kinetics of Ca²⁺ dissociation from the ATPase transport sites

SR vesicles were first derivatized for 30 min in the presence of 7 mM DEPC. After stopping the reaction with an imidazole medium, the incubation mixture consisted of 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 1 mM [³H]glucose, 0.1 mg of DEPC-treated protein/ml, 100 μM ⁴⁵Ca²⁺, 0.0512 mM EGTA, and 10 μM A23187. Aliquots of 1 ml were subjected to rapid filtration. The perfusion medium was 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM imidazole, 1 mM glucose and 1 mM EGTA (●). ⁴⁵Ca²⁺ and ³H on the filters were measured by scintillation counting. Parallel experiments were performed with untreated vesicles as a control (○).

profile similar to others previously reported [5,34]. The kinetic pattern of DEPC-treated vesicles shows a lower level of saturation, as expected from the equilibrium experiments; however, the kinetic profile is exactly the same as in the native samples (data not shown).

The Ca²⁺ dissociation kinetics were studied in SR vesicles equilibrated with 50 μM free Ca²⁺. This assay was accomplished by washing the vesicles with a medium containing 1 mM EGTA. The EGTA-dependent Ca²⁺ dissociation in SR vesicles incubated in the presence of a Ca²⁺ ionophore exhibits monophasic kinetics. Under the experimental conditions described in Figure 5 (open circles), the apparent rate constant has a value of approximately 13 s⁻¹. In this connection, we found the appearance of biphasic kinetics in Ca²⁺ dissociation after the DEPC treatment (closed circles in Figure 5). Half of the Ca²⁺ leaves the sites with an apparent rate constant of ~25 s⁻¹ whereas the other half corresponds to a slow component with an apparent rate constant of ~0.8 s⁻¹.

The Ca²⁺-binding sites can be experimentally distinguished by isotopic exchange [5,12]. The appearance of fast and slow components of Ca²⁺ exchange after partial labelling is illustrated in the inset of Figure 6. Labelling of the internal site (half of the sites) can be obtained by equilibration of the SR vesicles with ⁴⁵Ca²⁺ and then by controlled substitution of ⁴⁰Ca²⁺ for ⁴⁵Ca²⁺ at the external site. Thus, ⁴⁵Ca²⁺ at the internal site undergoes a slow substitution by ⁴⁰Ca²⁺ (open circles). However, when the radioactive Ca²⁺ labels the external site, a very fast exchange rate can be observed (open squares). Therefore, the Ca²⁺ exchange after addition of 1 mM ⁴⁰Ca²⁺ to SR vesicles equilibrated with saturating ⁴⁵Ca²⁺ shows a biphasic profile. This behaviour is also observed when the binding sites are partially saturated by Ca²⁺ [12]. We used SR vesicles previously treated with DEPC and then equilibrated in the presence of different free Ca²⁺ concentrations. The addition of the exchange medium (1 mM ⁴⁰Ca²⁺) to samples saturated with ⁴⁵Ca²⁺ at pCa 4.3 (closed circles in Figure 6)

Figure 6 Kinetics of Ca^{2+} exchange in derivatized SR with fractional saturation of the binding sites

SR vesicles were pretreated for 30 min with 7 mM DEPC. The chemical process was arrested and the reaction medium was supplemented with different EGTA concentrations to yield pCa values of 4.3 (●), 5.8 (▲), or 6.2 (■) as detailed in the Experimental section. Aliquots (1 ml) of the resulting media (each containing a specific free Ca^{2+} concentration), were layered on a filter and perfused with the aid of a rapid filtration apparatus. The flushing medium was 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl_2 , 5 mM imidazole, 1 mM glucose and 1 mM CaCl_2 . Ca^{2+} bound to the protein was deduced from the radioactive tracers retained by the filters. Inset, Ca^{2+} exchange was measured in native vesicles with partially labelled Ca^{2+} -saturated ATPase. The species $\text{E}^{45}\text{Ca}^{40}\text{Ca}$ (○) and $\text{E}^{40}\text{Ca}^{45}\text{Ca}$ (□) with the internal or the external labelled Ca^{2+} were generated as specified in the Experimental section. Rapid perfusion with a medium containing 50 mM Mops, pH 6.8, 80 mM KCl, 5 mM MgCl_2 , 1 mM glucose and 1 mM CaCl_2 evinced single exchange (fast or slow) components.

reveals biphasic kinetics with two components of equal amplitude. Half of the sites exhibit rapid exchange and the other half present a lower rate of exchange. Interestingly, this feature is observed even at submaximal saturation of the Ca^{2+} sites. Other kinetic profiles shown in this Figure correspond to samples previously equilibrated at pCa 5.8 (closed triangles) or pCa 6.2 (closed squares).

DISCUSSION

Our DEPC derivatization experiments included a reagent/ATPase molar ratio of approximately 1000:1, and the reaction was performed at room temperature. These drastic conditions preclude establishing any direct relationship between a defined structural change and a specific functional effect. In fact, the large excess of reagent makes possible irreversible bis-carbathoxylation and reaction with residues other than histidine [13]. However, this is a very convenient tool to manipulate the mechanism of Ca^{2+} binding to the Ca^{2+} -ATPase protein. The selected assay conditions may also explain some

apparent discrepancies of results with respect to stoichiometry, nature of the modification, or enzymic inhibition effect, when the Ca^{2+} -ATPase from SR was treated with DEPC [20–22]. It must be stressed that the observed fast-reacting amino acids associated with the loss of enzymic activity amount to ~ 103 nmol of reactive residues/mg of protein. This value corresponds to the sum of the fast- and the slow-reacting populations (106 nmol/mg of protein) obtained under milder experimental conditions [22].

In analysing our data of the non-cooperative Ca^{2+} isotherm we considered two different binding models accounting for sites that can be interacting or independent. The key question is to know how the loss of cooperative binding affects the individual Ca^{2+} -site affinities. We show that the fitting procedure does not provide a unique response and therefore it is not possible to distinguish between $K'_{d1} = 2.5 \mu\text{M}$ and $K'_{d2} = 8.3 \mu\text{M}$ [interacting sites, described by eqn. (3)] versus $K_{d1} = 1.34 \mu\text{M}$ and $K_{d2} = 16 \mu\text{M}$ [independent sites, described by eqn. (4)]. We assumed a 1:1 ratio [N_1 and N_2 values in eqn. (4)] between the amplitude of the two Ca^{2+} sites in the independent sites model, although deviation from this ratio may allow fitting with a completely different set of constants. Thus, by using $N_1 = 5.3$ nmol/mg of protein and $N_2 = 3.6$ nmol/mg of protein, it was concluded [22] that the sites are independent after the DEPC treatment, keeping the same binding affinities of the cooperative model ($K_{d1} = 14 \mu\text{M}$ and $K_{d2} = 0.5 \mu\text{M}$).

In the prestationary Ca^{2+} transport experiments, the sample filtration was performed a few seconds after the addition of the ATP/EGTA medium to allow the phosphoenzyme decomposition and the subsequent release of Ca^{2+} inside the vesicles. This value was correlated with the corresponding P_i released under the same experimental conditions. These assays confirm that the Ca^{2+} :ATP coupling is 2:1, independent of the Ca^{2+} concentration used to saturate the sites. Therefore, after elimination of the positive cooperativity, the enzyme still requires two Ca^{2+} to be phosphorylated by ATP at any Ca^{2+} concentration, as occurs with vesicles in the native state [3,11]. It means that even at the lower Ca^{2+} concentrations some ATPase molecules will be able to bind two Ca^{2+} . Assuming independent sites, the first Ca^{2+} site is expected to saturate at lower Ca^{2+} concentrations. This implies that the enzyme will be unable to be phosphorylated by one Ca^{2+} or alternatively a 1 Ca^{2+} :1 ATP coupling ratio must be expected. None of these possibilities is confirmed in Figure 4.

The mechanism of Ca^{2+} binding to the Ca^{2+} -ATPase has been analysed recently with respect to the Ca^{2+} dissociation kinetics [35]. This study shows that the cooperative character of Ca^{2+} binding in native SR vesicles is associated with a decrease in the dissociation constant of the second Ca^{2+} as the external Ca^{2+} concentration increases. Simulations also show that the referred rate constant is expected to increase in the absence of cooperativity (two sites with the same K_d). From the EGTA-induced Ca^{2+} dissociation of Figure 5 it is clear that the DEPC modification slowed down the exit of the internal (first ion to bind and second ion to release) Ca^{2+} , suggesting that the Ca^{2+} sites remain interdependent after derivatization.

Another important clue to confirm the Ca^{2+} binding model was provided by the partial isotopic exchange at the Ca^{2+} sites. This is a biphasic process when performed in untreated vesicles preincubated with saturating $^{45}\text{Ca}^{2+}$ and when the washing medium contains 1 mM $^{40}\text{Ca}^{2+}$ [5,12]. This biphasic profile was also obtained in DEPC-treated vesicles saturated with $^{45}\text{Ca}^{2+}$ or under different degrees of saturation. The 1:1 ratio between the fast and the slow component of Ca^{2+} exchange under conditions of partial Ca^{2+} saturation and in the absence of positive cooperativity can be explained in terms of the equilibrium constants by assuming two sites with the same K_d and no

interaction (eqn. 5) or two interacting sites with very close dissociation constants (eqn. 3). This latter possibility is consistent with the Ca²⁺ dissociation experiments. By contrast, in the independent sites model (eqn. 4) what can be expected is that the high-affinity site will fill selectively at lower Ca²⁺ concentrations and thereby the ⁴⁵Ca²⁺–⁴⁰Ca²⁺ exchange profile will contain a single (fast or slow) component. This is not supported by the present results.

The isotopic exchange experiments indicate an acceleration in the external (first ion to release) Ca²⁺ exchange after the chemical modification. The rapid exchange phase in treated vesicles (main frame of Figure 6), provides a k_{-2} of 25 s⁻¹ whereas the k_{-2} value is 10 s⁻¹ in untreated vesicles (fast component in the inset of the figure). This latter value of k_{-2} was also obtained by using the same protocol described in the main frame of Figure 6 but using native vesicles (data not shown). This increase in k_{-2} and the observed decrease in k_{-1} from the Ca²⁺ dissociation experiments are consistent with a decrease in K_{d1} and an increase in K_{d2} , as predicted by our model.

In conclusion, the chemical treatment with DEPC appears to modify the nature of the Ca²⁺ sites, producing an alteration in the Ca²⁺-binding affinities and a loss of the binding cooperativity. As a consequence, the Ca²⁺ sites present similar K_d s and act as interdependent sites, retaining the topology of the native enzyme, i.e. two binding domains with external (cytosolic) and internal (luminal) positions. It is suggested that the Ca²⁺ binding affinities are only sensitive to Ca²⁺ in the native enzyme and therefore, the binding of Ca²⁺ to the first site will increase the binding affinity of the second site.

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REFERENCES

- MacLennan, D. H., Clarke, D. M., Loo, T. W. and Skerjanc, I. S. (1992) *Acta Physiol. Scand.* **146**, 141–150
- Inesi, G., Lu, L., Kirtley, M. E. and Takeyasu, K. (1994) *Cell. Physiol. Biochem.* **4**, 135–147
- Inesi, G., Kurzmack, M., Coan, C. and Lewis, D. E. (1980) *J. Biol. Chem.* **255**, 3025–3031
- Fernandez-Belda, F., Kurzmack, M. and Inesi, G. (1984) *J. Biol. Chem.* **259**, 9687–9698
- Inesi, G. (1987) *J. Biol. Chem.* **262**, 16338–16342
- Forge, V., Mintz, E. and Guillaud, F. (1993) *J. Biol. Chem.* **268**, 10953–10960
- Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) *Nature (London)* **339**, 476–478
- Skerjanc, I. S., Toyofuku, T., Richardson, C. and MacLennan, D. H. (1993) *J. Biol. Chem.* **268**, 15944–15950
- Sumbilla, C., Cantilina, T., Collins, J. H., Malak, H., Lakowicz, J. R. and Inesi, G. (1991) *J. Biol. Chem.* **266**, 12682–12689
- Sumbilla, C., Lu, L., Lewis, D. E., Inesi, G., Ishii, T., Takeyasu, K., Feng, Y. and Fambrough, D. M. (1993) *J. Biol. Chem.* **268**, 21185–21192
- Petithory, J. R. and Jencks, W. P. (1988) *Biochemistry* **27**, 5553–5564
- Orlowski, S. and Champeil, P. (1991) *Biochemistry* **30**, 352–361
- Miles, E. W. (1977) *Methods Enzymol.* **47**, 431–442
- Morris, D. L. and McKinley-McKee, J. S. (1972) *Eur. J. Biochem.* **29**, 515–520
- Burstein, Y., Walsh, K. A. and Neurath, H. (1974) *Biochemistry* **13**, 205–210
- Carrillo, N. and Vallejos, R. H. (1983) *Biochemistry* **22**, 5889–5897
- Kirley, T. L. (1988) *J. Biol. Chem.* **263**, 12682–12689
- Cheng, K. C. and Nowak, T. (1989) *J. Biol. Chem.* **264**, 19666–19676
- Pelton, P. D. and Ganzhorn, A. J. (1992) *J. Biol. Chem.* **267**, 5916–5920
- Coffey, R. L., Lagwinska, E., Oliver, M. and Martonosi, A. (1975) *Arch. Biochem. Biophys.* **170**, 37–48
- Tenu, J. P., Ghelis, C., Saint Leger, D., Carrette, J. and Chevalier, J. (1976) *J. Biol. Chem.* **251**, 4322–4329
- Coan, C. and DiCarlo, R. (1990) *J. Biol. Chem.* **265**, 5376–5384
- Eletr, S. and Inesi, G. (1972) *Biochim. Biophys. Acta* **282**, 174–179
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Fabiato, A. and Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Schwartzbach, G., Senn, H. and Anderegg, G. (1957) *Helv. Chim. Acta* **40**, 1886–1900
- Blinks, J., Wier, W., Hess, P. and Prendergast, F. (1982) *Prog. Biophys. Mol. Biol.* **40**, 1–114
- Ovádi, J., Libor, S. and Elödi, P. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung.* **2**, 455–458
- Lin, T. and Morales, M. (1977) *Anal. Biochem.* **77**, 10–17
- Davidson, G. A. and Berman, M. C. (1988) *J. Biol. Chem.* **263**, 11786–11791
- Champeil, P. and Guillaud, F. (1986) *Biochemistry* **25**, 7623–7633
- Orlowski, S. and Champeil, P. (1991) *Biochemistry* **30**, 11331–11342
- Dupont, Y. and Moutin, M. J. (1987) *Methods Enzymol.* **148**, 675–683
- Caravaca, F., Soler, F., Gomez-Fernandez, J. C. and Fernandez-Belda, F. (1995) *Arch. Biochem. Biophys.* **318**, 97–104
- Henderson, I. M. J., Starling, A. P., Wictome, M., East, J. M. and Lee, A. G. (1994) *Biochem. J.* **297**, 625–636