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Ca^{2+} binding to sarcoplasmic reticulum ATPase phosphorylated by P_i reveals four thapsigargin-sensitive Ca^{2+} sites in the presence of ADP

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Abstract

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase was phosphorylated by P_i at pH 8.0 in the presence of dimethyl sulfoxide (Me_2SO). Under these conditions, it was possible to measure transient $^{45}Ca^{2+}$ binding to the phosphoenzyme. Binding reached 1.2 Ca^{2+} per phosphoenzyme (E-PCa_x) within 10 min in 30% Me_2SO, 20 mM $MgCl_2$ and 0.1 mM P_i and the phosphoenzyme only decreased by 23% during this period. This Ca^{2+} binding was abolished by thapsigargin, showing that it is associated with functional sites of the Ca^{2+}-ATPase. At 40% Me_2SO, simultaneous addition of Ca^{2+} and ADP increased Ca^{2+} binding up to almost four Ca^{2+} per phosphoenzyme (ADPE-PCa_y), revealing a species bearing simultaneously four Ca^{2+} sites. Both E-PCa_x and ADPE-PCa_y were further identified as distinct species by (2′,3′-*O*-2(2,4,6-trinitrophenyl)adenosine 5′-triphosphate) fluorescence, which revealed long-range modifications in the Ca^{2+}-transport sites induced by ADP binding to E-P. In addition, E-PCa_x was shown to be a functional intermediate of the cycle leading to ATP synthesis provided that Me_2SO was diluted. These findings indicate that more than two functional Ca^{2+}-sites exist on the functional Ca^{2+}-ATPase unit, and that the additional sites become accessible upon ADP addition. This is compatible with a four-site model of the SR Ca^{2+}-ATPase allowing simultaneous binding of Ca^{2+} at lumenal and cytosolic sites. The stoichiometries for Ca^{2+} binding found here could either be interpreted as binding of four Ca^{2+} on a Ca^{2+}-ATPase monomer considered as the functional unit or as binding of two Ca^{2+} per monomer of a functional dimer.

Keywords: Ca^{2+}-ATPase; Sarcoplasmic reticulum; Calcium binding site; Phosphorylation by P_i; ADP; TNP-ATP; Dimethyl sulfoxide

1. Introduction

The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase catalyzes active uptake of Ca^{2+} across the SR membrane with a stoichiometry of two Ca^{2+} ions transported per ATP hydrolyzed. For a number of years, the prevailing model for Ca^{2+} translocation proposed the interconversion of two high-affinity Ca^{2+} sites facing the cytosol into two low-affinity sites facing the lumen [1,2] (for a review, see Ref. [3]). Such a change in orientation and affinity for both Ca^{2+} sites should require conformational modifications of some Ca^{2+}-ATPase domains, particularly those involved in Ca^{2+} binding and translocation [4]. Interconversion between two main conformations has been proposed in more than one model. These conformations, often denoted as E_1 and E_2, are phosphorylated by ATP or P_i in forward and reverse cycles, respectively [2]. A crucial feature of the E_1/E_2 model is that the high- and low-affinity Ca^{2+}-binding sites are mutually exclusive, with the high-affinity sites being accessible to Ca^{2+} in the unphosphorylated enzyme and the low-affinity sites in the phosphorylated enzyme.

However, there is evidence that the E_1/E_2 model with only one pair of interconverting sites may not be sufficient to explain all events mediated by the Ca^{2+}-ATPase during Ca^{2+} transport (for details see Refs. [5,6]). For instance, during the initial phase of Ca^{2+} uptake into sarcoplasmic reticulum vesicles (SRV), Mészáros and Bak [7,8] observed simultaneous binding of two Ca^{2+} on the cytoplasmic side during internalization of two other Ca^{2+} bound to the

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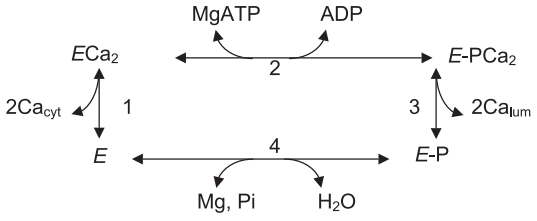
phosphoenzyme. This means that during Ca^{2+} uptake, there is an intermediate species which bears more than two Ca^{2+} ions, particularly at millimolar lumenal Ca^{2+} concentrations. On the other hand, Jencks and co-workers have shown that, on the lumenal side of the membrane, Ca^{2+} ions bind with low affinity to dephosphorylated Ca^{2+}-ATPase [9,10]. This indicates that the low-affinity sites are different from, and coexist with, the high-affinity sites detected in the unphosphorylated ATPase. More recently, Webb et al. [11] presented evidence that both unphosphorylated and phosphorylated forms of Ca^{2+}-ATPase bind Ca^{2+} from the lumenal side with similar affinities.

The simplified model of Scheme 1, which does not include the E_1/E_2 notation, only refers to the chemical state of the Ca^{2+}-ATPase and shows the main steps of the catalytic cycle.

The aim of this work was to measure Ca^{2+} binding directly when the Ca^{2+}-ATPase is phosphorylated by inorganic phosphate (P_i), i.e., when the Ca^{2+} ions bind with low affinity (step 3 in Scheme 1). To do this, we chose conditions that slow the dephosphorylation rate to allow Ca^{2+} to bind to *E*-P before dephosphorylation has occurred. Then, by changing the conditions, we sought evidence for or against the existence of an intermediate species having more than two Ca^{2+} bound. We describe here the transient binding of $^{45}Ca^{2+}$ to the Ca^{2+}-ATPase previously phosphorylated by P_i in the presence of 30% dimethyl sulfoxide (Me_2SO) at pH 8.0. Under these conditions the phosphoenzyme was stable and slowly bound Ca^{2+} up to 8.5±1.5 nmol/mg (at 100 μM free Ca^{2+}) with a high enough affinity to allow direct measurement of $^{45}Ca^{2+}$ binding. Increasing Me_2SO to 40% further stabilized the phosphoenzyme [12] and simultaneous addition of $^{45}Ca^{2+}$ and ADP increased the amount of Ca^{2+} bound up to 22 nmol/mg at 100 μM free Ca^{2+}. Moreover, we found that the catalytic site was sensitive to Ca^{2+} and ADP binding to the phosphoenzyme, as revealed by changes in the fluorescence of 2′,3′-*O*-2(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP). Once Me_2SO was diluted, the phosphoenzyme derived from P_i and having bound 8.5 nmol/mg of Ca^{2+} could transfer its covalently bound phosphate to ADP, indicating that it is a functional intermediate of the Ca^{2+}-ATPase cycle.

The results are discussed in terms of four-site models in which the four Ca^{2+} binding sites either belong to a Ca^{2+}-ATPase monomer or belong to two Ca^{2+}-ATPase monomers arranged in a functional dimer.



Scheme 1.

2. Materials and methods

2.1. Materials

ATP, ionophore A23187, EGTA, NADP and thapsigargin were from Sigma-Aldrich (Saint-Quentin Fallavier, France). $^{45}CaCl_2$, ^{3}H-glucose and $^{32}P_i$ were from Amersham Biosciences (Saclay, France) and Me_2SO was from Merck (Darmstadt, Germany). Acetate cellulose filters for $^{45}Ca^{2+}$-binding were DAWP (0.65 μm) and for synthesized ATP measurements were Millex (0.45 μm), both from Millipore (Saint-Quentin en Yvelines, France); glass-fiber filters for phosphoenzyme determination were A/E from Pall-Gelman (Saint-Germain en Laye, France). ADP, hexokinase and glucose-6-P dehydrogenase were from Roche Molecular Biochemicals (Meylan, France). All other reagents were of the greatest purity available.

2.2. General procedures

SRV were prepared and tested as described in Ref. [13]. All experiments were carried out at room temperature (22–26 °C) in 100 mM Tes-Tris buffer (pH 8.0). Unless otherwise specified, phosphorylation was achieved by incubating 0.3 mg/ml SRV in the presence of 40 μM EGTA, 0.1 mM P_i (or $^{32}P_i$, as indicated), 20 mM $MgCl_2$ and 30% (v/v) Me_2SO for 15 min, a time that is sufficient to reach equilibrium in spite of the slow rate of phosphorylation imposed by the presence of the cosolvent [14]. Vesicles were made leaky by addition of calcimycin (A23187) as specified. Free Ca^{2+} concentrations were calculated using the BAD program [15]. Cross-linking of the Ca^{2+}-ATPase was done following the method described by Ross and McIntosh [16] and phosphoenzyme formed from $^{32}P_i$ was determined by filtration as in Ref. [12].

2.3. Ca^{2+} binding to phosphoenzyme formed from P_i (E-PCa_x)

Ca^{2+}-binding levels were measured by filtration. SRV (0.2–0.4 mg/ml) were first phosphorylated for 15 min as above except that non-radioactive P_i was used and 1 mM ^{3}H-glucose was added to determine the wet volume of the filters (25–35 μl). Then the SRV suspended in phosphorylation buffer were supplied with $^{45}Ca^{2+}$ and filtered at the desired times. ^{3}H and ^{45}Ca retained on the filters were counted simultaneously by scintillation. The $^{45}Ca^{2+}$ contained in the wet volume was subtracted from the total $^{45}Ca^{2+}$ to evaluate the Ca^{2+} bound to the Ca^{2+}-ATPase.

2.4. Kinetics simulation

Data from Fig. 1B, in which both Ca^{2+} binding and phosphoenzyme are measured, have been simulated with Kinsim [17] according to Scheme 2. A nonspecific binding of Ca^{2+} of 1 nmol/mg improved the simulation of the Ca^{2+}

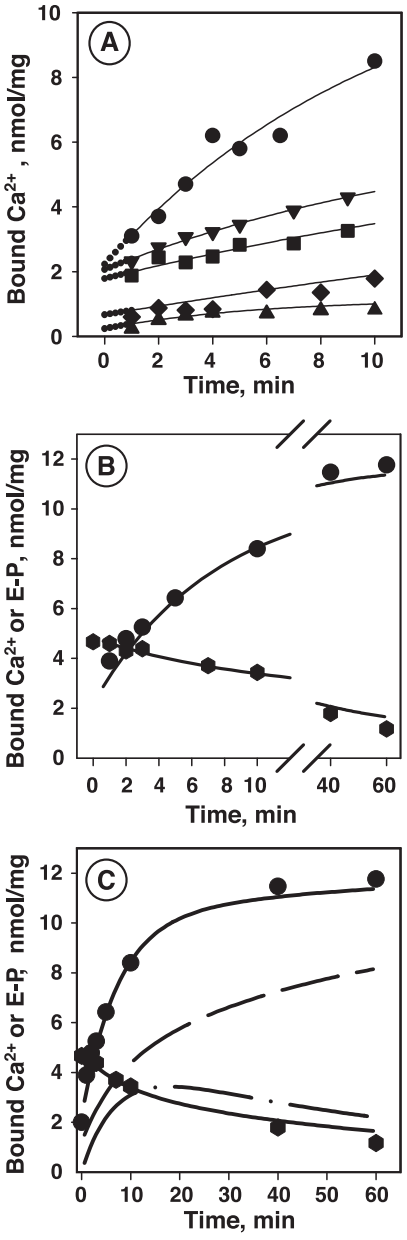
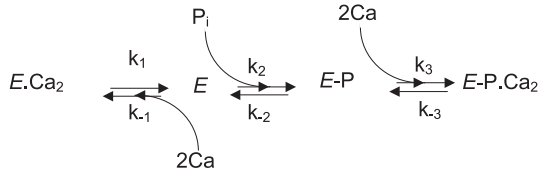


Fig. 1. $^{45}Ca^{2+}$ binding to *E*-P and stability of Ca^{2+}-ATPase phosphorylated by P_i. (A) Leaky SRV (0.3 mg/ml+6 μg/ml A23187) were phosphorylated by non-radioactive P_i as described under Materials and methods. Five minutes after phosphorylation reached equilibrium (time 0 on the abscissa), the samples were supplied with enough $^{45}CaCl_2$ to give the following free Ca^{2+} concentrations (in μM): 1 (▲), 10 (♦), 30 (■), 60 (▼) and 100 (●). At the times indicated on the abscissa, 1-ml aliquots were filtered to measure Ca^{2+} binding. Continuous lines are best fits using exponential rises to evaluate the initial burst of Ca^{2+} binding (dotted lines) by extrapolation to time 0. (B) Leaky SRV were phosphorylated and Ca^{2+} binding was measured as in (A) after addition of 100 μM Ca^{2+}. In a separate experiment, SRV were phosphorylated by $^{32}P_i$ under the same conditions and phosphoenzyme levels (⬣) were measured after addition of 100 μM non-radioactive free Ca^{2+}. (C) The experiment shown in B is simulated according to Scheme 2. Bound Ca^{2+} (●) is simulated by the Ca^{2+} content in ($E.Ca_2$+E-PCa_2) and phosphoenzyme level (⬣) by (E-P+E-PCa_2). The contributions of ECa_2 (dashed line) and E-PCa_2 (dashed-dotted line) to the total Ca^{2+} content are also shown.



Scheme 2.

measurements for short times. This nonspecific binding is nearly equivalent to binding to non-phosphorylated enzyme at t=0. However, because the nonspecific Ca^{2+} binding we measured in the presence of thapsigargin never exceeded 1 nmol/mg, we have maintained 1 nmol/mg E at t=0. In addition, the rate constants k_1 and k_{-1} do not influence the simulation as long as k_{-1} (Ca^{2+} binding) is fast in comparison to the other rate constants and as long as the dissociation rate constant k_1 is 100-fold higher than k_{-1}. The best fit (lines in Fig. 1C) has been obtained with the following rate constants: k_1=0.1 min^{-1}, k_{-1}=10 min^{-1}, k_2=10^3 min^{-1}, k_{-2}=0.05 min^{-1}, k_3=0.06 min^{-1}, k_{-3}=0.05 min^{-1}.

2.5. Thapsigargin sensitivity of Ca^{2+} binding to E-P

Leaky SRV (0.3 mg/ml+12 μg/ml A23187) were first phosphorylated with either $^{32}P_i$ or non-radioactive P_i (100 μM) as described above, and 15 min later 10 μM thapsigargin was added. Then, in the first case, phosphoenzyme was measured at the times indicated on the abscissa of Fig. 2 and in the second case, after a 45-min incubation in thapsigargin, the samples were supplied with 100 μM free $^{45}Ca^{2+}$. Aliquots were removed at different times to measure Ca^{2+} binding. In another series of experiments, leaky SRV

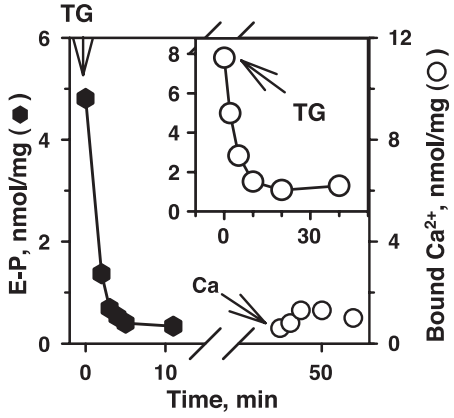


Fig. 2. Thapsigargin inhibits Ca^{2+} binding in the presence of Me_2SO and P_i, revealing specific binding of Ca^{2+} to *E*-P. Leaky SRV (0.3 mg/ml+6 μg/ml A23187) were first phosphorylated by P_i for 15 min and then additions were made as shown by the arrows: TG, 10 μM thapsigargin; Ca, 100 μM free $^{45}Ca^{2+}$. Inset: leaky SRV were first phosphorylated by P_i, then supplied with 100 μM free $^{45}Ca^{2+}$ and 2 min later with 10 μM thapsigargin (arrow at time 0). Dissociation of ^{45}Ca bound was evaluated at the times shown on the abscissa. For details, see Section 2.5.

were phosphorylated by P_i and supplied with 100 μM free $^{45}Ca^{2+}$. After 2 min, an aliquot was removed to measure Ca^{2+} binding and 10 μM thapsigargin was immediately added. Aliquots were then taken to measure thapsigargin-induced $^{45}Ca^{2+}$ dissociation from the enzyme. The thapsigargin-insensitive (i.e., nonspecific) Ca^{2+} binding in the presence of Me_2SO and P_i was less than 1 nmol Ca^{2+}/mg.

Although thapsigargin is soluble in Me_2SO, it still binds to the membrane at the Me_2SO concentrations used in the present work (30% and 40%). It has been shown that thapsigargin and Me_2SO have different and independent effects on the SR Ca^{2+}-ATPase even when added together [18].

2.6. Fluorescence of TNP-ATP

Spectra of bound TNP-ATP (10 μM in the assay solution) were recorded under different conditions which lead to formation of different Ca^{2+}-ATPase intermediates. These were (1) Ca^{2+}-deprived enzyme (*E*): leaky SRV (0.2 mg/ml+6 μg/ml A23187) incubated in 100 mM Tes-Tris buffer (pH 8.0), 30% Me_2SO, 20 mM $MgCl_2$ and 40 μM EGTA; (2) phosphoenzyme formed from P_i (*E*-P): addition of 100 μM P_i to *E* and spectrum acquired 10 min later; (3) phosphoenzyme with bound Ca^{2+} (*E*-PCa_x): addition of 100 μM free Ca^{2+} to *E*-P and spectra acquired at different times as indicated; (4) unphosphorylated ATPase with bound Ca^{2+} (ECa_2): incubation conditions as for *E* plus 100 μM free Ca^{2+}. For all fluorescence measurements, excitation was set at 408 nm and for kinetic recordings, emission was measured at 522 nm (Figs. 5A and 7).

2.7. ATP synthesis from E-PCa_x and ADP after dilution of Me_2SO

Leaky SRV (3 mg/ml+60 μg/ml A23187) were first phosphorylated by 100 μM P_i for 15 min in 100 mM Tes-Tris, pH 8.0, 30% Me_2SO, 20 mM $MgCl_2$, 40 μM EGTA and 5 mM glucose. Then 138 μM $CaCl_2$ (100 μM free Ca^{2+}) was added, and after 10 min the reaction mixture was diluted 10-fold in 100 mM Tes-Tris, pH 8.0, 20 mM $MgCl_2$, 5 mM glucose, 104 μM $CaCl_2$ (to maintain 100 μM free Ca^{2+} after dilution) and 0.5 mM ADP plus 4 U/ml hexokinase (to avoid ATP hydrolysis by the Ca^{2+}-ATPase). Aliquots were removed after 1 min, quenched with 1 ml of 6 M HCl, neutralized with equimolar NaOH and centrifuged for 20 min at 500×*g*. Finally, aliquots of the supernatant were filtered and supplied with 0.2 mM NADP and 1.8 U/ml glucose-6-P dehydrogenase for spectrophotometric measurement of synthesized ATP. The amount of ATP was calculated from the increase in the absorbance of the stoichiometrically (1:1) formed NADPH, recorded at 340 nm (ε=6300 M^{-1} cm^{-1}).

The same mixture (hexokinase, glucose-6-P dehydrogenase, glucose and NADP) was used to determine whether our SRV preparation displayed any myokinase activity in the presence of $MgCl_2$ and ADP. This control experiment showed no ATP synthesis, and therefore no myokinase activity, under our conditions.

3. Results

3.1. Calcium binding to Ca^{2+}-ATPase phosphorylated by P_i in the presence of Me_2SO

In purely aqueous medium, addition of micromolar Ca^{2+} promotes rapid dephosphorylation of the phosphoenzyme (*E*-P) formed from P_i [19,20] (step 4 in Scheme 1). This is attributed to a shift of the $E\text{-P} \leftrightarrow E \leftrightarrow ECa_2$ equilibrium towards the ECa_2 species, induced by the binding of two Ca^{2+} ions to the high-affinity sites (steps 1 and 2 in Scheme 2).

According to the E_1/E_2 and related models [1,2], during this transition the two low-affinity Ca^{2+} sites, which face the SR lumen, are converted into high-affinity sites which face the cytoplasm. In the presence of high Me_2SO, *E*-P decays slowly after addition of Ca^{2+}. In addition, the Ca^{2+} concentration required to saturate low-affinity sites prior to ATP synthesis from *E*-P decreases from millimolar to submillimolar range when the pH is raised from 6.0 to 8.0 [14]. Thus, the use of high Me_2SO, pH 8.0 and a Ca^{2+} ionophore, in order not to limit Ca^{2+} access to putative internal Ca^{2+}-binding sites, seems to be appropriate to directly measure by filtration the binding of radioactive $^{45}Ca^{2+}$ to *E*-P (step 3 in Scheme 2).

Fig. 1A shows that under these conditions, and in the presence of 20 mM $MgCl_2$ to strongly favor phosphorylation by P_i, there was a slow concentration-dependent Ca^{2+} binding to leaky SRV previously phosphorylated by P_i. As will be shown below, Ca^{2+} binding to *E*-P was found to be sensitive to thapsigargin (Fig. 2) and the Ca^{2+}-bound *E*-P species detected this way was found a true intermediate of the cycle (Scheme 1), since it allowed ATP synthesis after Me_2SO dilution (Fig. 8).

In Fig. 1B, 10 min after addition of 100 μM free $^{45}Ca^{2+}$, 8.5±1.5 nmol of Ca^{2+} was bound per miligram of protein. Correction for thapsigargin-insensitive Ca^{2+} binding (Fig. 2) revealed that at least 7.5–8.0 nmol/mg was specifically bound to Ca^{2+} sites of the Ca^{2+}-ATPase. To determine which species these Ca^{2+} ions were bound to, the Ca^{2+}-induced dephosphorylation of *E*-P must be taken into account. The concentration of 100 μM Ca^{2+} used in Fig. 1B is sufficient to saturate the high-affinity Ca^{2+} binding sites at pH 8 [1–3] and the burst of 2 nmol/mg (extrapolation represented by dotted lines in Fig. 1A) probably represents Ca^{2+} binding to the unphosphorylated fraction of Ca^{2+}-ATPase. Ten minutes after addition of 100 μM free Ca^{2+}, *E*-P had slowly decreased from 4.5 to 3.4 nmol/mg (hexagons in Fig. 1B). Taking these crude numbers, the Ca^{2+}-ATPase species that could bind Ca^{2+} were estimated as 2 nmol/mg *E* (the unphosphorylated

ATPase) and 3.4 nmol/mg *E*-P. Therefore, about 4 nmol Ca^{2+}/mg in *E*-P coexist with about 4 nmol Ca^{2+}/mg in *E* 10 min after $^{45}Ca^{2+}$ addition. The distribution of Ca^{2+} binding between *E*-P and *E* changes slowly as *E*-P dephosphorylates towards *E* with a half-life of 20 min (Figs. 1B and 5B). Within 40–60 min, i.e., after total dephosphorylation, Ca^{2+} binding reached 12 nmol/mg. Therefore, Ca^{2+} binding in Fig. 1A and B could be described as the result of competition between Ca^{2+} binding to *E*-P and Ca^{2+} binding to *E* after dephosphorylation. The fastest event corresponds to a small initial burst of Ca^{2+} binding to the high affinity sites of *E*, the fraction of enzyme which has not been phosphorylated (step 1 in Scheme 2). This induces slow dephosphorylation of *E*-P (step 2 in Scheme 2). Simultaneously Ca^{2+} binds to low affinity sites of *E*-P. The final 12 nmol/mg of Ca^{2+} bound reached after 1-h dephosphorylation corresponds to the usual saturation stoichiometry of 10–12 nmol Ca^{2+}/mg because the overall equilibrium constant favors $E.Ca_2$ [13,20–23]. This final stoichiometry reinforces the view that in this experiment Ca^{2+} transiently binds to low affinity sites on *E*-P.

Numerical simulation of the data according to Scheme 2 clearly shows the competitive aspect of Ca^{2+} binding (see Section 2.4). Although no attempt was made to go into the details of the Ca^{2+} binding steps in the numerical simulation, a simple comparison between the equilibrium constants k_1/k_{-1}=0.01 for Ca^{2+} binding to *E* and k_{-3}/k_3=0.83 for Ca^{2+} binding to *E*-P shows that at equilibrium ECa_2 is predominant at 100 μM Ca^{2+}. However, when at t=0, the reaction starts from addition of Ca^{2+} to *E-P* and because the rate of Ca^{2+} binding to *E*-P (0.06 min^{-1}) is of the same magnitude as that of dephosphorylation (0.05 min^{-1}), there is a transient and significant binding of Ca^{2+} to *E*-P. The numerical simulation also shows that Ca^{2+} binding to *E*-P is slow (0.06 min^{-1} for 100 μM Ca^{2+}) in Me_2SO and appears much slower in Fig. 1, particularly for the lowest Ca^{2+} concentrations, because the main part of Ca^{2+} binding occurs after dephosphorylation which is a combination of k_{-3} and k_{-2} and therefore a very slow process. That duality in Ca^{2+} binding will be illustrated again in Fig. 5. If we assume that apparent dissociation constants can be evaluated from the rate constants of the numerical simulation as if Ca^{2+} binding were a simple reaction, calculation yields K_1=1 μM and K_3=83 μM, two values which are in agreement with what is known for the cytoplasmic and the lumenal sites [13,14]. When vanadate, a P_i analogue, was used, Ca^{2+} in the 100 μM range was also assumed to bind to lumenal sites on the monovanadate–enzyme complex [24]. In the experiments reported in Fig. 1, saturation of these low-affinity sites could not be reached because measurements of Ca^{2+} binding at concentrations higher than 300 μM $^{45}Ca^{2+}$ are not technically possible. Therefore, we could not experimentally reach a 2 Ca^{2+}/*E*-P stoichiometry for Ca^{2+} binding and the species was denoted as *E*-PCa_x.

Here, however, arises the question about the meaning of a stoichiometry of 10–12 nmol/mg for Ca^{2+} binding whereas about 4–5 nmol/mg represents the maximum measurable phosphorylation by ATP or P_i for a native and membranous enzyme. The molecular weight of the Ca^{2+}-ATPase is 109.4 kDa and our preparation of SR vesicles contains more than 90% Ca^{2+}-ATPase. This means that the theoretical stoichiometry for phosphorylation should be about 8 nmol/mg if all Ca^{2+}-ATPase monomers were active and phosphorylatable. Are half of the Ca^{2+}-ATPase molecules inactive in an SR preparation [25–27]? This has never been shown. A simple arithmetic rather favors a dimer as active enzyme unit in the SR membrane but, since it has been shown that after solubilisation, and therefore membrane structure disruption, the soluble monomer is active [28], the hypothesis of a functional dimer in the SR membrane has been rarely favored in the literature. This will be discussed below.

3.2. Thapsigargin sensitivity of Ca^{2+} binding to E-P in the presence of Me_2SO

To test the specificity of Ca^{2+} binding, we first checked that retention of insoluble CaP_i complexes on filters during the filtration experiments was not the source of apparent Ca^{2+} binding, particularly in 30–40% Me_2SO [29]. With 0.1 mM P_i, which promoted the formation of 4.5 nmol *E*-P/mg, i.e., nearly maximal phosphorylation (Figs. 1A, 5B, 6 and 8) and up to 1 mM $CaCl_2$, no precipitated CaP_i complex was detected either by light scattering or by filtration (data not shown). Fig. 2 shows experiments in which thapsigargin, the Ca^{2+}-ATPase specific inhibitor [30], was used to evaluate any nonspecific Ca^{2+} binding under our conditions. Ca^{2+}-ATPase was first phosphorylated with 0.1 mM P_i and once *E*-P had reached its maximal value of 4.8 nmol/mg, 10 μM thapsigargin was added (TG). Thapsigargin induced dephosphorylation and subsequently impaired Ca^{2+} binding when Ca^{2+} was added. The same result was obtained when Ca^{2+} was added to unphosphorylated enzyme previously treated with thapsigargin (data not shown). In a third experiment (Fig. 2, inset), thapsigargin induced dissociation of Ca^{2+} bound to previously phosphorylated SRV. For all thapsigargin-treated samples, Ca^{2+} binding at equilibrium was less than 1 nmol/mg, and was taken as the nonspecific fraction in calculating the amount of Ca^{2+} bound to *E*-P in Fig. 1. In another experiment, Ca^{2+} was first bound to the high-affinity sites of Ca^{2+}-ATPase in Me_2SO-containing medium in the absence of P_i. Further addition of 0.1 mM P_i did not modify Ca^{2+}-binding stoichiometry (10–12 nmol Ca^{2+}/mg; data not shown). Therefore, various controls, including those shown in Fig. 2, confirm that most of the Ca^{2+} bound during the first 10 min following the initial burst in Fig. 1A is Ca^{2+} bound to specific sites on *E*-P.

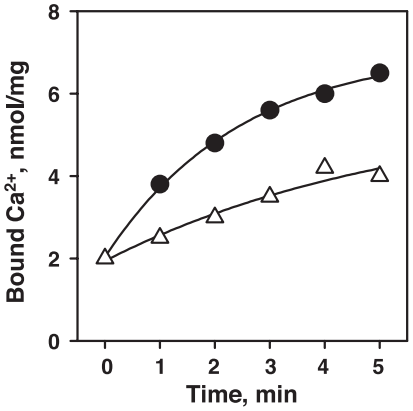


Fig. 3. Sidedness of Ca^{2+} binding to *E*-P: time course of Ca^{2+} binding to intact or A23187-treated SRV. Phosphorylation by non-radioactive P_i and Ca^{2+} binding (100 μM free Ca^{2+}) were carried out as in Fig. 1A, except that the Ca^{2+} ionophore was either present (●) or absent (△).

3.3. Sidedness of Ca^{2+} binding to E-P formed by P_i

Fig. 3 shows that the Ca^{2+} sites on *E*-P that are filled under the conditions of Fig. 1A are lumenal. In Fig. 1 experimental conditions included A23187, a Ca^{2+} ionophore that gives access to lumenal Ca^{2+} sites. When the experiment depicted in Fig. 1A with 100 μM free Ca^{2+} was carried out with intact vesicles, i.e., in the absence of ionophore (△), Ca^{2+} binding following the initial burst was much slower, probably reflecting the time necessary for Ca^{2+} to cross the SRV membrane and to enter the vesicles lumen. This reinforces the view that in Fig. 1 during the first 5 min, Ca^{2+} binding took place at lumenal sites of the phosphoenzyme formed from P_i. Extrapolation of the binding curves to time zero in Fig. 3 shows an amount of 2 nmol Ca^{2+}/mg, representing fast binding to cytoplasmic sites of 1 nmol/mg *E* as already shown in Fig. 1B. This burst is followed by Ca^{2+}-binding to the luminal sites of *E*-P at different rates with tight (△) or A23187-treated vesicles (●).

3.4. Conformational changes promoted by Ca^{2+} binding to E-P probed by TNP-ATP

To see whether Ca^{2+} binding to *E*-P induces modifications in the catalytic site, changes in the fluorescence of TNP-ATP, an ATP analogue, were examined. This nucleotide binds to the catalytic site with a dissociation constant K_d=0.1 μM [31]. It has been used to study polarity changes at the catalytic site upon phosphorylation by P_i [32]. The spectra in Fig. 4 confirm that the presence of Me_2SO does not change the main fluorescence characteristics of the interaction between TNP-ATP and the Ca^{2+}-ATPase (for species nomenclature see Materials and methods). The TNP-ATP*E* and TNP-ATP*E*Ca_2 complexes have the lowest fluorescence intensities and a maximum at 541 nm; TNP-ATP*E*-P has the highest fluorescence intensity and a maximum at 526 nm. Therefore, when these TNP-ATP complexes are compared to TNP-enzyme complexes in pure water [32], Me_2SO induces a general blue shift of about 10 nm.

Recording of spectra at different times after addition of 100 μM free Ca^{2+} to *E*-P shows that formation of *E*-PCa_x after 2 min is accompanied by a slightly lower fluorescence intensity and a red shift of 4 nm which brings the maximum to 530 nm. This decrease in fluorescence intensity is more pronounced after 10 min, with no change in the maximum. At 10 min approximately 80% of the initial phosphoenzyme is still present (Figs. 1B, filled hexagons and 5B, filled hexagons) and the TNP-ATP*E*-PCa_x spectrum cannot be fitted as a linear combination of those of TNP-ATP*E*-P and TNP-ATP*E*Ca_2. This specificity of the TNP-ATP*E*-PCa_x spectra recorded after 2 and 10 min indicates that Ca^{2+} binding to *E*-P is responsible for a conformational change at the TNP-ATP-binding site, which is known to be at least 40 Å away from the Ca^{2+}-binding sites [33].

Fig. 5A shows the kinetics of the fluorescence intensity decrease which occurs when Ca^{2+} is added to TNP-ATP*E*-P and Fig. 5B shows the concomitant Ca^{2+}-induced *E*-P breakdown in the absence of TNP-ATP. According to previous reports, nucleotides do not modify the rate of *E*-P dephosphorylation when Mg^{2+} is present [34], so that the experiments shown in Fig. 5 may be compared to each other. The fluorescence decrease is biphasic (Fig. 5A) and can be described by the sum of two exponentials with half-lives of 3 and 20 min. The slow component can be attributed to *E*-P dephosphorylation induced by Ca^{2+} binding, which fits a single exponential with a half-life of 21 min (Fig. 5B). Here, due to the fluorescence of bound TNP-ATP which reveals the different phosphoenzyme species, the two phases of Ca^{2+} binding clearly appear. Note that the fast component is at least six times faster than the slow one and this difference allows their clear separation. In Fig. 5A, 10 min after Ca^{2+} addition, the fast component of the fluorescence

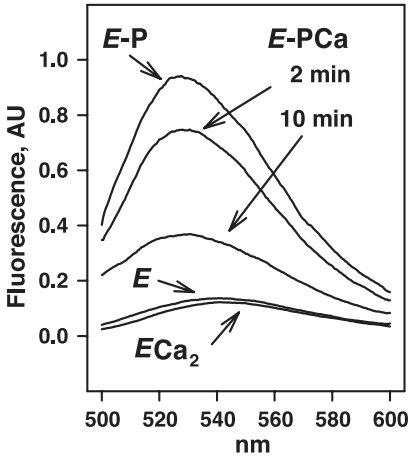


Fig. 4. Changes in TNP-ATP fluorescence spectra upon Ca^{2+} binding to *E*-P. Conditions were as described under Section 2.6. Successively, *E* (40 μM EGTA), *E*–P (after 10 min in the presence of 0.1 mM P_i), *E*-PCa (spectra were acquired 2 and 10 min after addition of 100 μM free Ca^{2+}), *E*Ca_2 (as for *E* plus 100 μM free Ca^{2+}). For all spectra, λ_{ex}=408 nm.

decrease is almost finished, although 3.4 nmol *E*-P/mg still remains. All this suggests that the fast fluorescence drop is due to Ca^{2+} binding to *E*-P.

3.5. Binding of ADP to E-PCa$_x$ allows occupancy of four Ca^{2+}-binding sites

Mészáros and Bak [7,8] have reported evidence that during the initial phase of a forward cycle, the Ca^{2+}-ATPase binds cytoplasmic Ca^{2+} during internalization of the Ca^{2+} ions which have initiated the cycle. These authors proposed that the Ca^{2+}-ATPase can bind Ca^{2+} simultaneously at cytoplasmic and lumenal sites. On the other hand, according to Myung and Jencks [35], the Ca^{2+}-ATPase must be ADP-sensitive when Ca^{2+} is bound at the lumenal sites. The experiment of Fig. 6 was designed to explore the possibility that the presence of ADP allows Ca^{2+} to occupy pre-existing but initially inaccessible Ca^{2+} sites. In this experiment we combined conditions to favor phosphoenzyme stability (40% Me_2SO [12]) and free ADP (5 mM $MgCl_2$ instead of 20 mM). Lower $MgCl_2$ concentration was necessary because *E*-P is sensitive to free ADP and not to MgADP [34]. We also used the purest available ADP to avoid traces of ATP which can induce ATPase turnover and Ca^{2+} accumulation, although ATPase turnover under these conditions is about 10 nmol/mg/min at 28 °C, i.e., 2–3 orders of magnitude slower than the physiological turnover.

Under these conditions, and probably because of a lower inhibition by Mg^{2+} at 5 mM than at 20 mM $MgCl_2$ at pH 8.0

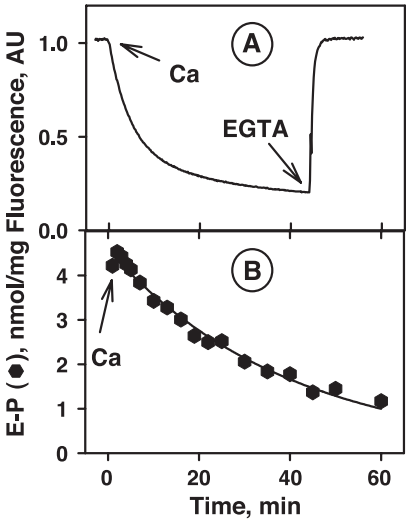


Fig. 5. Changes in TNP-ATP fluorescence and dephosphorylation upon binding of Ca^{2+} to *E*-P, λ_{ex}=408 nm and λ_{em}=522 nm. (A) Leaky SRV (0.2 mg/ml+6 μg/ml A23187) were phosphorylated and TNP-ATP added as in Fig. 4. Ca^{2+} binding to *E*-P was initiated after 15 min (time 0 on the abscissa) by addition of 100 μM free Ca^{2+} and 45 min later Ca^{2+} was removed by addition of 2 mM EGTA. Fluorescence decay was fitted by two exponentials: $0.55\exp(-0.23t)+0.30\exp(-0.043t)+0.16$. (B) Leaky SRV as in A were phosphorylated with 100 μM $^{32}P_i$ for 15 min, 100 μM free Ca^{2+} (Ca) was added 5 min later (t=0 on the abscissa) and *E*-P levels were measured. *E*-P (●) decay was fitted by a single exponential: $4.1\exp(-0.033t)+0.6$.

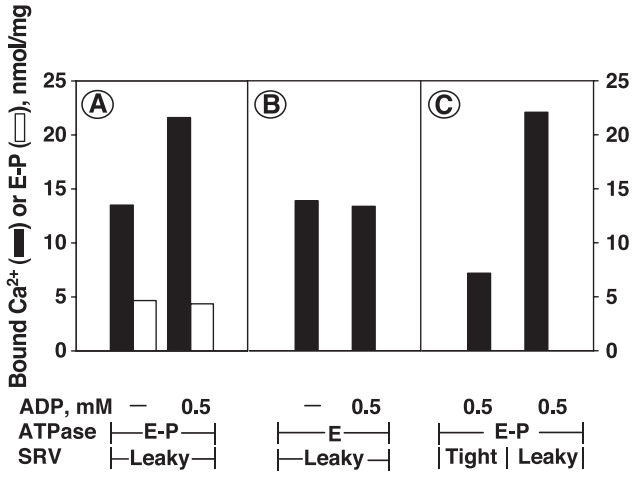


Fig. 6. Effects of ADP on Ca^{2+} binding to *E*-P or *E* and sidedness of Ca^{2+} binding. (A) Leaky SRV (0.3 mg/ml+6 μg/ml A23187) were phosphorylated as described under Section 2.2, except that here 40% Me_2SO and 5 mM $MgCl_2$ were present. Ca^{2+} binding was initiated by addition of 100 μM free $^{45}Ca^{2+}$ alone or 100 μM free $^{45}Ca^{2+}$ plus 0.5 mM ADP, and bound Ca^{2+} (solid bars) and *E*-P levels (empty bars) were measured 1 min later. (B) Ca^{2+} binding to unphosphorylated Ca^{2+}-ATPase under the same conditions as in A, except for the absence of P_i. (C) Ca^{2+} binding to *E*-P measured 1 min after addition of 100 μM free $^{45}Ca^{2+}$ plus 0.5 mM ADP in the absence or presence of A23187, as indicated.

[36], Ca^{2+} bound faster to the P_i-derived phosphoenzyme (data not shown) and a stable level of bound Ca^{2+} was reached at 1 min. The amount of Ca^{2+} bound to *E*-P significantly increased from 13 to 22 nmol Ca^{2+}/mg in the presence of 0.5 mM ADP (Fig. 6A), whereas there was no nucleotide effect with the use of unphosphorylated Ca^{2+}-ATPase (*E*) (Fig. 6B). In a control experiment conducted with glutaraldehyde cross-linked Ca^{2+}-ATPase, which cannot be phosphorylated by P_i [37], Ca^{2+} binding was the same in the absence or presence of ADP (data not shown). It should be mentioned that in 40% Me_2SO and 0.5 mM ADP, the Ca^{2+}-ATPase remained almost fully phosphorylated throughout the assay (Fig. 6A). Therefore, this experiment confirms that 40% Me_2SO impairs phosphoryl transfer to ADP to synthesize ATP [14] and showed that under these conditions, Ca^{2+}-binding reached nearly 4 Ca^{2+}/*E*-P. Such extra binding is not likely to come from CaADP because there were 100 μM Ca^{2+} and 5 mM $MgCl_2$ in the medium (MgADP/CaADP>100). Therefore, this extra binding is probably Ca^{2+} binding to extra Ca^{2+} sites, revealed by the binding of ADP to *E*-PCa$_x$. This new species is now denoted as ADP*E*-PCa$_y$, where y stands for higher stoichiometry of Ca^{2+} binding. Fig. 6C also shows that the ADP-induced additional Ca^{2+}-binding to *E*-P, measured 1 min after Ca^{2+} addition, is much lower in tight vesicles, adding support to the view that Ca^{2+} binds to *E*-P from the lumenal side of the membrane (see also Fig. 3). It is interesting to note here that binding of ADP could increase the affinity for Ca^{2+} as it leads the cycle towards ATP synthesis and, at the same time, changes the low affinity Ca^{2+} sites in high affinity sites. This effect favors Ca^{2+}

binding and could increase an apparent stoichiometry. However, according to the E_1/E_2 model Ca^{2+} binding could never exceed 10–12 nmol/mg proteins, i.e., 2 Ca^{2+}/E-P, whereas in Fig. 6 Ca^{2+} binding reaches 22 nmol/mg, i.e., 4 Ca^{2+}/E-P (y=4).

3.6. Changes in TNP-ATP Fluorescence upon Addition of ADP to E-PCa$_x$

When repeated in the presence of TNP-ATP and 30% Me_2SO instead of 40% to avoid SRV coalescence and optical problems, the effect of ADP on Ca^{2+}-binding to the E-P complex is followed by recording the TNP-ATP fluorescence at fixed wavelengths. Fig. 7 shows that Ca^{2+} alone (panel A) or ADP alone (panel B) induced a decrease in the TNP-ATP fluorescence of TNP-ATPE-P, whereas the presence of both ADP and Ca^{2+}, added either together (panel C) or sequentially (panels A and B; see figure legend), caused a significant increase in the fluorescence signal. In all three traces, the fluorescence increase was reversed upon chelation of free Ca^{2+} by EGTA, and the fluorescence decreased to the low TNP-ATPE level when dephosphorylation was induced by removal of Mg^{2+} by EDTA (EG and ED, respectively, as indicated by arrows in Fig. 7). Although it does not give any information about Ca^{2+}-binding stoichiometry, this experiment is in line with the observation that in the presence of Me_2SO, the Ca^{2+}-ATPase remained phosphorylated after Ca^{2+} and ADP addition (Fig. 6A). It also implies that ADP has a significant effect on E-P and E-PCa$_x$. It should also be recalled that, up to now, all interpretations of TNP-nucleotide fluorescence consider the low fluorescence level (E and ECa$_2$ in Fig. 4) as reporting Ca^{2+}-ATPase conformations having a hydrophilic nucleotide site. In contrast, the high fluorescence level (E-P in Fig. 4) reports Ca^{2+}-ATPase conformations having a hydrophobic nucleotide site [31,32]. Fig. 7 suggests that, when the concentration of Me_2SO is high enough to inhibit ATP synthesis, simultaneous binding of Ca^{2+} and ADP to TNP-ATPE-P drives the enzyme molecules in the SR membrane into a conformation in which the nucleotide sites have an unexpectedly high hydrophobicity.

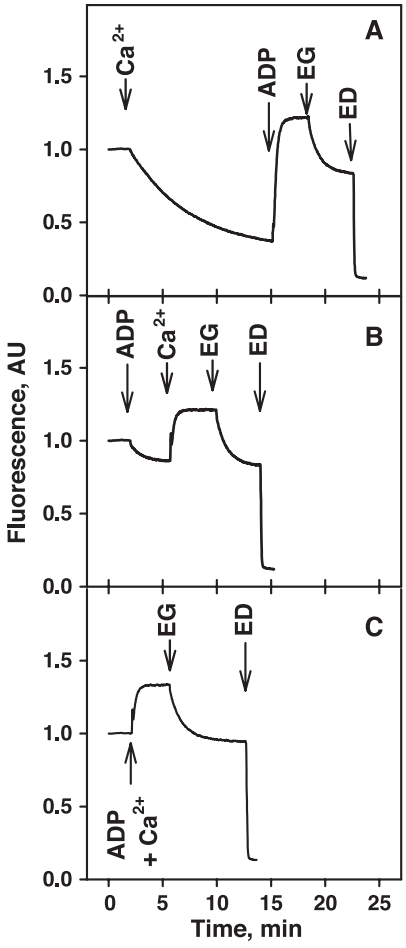


Fig. 7. Changes in TNP-ATP fluorescence upon addition of Ca^{2+} and ADP to E-P. Leaky SRV (0.3 mg/ml+6 μg/ml A23187) were incubated with TNP-ATP and phosphorylated from P_i during 15 min as described under Materials and methods, and fluorescence has been measured as in Fig. 5A. When phosphorylation reached a plateau, a baseline was maintained at an arbitrary fluorescence level F=1. Fifteen minutes after addition of P_i (2 min on the abscissa), 100 μM free Ca^{2+} and 0.5 mM ADP were added as follows: (A) first Ca^{2+} and ADP 13 min later; (B) first ADP and Ca^{2+} 4 min later; (C) Ca^{2+} and ADP simultaneously. Then, for all three traces, Ca^{2+} was removed by addition of 2 mM EGTA (EG) and Mg^{2+} was removed by addition of 25 mM EDTA (ED).

Here again, it is interesting to note that the crystallographic structure published by Toyoshima et al. [33] only shows one nucleotide binding site. In Fig. 7, at 10 μM TNP-ATP, a concentration which is supposed to saturate the unique ATP binding site, the fluorescence of the TNP-ATPE complex (in the presence or absence of Ca^{2+}) is sensitive to ADP (Fig. 7A, B and C). The solving to this apparent contradiction probably resides in the structure (monomers, dimers or equilibrium between monomers and dimers) of the functional unit of the Ca^{2+}-ATPase. This point will be also discussed below.

3.7. The E-PCa$_x$ complex formed in Me_2SO is a functional intermediate of the reverse cycle

Fig. 8 shows that a 10-fold dilution of Me_2SO allowed the E-PCa$_x$ intermediate formed after incubation of E-P with 100 μM Ca^{2+} to transfer its phosphoryl group to ADP, thus synthesizing ATP. When the dilution was carried out keeping the free Ca^{2+} concentration at 100 μM, there was as much ATP synthesized as E-P formed (compare panels A and B in Fig. 8). Therefore, in the presence of ADP, the phosphorylated intermediate which had bound nearly four Ca^{2+} per E-P (Fig. 6) accumulated because the high Me_2SO concentration impaired its phosphoryl transfer to ADP [14]. In Fig. 8, formation of ATP after Me_2SO dilution was measured by trapping its γ-phosphoryl group as glucose-6-phosphate, thereby precluding Ca^{2+}-ATPase turnover. The

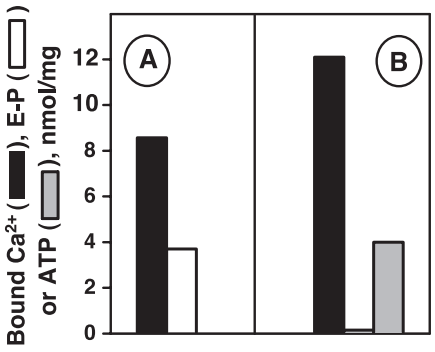


Fig. 8. ATP synthesis from E-PCa$_x$ and ADP. (A) Measurement of either E-P, using $^{32}P_i$ and non-radioactive Ca^{2+} or Ca^{2+} binding, using $^{45}Ca^{2+}$ and non-radioactive P_i. Leaky SRV (0.3 mg/ml+6 μg/ml A23187) were first phosphorylated by P_i in the presence of 30% Me_2SO for 15 min, then supplied with 100 μM free Ca^{2+}; Ca^{2+} binding (solid bar) or E-P (empty bar) was measured 10 min later. (B) ATP synthesis. Leaky SRV (3 mg/ml+60 μg/ml A23187) were phosphorylated and supplied with Ca^{2+} as in (A). After 10-min incubation of E-P in 100 μM free Ca^{2+}, the reaction mixture was diluted 10-fold in the same medium, except for omission of Me_2SO and addition of 0.5 mM ADP, glucose and hexokinase. Ca^{2+} binding, E-P and newly synthesized ATP (gray bar) were measured 1 min after dilution. For details, see Section 2.7.

dephosphorylated enzyme recovered its micromolar affinity for Ca^{2+} and bound 12 nmol Ca/mg.

4. Discussion

Under appropriate conditions favoring both P_i phosphorylation (Me_2SO) and Ca^{2+} binding (pH 8.0), we were able to directly measure transient $^{45}Ca^{2+}$ binding to the phosphoenzyme derived from P_i (E-PCa$_x$). This is, to our knowledge, the first direct measurement of Ca^{2+} binding to E-P, probably because of the low affinity of E-P for Ca^{2+} and the instability of E-P in the presence of Ca^{2+} in purely aqueous medium [19,20]. The sidedness of Ca^{2+} binding to E-P (Fig. 3), the response to ADP (Figs. 6 and 7) and the functionality of E-PCa$_x$ (Fig. 8) indicate that Ca^{2+} binding to E-P occurs at lumenal low-affinity sites.

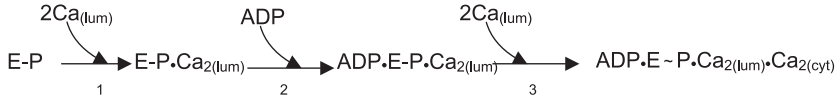
In the present work, measurement of a transient binding of Ca^{2+} to the lumenal sites of E-P has been possible because in the presence of Me_2SO the rate of Ca^{2+} binding to E-P occurred at a rate comparable to the dephosphorylation rate (k_3 and k_{-2} in Scheme 2) at 100 μM Ca^{2+}. It is known that Me_2SO modifies protein conformations [38] and substrate binding rates [39], two changes that can decrease the overall rate of enzyme-catalyzed reactions as shown for phosphorylation of the SR Ca^{2+}-ATPase by P_i [14] and Ca^{2+} transport in SRV [40]. According to various reports, these phenomena can be related to modifications in the protein hydration and to solvent-induced perturbations of substrate-binding sites [41–43].

Even in Me_2SO, E-P is not stable in the presence of Ca^{2+} and Ca^{2+} binding to E-P cannot be measured at equilibrium. Still, we were able to measure a transient stoichiometry of about 1.2 Ca^{2+}/E-PCa$_x$ and because the crystallographic structure of the Ca^{2+}-ATPase shows two Ca^{2+} binding sites [33], it is reasonable to assume that x=2. We also propose the value y=4 for the stoichiometry of Ca^{2+} in the ADPE-PCa$_y$ complex. There are not many values in the literature that can be compared with the 22 nmol/mg measured in Fig. 6, but if 10–12 nmol Ca^{2+} bound per millligram of protein corresponds to one pair of Ca^{2+} sites, it is reasonable to propose that 22 nmol/mg corresponds to two pairs of Ca^{2+} sites, i.e., y=4.

ADP is supposed to increase the apparent affinity of E-P for Ca^{2+} [14] by pulling the enzyme phosphorylated by P_i towards a conformation that allows ATP synthesis after a water jump [44]. This could be one reason for higher Ca^{2+} binding in the presence of ADP. However, the increase in Ca^{2+} binding that could result from this change in affinity is limited to 10–12 nmol Ca^{2+}/mg, the maximal occupancy of the high-affinity Ca^{2+}-binding sites. Therefore, among the 22 nmol/mg Ca^{2+} bound after ADP addition (Fig. 6), at least 10–12 nmol/mg is due to binding to an additional pair of sites, according to Scheme 3.

In Fig. 6, it can be assumed that Me_2SO inhibits phosphoryl transfer and ADP binding to E-P promotes sequential movement of two Ca^{2+} from low- to high-affinity sites (step 3 in Scheme 3), thus leaving the lumenal sites vacant and accessible for two additional Ca^{2+} ions. This movement of Ca^{2+} from low- to high-affinity sites (i.e., from high energy potential to low energy potential [45]) could provide the energy necessary for ATP synthesis [35] after dilution of Me_2SO [14] with a tight stoichiometry of 1 E-P/ATP (Fig. 8). Due to the coupling between Ca^{2+} movement within the ATPase molecule and simultaneous interconversion of the phosphoenzyme forms (E-P→E~P), ADP can readily interact with the high-energy phosphorylated intermediate and ATP is synthesized [35]. This is the reason why Ca^{2+} ions do not dissociate from the low-affinity sites and P_i is not released to the medium when dilution takes place.

The fluorescence of TNP-ATP bound to E-P reveals that the catalytic site of the Ca^{2+}-ATPase responds to lumenal Ca^{2+} binding by a modification in its hydrophobicity, which is different in E-P, E-PCa$_x$ and ADPE-PCa$_y$. Therefore, these three phosphorylated species are chemically different. Webb et al. [11] have shown that binding of nucleotides to the ATPase promotes conformational changes in the lumenal Ca^{2+} sites of the SR membrane. This observation



Scheme 3.

can relate to the increase in Ca^{2+} binding observed herein upon ADP addition to form the ADP · *E*-P · $Ca_{2(lum)}$ · $Ca_{2(cyt)}$ complex (or ADP*E*-PCa_y) depicted in Scheme 3. The formation of this transient intermediate can therefore be associated with the increase in fluorescence shown in Fig. 7, when both Ca^{2+} and ADP are present.

As mentioned above, several groups have proposed more than two Ca^{2+} sites in the Ca^{2+}-ATPase [5–11]. Jencks et al. [9] and Myung and Jencks [10,35], in their studies about the influence of lumenal Ca^{2+} on the phosphorylation by P_i, came to the conclusion that the Ca^{2+}-ATPase has two pairs of independent Ca^{2+}-binding sites. Mészáros and Bak [7,8], to explain simultaneous Ca^{2+} binding and Ca^{2+} internalization, also proposed the coexistence of four Ca^{2+} sites. In the same line, Webb et al. [11] have shown that chemical modification of the Ca^{2+}-ATPase by 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide inhibits lumenal Ca^{2+} binding whereas the cytoplasmic Ca^{2+}-binding sites remained unchanged. These authors also came to the conclusion that Ca^{2+} ions bind to two pairs of sites.

More recently, Champeil et al. [46] described a stable phosphoenzyme having a Ca^{2+} binding stoichiometry greater than 10 nmol/mg and which may be related to the phosphoenzyme described here. They obtained this phosphoenzyme by the reaction of a fluorescein 5′ -isothiocyanate-labeled Ca^{2+}-ATPase with either acetyl phosphate or P_i. This stable FITC-labeled phosphoenzyme—i.e., a conformation similar to the ADP-bound form found herein—bound 10 nmol Ca/mg to high-affinity Ca^{2+}-binding sites, and bound an extra amount of 5 nmol/mg after addition of 50 μM free Ca^{2+}. This extra binding was still increasing after 4 min and was attributed to part of a single turnover accompanying a small enzyme dephosphorylation. Instead, because under our experimental conditions there is no turnover, we propose that the functional unit of the Ca^{2+}-ATPase possesses two pairs of functional Ca^{2+} sites to explain the extra binding shown in Fig. 6. These results are in agreement with the hypothesis that *E*-P with Ca^{2+} bound to lumenal sites is able to react with ADP during the reversal cycle, as proposed by Myung and Jencks [35].

Even though the stoichiometry shown in Fig. 6C agrees with the proposal of simultaneous occupancy of four Ca^{2+} sites in the phosphoenzyme (lumenal and cytosolic pairs of sites), the fact that the protein is only 50% active based on phosphorylation measurements deserves additional consideration. We already mentioned that a maximal phosphorylation level of 4.5–5 nmol/mg (Figs. 1A and 6A) roughly corresponds to 50% of the Ca^{2+} pump units in SRV [47]. The remaining 50% are not without biological activity since they can bind nucleotide analogues to reach a maximal binding ratio of 1 mol/mol ATPase [26] and can alternate with the other phosphorylated units during the catalytic cycle [27]. This hypothesis particularly applies to the present work in Fig. 7 in which the phosphoenzyme having bound TNP-ATP, supposedly at the catalytic site, is still sensitive to ADP. In addition, interactions between phosphorylated and non-phosphorylated subunits have been postulated to be needed for the proper function of the Ca^{2+}-ATPase in the SR membrane [27,48]. Thus, an alternative hypothesis to a monomeric Ca^{2+}-ATPase considered as the functional unit could be a dimeric Ca^{2+}-ATPase in which each monomer would possess one pair of Ca^{2+} sites alternatively facing the cytoplasm or the lumen.

In the presence of ADP, after movement of the first pair of Ca^{2+} ions from lumenal to cytosolic sites (with simultaneous *E*-P→*E*~P transition), the second pair of Ca^{2+} ions could enter the lumenal sites of a different, adjacent, non-phosphorylated Ca^{2+}-ATPase molecule. Interactions between phosphorylated and non-phosphorylated subunits could be modulated by simultaneous binding of Ca^{2+} to lumenal and cytosolic sites of different subunits of a dimeric Ca^{2+}-ATPase during the transient part of the cycle, represented by step 3 in Scheme 1. In the present work, it is possible that ADP modifies the fluorescence signal of TNP-ATP (Fig. 7) by binding to a neighboring non-phosphorylated subunit, where it could accept the phosphoryl group during the reversal cycle (Fig. 8). These conclusions are compatible with the proposals that the minimal functional unit of the Ca^{2+}-ATPase is a dimer with the nucleotide-binding sites facing each other in close proximity [26] and that their interactions are needed to improve free energy exchange and catalytic performance [27,48].

Acknowledgements

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