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The vectorial specificity for calcium binding to the CaATPase of sarcoplasmic reticulum is controlled by phosphorylation, not by an E–E* conformational change

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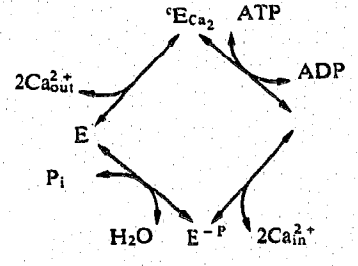
The E–E* model for calcium pumping by the CaATPase of sarcoplasmic reticulum includes two distinct conformational states of the enzyme, E and E*. Exterior Ca^{2+} binds only to E and interior Ca^{2+} binds only to E*. Therefore, it is expected that there will be competition between the binding of calcium to the unphosphorylated enzyme from the two sides of the membrane. The equilibrium concentration of $^cE_{Ca_2}$, the enzyme with Ca^{2+} bound at the exterior site, was measured at different Ca^{2+} concentrations with empty sarcoplasmic reticulum vesicles (SRV) and with SRV loaded with 40 mM Ca^{2+} by reaction with 0.5 mM [γ-^{32}P]ATP plus 20 mM EGTA for 13 ms (100 mM KCl, 5 mM $MgSO_4$, 40 mM Mops/KOH, pH 7.0, 25°C). The sigmoidal dependence on free exterior calcium concentration of the concentration of $^cE_{Ca_2}$, measured as [^{32}P]phosphoenzyme, is identical with empty and loaded SRV, within experimental error. The value of $K_{0.5}$ is 2.8 μM, and the Hill coefficient is 2. This result shows that there is no competition between binding of Ca^{2+} to the outside and the inside of the membrane. This is consistent with a model in which the vectorial specificity for calcium binding is controlled by the chemical state of the enzyme, rather than a simple conformational change. It is concluded that there are not two interconverting forms of the free enzyme, E and E*, instead the vectorial specificity for binding and dissociation of Ca^{2+} is determined by the state of phosphorylation of the CaATPase.

Calcium ATPase; Vectorial specificity; Calcium binding

1. INTRODUCTION

The calcium ATPase of SR pumps Ca^{2+} from the cytoplasm of muscle to the inside of the SR in order to bring about muscle relaxation [1]. The CaATPase uses the energy derived from the hydrolysis of ATP to pump Ca^{2+} into the vesicles and to maintain the steep Ca^{2+} concentration gradient that is formed across the membrane. We are interested in understanding the mechanism of coupling between a chemical process, the hydrolysis of ATP, and a vectorial process, the transport of two Ca^{2+} ions. This coupling has usually been described by the E–E* model, or a similar E–E′ model [2,3], in which the specificities for catalysis of chemical and vectorial steps are determined by different conformations of the enzyme. The E–E* model includes two distinct conformational states of the enzyme, E and E*: the high affinity Ca^{2+} binding site of E faces the outer surface of the vesicle and the low affinity Ca^{2+} binding site of E* faces the inner surface of the vesicle [2]. It appears that several predictions of certain of these models are not consistent with experimental results [4–6].

We have suggested that the coupling is accounted for by changes in chemical and vectorial specificities that are determined by the chemical state of the enzyme, as defined in eqn 1 [7].



eqn 1

Calcium acts as a chemical switch that determines whether the enzyme reacts with ATP or P_i, and covalently bound phosphate acts as a vectorial switch that determines whether the enzyme binds and dissociates calcium at the outside or the inside surface of the vesicle. The model of eqn 1 was first proposed by Makinose in 1973 [8].

If the vectorial specificity for the binding of calcium to the outside or inside of the enzyme is controlled by a change in conformation between E and E*, as described by eqn 2 [1,9,10], then there will be competition between the binding of calcium to the un-

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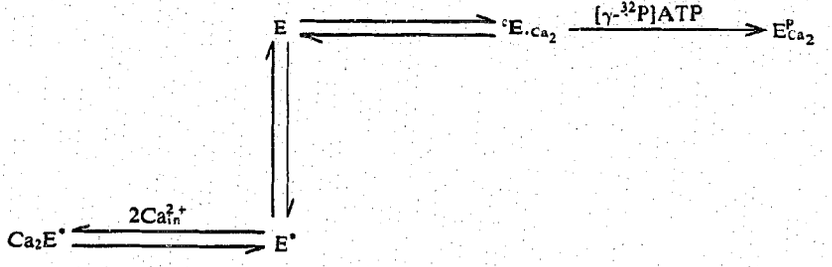
Abbreviations: SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles; E, calcium adenosinetriphosphatase; EGTA, ethylene glycol bis(beta-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid



eqn 2

phosphorylated enzyme from the two sides of the membrane, as shown in eqn 2. However, if the vectorial specificity for calcium binding and dissociation is controlled by the state of phosphorylation of the enzyme, as described in eqn 1, there will be no such competition in the absence of ATP or P_i. We report here that there is no such competition, within experimental error. This result is consistent with a model in which the vectorial specificity for calcium binding is controlled by the phosphorylation state of the enzyme, as described by eqn 1.

2. EXPERIMENTAL

Tightly sealed sarcoplasmic reticulum vesicles were prepared from rabbit white back and hind leg muscles by a slight modification of the procedure of MacLennan [11], as described previously [12], and were stored at −80°C. The SRV preparations were ~95% sealed, as shown by a 20–25-fold increase in the steady state activity upon the addition of A23187 in the standard assay. $[E]_{tot}$ was taken as the amount of [^{32}P]phosphoenzyme formed after a 5-s reaction of loaded and empty vesicles with ~15 μM free Ca_{out} and 0.5 mM [γ-^{32}P]ATP. The concentrations of free calcium were calculated from an apparent dissociation constant of 3.9×10^{-7} M for CaEGTA [13] using the computer program of Fabiato and Fabiato [14].

The SRV preparations were dialyzed at 4°C overnight against 400 ml of solutions containing 0.4 M sucrose, 100 mM KCl, 5 mM $MgSO_4$, 40 mM Mops/KOH, pH 7.0, and either no added Ca^{2+} or 40 mM Ca^{2+}. For each reaction, 10 μl of this stock solution of SRV was mixed with 0.99 ml of a CaEGTA-buffered solution to lower the free exterior Ca^{2+} concentration to the desired concentration, and was loaded into syringe A of a rapid mixing apparatus with three syringes [5,15]. The reaction was started within 10 s. The amount of radiolabeled phosphoenzyme was measured essentially as described by Verjovski-Almeida et al. [9].

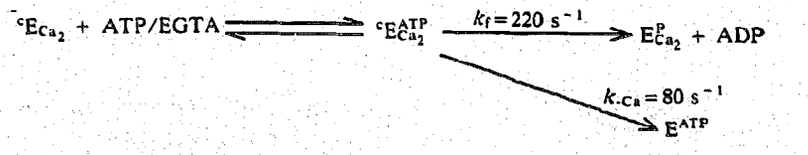
3. RESULTS AND DISCUSSION

3.1. *Assay of* $^cE_{Ca_2}$

The concentration of $^cE_{Ca_2}$, the stable form of the enzyme with bound Ca^{2+}, was measured by the addition of a solution containing [γ-^{32}P]ATP and EGTA to the vesicles, followed in 13 ms by an acid quench. Under the conditions of this assay, $^cE_{Ca_2}$ is phosphorylated with a rate constant of 220 s^{-1} and Ca^{2+} dissociates to give inactive enzyme with a rate constant of 80 s^{-1} (eqn 3). Thus, $^cE_{Ca_2}$ disappears rapidly, with a rate constant of 220 + 80 = 300 s^{-1}, and $E^P_{Ca_2}$ is formed with a yield of 220/300 = 73% [16].

3.2. *Measurement of the amount of* $^cE_{Ca_2}$, *the ATP-reactive species, from empty and loaded SRV*

Formation of the ATP-reactive species after the addition of Ca^{2+} to empty SRV and SRV loaded with 40 mM Ca^{2+} was assayed by reaction with 0.5 mM [γ-^{32}P]ATP plus 20 mM EGTA for 13 ms, as described above. Fig. 1 shows that the sigmoidal dependence on free exterior calcium concentration of the concentration of $^cE_{Ca_2}$, measured as phosphoenzyme, is identical with empty and loaded SRV, within experimental error. The line drawn in Fig. 1 is the best fit to the open and closed circles with a Hill coefficient of $n = 2$, $K_{0.5} = K_{ov}^{1/2} = 2.8$ μM, and $K_{ov} = 8.0 \times 10^{-12}$ M^2, based on free calcium concentrations that were calculated from $K_{diss} = 3.9 \times 10^{-7}$ M for CaEGTA [13]. This result shows that the competition between the binding of calcium to the unphosphorylated enzyme from the two sides of the membrane, according to the E–E* model and eqn 2, is not observed. The result is not consistent with a prediction based on the assumption that reactivity of the enzyme toward exterior or interior Ca^{2+} is determined by the conformation of the free enzyme, as suggested by the E–E* model. It is concluded that there are not two interconverting forms of the free enzyme, E and E*. Instead, the vectorial specificity for binding of Ca^{2+} to the CaATPase is determined by the chemical state of the enzyme. Ca^{2+} binds and dissociates at the high affinity cytoplasmic site only with free enzyme and at the low affinity inside site only with phosphoenzyme, as shown in eqn 1. There is no clear evidence for a change in vectorial specificity of the phosphoenzyme, or for the existence of more than a single species of the



eqn 3

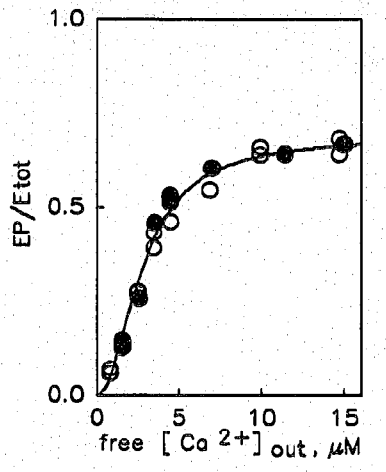


Fig. 1. Dependence on exterior calcium concentration of the equilibrium concentration of $^{c}E_{Ca_2}$, measured as [^{32}P]phosphoenzyme, with empty SRV (○) and loaded SRV (●). Empty and passively loaded ($[Ca]_{in}$ = 40 mM) vesicles were incubated in CaEGTA buffers for 10 s to give the indicated free $[Ca]_{out}$ and then reacted with 0.5 mM [γ-^{32}P]ATP plus 20 mM EGTA for 13 ms, followed by an acid quench. Syringes A and B contained 40 mM Mops/KOH, pH 7.0, 100 mM KCl, and 5 mM $MgSO_4$ at 25°C. In addition, syringe A contained 0.19 mg/ml empty SRV, 20 mM $CaCl_2$, and either 31 mM (0.80 μM), 25.8 mM (1.5 μM), 23.6 mM (2.5 μM), 22.6 mM (3.5 μM), 22.0 mM (4.5 μM), 21.3 mM (6.9 μM), 20.9 mM (9.9 μM), or 20.6 mM (14.7 μM) EGTA to give the final free $[Ca^{2+}]_{out}$ indicated in parentheses. Alternatively, syringe A contained 0.19 mg/ml passively loaded SRV, 20 mM $CaCl_2$, and either 26.3 mM (1.5 μM), 24.0 mM (2.5 μM), 23.0 mM (3.5 μM), 22.5 mM (4.5 μM), 21.7 mM (7.0 μM), 21.2 mM (11.4 μM), or 21.0 mM (15.0 μM) EGTA to give the final free $[Ca]_{out}$ indicated in parentheses. The contribution of Ca^{2+} from the medium of loaded SRV was considered to calculate free $[Ca]_{out}$. Syringe B in these experiments contained 1.0 mM [γ-^{32}P]ATP plus 20 mM EGTA. Syringe C contained 1.5 M HCl and 40 mM KH_2PO_4. The line is drawn for $K_{0.5} = K_{ov}^{1/2} = 2.8\,\mu M$ and a Hill coefficient of 2.0, obtained from a Hill plot of the data. $[E]_{tot}$ of empty and loaded SRV was 2.34 nmol/mg.

phosphorylated enzyme with bound calcium. It is well known that the calcium in $E^{P}_{Ca_2}$ is occluded, so that it does not dissociate to the outside of the vesicle unless the enzyme is dephosphorylated [17]. The calcium ions dissociate from $E^{P}_{Ca_2}$ to the inside of the vesicle with an overall rate constant of ~20–30 s^{-1}.

Petithory and Jencks [5] have reported previously that the presence or absence of interior calcium has little or no effect on the burst size or the rate constant of k_{obsd} = 38–40 s^{-1} for the binding of calcium to form ATP-reactive enzyme upon the addition of 200 μM Ca^{2+} to loaded SRV or leaky SRV. This shows that a high concentration of interior Ca^{2+} in loaded SRV does not have a significant effect on the rate of Ca^{2+} binding from the cytoplasm to the outside of the vesicle or on the reactivity of $^{c}E_{Ca_2}$ toward ATP. It is also inconsistent with a prediction of the E–E* model that interior Ca^{2+} will inhibit the binding of exterior Ca^{2+} to E (eqn 2). The existence of an interior calcium binding site on free E has been suggested by several kinds of evidence [10,18,19]. The properties of this site are not yet clear, but if it does exist the binding of interior Ca^{2+} to this site has no effect on the equilibrium or rate constant for binding of exterior Ca^{2+} to the high affinity site of E or on the reactivity of $^{c}E_{Ca_2}$ toward ATP.

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