Lumenal and Cytoplasmic Binding Sites for Calcium on the Calcium ATPase of Sarcoplasmic Reticulum Are Different and Independent[†,‡]

Jayhyuk Myung and William P. Jencks*

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254-9110

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ABSTRACT: The calcium ATPase of sarcoplasmic reticulum reacts with inorganic phosphate (P_i) to form phosphoenzyme that can bind two Ca^{2+} ions from the lumen of intact vesicles. Therefore, as the concentration of lumenal Ca^{2+} is increased, the concentration of phosphoenzyme at equilibrium increases; however, it levels off at lower maximal concentrations with decreasing concentrations of P_i. This requires that two Ca^{2+} ions can bind to lumenal binding sites of both the phosphoenzyme and the unphosphorylated enzyme. If lumenal Ca^{2+} could bind only to the phosphoenzyme, saturating concentrations of lumenal Ca^{2+} would drive phosphoenzyme formation to completion even at low concentrations of P_i. Phosphorylation is inhibited by cytoplasmic Ca^{2+} with $K_{0.5}$ = 2.1 and 4 μM in the absence and in the presence of 40 mM lumenal Ca^{2+}, respectively. $K_{0.5}$ = 4 μM is much lower than $K_{0.5}$ = 70 μM, which is expected if lumenal Ca^{2+} could bind only to the phosphoenzyme. Occupancy of the lumenal sites on the unphosphorylated enzyme by Ca^{2+} does not significantly change the rate constants of k_p = 220 s^{-1} for phosphorylation by ATP, k_{Ca} = 90 s^{-1} for dissociation of Ca^{2+}, and k_{Mg} = 50 s^{-1} for dissociation of Mg^{2+}. We conclude that the calcium ATPase has two low-affinity lumenal Ca^{2+}-binding sites that are independent of the high-affinity cytoplasmic Ca^{2+}-binding sites. The results are consistent with a mechanism of Ca^{2+} transport in which phosphorylation of the enzyme by ATP drives the translocation of two Ca^{2+} ions from the high-affinity to the low-affinity sites.

The Ca^{2+}-ATPase[1] of sarcoplasmic reticulum transports two Ca^{2+} ions from the cytoplasm of muscle to the lumen of the sarcoplasmic reticulum at the expense of hydrolysis of one molecule of ATP, in order to bring about relaxation of contracted muscle (de Meis, 1981; Martonosi & Beeler, 1983). The vectorial reaction of Ca^{2+} transport and the chemical reaction of ATP hydrolysis are tightly coupled according to a set of specificity rules, in such a way that neither reaction occurs unless the other reaction also occurs (Pickart & Jencks, 1984; Jencks, 1989). Additionally, there is a coupling of binding energies, which avoids the formation of intermediate species with high or low energies that would result in large kinetic or thermodynamic barriers in the reaction cycle (Pickart & Jencks, 1984).

The putative secondary structure of the Ca^{2+}-ATPase has been predicted on the basis of the primary amino acid sequence deduced from the cDNA clone and the hydrophobicity of the amino acid residues (MacLennan et al., 1985; Brandl et al., 1986). The predicted structure has two cytoplasmic domains that are joined to 10 transmembrane helices by a pentahelical stalk. Recently it has been concluded from a three-dimensional structure at 14-Å resolution, determined by cryoelectron microscopy and helical image analysis, that ~70% of the mass of the enzyme consists of the cytoplasmic domains and ~25% consists of the transmembrane domains (Toyoshima et al., 1993). Clarke et al. (1989, 1990) have proposed from the results of site-directed mutagenesis that high-affinity Ca^{2+}-binding sites are located in the putative transmembrane helices. We would like to understand the mechanism of translocation, in which Ca^{2+} ions bind to the cytoplasmic side of the SR membrane, move through the transmembrane protein domains upon phosphorylation of the enzyme by ATP, and dissociate from the lumenal side of the membrane.

Recently we have shown that as the concentration of lumenal Ca^{2+} is increased the concentration of phosphoenzyme that is formed at equilibrium from P_i and Mg^{2+} increases but then levels off to give different maximal concentrations in the presence of different concentrations of Mg^{2+} (Jencks et al., 1993). This result requires that Ca^{2+} ions can bind to low-affinity Ca^{2+}-binding sites on the lumenal side of the SR membrane in the unphosphorylated enzyme, as well as in the phosphoenzyme. Several other investigators have also proposed the existence of low-affinity lumenal Ca^{2+}-binding sites on the unphosphorylated Ca^{2+}-ATPase (Makinose & Hasselbach, 1965; Dupont, 1978; Suko et al., 1981). However, these lumenal Ca^{2+}-binding sites on the unphosphorylated enzyme are not sites that can bind Ca^{2+} ions from the lumen in one conformation and from the cytoplasm in another conformation, as proposed in the E1–E2 and related models. According to the E1–E2 model, cytoplasmic Ca^{2+} binds to the cytoplasmic sites of the E1 conformation, with a high affinity for Ca^{2+}, whereas lumenal Ca^{2+} binds to the same sites when they are exposed to the lumen in the E2 conformation, with a low affinity for Ca^{2+} (Verjovski-Almeida et al., 1978; Chaloub et al., 1979; de Meis, 1981; de Meis, 1988). Therefore, it is expected that there will be competition between the binding of Ca^{2+} ions to the unphosphorylated enzyme from the two sides of the membrane. However, we have shown previously that there is no competition between the binding of Ca^{2+} from the cytoplasm and the lumen to the unphosphorylated enzyme, because high concentrations of lumenal Ca^{2+} have no detectible effect on the rate constant and on the equilibrium constant for the binding of cytoplasmic Ca^{2+} to

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[1] Abbreviations: Ca^{2+}-ATPase, calcium-transporting ATPase; SR, sarcoplasmic reticulum; SRV, sarcoplasmic reticulum vesicles; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-morpholinopropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NADH, α-nicotinamide adenine dinucleotide (reduced form).

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the enzyme (Petithory & Jencks, 1988b; Myung & Jencks, 1991). These results show that there must be high-affinity cytoplasmic Ca^{2+}-binding sites on the unphosphorylated enzyme that are different from the low-affinity lumenal Ca^{2+}-binding sites.

In this paper we describe the properties of the two low-affinity lumenal binding sites for Ca^{2+}. These sites are different and independent of the two high-affinity cytoplasmic binding sites; the high-affinity Ca^{2+}-binding sites and the low-affinity Ca^{2+}-binding sites can bind Ca^{2+} ions simultaneously and independently. We conclude that the high-affinity sites and the low-affinity sites form a channel-like pathway in the Ca^{2+}-ATPase that allows translocation of Ca^{2+} ions from the high- to the low-affinity sites when the enzyme is phosphorylated by ATP.

EXPERIMENTAL PROCEDURES

Materials. MOPS, KCl, Tris, EGTA, and EDTA were purchased from Fluka, $Na_2ATP \cdot 3H_2O$ ("Sonderqualitat") was from Boehringer Mannheim, K-ADP and $CaCl_2 \cdot 2H_2O$ were from Sigma, $MgCl_2 \cdot 6H_2O$ was from Aldrich, and calcium ionophore A23187 was from Calbiochem. Carrier-free [^{32}P]-P_i and [γ-^{32}P]ATP (>99% pure) were obtained from New England Nuclear. All solutions were prepared with Milli-Q-grade water (Millipore Co.) and stored in polypropylene bottles (Nalgene Co.).

Tightly sealed sarcoplasmic reticulum vesicles were prepared from rabbit back and hind leg white muscles by a slight modification of the procedure of MacLennan (1970) as described previously (Pickart & Jencks, 1982) and were stored at −80 °C. The Ca^{2+}-ATPase in SRV preparations was found to hydrolyze 4–4.5 μmol of ATP·min^{-1}·(mg of total protein)$^{-1}$ under the standard conditions described below when the SRV were made permeable with 2 μM calcium ionophore A23187. The SRV preparations were tightly sealed, as shown by a 20–25-fold increase in the steady-state Ca^{2+}-ATPase activity upon the addition of the calcium ionophore A23187 to the standard assay solution.

Methods. Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as protein standard. The steady-state Ca^{2+}-ATPase activity was measured spectrophotometrically by coupling ADP production to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). The standard conditions were 0.01 mg/mL SRV, 100 mM KCl, 2 mM $MgCl_2$, ~25 μM $CaCl_2$, 40 mM MOPS/KOH, pH 7.0, 1.0 mM phosphoenolpyruvate, 0.15 mM NADH, 0.025 mg of pyruvate kinase, 0.025 mg of lactate dehydrogenase, and 2 μM calcium ionophore A23187 in a total volume of 2 mL at 25 °C.

The tightly sealed SRV preparations were passively loaded with Ca^{2+} by dialysis at 4 °C overnight against 400 mL of solutions containing 0.4 M sucrose, 100 mM KCl, 40 mM MOPS/Tris, pH 7.0, and different concentrations of $CaCl_2$. The activities of Ca^{2+} inside and outside the vesicles are the same after dialysis, and we used the concentrations of Ca^{2+} in the dialysis solutions as a measure of the concentration of free Ca^{2+} inside the SRV after dialysis.

Norit A charcoal was heated for 5 min with 1 N HCl in boiling water, filtered on a Büchner funnel, washed with water to remove acid, and dried in an oven (Boyer & Bryan, 1967). Carrier-free [^{32}P]P_i was treated with 1 N HCl for 2 h at room temperature and neutralized with Tris. Acid-washed Norit A charcoal (0.1 g) was added, and the mixture was forced through a Whatman GF/C glass microfiber filter and a 22-μm Millex-GV filter in a 10-mL plastic syringe.

The formation of ^{32}P-labeled phosphoenzyme from [^{32}P]P_i at equilibrium and the inhibition by cytoplasmic Ca^{2+} of ^{32}P-labeled phosphoenzyme formation from [^{32}P]P_i at equilibrium were measured by manually mixing and quenching the reaction solutions. For each reaction, 10 μL of the stock solution of SRV was added to 0.49 mL of reaction solution. Reaction mixtures were quenched with 0.5 mL of 1.2 N HCl and 48 mM unlabeled P_i at 20 s, at which time the concentration of phosphoenzyme was shown to have reached an equilibrium level. Bovine serum albumin and KH_2PO_4 were added to the acid-quenched reaction solutions to give final concentrations of ~0.3 mg/mL bovine serum albumin and ~25 mM KH_2PO_4; this was followed by the addition of trichloroacetic acid to give a final concentration of ~12% trichloroacetic acid (w/v).

The rate of formation of ^{32}P-labeled phosphoenzyme from [γ-^{32}P]ATP was measured with a rapid-mixing-quench apparatus that can be used with three syringes, as described previously (Stahl & Jencks, 1984; Petithory & Jencks, 1988a). The reaction times were calibrated from measurements of the rate of alkaline hydrolysis of 2,4-dinitrophenyl acetate (Barman & Gutfreund, 1964). For each reaction, 10 μL of the stock solution of SRV was mixed with 0.99 mL of a Ca^{2+}·EGTA buffer solution, to give ~25 μM free Ca^{2+}, and loaded into syringe A of the rapid-mixing-quench apparatus. Reactions were started within ~10 s. The solutions of syringes A and B were mixed and allowed to react in an aging tube; the reactions were quenched by the addition of quench solution from syringe C. Bovine serum albumin and ATP were added to the acid-quenched reaction solutions to give final concentrations of ~0.3 mg/mL bovine serum albumin and ~1 mM unlabeled ATP; this was followed by the addition of trichloroacetic acid to give a final concentration of ~12% trichloroacetic acid (w/v).

The concentration of ^{32}P-labeled phosphoenzyme was measured essentially as described by Verjovski-Almeida et al. (1978). The acid-quenched solutions were kept on ice not longer than 2 h and were then centrifuged at 1500*g* for 15 min at 4 °C. The supernatant solutions were decanted and the pellets were resuspended in 5 mL of ice-cold 5% trichloroacetic acid and 10 mM KH_2PO_4. The proteins were collected by vacuum filtration with Whatman GF/C glass microfiber filters and were rinsed with 15 mL of resuspension solution. The filters had been soaked in resuspension solution containing either ~10 mM ATP or ~50 mM KH_2PO_4. ^{32}P-labeled phosphoenzyme was measured by liquid scintillation counting of the samples in a glass vial containing ~7 mL of Aquasol-2.

The concentrations of free Ca^{2+}, Mg^{2+}, and P_i were calculated by using apparent dissociation constants of 3.9×10^{-7} M for Ca^{2+}·EGTA, 3×10^{-5} M for Mg^{2+}·EGTA, and 2.2×10^{-2} M for Mg^{2+}·P_i, with the computer program of Fabiato (1979, 1981, 1985, 1988).

RESULTS

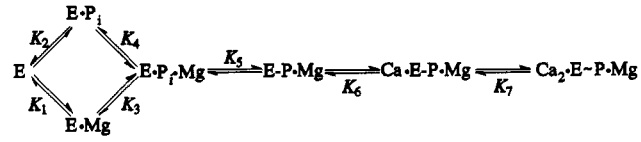
Phosphorylation of the Enzyme by P_i at Equilibrium. Figure 1 shows the dependence of the concentration of phosphoenzyme that was formed at equilibrium from P_i and Mg^{2+} on the concentration of lumenal Ca^{2+} in tightly sealed SRV. Phosphoenzyme was formed from the reaction of intact SRV, which were passively loaded with different concentrations of Ca^{2+}, with 4 mM $MgCl_2$ and 0.25, 1, or 5 mM [^{32}P]P_i for 20 s. The concentrations of phosphoenzyme were found to be stable between 20 and 40 s.

As the concentration of lumenal Ca^{2+} is increased, the concentration of phosphoenzyme increases and then levels off

at different maximal concentrations in the presence of different concentrations of P_i. We have shown previously that the concentration of phosphoenzyme at equilibrium also levels off at different maximal concentrations in the presence of different concentrations of Mg^{2+} (Jencks et al., 1993). These results show that increasing the concentration of lumenal Ca^{2+} does not drive the formation of phosphoenzyme to completion if the concentration of either P_i or Mg^{2+} is subsaturating.

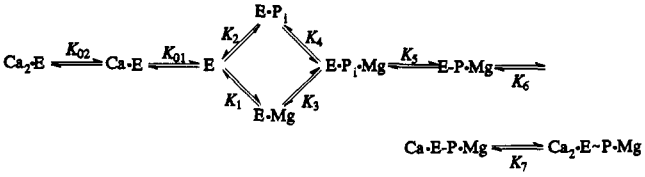
If there are no low-affinity lumenal binding sites for Ca^{2+} on the unphosphorylated enzyme, the binding of lumenal Ca^{2+} to form Ca_2·E~P·Mg can be described by Scheme 1.

Scheme 1



According to this mechanism, high concentrations of lumenal Ca^{2+} will drive the formation of phosphoenzyme from P_i and Mg^{2+} to completion even at subsaturating concentrations of P_i and Mg^{2+}, because lumenal Ca^{2+} can bind only to the phosphoenzyme and will convert all of the enzyme to Ca_2·E~P·Mg. We have shown that this is not observed (Figure 1; Jencks et al., 1993).

Scheme 2



Scheme 2 describes the equilibrium constants for a simple model in which lumenal Ca^{2+} can bind to the unphosphorylated enzyme as well as to the phosphoenzyme. According to this mechanism, an increase in the concentration of lumenal Ca^{2+} will not drive the formation of phosphoenzyme to completion at subsaturating concentrations of P_i or Mg^{2+}, because lumenal Ca^{2+} can bind to lumenal sites of the unphosphorylated enzyme as well as the phosphoenzyme. It should be noted that Ca_2·E~P·Mg can also be formed from Ca_2·E and P_i. This reaction is omitted from Schemes 2 and 5 for simplicity and because an equilibrium constant is independent of the pathway by which equilibrium is reached.

The solid lines in Figure 1 are the best fit to Scheme 2; they were drawn with values of K_{01} = 20 mM, K_{02} = 30 mM, K_1 = 8.7 mM, K_2 = 7.2 mM, K_3 = 1.5 mM, K_4 = 1.9 mM, K_5 = 0.6, K_6 = 2 mM, K_7 = 3.5 mM, and E_{tot} = 2.45 nmol/mg. The values of K_1, K_2, K_3, K_4, and K_5 were determined under similar conditions by Punzengruber et al. (1978) and the values of K_{01}, K_{02}, K_6, and K_7 were obtained from a quantitative analysis of the data. This set of values of K_{01}, K_{02}, K_6, and K_7 is not unique; several sets of values could fit the experimental results to Scheme 2. However, it is certain that lumenal Ca^{2+} ions bind to the unphosphorylated enzyme more weakly than to the phosphoenzyme; if they bound more strongly to the unphosphorylated enzyme, the concentration of phosphoenzyme would decrease as the concentration of lumenal Ca^{2+} is increased.

Inhibition by Cytoplasmic Ca^{2+} of Phosphorylation of the Enzyme by P_i. Figure 2 shows the inhibition by cytoplasmic Ca^{2+} of phosphoenzyme formation from P_i at equilibrium, with empty SRV (○) and with inact SRV that were passively loaded with 40 mM Ca^{2+} (●). Phosphoenzyme was formed by incubation of empty SRV or loaded SRV with 5 mM [^{32}P]-P_i for 20 s in the presence of different concentrations of cytoplasmic Ca^{2+}. The solid lines in Figure 2 were drawn for

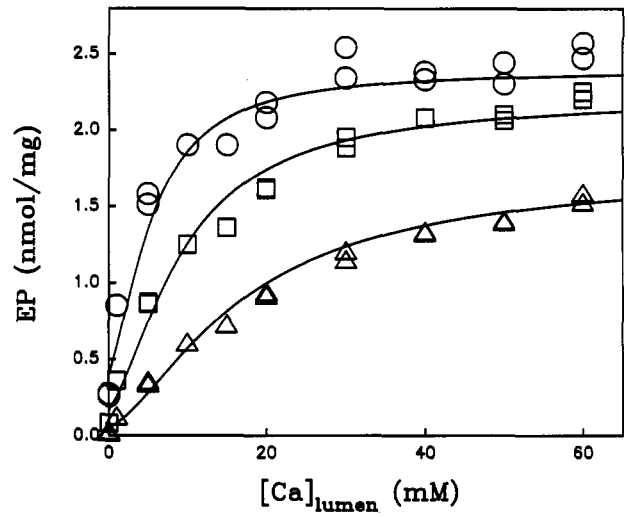


FIGURE 1: Dependence of the concentration of phosphoenzyme formed at equilibrium from different concentrations of P_i on the concentration of lumenal Ca^{2+}. The concentration of phosphoenzyme was measured 20 s after the addition of 4 mM $MgCl_2$ and 0.25 mM (△), 1 mM (□), or 5 mM (○) [^{32}P]P_i to 0.9 mg/mL SRV in the presence of 40 mM MOPS/Tris buffer, pH 7.0, 10 mM EGTA, and 100 mM KCl at 25 °C. SRV were passively loaded with the indicated concentrations of calcium. The solid lines were drawn according to Scheme 2, with K_{01} = 20 mM, K_{02} = 30 mM, K_1 = 8.7 mM, K_2 = 7.2 mM, K_3 = 1.5 mM, K_4 = 1.9 mM, K_5 = 0.6, K_6 = 2 mM, K_7 = 3.5 mM, and E_{tot} = 2.45 nmol/mg. The concentrations of free P_i and Mg^{2+} were calculated by using an apparent dissociation constant of 2.2×10^{-2} M for Mg^{2+}·P_i with the computer program of Fabiato (1988): free [P_i] = 0.22 (△), 0.88 (□), and 4.47 mM (○); free [Mg^{2+}] = 3.1 (△), 2.98 (□), and 2.65 mM (○).

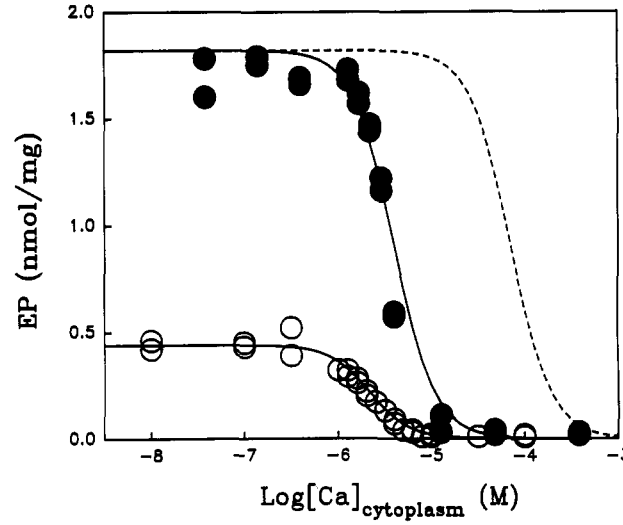
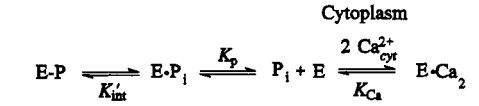


FIGURE 2: Inhibition by cytoplasmic Ca^{2+} of phosphorylation by P_i with empty SRV (○) and intact SRV passively loaded with 40 mM Ca^{2+} (●). The concentration of phosphoenzyme was measured 20 s after the addition of 10 mM $MgCl_2$ and 5 mM [^{32}P]P_i to 0.9 mg/mL empty or loaded SRV in the presence of 40 mM MOPS/Tris buffer, pH 7.0, 100 mM KCl, 10 mM EGTA, and different [Ca^{2+}] to give the desired free [Ca^{2+}] in the reaction solutions at 25 °C. The concentrations of free Ca^{2+} were calculated by using an apparent dissociation constant of 3.9×10^{-7} M for Ca^{2+}·EGTA with the computer program of Fabiato (1988) and taking into account the contribution of Ca^{2+} from the stock solution of loaded SRV. The solid lines were drawn for $K_{0.5}$ = 2.1 μM (○), $K_{0.5}$ = 4 μM (●), and a Hill coefficient of n_H = 2, obtained from a Hill plot of the data. The dashed line was drawn for 40 mM lumenal Ca^{2+} with $K_{0.5}$ = 70 μM and a Hill coefficient of n_H = 2.

$K_{0.5}$ = 2.1 μM cytoplasmic Ca^{2+} with empty SRV (○), $K_{0.5}$ = 4 μM cytoplasmic Ca^{2+} with loaded SRV (●), and a Hill coefficient of n_H = 2, which corresponds to the binding of two Ca^{2+} ions to the cytoplasmic sites with positive cooperativity.

It is well-known that cytoplasmic Ca^{2+} inhibits phosphorylation of the enzyme by P_i at equilibrium (Kanazawa & Boyer, 1973) by binding to the high-affinity cytoplasmic sites, as shown in Scheme 3. The concentration of cytoplasmic

Scheme 3



Ca^{2+} that causes half-maximal inhibition of phosphorylation of the enzyme by P_i at equilibrium, $K_{0.5}$, is determined by the dissociation constant for cytoplasmic Ca^{2+} from the unphosphorylated enzyme, K_{Ca}, the values of K'_{int} and K_p, and the concentration of P_i; it is described by eq 1, which is derived in the Appendix.

$$K_{0.5} = \left[K_{Ca}\left(K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1\right)\right]^{1/2} \quad (1)$$

The open circles in Figure 2 show that phosphorylation of the enzyme by P_i in empty SRV is inhibited by cytoplasmic Ca^{2+} and that this inhibition is consistent with a value of $K_{0.5}$ = 2.1 μM and a Hill coefficient of n_H = 2, as shown by the lower solid line. This agrees with the results of Kanazawa and Boyer (1973), who have shown that phosphorylation of the enzyme by P_i is inhibited by cytoplasmic Ca^{2+} with a value of $K_{0.5}$ = 2 μM and a Hill coefficient of n_H = 2 under similar conditions. The observed value of $K_{0.5}$ = 2.1 μM gives a value of $K_{Ca} = 3.2 \times 10^{-12}$ M^2 according to eq 1 and values of K'_{int} = 1.7 and K_p = 37 mM (Pickart & Jencks, 1984) in the presence of 5 mM P_i. These values of K'_{int} and K_p were determined under conditions very similar to those described here. Therefore, the inhibition by cytoplasmic Ca^{2+} of phosphorylation of the enzyme by P_i in the absence of lumenal Ca^{2+}, shown by the open circles in Figure 2, is consistent with binding of two cytoplasmic Ca^{2+} ions to the unphosphorylated enzyme with a value of $K_{Ca} = 3.2 \times 10^{-12}$ M^2. We have reported previously that binding of the two cytoplasmic Ca^{2+} ions to the enzyme to form E·Ca_2 is consistent with values of $K_{Ca} = 4.0$–7.8×10^{-12} M^2 (Petithory & Jencks, 1988a; Hanel & Jencks, 1990; Myung & Jencks, 1991).[2]

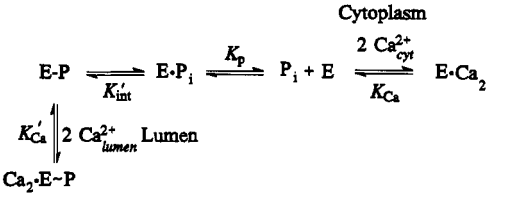
If lumenal Ca^{2+} could bind only to the phosphoenzyme, the concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation by P_i would be increased in the presence of lumenal Ca^{2+} by competition between the binding of lumenal Ca^{2+} to the phosphoenzyme and the binding of cytoplasmic Ca^{2+} to the unphosphorylated enzyme, as shown in Scheme 4. Equation 2, which is derived in the Appendix,

$$K_{0.5} = \left[K_{Ca}\left(\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1\right)\right]^{1/2} \quad (2)$$

describes the effect of lumenal Ca^{2+} on the value of $K_{0.5}$ for inhibition by cytoplasmic Ca^{2+} of phosphorylation of the enzyme by P_i, according to Scheme 4.

The presence of 40 mM lumenal Ca^{2+} is calculated to increase the value of $K_{0.5}$ by 35-fold if lumenal Ca^{2+} binds only to the phosphoenzyme and the phosphoenzyme is saturated with 40 mM lumenal Ca^{2+}. Figure 1 shows that 40 mM lumenal Ca^{2+} is saturating in the presence of 5 mM P_i and 4 mM Mg^{2+}. It is also known that Ca^{2+} inhibits the steady-state activity of the Ca^{2+}-ATPase in leaky SRV with $K_{1/2}$ = 0.5 mM (Bodley & Jencks, 1987; Khananshvili et al., 1990), which corresponds to a dissociation constant for Ca^{2+} from the phosphoenzyme of $K'_{Ca} = (K_{1/2})^2 = (0.5\ mM)^2 = 2.5 \times 10^{-7}$ M^2.

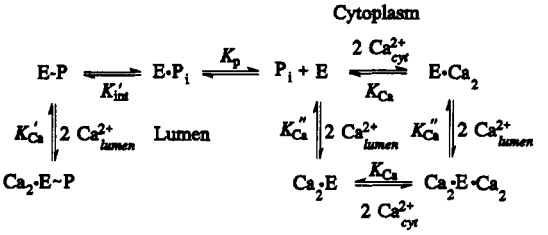
Scheme 4



We calculated a value of $K_{0.5}$ = 70 μM for inhibition by cytoplasmic Ca^{2+} of phosphorylation by P_i in the presence of 40 mM lumenal Ca^{2+} at equilibrium, from eq 2 and the values of $K_{Ca} = 3.2 \times 10^{-12}$ M^2, $K'_{Ca} = 2.5 \times 10^{-7}$ M^2, K'_{int} = 1.7, K_p = 37 mM, and $[P_i]$ = 5 mM. The dashed line in Figure 2 was drawn according to a model in which lumenal Ca^{2+} binds only to the phosphoenzyme (Scheme 4) and the calculated value of $K_{0.5}$ = 70 μM. However, the closed circles in Figure 2 show that the concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation of the enzyme by P_i is only $K_{0.5}$ = 4 μM, which is at least 15-fold smaller than the calculated value of $K_{0.5}$ = 70 μM for inhibition of phosphorylation in the presence of 40 mM lumenal Ca^{2+}. Therefore, the binding of lumenal Ca^{2+} only to the phosphoenzyme, as shown as K'_{Ca} in Scheme 4, does not account for the observed value of $K_{0.5}$ = 4 μM for inhibition by cytoplasmic Ca^{2+} of phosphorylation by P_i in the presence of 40 mM lumenal Ca^{2+}.

We have shown previously that lumenal Ca^{2+} binds to the unphosphorylated enzyme as well as to the phosphoenzyme (Figure 1; Jencks et al., 1993). Therefore, the inhibition by cytoplasmic Ca^{2+} of phosphorylation of the enzyme by P_i can be described by Scheme 5. The observation that high

Scheme 5



concentrations of lumenal Ca^{2+} do not change the equilibrium constant for the binding of cytoplasmic Ca^{2+} to the enzyme (Myung & Jencks, 1991) requires that lumenal Ca^{2+} must

[2] Petithory and Jencks (1988a) have shown that two cytoplasmic Ca^{2+} ions bind to the enzyme to form E·Ca_2 with $K_{Ca} = 1.2 \times 10^{-11}$ M^2; however, this value was calculated from a value of $K_d = 7.4 \times 10^{-7}$ M for the apparent dissociation constant of the Ca^{2+}·EGTA complex, which was determined at pH 7.0 in the presence of 100 mM KCl, 5 mM $MgCl_2$, and 5 mM ATP (Godt, 1974). This value is appropriate only under these conditions and it is ~2-fold weaker than the value of $K_d = 3.9 \times 10^{-7}$ M that was used in this study and corrected for the effects of other ions on the binding by EGTA by the computer program of Fabiato (1988). Hanel and Jencks (1990) calculated a value of $K_{Ca} = 4.0 \times 10^{-12}$ M^2 for the formation of E·Ca_2 from the data of Petithory and Jencks and $K_d = 3.9 \times 10^{-7}$ M for Ca^{2+}·EGTA.

bind to E and E·Ca_2 with the same dissociation constant, K''_{Ca}. Equation 3, which is derived in the Appendix, describes

$$K_{0.5} = \left[\frac{K_{Ca}}{1 + \frac{([Ca]_{lumen})^2}{K''_{Ca}}} \left(\frac{([Ca]_{lumen})^2}{K'_{Ca}} K'_{int} \frac{[P_i]}{K_p} + K'_{int} \frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{lumen})^2}{K''_{Ca}} \right) \right]^{1/2} \quad (3)$$

the effect of lumenal Ca^{2+} on the value of $K_{0.5}$ for inhibition by cytoplasmic Ca^{2+} of phosphorylation of the enzyme by P_i, according to Scheme 5: $K_{0.5}$ is determined by the dissociation constant for cytoplasmic Ca^{2+} from the unphosphorylated enzyme, K_{Ca}, as well as by the dissociation constants for lumenal Ca^{2+} from the unphosphorylated enzyme, K''_{Ca}, and from the phosphoenzyme, K'_{Ca}.

The closed circles in Figure 2 show that phosphorylation of the enzyme in intact SRV that were passively loaded with 40 mM Ca^{2+} is inhibited by cytoplasmic Ca^{2+} with $K_{0.5}$ = 4 μM. The observed value of $K_{0.5}$ = 4 μM for inhibition of phosphorylation in the presence of 40 mM lumenal Ca^{2+} is consistent with values of $K'_{Ca} = 2.5 \times 10^{-7}$ M^2 for dissociation of lumenal Ca^{2+} from the phosphoenzyme, $K''_{Ca} = 4.3 \times 10^{-6}$ M^2 for dissociation of lumenal Ca^{2+} from the unphosphorylated enzyme, and $K_{Ca} = 3.2 \times 10^{-12}$ M^2 for dissociation of cytoplasmic Ca^{2+} from the unphosphorylated enzyme that were calculated according to eq 3 and values of K'_{int} = 1.7 and K_p = 37 mM (Pickart & Jencks, 1984) in the presence of 5 mM P_i. The small increase in the value of $K_{0.5}$ = 4 μM for inhibition of phosphorylation in the presence of 40 mM lumenal Ca^{2+}, compared to $K_{0.5}$ = 2.1 μM in the absence of lumenal Ca^{2+}, can be accounted for by weaker binding of lumenal Ca^{2+} to the unphosphorylated enzyme, with $K''_{Ca} = 4.3 \times 10^{-6}$ M^2, than to the phosphoenzyme, with $K'_{Ca} = 2.5 \times 10^{-7}$ M^2. If lumenal Ca^{2+} bound to the unphosphorylated enzyme more strongly than to the phosphoenzyme, the value of $K_{0.5}$ for inhibition by cytoplasmic Ca^{2+} of phosphorylation by P_i would be decreased in the presence of lumenal Ca^{2+}.

Rate Constant for Dissociation of Ca^{2+} from the Cytoplasmic Sites. Figure 3 shows the rate of phosphorylation of enzyme that was incubated with 25 μM cytoplasmic Ca^{2+} upon the addition of 0.5 mM [γ-^{32}P]ATP and 10 mM EGTA, with empty SRV (○) and with intact SRV that were passively loaded with 40 mM Ca^{2+} (●). Phosphorylation of the enzyme in the presence of ATP and EGTA proceeds with the same rate constant of 310 s^{-1} with empty SRV and with loaded SRV.

The simultaneous addition of ATP and EGTA to enzyme with Ca^{2+} bound at the cytoplasmic sites, E·Ca_2·Mg, results in rapid binding of ATP to form E·Ca_2·Mg·ATP, which then partitions between phosphorylation, to give Ca_2·E~P·Mg, and irreversible dissociation of Ca^{2+}, which prevents rephosphorylation of the enzyme by bound ATP (Scheme 6). The binding of 0.5 mM ATP is fast, because there is no detectible lag for phosphorylation. This is consistent with the second-order rate constant for binding of ATP to E·Ca_2·Mg of ~10^7 M^{-1} s^{-1} (Petithory & Jencks, 1988a).

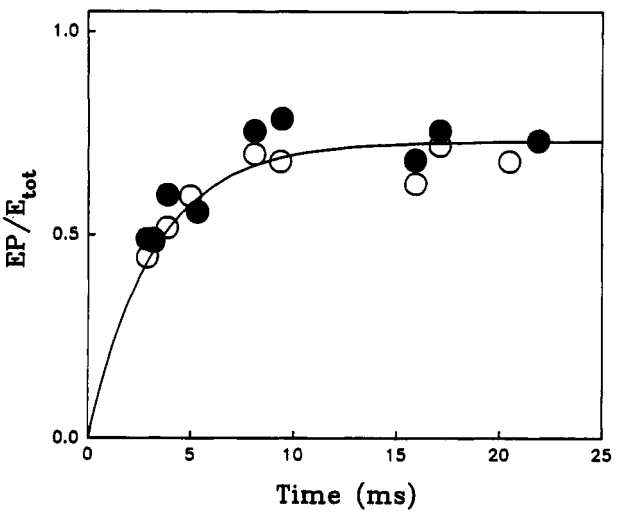
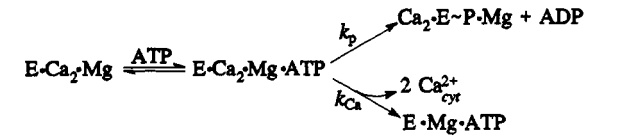


FIGURE 3: Phosphorylation of the enzyme in empty and loaded vesicles by ATP in the presence of EGTA. Syringes A and B contained 40 mM MOPS/Tris buffer, pH 7.0, 0.1 M KCl, and 5 mM $MgCl_2$ at 25 °C. In addition, syringe A contained 0.35 mg/mL empty SRV, 1.3 mM $CaCl_2$, and 1.3 mM EGTA to give ~25 μM free Ca^{2+} (○). Alternatively, syringe A contained 0.35 mg/mL loaded SRV (40 mM $CaCl_2$), 1.3 mM $CaCl_2$ (0.28 mM from the enzyme solution), and 1.3 mM EGTA to give ~25 μM free Ca^{2+} (●). Syringe B contained 1.0 mM [γ-^{32}P]ATP and 20 mM EGTA. Syringe C contained 1.5 N HCl and 60 mM P_i. The solid line was drawn for a rate constant of 310 s^{-1} and an end point of 0.73. E_{tot} = 1.47 nmol/mg.

Scheme 6



Scheme 7

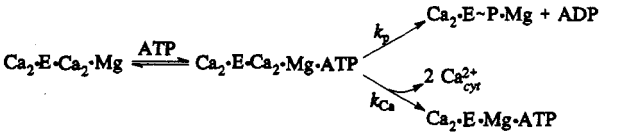


Figure 1 shows that lumenal Ca^{2+} can bind to the lumenal Ca^{2+}-binding sites of both the phosphoenzyme and the unphosphorylated enzyme and that both sites are saturated at high concentrations of lumenal Ca^{2+}. In Scheme 7 we designate enzyme that is saturated with 25 μM Ca^{2+} at the cytoplasmic sites as well as with 40 mM Ca^{2+} at the lumenal sites as Ca_2·E·Ca_2·Mg. The simultaneous addition of ATP and EGTA to Ca_2·E·Ca_2·Mg results in rapid binding of ATP to form Ca_2·E·Ca_2·Mg·ATP, which then partitions between phosphorylation to give Ca_2·E~P·Mg and dissociation of Ca^{2+} from the high-affinity cytoplasmic sites to give unreactive enzyme, as shown in Scheme 7.

According to Schemes 6 and 7, the observed first-order rate constant for phosphorylation of the enzyme in the presence of ATP and EGTA is the sum of the rate constants of k_p = 220 s^{-1} for phosphorylation and k_{Ca} for the irreversible dissociation of Ca^{2+}. Therefore, the rate constant for dissociation of Ca^{2+} from the cytoplasmic sites is $k_{Ca} = k_{obs} - k_p$ = 310 s^{-1} − 220 s^{-1} = 90 s^{-1} for both empty SRV and loaded SRV. The fraction of total enzyme that undergoes phosphorylation is equal to $k_p/(k_p + k_{Ca})$. Therefore, the values of k_p = 220 s^{-1} and k_{Ca} = 90 s^{-1} are expected to result in a yield of 220 s^{-1}/(220 + 90) s^{-1} = 71% phosphoenzyme, which agrees with the observed yield of 73% phosphoenzyme.

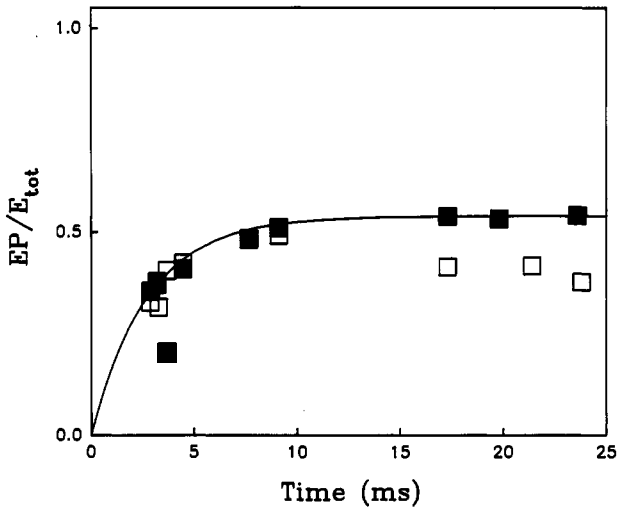


FIGURE 4: Phosphorylation of the enzyme in empty and loaded vesicles by ATP in the presence of EDTA. Syringes A and B contained 40 mM MOPS/Tris buffer, pH 7.0, and 0.1 M KCl at 25 °C. In addition, syringe A contained 0.35 mg/mL empty SRV, 5 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 1.3 mM EGTA to give ~25 μM free Ca^{2+} (□). Alternatively, syringe A contained 0.35 mg/mL loaded SRV (40 mM $CaCl_2$), 5 mM $MgCl_2$, 1.3 mM $CaCl_2$ (0.28 mM from the enzyme solution), and 1.3 mM EGTA to give ~25 μM free Ca^{2+} (■). Syringe B contained 1.0 mM [γ-^{32}P]ATP and 40 mM EDTA. Syringe C contained 1.5 N HCl and 60 mM P_i. The solid line was drawn for a rate constant of 360 s^{-1} and an end point of 0.54. E_{tot} = 1.47 nmol/mg.

Petithory and Jencks (1986) found that Ca^{2+} dissociates from the high-affinity cytoplasmic sites with a rate constant of k_{Ca} = 80 s^{-1} under similar conditions.

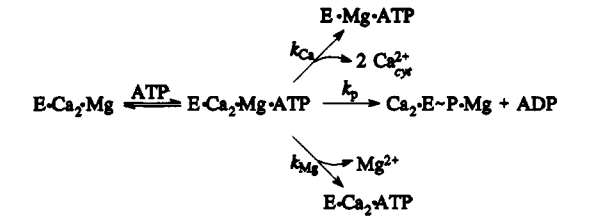
This result shows that binding of Ca^{2+} to the lumenal sites of the unphosphorylated enzyme does not cause a significant change in the rate constants of k_{Ca} = 90 s^{-1} for dissociation of Ca^{2+} from the high-affinity cytoplasmic sites and k_p = 220 s^{-1} for phosphorylation by ATP. We have shown previously that high concentrations of lumenal Ca^{2+} have no effect on the rate constant and the equilibrium constant for binding of cytoplasmic Ca^{2+} to the high-affinity sites (Petithory & Jencks, 1988b; Myung & Jencks, 1991). These results require that the cytoplasmic sites and the lumenal sites for Ca^{2+} are different and independent of each other.

Rate Constant for Dissociation of Mg^{2+} from the Catalytic Site. Figure 4 shows the rate of phosphorylation of enzyme that was incubated with 25 μM cytoplasmic Ca^{2+} and 5 mM Mg^{2+} upon the addition of 0.5 mM [γ-^{32}P]ATP and 20 mM EDTA, with empty SRV (□) and with intact SRV that were passively loaded with 40 mM Ca^{2+} (■). These data are consistent with phosphorylation of the enzyme in empty SRV and loaded SRV with the same rate constant of 360 s^{-1} in the presence of ATP and EDTA. The level of phosphoenzyme formed with empty SRV decreases after ~15 ms (□) because of hydrolysis of the phosphoenzyme.

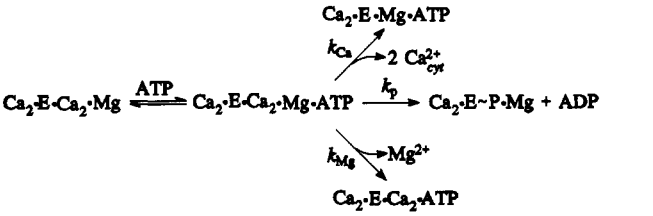
The simultaneous addition of ATP and EDTA to E·Ca_2·Mg results in rapid binding of ATP to form E·Ca_2·Mg·ATP, which then partitions between phosphorylation to give Ca_2·E~P·Mg, dissociation of Ca^{2+}, and dissociation of Mg^{2+} to give unreactive enzyme, as shown in Scheme 8.

In the presence of 40 mM lumenal Ca^{2+}, E·Ca_2·Mg is saturated with Ca^{2+} at the lumenal sites and exists as Ca_2·E·Ca_2·Mg. The simultaneous addition of ATP and EDTA to Ca_2·E·Ca_2·Mg results in rapid binding of ATP to form Ca_2·E·Ca_2·Mg·ATP, which then partitions between phosphorylation to give Ca_2·E~P·Mg, dissociation of Ca^{2+}, and

Scheme 8



Scheme 9



dissociation of Mg^{2+} to give unreactive enzyme, as shown in Scheme 9.

According to Schemes 8 and 9, the observed first-order rate constant for phosphorylation of the enzyme in the presence of ATP and EDTA is the sum of the rate constants of k_p = 220 s^{-1} for phosphorylation, k_{Ca} = 90 s^{-1} for dissociation of Ca^{2+}, and k_{Mg} for dissociation of Mg^{2+}. Therefore, the rate constant for dissociation of Mg^{2+} from the catalytic site is $k_{Mg} = k_{obs} - (k_p + k_{Ca})$ = 360 s^{-1} − (220 + 90) s^{-1} = 50 s^{-1} for both empty SRV and loaded SRV. The dissociation constant for Mg^{2+} from the catalytic site is K_d = 0.94 mM (Reinstein & Jencks, 1993), so that the catalytic site is not completely saturated in the presence of 5 mM Mg^{2+}; the fraction of total enzyme that undergoes phosphorylation is equal to $k_p/(k_p + k_{Ca} + k_{Mg}) \times [Mg^{2+}]/([Mg^{2+}] + K_d)$. The values of k_p = 220 s^{-1}, k_{Ca} = 90 s^{-1}, k_{Mg} = 50 s^{-1}, and K_d = 0.94 mM give 220 s^{-1}/(220 + 90 + 50) s^{-1} × 5 mM/(5 mM + 0.94 mM) = 51% phosphoenzyme, which agrees with the observed yield of 54% phosphoenzyme. Reinstein and Jencks (1993) obtained a rate constant for dissociation of Mg^{2+} from the enzyme of k_{Mg} = 60 s^{-1} under similar conditions.

This result shows that binding of Ca^{2+} to the lumenal sites of the unphosphorylated enzyme does not cause a significant change in the rate constant of k_{Mg} = 50 s^{-1} for dissociation of Mg^{2+} from the catalytic site. This is in contrast to the strong interaction between the cytoplasmic Ca^{2+}-binding sites and the catalytic site: (1) Phosphorylation of the enzyme by ATP occurs when the cytoplasmic sites are occupied by Ca^{2+}, while phosphorylation of the enzyme by P_i occurs when the cytoplasmic sites are free from Ca^{2+} (Yamamoto & Tonomura, 1967; Makinose, 1969; Kanazawa & Boyer, 1973; Pickart & Jencks, 1984). (2) Mg^{2+} dissociates from the catalytic site with K_d = 8.7 mM in the absence of Ca^{2+} (Punzengruber et al., 1978), which is ~10 times weaker than K_d = 0.94 mM from the enzyme with Ca^{2+} bound at the cytoplasmic sites (Reinstein & Jencks, 1993).

DISCUSSION

Low-Affinity Ca^{2+}-Binding Sites Exist on the Lumenal Side of the SR Membrane in the Unphosphorylated Enzyme, as Well as in the Phosphoenzyme. It is well-established that lumenal Ca^{2+} binds to the phosphorylated Ca^{2+}-ATPase. Several investigators have shown that the concentration of phosphoenzyme that is formed at equilibrium from P_i and Mg^{2+} increases as the concentration of lumenal Ca^{2+} is increased (Yamada et al., 1972; Prager et al., 1979); if lumenal

Ca^{2+} did not bind to the phosphoenzyme, the concentration of phosphoenzyme would remain constant when the concentration of lumenal Ca^{2+} is increased. Furthermore, Ca^{2+} inhibits the steady-state activity of the Ca^{2+}-ATPase in leaky SRV by binding to the phosphoenzyme to regenerate Ca_2·E~P·Mg, which undergoes hydrolysis very slowly (Souza & de Meis, 1976; Khananshvili et al., 1990). It is known that Ca^{2+} dissociates from the phosphoenzyme with a rate constant of ~30 s^{-1} and that this step is largely rate-limiting for the steady-state activity of the enzyme (Hanel & Jencks, 1991).

Figure 1 shows that the concentration of phosphoenzyme that is formed at equilibrium from P_i and Mg^{2+} increases as the concentration of lumenal Ca^{2+} is increased. However, the concentration of phosphoenzyme levels off to give different maximal concentrations at different concentrations of P_i. We have shown previously that when the concentration of lumenal Ca^{2+} is saturating the concentration of phosphoenzyme that is formed at equilibrium from P_i and Mg^{2+} decreases as the concentration of Mg^{2+} decreases (Jencks et al., 1993). These results show that an increase in the concentration of lumenal Ca^{2+} does not drive the formation of phosphoenzyme to completion when the concentration of P_i or Mg^{2+} is not saturating.

If lumenal Ca^{2+} ions bound only to the phosphoenzyme at equilibrium, as shown in Scheme 1, an increase in the concentration of lumenal Ca^{2+} would drive the formation of phosphoenzyme to completion even when the concentration of P_i or Mg^{2+} is subsaturating, because lumenal Ca^{2+} would bind only to the phosphoenzyme and would convert all of the enzyme to Ca_2·E~P·Mg.

However, the results reported here and previously (Figure 1; Jencks et al., 1993) show that increasing the concentration of lumenal Ca^{2+} does not drive the formation of phosphoenzyme to completion in the presence of subsaturating concentrations of P_i or Mg^{2+}. This shows that lumenal Ca^{2+} can bind to the phosphoenzyme, E–P·Mg, to form Ca_2·E~P·Mg in the presence of high concentrations of P_i and Mg^{2+} and can also bind to the unphosphorylated enzyme, E, to form Ca_2·E in the presence of low concentrations of P_i and Mg^{2+}. Therefore, there must be lumenal binding sites for Ca^{2+} on the unphosphorylated enzyme, as well as on the phosphoenzyme, as shown in Scheme 2.

The binding of a single Ca^{2+} ion to the lumenal sites of the unphosphorylated enzyme does not explain the results, because it is known that two Ca^{2+} ions bind to the enzyme and are transported (Hasselbach, 1978). If only one Ca^{2+} ion bound to the unphosphorylated enzyme and two Ca^{2+} ions bound to the phosphoenzyme, then the concentration of phosphoenzyme would not level off to give low maximal concentrations at high concentrations of lumenal Ca^{2+} because the binding of one Ca^{2+} ion to the unphosphorylated enzyme would not compete effectively with the binding of two Ca^{2+} ions to the phosphoenzyme. Similarly, the possibility of binding of three Ca^{2+} ions to the lumenal sites of the unphosphorylated enzyme is excluded, because the binding of three Ca^{2+} ions to the unphosphorylated enzyme and two Ca^{2+} ions to the phosphoenzyme would result in conversion of all of the enzyme to Ca_3·E at high concentrations of lumenal Ca^{2+}.

Suko et al. (1981) also concluded that there are low-affinity lumenal binding sites for Ca^{2+} on the unphosphorylated enzyme, as well as on the phosphoenzyme. This conclusion is based on a series of measurements of the concentration of phosphoenzyme that is formed at equilibrium from different concentrations of P_i and Mg^{2+} in the presence of 40 mM lumenal Ca^{2+}. They showed that the double reciprocal plots of phosphoenzyme concentration versus the concentration of P_i or Mg^{2+} are consistent with the characteristic patterns that are predicted from a model in which lumenal Ca^{2+} binds to the unphosphorylated enzyme, as well as to the phosphoenzyme, but are not consistent with the prediction from a model in which lumenal Ca^{2+} binds only to the phosphoenzyme. In particular, the fact that the dependence of phosphoenzyme concentration on the concentration of P_i in the presence of 40 mM lumenal Ca^{2+} at equilibrium is different in the presence of different concentrations of Mg^{2+} shows that lumenal Ca^{2+}-binding sites exist on the unphosphorylated enzyme, as well as on the phosphoenzyme.

There Are Both Cytoplasmic and Lumenal Ca^{2+}-Binding Sites on the Unphosphorylated Enzyme. We have shown here and previously (Figure 1; Jencks et al., 1993) that two Ca^{2+} ions from the lumen can bind to the unphosphorylated Ca^{2+}-ATPase, as well as to the phosphoenzyme. We have tested the possibility that there is only one pair of Ca^{2+}-binding sites on the unphosphorylated enzyme, which can bind two Ca^{2+} ions from the lumen in one conformation or from the cytoplasm in a different conformation of the enzyme (Verjovski-Almeida et al., 1978; Chaloub et al., 1979; de Meis, 1981; de Meis, 1988). If there were only one pair of Ca^{2+}-binding sites, there should be competition between the binding of Ca^{2+} to the enzyme from the cytoplasm and from the lumen. However, we have shown previously that there is no such competition because high concentrations of lumenal Ca^{2+} have no detectible effect on the rate constant and on the equilibrium constant for the binding of cytoplasmic Ca^{2+} to the enzyme (Petithory & Jencks, 1988b; Myung & Jencks, 1991). Therefore, there must be both cytoplasmic and lumenal binding sites for Ca^{2+} on the unphosphorylated enzyme, and these sites do not interact with each other.

It is well-known that two Ca^{2+} ions from the cytoplasm bind to the high-affinity sites of the enzyme with positive cooperativity (Inesi et al., 1980; Dupont, 1982) and activate the enzyme for phosphorylation by ATP (Yamamoto & Tonomura, 1967; Makinose, 1969; Petithory & Jencks, 1988a). The binding of cytoplasmic Ca^{2+} to the enzyme can be determined by measuring the concentration of ^{32}P-labeled phosphoenzyme that is formed upon the addition of [γ-^{32}P]-ATP and EGTA; the simultaneous addition of EGTA and ATP to E·Ca_2 prevents further binding of Ca^{2+} to the enzyme and results in the formation of E·Ca_2·ATP, which partitions between the formation of phosphoenzyme and irreversible dissociation of Ca^{2+}. The binding of two cytoplasmic Ca^{2+} ions to the enzyme to form E·Ca_2 is consistent with values of $K_{Ca} = 4.0–7.8 \times 10^{-12}$ M^2 (Petithory & Jencks, 1988a; Hanel & Jencks, 1990; Myung & Jencks, 1991).[2] The binding of cytoplasmic Ca^{2+} to the enzyme prevents phosphorylation by P_i, so that the concentration of phosphoenzyme that is formed at equilibrium from P_i and Mg^{2+} decreases as the concentration of cytoplasmic Ca^{2+} increases. Figure 2 shows that phosphorylation of the enzyme at equilibrium by 5 mM P_i is inhibited by cytoplasmic Ca^{2+}, with a value of $K_{0.5} = 2.1$ μM in the absence of lumenal Ca^{2+}. Because P_i competes with Ca^{2+} for the enzyme, the concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation by P_i is higher than the concentration of cytoplasmic Ca^{2+} that results in half-maximal binding to the enzyme, as described by eq 1. Therefore, the value of $K_{0.5} = 2.1$ μM for the inhibition of phosphorylation is consistent with the binding of cytoplasmic Ca^{2+} to the enzyme with the value of $K_{Ca} = 3.2 \times 10^{-12}$ M^2 that is obtained from eq 1.

The Hill coefficient of $n_H = 2$ (Figure 2) for the inhibition of phosphorylation by cytoplasmic Ca^{2+}, with both empty SRV and loaded SRV, agrees with Hill coefficients in the range of $n_H = 1.6$–2.0 for the binding of Ca^{2+} to the cytoplasmic sites of the unphosphorylated enzyme at equilibrium that have been determined by several methods (Inesi et al., 1980; Dupont, 1982; Petithory & Jencks, 1988a). Therefore, two cytoplasmic Ca^{2+} ions bind to the enzyme with positive cooperativity and inhibit phosphorylation by P_i.

If lumenal Ca^{2+} could bind only to the phosphoenzyme (Scheme 4), a much higher concentration of cytoplasmic Ca^{2+} would be required for half-maximal inhibition of phosphorylation of the enzyme by P_i in the presence of saturating lumenal Ca^{2+} than in the absence of lumenal Ca^{2+}. However, Figure 2 shows that phosphorylation of the enzyme by P_i is inhibited by similar concentrations of cytoplasmic Ca^{2+} in the absence and in the presence of 40 mM lumenal Ca^{2+}. The values of $K_{0.5} = 2.1$ μM for inhibition of phosphorylation in the absence of lumenal Ca^{2+} and $K_{0.5} = 4$ μM in the presence of 40 mM lumenal Ca^{2+} show that Ca^{2+} binds to the lumenal sites of both the phosphoenzyme and the unphosphorylated enzyme, as well as to the cytoplasmic sites of the unphosphorylated enzyme, as described in Results.

The independent and simultaneous binding of Ca^{2+} to the cytoplasmic sites and to the lumenal sites of the enzyme (Figure 2) is not consistent with the E1–E2 and related models. According to the E1–E2 model, cytoplasmic Ca^{2+} binds to the cytoplasmic sites of the E1 conformation, with a high affinity for Ca^{2+}, whereas lumenal Ca^{2+} binds to the same sites when they are exposed to the lumen in the E2 conformation, with a low affinity for Ca^{2+} (Verjovski-Almeida et al., 1978; Chaloub et al., 1979; de Meis, 1981; de Meis, 1988). Thus, cytoplasmic and lumenal Ca^{2+} ions compete for binding to the enzyme and cannot bind to the enzyme simultaneously. However, we have shown previously that there is no such competition between the binding of Ca^{2+} from the cytoplasm and the lumen (Petithory & Jencks, 1988b; Myung & Jencks, 1991). This requires that Ca^{2+} binds to the enzyme simultaneously from the cytoplasm and the lumen and that occupancy of the lumenal binding sites by Ca^{2+} does not cause a significant change in the affinity of the cytoplasmic binding sites for Ca^{2+}. We have shown previously that several other predictions of the E1–E2 model are not consistent with experimental results (Pickart & Jencks, 1984; Petithory & Jencks, 1988b; Stahl & Jencks, 1987).

It should be noted that lumenal Ca^{2+} ions cannot bind to the high-affinity cytoplasmic sites of the unphosphorylated enzyme. If lumenal Ca^{2+} ions were allowed to bind to the cytoplasmic sites, these Ca^{2+} ions would dissociate from the cytoplasmic sites in the presence of EGTA, which would result in rapid leakage of Ca^{2+} from the vesicles. However, it was shown that at 5 °C there is no significant release of Ca^{2+} over a period of 1 min in the presence of EGTA and at 20 °C there is only a slow leakage of Ca^{2+} from the loaded vesicles (Prager et al., 1979). We also found that although the concentration of phosphoenzyme formed from P_i and Mg^{2+} decreased by 35% between 40 and 120 s because of slow leakage of Ca^{2+} from loaded vesicles, there was little or no decrease between 20 and 40 s. This also shows that there is no rapid leakage of Ca^{2+} from the SR vesicles.

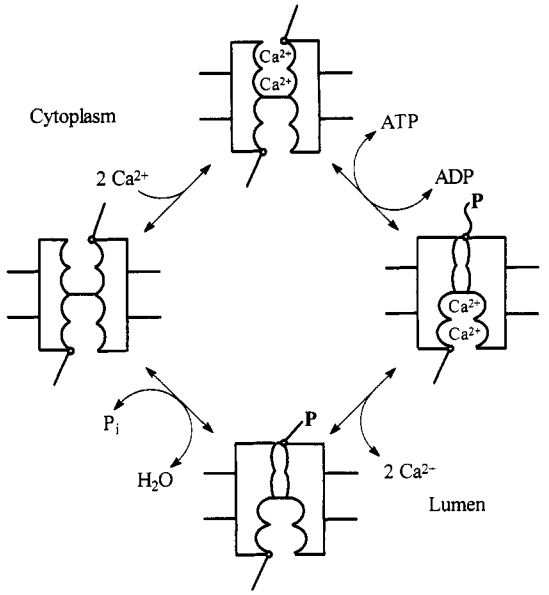
Figure 1 shows that movement of Ca^{2+} ions from the lumenal sites to the cytoplasmic sites of the phosphoenzyme is not allowed. If such movement could occur, an increase in the concentration of lumenal calcium would convert all of the enzyme to phosphoenzyme with four bound Ca^{2+} ions, $Ca_2{\cdot}E{\sim}P{\cdot}Ca_2{\cdot}Mg$, at equilibrium and would drive the formation of phosphoenzyme from P_i and Mg^{2+} to completion even in the presence of subsaturating concentrations of P_i and Mg^{2+}. However, Figure 1 shows that increasing the concentration of lumenal Ca^{2+} does not drive the phosphorylation by P_i to completion in the presence of subsaturating concentrations of P_i. We conclude that the two Ca^{2+} ions are transported from the high-affinity cytoplasmic sites to the low-affinity lumenal sites when the enzyme is phosphorylated by ATP.

Cytoplasmic and Lumenal Ca^{2+}-Binding Sites Are Different and Independent. Figure 3 shows that the presence or absence of 40 mM lumenal Ca^{2+} has no detectible effect on the rate constant of $k_{Ca} = 90$ s^{-1} for the dissociation of Ca^{2+} from the high-affinity cytoplasmic sites. Petithory and Jencks (1988b) have shown previously that the rate constant for binding of cytoplasmic Ca^{2+} to the enzyme is not changed by lumenal Ca^{2+}. These results show that there is no significant effect of the presence of lumenal Ca^{2+} on the equilibrium constant for the binding of cytoplasmic Ca^{2+} to the high-affinity sites (Myung & Jencks, 1991). We conclude that the lumenal binding sites and the cytoplasmic binding sites for Ca^{2+} are independent because cytoplasmic Ca^{2+} ions bind and dissociate at the high-affinity cytoplasmic sites with rate and equilibrium constants that are identical in the presence and in the absence of lumenal Ca^{2+}.

Figure 3 also shows that the rate constant of $k_p = 220$ s^{-1} for phosphorylation by ATP is not changed by the presence of 40 mM lumenal Ca^{2+}; *i.e.*, the occupancy of lumenal sites by Ca^{2+} does not inhibit phosphorylation. This requires that the Ca^{2+} ions that are bound at the low-affinity sites do not inhibit the translocation of Ca^{2+} from the high-affinity sites to the low-affinity sites upon phosphorylation by ATP. The rate-limiting step for phosphorylation by ATP has been shown to be a conformational change from the unreactive enzyme, $^{c}E{\cdot}Ca_2{\cdot}ATP$, to the active species, $^{a}E{\cdot}Ca_2{\cdot}ATP$, with a rate constant of $k = 220$ s^{-1}; this is followed by rapid phosphoryl transfer with a rate constant of $k \geq 1000$ s^{-1} (Petithory & Jencks, 1986). Therefore, the absence of inhibition of the rate of phosphorylation by ATP in the presence of lumenal Ca^{2+} requires that the dissociation of Ca^{2+} from lumenal sites of the unphosphorylated enzyme must be faster than the rate-limiting conformational change for phosphorylation by ATP; it may be combined with the translocation of Ca^{2+} from cytoplasmic to lumenal sites in one kinetically significant step.

Figure 4 shows that occupancy of the lumenal sites by Ca^{2+} has no significant effect on the rate constant for dissociation of Mg^{2+} from the catalytic site; *i.e.*, there is no detectible interaction between the lumenal binding sites for Ca^{2+} and the catalytic site. This is in contrast with the strong interaction that exists between the cytoplasmic high-affinity sites for Ca^{2+} and the catalytic site, although these sites are separated by at least 30 Å (Highsmith & Murphy, 1984; Scott, 1985; Toyoshima et al., 1993). The enzyme is phosphorylated only by ATP when cytoplasmic Ca^{2+} is bound to the high-affinity sites, whereas it is phosphorylated only by P_i in the absence of cytoplasmic Ca^{2+} (Yamamoto & Tonomura, 1967; Makinose, 1969; Kanazawa & Boyer, 1973; Pickart & Jencks, 1984). The dissociation constant of Mg^{2+} from the catalytic site is decreased by ~10-fold, from 8.7 mM (Punzengruber et al., 1978) to 0.94 mM (Reinstein & Jencks, 1993), when cytoplasmic Ca^{2+} is bound to the high-affinity sites. This also shows that there is an interaction between the cytoplasmic sites and the catalytic site and confirms the conclusion that the lumenal binding sites and the cytoplasmic binding sites for Ca^{2+} are different.

Scheme 10



A Model for the Active Transport of Ca^{2+} by the Ca^{2+}-ATPase. The model shown in Scheme 10 describes a mechanism for the transport of Ca^{2+} from one side to the other side of the SR membrane by the Ca^{2+}-ATPase that involves four binding sites for Ca^{2+}. Two Ca^{2+} ions from the cytoplasm bind to the high-affinity sites, and this binding activates the enzyme for phosphorylation by ATP. Phosphorylation of the enzyme results in translocation of the two Ca^{2+} ions from the high-affinity to the low-affinity sites. The Ca^{2+} ions dissociate from these sites into the lumen of the SR vesicles and the resulting phosphoenzyme is hydrolyzed to complete the transport cycle.

The high-affinity sites and the low-affinity sites must form a channel-like pathway in the Ca^{2+}-ATPase to allow translocation of Ca^{2+} ions; however, the translocation of Ca^{2+} ions from the high-affinity sites to the low-affinity sites occurs only upon phosphorylation of the enzyme by ATP, and translocation in the reverse direction occurs only when ADP is phosphorylated by the phosphoenzyme. If this were not the case, the Ca^{2+}-ATPase would become a Ca^{2+} channel and would allow rapid leakage of Ca^{2+} from the sarcoplasmic reticulum vesicles. Clarke et al. (1989, 1990) have shown that changes in six polar amino acid residues by site-directed mutagenesis in four putative transmembrane helices (M4, M5, M6, and M8) result in the loss of Ca^{2+} transport, Ca^{2+} activation of phosphorylation by ATP, and Ca^{2+} inhibition of phosphorylation by P_i and have proposed that the M4, M5, M6, and M8 helices form high-affinity Ca^{2+}-binding sites. However, it is not yet clear how these transmembrane helices are arranged to form the pathway for ion transport through the enzyme that is controlled by phosphorylation of the enzyme.

APPENDIX: DERIVATION OF EQUATIONS

Scheme 3 in Results describes a model in which the binding of cytoplasmic Ca^{2+} inhibits phosphorylation of the enzyme by P_i. The corresponding equilibrium constants are defined in eq A.1.

$$K_p = \frac{[E][P_i]}{[E{\cdot}P_i]}, \quad K'_{int} = \frac{[E{-}P]}{[E{\cdot}P_i]}, \quad K_{Ca} = \frac{[E]([Ca]_{cyt})^2}{[E{\cdot}Ca_2]} \tag{A.1}$$

The fraction of phosphoenzyme that is formed from P_i at equilibrium in the presence of cytoplasmic Ca^{2+} is described by eq A.2.

$$\frac{\sum E{-}P}{E_{tot}} = \frac{[E{-}P]}{[E{-}P] + [E{\cdot}P_i] + [E] + [E{\cdot}Ca_2]} \tag{A.2}$$

Substituting from eq A.1 into eq A.2 gives

$$\frac{\sum E{-}P}{E_{tot}} = \frac{K'_{int}\frac{[P_i]}{K_p}}{K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{cyt})^2}{K_{Ca}}} \tag{A.3}$$

The fraction of maximal phosphoenzyme that is formed from P_i in the absence of cytoplasmic Ca^{2+} is given by eq A.4.

$$\frac{\sum E{-}P_{max}}{E_{tot}} = \frac{[E{-}P]}{[E{-}P] + [E{\cdot}P_i] + [E]} \tag{A.4}$$

Substituting from eq A.1 into eq A.4 gives

$$\frac{\sum E{-}P_{max}}{E_{tot}} = \frac{K'_{int}\frac{[P_i]}{K_p}}{K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1} \tag{A.5}$$

The value of Y is defined as the concentration of phosphoenzyme formed divided by the maximal concentration of phosphoenzyme, $\sum E{-}P/\sum E{-}P_{max}$, and is obtained by dividing eq A.3 by eq A.5.

$$Y = \frac{\sum E{-}P}{\sum E{-}P_{max}} = \frac{K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1}{K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{cyt})^2}{K_{Ca}}} \tag{A.6}$$

The concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation by P_i is calculated by substituting $Y = {}^1/_2$ and $[Ca]_{cyt} = K_{0.5}$ into eq A.6.

$$\frac{1}{2} = \frac{K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1}{K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{(K_{0.5})^2}{K_{Ca}}} \tag{A.7}$$

Equation A.7 is rearranged to solve for $K_{0.5}$:

$$K_{0.5} = \left[K_{Ca}\left(K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1\right)\right]^{1/2} \tag{A.8}$$

Scheme 4 in Results describes a model in which the binding of cytoplasmic Ca^{2+} inhibits phosphorylation of the enzyme by P_i in the presence of lumenal Ca^{2+}. According to Scheme 4 the concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation by P_i is increased in the presence of lumenal Ca^{2+} by competition between the binding of lumenal Ca^{2+} to the phosphoenzyme and the binding of cytoplasmic Ca^{2+} to the unphosphorylated enzyme. The corresponding equilibrium constants are defined in eqs A.1 and A.9.

$$K'_{Ca} = \frac{[E{-}P]([Ca]_{lumen})^2}{[Ca_2{\cdot}E{\sim}P]} \tag{A.9}$$

The fraction of phosphoenzyme that is formed from P_i at equilibrium in the presence of both cytoplasmic Ca^{2+} and lumenal Ca^{2+} is described by eq A.10.

$$\frac{\sum E\text{–}P}{E_{tot}} = \frac{[Ca_2\cdot E\sim P] + [E\text{–}P]}{[Ca_2\cdot E\sim P] + [E\text{–}P] + [E\cdot P_i] + [E] + [E\cdot Ca_2]} \quad (A.10)$$

Substituting from eqs A.1 and A.9 into eq A.10 gives

$$\frac{\sum E\text{–}P}{E_{tot}} = \frac{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p}}{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{cyt})^2}{K_{Ca}}} \quad (A.11)$$

The fraction of maximal phosphoenzyme that is formed from P_i in the absence of cytoplasmic Ca^{2+} and in the presence of lumenal Ca^{2+} is given by eq A.12.

$$\frac{\sum E\text{–}P_{max}}{E_{tot}} = \frac{[Ca_2\cdot E\sim P] + [E\text{–}P]}{[Ca_2\cdot E\sim P] + [E\text{–}P] + [E\cdot P_i] + [E]} \quad (A.12)$$

Substituting from eqs A.1 and A.9 into eq A.12 gives

$$\frac{\sum E\text{–}P}{E_{tot}} = \frac{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p}}{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1} \quad (A.13)$$

The value of Y is defined as the concentration of phosphoenzyme formed divided by the maximal concentration of phosphoenzyme, $\sum E\text{–}P/\sum E\text{–}P_{max}$, and is obtained by dividing eq A.11 by eq A.13.

$$Y = \frac{\sum E\text{–}P}{\sum E\text{–}P_{max}} = \frac{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1}{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{cyt})^2}{K_{Ca}}} \quad (A.14)$$

The concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation by P_i is calculated by substituting $Y = {}^1/_2$ and $[Ca]_{cyt} = K_{0.5}$ into eq A.14.

$$\frac{1}{2} = \frac{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1}{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{(K_{0.5})^2}{K_{Ca}}} \quad (A.15)$$

Equation A.15 is rearranged to solve for $K_{0.5}$:

$$K_{0.5} = \left[K_{Ca}\left(\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1\right)\right]^{1/2} \quad (A.16)$$

Scheme 5 in Results describes a model in which the binding of cytoplasmic Ca^{2+} inhibits phosphorylation of the enzyme by P_i in the presence of lumenal Ca^{2+}. According to Scheme 5 lumenal Ca^{2+} can bind to both the phosphoenzyme and the unphosphorylated enzyme and cytoplasmic Ca^{2+} can bind to the unphosphorylated enzyme. The corresponding equilibrium constants are defined in eqs A.1, A.9, and A.17.

$$K''_{Ca} = \frac{[E](([Ca]_{lumen})^2}{[Ca_2\cdot E]} = \frac{[E\cdot Ca_2](([Ca]_{lumen})^2}{[Ca_2\cdot E\cdot Ca_2]} \quad (A.17)$$

The fraction of phosphoenzyme that is formed from P_i at equilibrium in the presence of both cytoplasmic Ca^{2+} and lumenal Ca^{2+} is described by eq A.18.

$$\frac{\sum E\text{–}P}{E_{tot}} = ([Ca_2\cdot E\sim P] + [E\text{–}P])/([Ca_2\cdot E\sim P] + [E\text{–}P] + [E\cdot P_i] + [E] + [E\cdot Ca_2] + [Ca_2\cdot E] + [Ca_2\cdot E\cdot Ca_2]) \quad (A.18)$$

Substituting from eqs A.1, A.9, and A.17 into eq A.18 gives

$$\frac{\sum E\text{–}P}{E_{tot}} = \left[\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{ir}\frac{[P_i]}{K_p}\right]\Big/ \left[\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{cyt})^2}{K_{Ca}} + \frac{([Ca]_{lumen})^2}{K''_{Ca}} + \frac{([Ca]_{cyt})^2}{K_{Ca}}\frac{([Ca]_{lumen})^2}{K''_{Ca}}\right] \quad (A.19)$$

The fraction of maximal phosphoenzyme that is formed from P_i in the absence of cytoplasmic Ca^{2+} and in the presence of lumenal Ca^{2+} is given by eq A.20.

$$\frac{\sum E\text{–}P_{max}}{E_{tot}} = \frac{[Ca\cdot E\sim P] + [E\text{–}P]}{[Ca_2\cdot E\sim P] + [E\text{–}P] + [E\cdot P_i] + [E] + [Ca_2\cdot E]} \quad (A.20)$$

Substituting from eqs A.1, A.9, and A.17 into eq A.20 gives

$$\frac{\sum E\text{–}P_{max}}{E_{tot}} = \frac{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p}}{\frac{([Ca]_{lumen})^2}{K'_{Ca}}K'_{int}\frac{[P_i]}{K_p} + K'_{int}\frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([Ca]_{lumen})^2}{K''_{Ca}}} \quad (A.21)$$

The value of Y is defined as the concentration of phosphoenzyme formed divided by the maximal concentration of phosphoenzyme, $\sum E\text{–}P/\sum E\text{–}P_{max}$, and is obtained by dividing eq A.19 by eq A.21.

$$Y = \frac{\sum \text{E–P}}{\sum \text{E–P}_{max}} = \left[\frac{([\text{Ca}]_{lumen})^2}{K'_{Ca}} K'_{int} \frac{[P_i]}{K_p} + K'_{int} \frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} \right] \Big/ \left[\frac{([\text{Ca}]_{lumen})^2}{K'_{Ca}} K'_{int} \frac{[P_i]}{K_p} + K'_{int} \frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([\text{Ca}]_{cyt})^2}{K_{Ca}} + \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} + \frac{([\text{Ca}]_{cyt})^2}{K_{Ca}} \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} \right] \quad \text{(A.22)}$$

The concentration of cytoplasmic Ca^{2+} that causes half-maximal inhibition of phosphorylation by P_i is calculated by substituting $Y = {}^1/_2$ and $[\text{Ca}]_{cyt} = K_{0.5}$ into eq A.22.

$$\frac{1}{2} = \left[\frac{([\text{Ca}]_{lumen})^2}{K'_{Ca}} K'_{int} \frac{[P_i]}{K_p} + K'_{int} \frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} \right] \Big/ \left[\frac{([\text{Ca}]_{lumen})^2}{K'_{Ca}} K'_{int} \frac{[P_i]}{K_p} + K'_{int} \frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{(K_{0.5})^2}{K_{Ca}} + \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} + \frac{(K_{0.5})^2}{K_{Ca}} \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} \right] \quad \text{(A.23)}$$

Equation A.23 is rearranged to solve for $K_{0.5}$:

$$K_{0.5} = \left[\frac{K_{Ca}}{1 + \dfrac{([\text{Ca}]_{lumen})^2}{K''_{Ca}}} \left(\frac{([\text{Ca}]_{lumen})^2}{K'_{Ca}} K'_{int} \frac{[P_i]}{K_p} + K'_{int} \frac{[P_i]}{K_p} + \frac{[P_i]}{K_p} + 1 + \frac{([\text{Ca}]_{lumen})^2}{K''_{Ca}} \right) \right]^{1/2} \quad \text{(A.24)}$$

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