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Binding of Ca^{2+} to the Calcium Adenosinetriphosphatase of Sarcoplasmic Reticulum†

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ABSTRACT: The binding of Ca^{2+} and the resulting change in catalytic specificity that allows phosphorylation of the calcium ATPase of sarcoplasmic reticulum by ATP were examined by measuring the amount of phosphoenzyme formation from [^{32}P]ATP, or ^{45}Ca incorporation into vesicles, after the simultaneous addition of ATP and EGTA at different times after mixing enzyme and Ca^{2+} (25 °C, pH 7.0, 5 mM $MgSO_4$, 0.1 M KCl). A "burst" of calcium binding in the presence of high [Ca^{2+}] gives ~12% phosphorylation and internalization of two Ca^{2+} at very short times after the addition of Ca^{2+} with this assay. This shows that calcium binding sites are available on the cytoplasmic-facing side of the free enzyme. Calcium binding to these sites induces the formation of $^{c}E{\cdot}Ca_2$, the stable high-affinity form of the enyzme, with $k = 40\ s^{-1}$ at saturating [Ca^{2+}] and a half-maximal rate at approximately 20 μM Ca^{2+} (from $K_{diss} = 7.4 \times 10^{-7}$ M for Ca·EGTA). The formation of $^{c}E{\cdot}Ca_2$ through a "high-affinity" pathway can be described by the scheme $E \overset{1}{\rightleftharpoons} {}^{c}E{\cdot}Ca_1 \overset{2}{\rightleftharpoons} {}^{c}E{\cdot}Ca_2$, with $k_1 = 3 \times 10^6\ M^{-1}\ s^{-1}$, $k_2 = 4.3 \times 10^7\ M^{-1}\ s^{-1}$, $k_{-1} = 30\ s^{-1}$, $k_{-2} = 60\ s^{-1}$, $K_1 = 9 \times 10^{-6}$ M, and $K_2 = 1.4 \times 10^{-6}$ M. The approach to equilibrium from E and 3.2 μM Ca^{2+} follows $k_{obsd} = k_f + k_r = 18\ s^{-1}$ and gives $k_f = k_r = 9\ s^{-1}$. The rate of exchange of ^{45}Ca into the inner position of $^{c}E{\cdot}Ca_2$ shows an induction period and is not faster than the approach to equilibrium starting with E and ^{45}Ca. The dissociation of ^{45}Ca from the inner position of $^{c}E{\cdot}{}^{45}Ca{\cdot}Ca$ in the presence of 3.2 μM Ca^{2+} occurs with a rate constant of 7 s^{-1}. These results are inconsistent with a slow conformational change of free E to give ^{c}E, followed by rapid binding–dissociation of Ca^{2+}.

The binding of two calcium ions to cytoplasmic-facing sites on the calcium ATPase of SRV[1] is of interest because it is a critical vectorial step in the transport process and it initiates the change in catalytic specificity of the enzyme that allows

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[1] Abbreviations: SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; P_i, inorganic phosphate.

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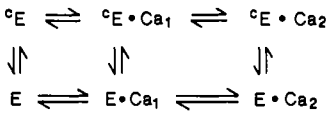
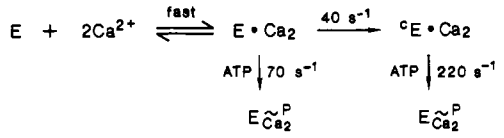
Scheme I



Scheme II



phosphorylation by ATP, the first chemical step in the transport process (de Meis & Vianna, 1979; Pickart & Jencks, 1984). Several pathways for the binding of two Ca^{2+} ions are shown in Scheme I, in which E is the free enzyme and $^cE{\cdot}Ca_2$ is defined simply as the stable species of enzyme with bound calcium; it corresponds to the classical "$E_1{\cdot}Ca_2$", but we avoid this nomenclature because of the several different meanings that have been ascribed to the species "E_1", which does not seem to have been observed directly. There is abundant physical and chemical evidence that the conversion of E to $^cE{\cdot}Ca_2$ involves at least one conformational change, which is presumably responsible for the well-known cooperativity of calcium binding (Dupont, 1976; Dupont & Leigh, 1978; Ikemoto et al., 1978; Murphy, 1978; Guillain et al., 1980, 1981; Inesi et al., 1980; Champeil et al., 1983; Fernandez-Belda et al., 1984; Kawakita et al., 1985; Anderson & Jørgensen, 1985; Tanford et al., 1985; Inesi, 1987).

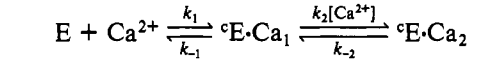
All of the several pathways for calcium binding in Scheme I have been proposed previously (Dupont & Leigh, 1978; Sumida et al., 1978; Guillain et al., 1980, 1981; Inesi et al., 1980; Dupont et al., 1985), but there is still uncertainty as to which of them are actually followed. An important experimental observation is that calcium dissociation to give E is inhibited by very low concentrations of calcium, which provides evidence for a dissociation pathway through a high-affinity species, $^cE{\cdot}Ca_1$ or ^cE (Guillain et al., 1980, 1981; Dupont, 1982; Petithory & Jencks, 1988). There is also strong evidence that binding and dissociation are sequential and that the two Ca^{2+} ions do not interchange during the reaction (Ikemoto et al., 1981; Dupont, 1982; Dupont et al., 1985; Inesi, 1987; Petithory & Jencks, 1988). A recent investigation in this laboratory has utilized a chemical assay for following the dissociation of Ca^{2+} by measuring either phosphorylation by [^{32}P]ATP or internalization of ^{45}Ca after the simultaneous addition of ATP and EGTA. It was shown that dissociation of the first Ca^{2+} ion is sufficient to prevent phosphorylation by ATP, and rate constants for Ca^{2+} dissociation steps through a high-affinity pathway were determined (Petithory & Jencks, 1988).

The results reported here were obtained with the same assay and show that the binding of Ca^{2+} to the calcium ATPase can be described by two relatively simple mechanisms:

(1) Two Ca^{2+} ions bind rapidly to preexisting, low-affinity sites with $K_{0.5} \sim 20\ \mu M$ on the cytoplasmic side of the vesicular membrane (Scheme II). The resulting $E{\cdot}Ca_2$ is converted to the more stable $^cE{\cdot}Ca_2$ species with a rate constant of 40 s^{-1}. This species is phosphorylated in the presence of saturating ATP with $k = 220\ s^{-1}$ (Petithory & Jencks, 1986). Alternatively, the low-affinity species $E{\cdot}Ca_2$ can itself bind ATP and undergo phosphorylation with $k = 70\ s^{-1}$ (Stahl & Jencks, 1987).

(2) Two Ca^{2+} ions bind sequentially to E through a high-affinity pathway with two kinetically significant steps, according to the mechanism of Scheme III. Although the species $^cE{\cdot}Ca_1$ is not competent to support phosphorylation, it does have a high-affinity binding site for the second Ca^{2+} ion, with $K_2 = 1.4\ \mu M$. This suggests that it has undergone the conformational change that is responsible for the development of cooperativity.

Scheme III



It is concluded that binding sites for two calcium ions exist on the cytoplasmic surface of the free enzyme and that binding of one Ca^{2+} brings about a conformational change that increases the affinity for the second Ca^{2+}; rapid binding of Ca^{2+} ions to a low-affinity site induces a conformational change with a rate constant of 40 s^{-1}. These changes could be brought about by twisting or other rearrangements of α-helices surrounding the bound Ca^{2+} ions (Tanford, 1982; Brandl et al., 1986). There is no evidence for the formation of free ^cE (or E_1).

Materials and Methods

Reagents and experimental procedures were the same as those described in a previous paper (Petithory & Jencks, 1988), unless indicated otherwise. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a slight modification of the MacLennan (1970) procedure, as described previously (Khananshvili & Jencks, 1988). The preparations hydrolyzed ATP at 3.5–5.0 μmol/(mg of total protein·min) when the vesicles were made permeable with the calcium ionophore A23187 (2 μL of 1–2 mM in ethanol for 2 mL of reaction solution). SRV as isolated were ~98% sealed, as shown by an increase of ~50-fold in the steady-state ATP hydrolysis rate upon addition of ionophore in the standard assay. The amount of phosphoenzyme observed with intact vesicles at saturating [Ca^{2+}] and [ATP] was 2.0–4.0 nmol/mg of total protein.

Leaky Vesicles. Vesicles were made permeable to Ca^{2+} by incubation of ~6 mg/mL SR protein with 1.0 mM EGTA plus 0.17 M Tris base (added dropwise, to give pH $\simeq$ 9) for ~2 h at room temperature (de Meis & Carvahlo, 1974). Vesicles were considered to be leaky when addition of ionophore A23187 to the standard ATPase assay failed to give a rate increase for steady-state hydrolysis. $CaCl_2$ (0.5 mM) was added to give [Ca^{2+}] $\simeq$ 20 μM in the 2-fold diluted vesicle suspension, and the pH was adjusted to 7.0 by dropwise addition of 0.2 M maleic acid and 0.17 M Tris (pH 2.5).

Calcium ATPase activity was measured spectrophotometrically by coupling ADP production to NADH oxidation by using pyruvate kinase and lactate dehydrogenase (Rossi et al., 1979). Standard assay conditions were 40 mM MOPS, 100 mM KCl, 5.0 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.40 mM EGTA (23 μM free Ca^{2+}), and 1.5 mM ATP, pH 7.0, 25 °C.

For experiments in which high concentrations of Ca^{2+} and EGTA were mixed, proton release upon formation of the Ca·EGTA complex was neutralized by the addition of 1.9 equiv of Tris base to the substrate solution.

Concentrations of free Ca^{2+} were calculated from a dissociation constant of 7.4×10^{-7} M for Ca·EGTA (Godt, 1974). This value was determined under conditions (pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP) similar to those used in our experiments.

Rapid Mix–Quench Experiments. The formation or decay of covalent phosphoenzyme was followed by using a rapid mix–quench apparatus that can be used with either three or four syringes of 1-mL volume, as described previously (Stahl

& Jencks, 1984; Petithory & Jencks, 1988).

Passively Loaded Vesicles. SRV were passively loaded with high [Ca^{2+}] in some experiments in order to inhibit phosphoenzyme hydrolysis. SRV were incubated for 12–16 h at 4 °C in a solution containing ~30 mg/mL SRV protein, 40 mM MOPS, pH 7.0, 100 mM KCl, 5.0 mM $MgSO_4$, 0.2 M sucrose, and 20 mM $CaCl_2$. For each reaction, 10 μL of this stock solution of SRV was mixed with 0.99 mL of a solution containing appropriate concentrations of calcium or EGTA. This solution was then loaded into syringe A of the rapid-mixing apparatus, and the reaction was started within 15–30 s. It was shown previously that the levels of phosphorylation remain constant for the duration of the experiment after phosphorylation is complete; i.e., there is no significant hydrolysis of phosphoenzyme that arises from calcium leakage out of the loaded vesicles (Petithory & Jencks, 1986). Filtration of the vesicles was carried out within 5 s in experiments in which uptake of ^{45}Ca was measured. Control experiments in which filtration was delayed for 10 s showed no detectable leakage of ^{45}Ca from the vesicles in this time period.

Computer Programs. Rate constants were estimated by using programs written for IBM-compatible microcomputers that allow simulation of reaction progress and fitting of experimental data by a nonlinear least-squares procedure, as described previously (Stahl & Jencks, 1987). The time course for chelation of Ca^{2+} by EGTA at various concentrations (Table I) was calculated according to

$$Ca^{2+} + EGTA \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ca{\cdot}EGTA \qquad (1)$$

with $k_f = 1.8 \times 10^6\ M^{-1}\ s^{-1}$ (Smith et al., 1977) and $k_r = 1.3\ s^{-1}$, obtained from $(K_{diss})(k_f)$ with $K_{diss} = 7.4 \times 10^{-7}$ M (Godt, 1974).

RESULTS

Assay of $^cE{\cdot}Ca_2$. The formation of the ATP-reactive species $^cE{\cdot}Ca_2$ was measured by the addition of a solution containing ATP and EGTA to the vesicles. The reaction was monitored with [^{32}P]ATP, followed by an acid quench in ~30 ms and measurement of the amount of [^{32}P]E~P·Ca_2 that was formed, or by use of ^{45}Ca and measurement of the amount of labeled calcium that was retained in the vesicles after filtration. Under the conditions of the assay $^cE{\cdot}Ca_2$ 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} (eq 2). Thus,

$$^cE \cdot Ca_2 \cdot ATP \xrightarrow[80\ s^{-1}]{220\ s^{-1}} E{\sim}P \cdot Ca_2 \qquad (2)$$
$$E \cdot ATP + 2Ca^{2+}$$

$^cE{\cdot}Ca_2$ 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% (Petithory & Jencks, 1986).

Formation of ATP-Reactive Enzyme at High [Ca^{2+}]. The formation of $^cE{\cdot}Ca_2$ after the addition of calcium to free enzyme was assayed by the addition of ATP plus EGTA at various times, t_1, as described above. A burst of phosphorylation corresponding to 12 ± 2% of E_{tot}, followed by a first-order increase in EP with k_{obsd} = 38–40 s^{-1}, was observed when vesicles passively loaded with 20 mM calcium (Figure 1, closed symbols) or leaky vesicles (open symbols) were preincubated with EGTA for 15 s and then mixed with sufficient calcium to give a final free [Ca^{2+}] during t_1 in the range 150–300 μM. The line drawn in Figure 1 is the best fit to the closed circles (final [Ca^{2+}] in t_1 = 200 μM) with k_{obsd} = 38 s^{-1} and a burst of EP/E_{tot} = 0.12. The closed triangles [[Ca^{2+}] in t_1 = 342 μM (▲) and 156 μM (▼)] fit best to a burst of 0.11 and k_{obsd} = 40 s^{-1} (line not shown). The results with leaky vesicles (open squares, [Ca^{2+}] in t_1 = 197 μM) give a best fit to k_{obsd} = 38 s^{-1} and a burst of ~0.15; these points show more scatter because phosphoenzyme hydrolysis is not inhibited in leaky vesicles.

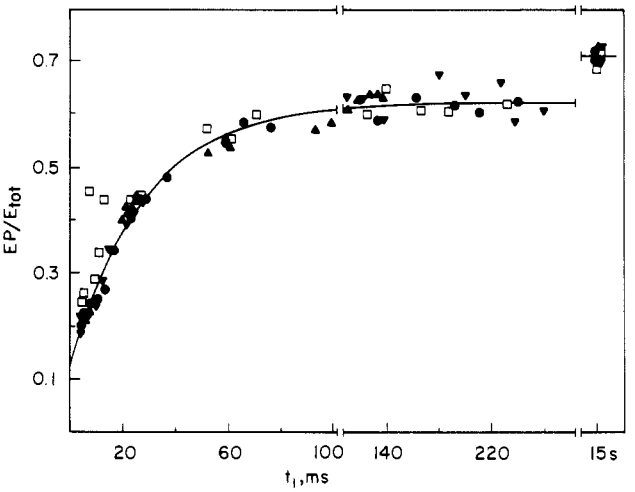


FIGURE 1: Reaction of free enzyme with 150–300 μM Ca^{2+} assayed with [^{32}P]ATP plus EGTA. By use of the four-syringe configuration on the rapid mix–quench apparatus, passively loaded vesicles (▲, ▼, ●) or leaky vesicles (□) were mixed with $CaCl_2$ for varying times t_1 and then reacted with [γ-^{32}P]ATP plus 5 mM EGTA for t_2 = 25–35 ms, followed by acid quench. Syringe A contained ~0.25 mg/mL passively loaded SRV and either 4.30 mM EGTA plus 0.17 mM $CaCl_2$ (0.03 μM free Ca^{2+}) (▲); 1.72 mM EGTA plus 0.17 mM $CaCl_2$ (0.08 μM free Ca^{2+}) (▼, ●); or 0.2 mg/mL leaky SRV in 0.91 mM EGTA plus 0.04 mM $CaCl_2$ (0.03 μM free Ca^{2+}) (□). Syringe B contained 4.80 mM $CaCl_2$ (▲), 1.85 mM $CaCl_2$ (▼), 1.94 mM $CaCl_2$ (●), or 1.26 mM $CaCl_2$ (□). Syringe C contained 0.9 mM [γ-^{32}P]ATP plus 15 mM EGTA. Syringe D contained 2.0 M HCl and 60 mM KH_2PO_4. All syringes except D also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM $MgSO_4$. Final free [Ca^{2+}] in t_1 (with the concentration of free Ca^{2+} immediately after mixing the contents of syringes A and B in parentheses): 342 μM (2.4 mM) (▲); 200 μM (0.97 mM) (●); 156 μM (0.92 mM) (▼); 197 μM (0.63 mM) (□). The line is drawn for a burst of EP/E_{tot} = 0.12 followed by a first-order rate constant of 38 s^{-1} to an end point of EP/E_{tot} = 0.63.

Since the passively loaded vesicles and the leaky vesicles were preincubated with ~1 mM EGTA to ensure a very low exterior free calcium concentration before the reaction, a large concentration of unbuffered calcium was added in order to obtain a final free [Ca^{2+}] of >150 μM during t_1. When high concentrations of calcium and EGTA are mixed together such that neither is in large excess, chelation is not instantaneous; the second-order rate constant for calcium chelation by EGTA is $1.8 \times 10^6\ M^{-1}\ s^{-1}$ under conditions similar to those of our experiments (Smith et al., 1977), and transient changes in free [Ca^{2+}] occur upon mixing. In the experiments shown in Figure 1, there was a very high concentration of free calcium present immediately after mixing, ranging from 0.9 to 2.4 mM, which did not decrease to its final calculated value until after ~5 ms in t_1. This exposure of the enzyme to a transient high concentration of free calcium at very short t_1 can be diminished if the calcium in syringe B is itself buffered with EGTA, but it cannot be totally eliminated if the desired final free [Ca^{2+}] in t_1 is greater than ~6 μM. However, the quench with 5 mM EGTA at the end of t_1 decreases the free calcium concentration with a half-time of <0.1 ms.

Dependence of the Burst Size on [Ca^{2+}]. Table I shows that the amount of phosphorylation in the burst phase is dependent on the concentration of free calcium immediately after mixing, not on the final free [Ca^{2+}] after chelation is complete. When

Table I: Dependence on Free $[Ca^{2+}]$ of the Burst of ATP-Reactive Enzyme[a]

syringe A (mM)	syringe B (mM)	$[Ca^{2+}]_0$[b] (μM)	$[Ca^{2+}]_{5ms}$ (μM)	$[Ca^{2+}]_{final}$ (μM)	burst EP/E_{tot}
0.17 Ca	4.80 Ca	2400	350	340	0.12
4.30 EGTA					
0.04 Ca	1.26 Ca	630	201	197	~0.15[c]
0.91 EGTA					
0.17 Ca	1.85 Ca	925	190	155	0.12
1.72 EGTA					
0.043 EGTA	0.40 Ca	200	182	178	0.11[d]
1.72 EGTA	1.32 Ca	660	24	4.6	0.11
0.17 Ca	1.25 Ca	625	35	3.6	0.10[e]
1.72 EGTA					±0.02
0.09 Ca	5.20 Ca	108	10.4	6.6	0.08
0.86 EGTA	5.00 EGTA				
0.17 Ca	10.0 Ca	43	4.8	4.8	0.06
1.72 EGTA	10.0 EGTA				
0.09 Ca	4.90 Ca	14	4.3	4.2	~0.02
0.86 EGTA	5.00 EGTA				
0.17 Ca	11.0 Ca	4.0	3.2	3.2	0
1.72 EGTA	12.0 EGTA				

[a] Intact vesicles passively loaded with 10–20 mM $CaCl_2$, except as noted. [b] $[Ca^{2+}]_0$ is the concentration of free Ca^{2+} immediately after mixing the contents of syringes A and B (half the concentration of free Ca^{2+} in syringe B). [c] Leaky vesicles. [d] Intact vesicles, not loaded with calcium. [e] The time course for this reaction showed a burst followed by a decrease in ATP-reactive enzyme.

enzyme preincubated with EGTA in syringe A was mixed with 1.32 mM unbuffered calcium from syringe B to give 660 μM free Ca^{2+} before chelation with EGTA and 4.8 μM free calcium after chelation was complete, a burst amounting to ~12% of E_{tot} was observed (Table I). When enzyme was mixed with buffered calcium to give an initial free $[Ca^{2+}]$ of 43 μM and a final free $[Ca^{2+}]$ of 4.8 μM, the burst was about half as large. No burst was observed when the concentration of calcium immediately after mixing was 4 μM. The concentration of free calcium immediately after mixing is an upper limit for the amount of free calcium "seen" by the enzyme. Since the burst phase appeared to be complete by 5 ms, the concentration of free calcium 5 ms after mixing was taken as a lower limit for the calcium dependence of the burst (Table I).

Ca/EP Stoichiometry in the Burst. The reaction of enzyme with a high concentration of free calcium for varying t_1, followed by addition of ATP plus EGTA, was also examined with ^{45}Ca instead of [^{32}P]ATP in order to determine the Ca/EP stoichiometry of the phosphoenzyme formed in the burst and the subsequent slow phase (Figure 2, diamonds). Enzyme preincubated with 0.9 mM EGTA was mixed with an equal volume containing 1.10 mM ^{45}Ca for varying times t_1 (final free $[Ca^{2+}]$ in t_1 = 120 μM), followed by 900 μM ATP plus 15 mM EGTA. The data fit a burst size of ~24% $^{45}Ca/E_{tot}$ and an exponential with k_{obsd} = 38 s^{-1} to an end point of ~140% $^{45}Ca/E_{tot}$. The reaction of enzyme with 200 μM free Ca^{2+}, assayed with [^{32}P]ATP, is shown for comparison (Figure 2, circles).

The identical time courses for the formation of ATP-reactive enzyme at high $[Ca^{2+}]$ monitored by [^{32}P]EP formation and by ^{45}Ca accumulation and the 2:1 Ca/EP stoichiometry that is maintained throughout (Figure 2) show that the phosphoenzyme formed in the burst and in the exponential phase contains two bound Ca^{2+} ions.

The burst in the formation of ATP-reactive enzyme is attributed to the rapid binding of calcium at high concentrations to low-affinity sites on the exterior of the vesicle to form $E{\cdot}Ca_2$, which is an ATP-reactive species that is phosphorylated by a pathway different from that followed by $^cE{\cdot}Ca_2$. The simultaneous addition of ATP and calcium to E gives an $E{\cdot}Ca_2{\cdot}$ATP intermediate that undergoes phosphorylation through this alternative pathway with a first-order rate constant of 70 s^{-1}, as described previously (Stahl & Jencks, 1987). This

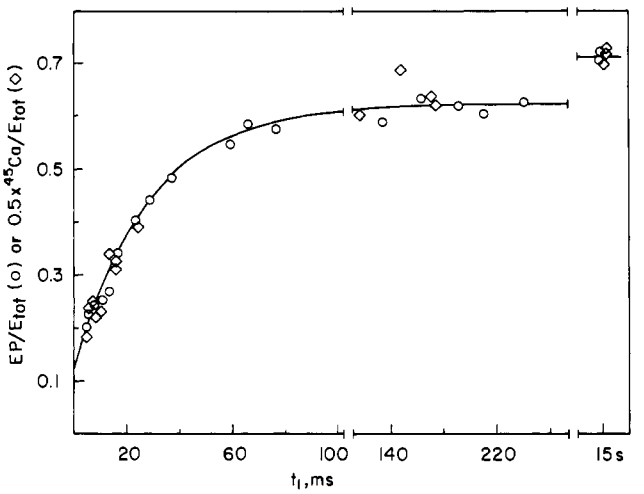


FIGURE 2: Reaction of free enzyme with high concentrations of calcium, assayed by using ^{45}Ca (◇) or [^{32}P]ATP (○, data of Figure 1). For (◇), intact vesicles preincubated in EGTA were mixed with ^{45}Ca to give 120 μM free ^{45}Ca in t_1. Syringe A contained 0.3 mg/mL SRV plus 0.86 mM EGTA. Syringe B contained 1.10 mM $^{45}CaCl_2$. Syringe C contained 0.9 mM ATP and 15 mM EGTA. Blanks were performed by mixing the contents of syringe A with 1.10 mM $^{45}CaCl_2$ plus 15 mM EGTA from syringe B, followed by 0.9 mM ATP from syringe C. The final concentration of reactants in the blank is the same as for the experimental points, so that the amount of ^{45}Ca uptake due to turnover in the collection tube prior to filtration over Millipore filters will be the same for both blanks and reaction points. Blanks performed by simply reversing the order of addition of ATP/EGTA and ^{45}Ca are not satisfactory, because immediately after mixing the conditions are reproduced that result in the transiently high concentration of free Ca^{2+} before chelation by EGTA, so that these blanks would also include the burst phase. The reaction assayed with [^{32}P]ATP was performed as described in the legend to Figure 1 (○). The line is drawn for a burst of EP/E_{tot} (=0.5 × $^{45}Ca/E_{tot}$) = 0.12 followed by a first-order rate constant of 38 s^{-1} and an end point of EP/E_{tot} (=0.5 × $^{45}Ca/E_{tot}$) = 0.63.

Scheme IIa

$$E \underset{K_{Ca}}{\overset{K_{-Ca}}{\rightleftharpoons}} E \cdot Ca_2 \xrightarrow[38\text{-}40\ s^{-1}]{k_c'} {}^cE \cdot Ca_2$$

$E{\cdot}Ca_2{\cdot}$ATP intermediate binds exterior calcium weakly, compared with $^cE{\cdot}Ca_2$, and partitions between phosphorylation and irreversible calcium dissociation in the presence of EGTA to give 17–20% covalent phosphoenzyme (Stahl & Jencks, 1987).

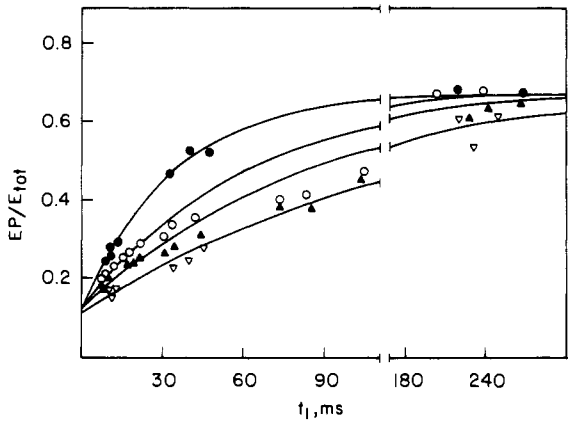
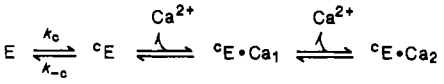


FIGURE 3: Reaction of free enzyme with 10–50 μM Ca^{2+}. Reactions were carried out essentially as described in Figure 1. Syringe A contained 0.3 mg/mL passively loaded SRV and 0.86 mM EGTA plus 0.087 mM $CaCl_2$. Syringe B contained 5.0 mM EGTA plus either 5.4 mM (▽), 5.5 mM (▲), 5.6 mM (○), or 5.8 mM (●) $CaCl_2$. Syringe C contained 0.9 mM [^{32}P]ATP plus 15 mM EGTA. Syringe D contained 2 M HCl and 60 mM KH_2PO_4. Final free [Ca^{2+}] in t_1 (with concentration of free Ca^{2+} immediately after mixing in parentheses): 10 μM (205 μM) (▽), 14 μM (250 μM) (▲), 20 μM (300 μM) (○), and 53 μM (400 μM) (●). The lines are drawn for first-order rate constants, fit to the reaction points up to 50 ms, of 8 (▽), 12 (▲), 16 (○), and 29 s^{-1} (●).

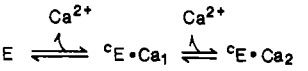
These results are consistent with the mechanism shown in Scheme IIa for the formation of $^cE{\cdot}Ca_2$ at high concentrations of Ca^{2+}. The free enzyme, E, binds calcium very rapidly to form $E{\cdot}Ca_2$. This gives $E{\cdot}Ca_2{\cdot}$ATP upon addition of saturating ATP plus EGTA during t_2 and results in the phosphorylation of ~12% of E_{tot}.

The participation of the low-affinity $E{\cdot}Ca_2$ species as an intermediate in the calcium-binding pathway was further characterized by measuring the time courses for formation of ATP-reactive enzyme at different calcium concentrations. Figure 3 shows that when calcium is added to free enzyme to give final concentrations of free calcium in the range of 10–50 μM during t_1, the formation of ATP-reactive enzyme, assayed as described above, proceeds essentially to completion (EP_{max} = 70% of E_{tot} in the presence of ATP plus EGTA). The free [Ca^{2+}] immediately after mixing ranged from 200 (▽) to 400 μM (●) in these experiments, and the burst size remained constant, within experimental error, at ~12 ± 2% of E_{tot}. The reactions do not show satisfactory first-order kinetics, but it is clear that the rate is sensitive to changes in Ca^{2+} concentration in the range 10–50 μM, and a rough estimate of the dependence of the rates on calcium concentration can be obtained from the initial time points. The solid lines in Figure 3 are drawn through the initial points with (pseudo-) first-order rate constants of 8, 12, 16, and 29 s^{-1} for final free calcium concentrations of 10, 14, 20, and 53 μM, respectively. Although these rate constants are too crude for quantitative analysis, they do fit a Hill plot with a slope of 1.5 and a midpoint at 24 μM Ca^{2+}; they give a somewhat better fit to a dependence on [Ca^{2+}]^2 than on [Ca^{2+}] (not shown). All of these concentrations are much larger than the $(K_{ov})^{0.5}$ concentration of 3.4 μM that is observed for Ca^{2+} binding and activation of reactivity toward ATP at equilibrium (Petithory & Jencks, 1988). They establish that the rate of formation of $^cE{\cdot}Ca_2$ depends on the occupancy of a low-affinity calcium binding site on the cytoplasmic side of the membrane with a dissociation constant on the order of $(K'_{Ca})^{0.5}$ = 20 μM. A dissociation constant of this magnitude is also consistent with the dependence of the size of the initial burst on the calcium concentration from 0 to 5 ms after the addition of calcium to enzyme and EGTA (Table I).

Scheme IV



Scheme IIIa



The High-Affinity Pathway for Calcium Binding. The reaction of enzyme with 3.2 μM Ca^{2+} was followed with the same assay of [^{32}P]ATP plus EGTA under conditions in which there is no transient increase in free [Ca^{2+}] upon mixing. The reaction represents an approach to equilibrium, rather than to completion, and follows a first-order time course with a rate constant of k_{obsd} = 18 s^{-1} and no initial burst, as shown in Figure 4. The final EP level is 48% of EP_{max} (34% EP/E_{tot}), which is in good agreement with the expected value from the previously determined value of $(K_{ov})^{0.5}$ = 3.4 μM Ca^{2+} (Petithory & Jencks, 1988). It is important to note that the calcium concentrations in these experiments were calculated from a dissociation constant of 7.4 × 10^{-7} M for Ca·EGTA that was determined under conditions similar to those used in this work (Godt, 1974); this gives free Ca concentrations that are approximately 2-fold larger than those obtained from dissociation constants used by some other workers, as described previously (Petithory & Jencks, 1988).

The observed rate constant cannot be accounted for by the low-affinity binding pathway for 2 Ca^{2+} described in the preceding section. The rate constant for the formation of ATP-reactive enzyme at 3.2 μM Ca^{2+} by a path involving Ca^{2+} binding sites with $K_{app} \geq (20\ \mu M)^2$ followed by a conformational change at 40 s^{-1} (Scheme I) is equal to 40 s^{-1} times the fraction of enzyme that is $E{\cdot}Ca_2$ at 3.2 μM Ca^{2+}. Assuming a value of $K'_{Ca} = (20\ \mu M)^2$, $k_c' = 40\ s^{-1}$ gives a value of k_{obsd} equal to only ~1 s^{-1} for the formation of $^cE{\cdot}Ca$ from E by the pathway of Scheme I in the presence of 3.2 μM Ca^{2+}; the calculated value from the Hill slope of n = 1.5 is 2 s^{-1}.

This result requires that the formation of ATP-reactive enzyme at low [Ca] must proceed through a "high-affinity" pathway that is different from the pathway followed in the presence of high [Ca]. The formation of ATP-reactive enzyme proceeds to equilibrium, not to completion, so that k_{obsd} represents the sum of the first-order rate constants k_f and k_r for the forward and reverse reactions, respectively (eq 3). Since

$$E + 2Ca^{2+} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} {}^cE{\cdot}Ca_2 \qquad (3)$$

the reaction proceeds to ~50% completion, it is described by k_f/k_r = 1 and $k_f = k_r$ = 9 s^{-1} at 3.2 μM Ca. Correcting for a contribution of 1 s^{-1} from the low-affinity pathway, in both directions, gives $k_f = k_r$ = 8 s^{-1}.

Two general mechanisms for the high-affinity pathway are consistent with these data. According to the first mechanism, shown in Scheme IV, the slow, rate-limiting step is a conformational change between free E and the high-affinity form ^cE, followed by fast binding and dissociation of Ca^{2+}. According to the second mechanism, shown in Scheme IIIa, there is no kinetically significant conformational change of the free enzyme; the conformational change accompanies or follows the binding of Ca^{2+}.

Two experiments were carried out to distinguish between these two mechanisms. In the first experiment the rate of incorporation of ^{45}Ca into the inner calcium binding site was measured, starting with $^cE{\cdot}Ca_2$, in the presence of low [Ca^{2+}]

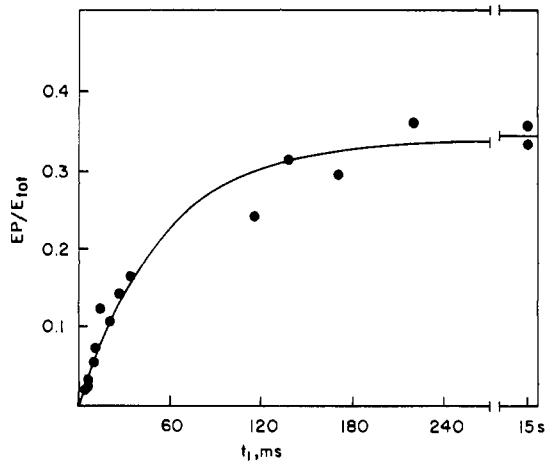


FIGURE 4: Reaction of free enzyme with 3.2 μM free Ca^{2+}. Reactions were performed essentially as described in Figure 1. Syringe A contained 0.3 mg/mL passively loaded SRV in 1.72 mM EGTA and 0.17 mM $CaCl_2$. Syringe B contained 10.0 mM EGTA and 10.0 mM $CaCl_2$ (free [Ca^{2+}] in syringe B = 8 μM). Syringe C contained 0.9 mM [^{32}P]ATP plus 15 mM EGTA. Syringe D contained 2 M HCl and 60 mM KH_2PO_4. All syringes except D also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM $MgSO_4$. Final [Ca^{2+}] in t_1 was 3.2 μM (4.0 μM immediately after mixing). The line is drawn for a first-order rate constant of 18 s^{-1} and an end point of EP/E_{tot} = 0.34.

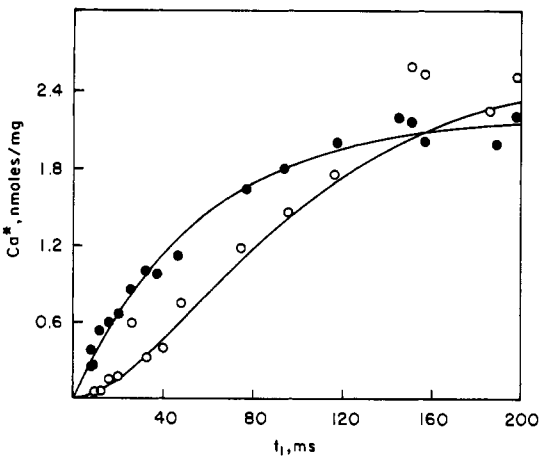


FIGURE 5: Exchange of the inner Ca^{2+} on $^cE{\cdot}Ca_2$ with ^{45}Ca in solution. Intact vesicles were preincubated with unlabeled calcium (○) or EGTA (●) and reacted with ^{45}Ca-EGTA buffers for t_1 [free [Ca^{2+}] = 2.3 μM (○) or 2.0 μM (●)], followed by a quench with excess unlabeled calcium for t_2 = 120–180 ms, followed by ATP. Syringe A contained ~0.2 mg/mL intact vesicles plus 15 μM unlabeled calcium (○) or 0.09 mM EGTA (●). Syringe B contained 0.40 mM $^{45}CaCl_2$ plus 0.54 mM EGTA (free [Ca^{2+}] = 2.1 μM) (○) or 0.40 mM $^{45}CaCl_2$ plus 0.46 mM EGTA (free [Ca^{2+}] = 4.6 μM) (●). Syringe C contained unlabeled calcium quench, 21 mM $CaCl_2$ plus 20 mM EGTA. Syringe D contained 1.2 mM ATP. Blanks were obtained by reversing the order of addition of syringes B and C. All syringes also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5 mM $MgSO_4$. The collection tube contained 1.0 mL of this buffer plus 10 mM $CaCl_2$. E_{tot} was determined by preincubating E with ^{45}Ca to give $^cE{\cdot}Ca_2{}^*$ and reacting with an unlabeled calcium quench for 160–190 ms in order to exchange the outer bound ^{45}Ca for unlabeled Ca^{2+} prior to addition of ATP, which gave E_{tot} as $^cE{\cdot}Ca_1{}^*{\cdot}Ca_1$ = 4.6 nmol/mg. The upper line (●) is calculated for a single exponential of 19 s^{-1} and an end point of 2.2 nmol/mg.

to give ~50% $^cE{\cdot}Ca_2$ at equilibrium. Incorporation of ^{45}Ca into the inner binding site requires dissociation of both unlabeled Ca^{2+} ions. If the mechanism of Scheme IV is correct, the slow step of the overall reaction is the conformational change of the free enzyme, with $k_f = k_c = 8\ s^{-1}$, and the exchange of enzyme-bound unlabeled Ca^{2+} with ^{45}Ca should occur with a rate constant of $\gg 8\ s^{-1}$ because it does not require this slow step. If the reaction follows the mechanism of Scheme III, the rate constant for exchange will be similar to that for the overall reaction.

The exchange reaction was followed by measuring the amount of ^{45}Ca incorporated into vesicles after incubation of 2.3 μM ^{45}Ca with $^cE{\cdot}Ca_2$, removal of the outer ^{45}Ca by exchange with unlabeled Ca^{2+}, and internalization of both Ca^{2+} after reaction with ATP as shown in eq 4. The addition of unlabeled Ca^{2+} inhibits further exchange of the inner Ca^{2+} (Petithory & Jencks, 1988).

$$^cE{\cdot}Ca_2 \xrightarrow{^{45}Ca} \longrightarrow {}^cE_{Ca^*Ca^*} \xrightarrow{Ca^{2+}} {}^cE_{Ca^*Ca} \xrightarrow{ATP} \quad (4)$$

The closed circles in Figure 5 show the approach to equilibrium with a rate constant of 19 s^{-1} after the addition of 2.0 μM ^{45}Ca to the free enzyme, which is similar to the result shown in Figure 4. The reaction follows satisfactory first-order kinetics, with no induction period. This control experiment is consistent with a rate-limiting conformational change of E according to Scheme IV or with the mechanism of Scheme III, as noted above. In the exchange experiment, shown by the lower curve and the open circles, the incorporation of ^{45}Ca into the inner site of $^cE{\cdot}Ca_2$ under the same conditions is slower and occurs with an induction period. This experiment is technically difficult, and the data are not adequate for quantitative analysis; however, the results show that the exchange reaction (open circles) is not fast compared with the overall reaction (closed circles); they are not consistent with rapid exchange and a slow conformational change in the k_c–k_{-c} step, according to Scheme IV. The biphasic reaction is consistent with the dissociation of 2 Ca^{2+} from $^cE{\cdot}Ca_2$ to give E in the first phase, followed by addition of ^{45}Ca to E to give $^cE{\cdot}Ca^*{}_2$ in the second phase, according to Scheme III; both steps are slow enough to be kinetically significant.

In the second experiment the rate of loss of the inner Ca from $^cE{\cdot}Ca^*{\cdot}Ca^*$ was determined in the presence of 3.3 μM unlabeled Ca^{2+}. According to the mechanism of Scheme IV, the slow step in the overall reverse reaction is the conversion of ^cE to E, with $k_r = k_{-c} = 8\ s^{-1}$. This step is not required for the dissociation of Ca^{2+}, so that the loss of ^{45}Ca from E·Ca*·Ca* should occur rapidly at $\gg 8\ s^{-1}$. According to the mechanism of Scheme III, the Ca^{2+} does not dissociate until the conformational change occurs and the rate constant for loss of ^{45}Ca should be the same as for the formation of E, i.e., $k_r = 8\ s^{-1}$.

The reaction was followed by quenching with a large excess of unlabeled Ca^{2+}, followed by phosphorylation with ATP plus EGTA and measurement of the amount of internalized ^{45}Ca. Figure 6 shows that the reaction is first order with a rate constant of 7 s^{-1}. This is not significantly different from k_r = 8 s^{-1}; it is certainly not larger than 8 s^{-1}, as would be required for the mechanism of Scheme IV. This experiment shows that the slow step of the overall reaction involves a species in which the inner Ca^{2+} is still bound to the enzyme, as in Scheme III; it is inconsistent with the mechanism of Scheme IV, with a kinetically significant conformational change from ^cE to E.

We conclude that the data are consistent with the mechanism of Scheme III, in which the conformational change does not occur in a kinetically significant step before Ca^{2+} binding or after Ca^{2+} dissociation.

Differences in the Partitioning of $^cE{\cdot}Ca_2{\cdot}ATP$. There is a small, but reproducible, increase in the yield of phosphoenzyme, from 62–65% to 70–72%, upon reaction of $^cE{\cdot}Ca_2$ with ATP plus EGTA when the enzyme is incubated with Ca^{2+} for 15 s instead of ~200 ms (Figure 7). The rate constant of 220 s^{-1} for phosphorylation after 200-ms exposure to Ca^{2+} is the same as has been observed many times after long exposure

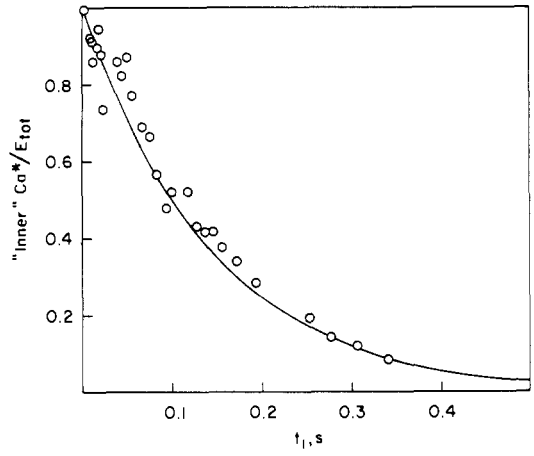


FIGURE 6: Dissociation of the inner Ca^{2+} from $^{c}E{\cdot}Ca_2$ in the presence of 3.3 μM exterior free Ca^{2+}. Intact vesicles were preincubated with $^{45}CaCl_2$ for at least 15 s and then reacted with an unlabeled Ca·EGTA buffer (~20-fold excess of unlabeled over labeled Ca^{2+}) to give 3.3 μM free $[Ca^{2+}]$ in t_1. This was followed by a quench of excess unlabeled calcium to give free $[Ca^{2+}] > 200$ μM for t_2 = 120–180 ms, followed by ATP at a final concentration of 300 μM. Syringe A contained ~0.2 mg/mL intact vesicles plus 15 μM $^{45}CaCl_2$. Syringe B contained 4.0 mM Ca^{2+} and 5.0 mM EGTA (free $[Ca^{2+}]$ in t_1 = 3.3 μM). Syringe C contained unlabeled Ca^{2+} quench consisting of 4.0 mM Ca^{2+} and 2.5 mM EGTA (free Ca^{2+} in t_2 = 210 μM). Syringe D contained 1.2 mM ATP. For t_1 = 0, the order of addition of substrates was reversed, corresponding to syringes A, C, D, and B. The single data point at t_1 = 0 represents the average of five reactions for the exchange of a single ^{45}Ca in the presence of >200 μM unlabeled calcium over a range of t_1 = 140–300 ms, without prior reaction with 3.3 μM Ca^{2+} buffer. All syringes also contained 40 mM MOPS buffer, pH 7.0, 100 mM KCl, and 5.0 mM $MgSO_4$. The collection tube contained 1.0 mL of this buffer plus 10 mM $CaCl_2$ to minimize any turnover with ^{45}Ca prior to filtration. The line is drawn for a first-order rate constant of 7 s^{-1}.

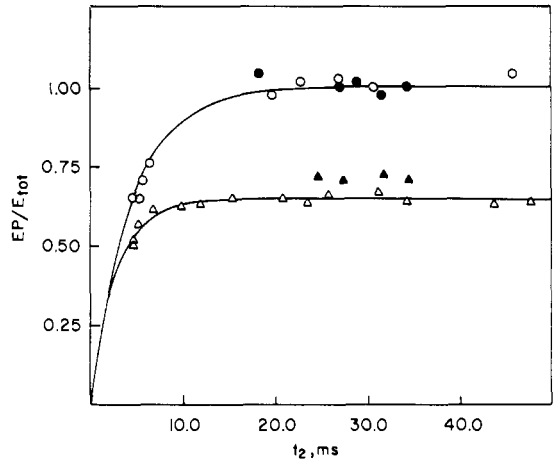


FIGURE 7: The partitioning ratio from ATP plus EGTA varies with the length of exposure of free enzyme to calcium. Loaded vesicles were preincubated in EGTA and then mixed with calcium for either 150–250 ms (○, △) or ~15 s (●, ▲) before reaction with [^{32}P]ATP (○, ●) plus EGTA (△, ▲) for varying times t_2. Syringe A contained 0.18 mg/mL passively loaded SRV and either 1.70 mM EGTA plus 0.17 mM $CaCl_2$ (0.08 μM free Ca^{2+}) (○, △) or 0.85 mM EGTA plus 0.99 mM $CaCl_2$ (140 μM free Ca_{2+}), from preincubating SRV with 2.0 mM EGTA and then adding 1.90 mM $CaCl_2$ prior to loading into syringe A (●, ▲). Syringe B contained either 2.00 mM $CaCl_2$ (○, △) or 0.85 mM EGTA and 0.99 mM $CaCl_2$ (●, ▲) [free Ca^{2+} during t_1 = 154 μM (○, △) or 144 μM (●, ▲)]. Syringe C contained either 0.9 mM [^{32}P]ATP (○, ●) or 0.9 mM [^{32}P]ATP plus 15 mM EGTA (△, ▲). All syringes except D also contained 40 mM MOPS, pH 7.0, 100 mM KCl, and 5.0 mM $MgSO_4$. The lines are drawn for first-order rate constants of 220 s^{-1} to EP/E_{tot} = 1.0 (○) and 350 s^{-1} to EP/E_{tot} = 0.65 (△).

to Ca (Petithory & Jencks, 1986). Figure 7 also shows that the same amount of enzyme is phosphorylated by ATP after incubation with Ca^{2+} for 150–250 ms and for 15 s (open and closed circles). This shows that all of the enzyme becomes ATP reactive in 200 ms and that brief treatment with EGTA does not denature the enzyme [cf. McIntosh and Berman (1978) and Berman (1982)]. Therefore, the smaller yield of phosphoenzyme from enzyme that is incubated only briefly with Ca^{2+} arises from a larger rate constant for Ca^{2+} dissociation in the presence of EGTA (open triangles, Figure 7). Enzyme that has been preincubated with Ca^{2+} for ≥15 s is phosphorylated at 300 s^{-1} in the presence of ATP plus EGTA, which corresponds to 220 + 80 = 300 s^{-1} for phosphorylation and calcium dissociation. The ratio 220/300 = 0.73 agrees with the observed yield of 70% phosphoenzyme under these conditions (Petithory & Jencks, 1986). The lower curve in Figure 7 is consistent with a rate constant of 220 + 120 = 340 s^{-1}, which gives a calculated yield of 220/340 = 0.65 phosphoenzyme that agrees with the observed yield of 62–65% for enzyme that was incubated for ~200 ms with calcium. These results suggest that the enzyme slowly undergoes a small additional conformational change that decreases the rate of Ca^{2+} dissociation after prolonged incubation with calcium.

Discussion

The Low-Affinity Pathway for Calcium Binding. It has been shown previously that the dependence on $[Ca^{2+}]$ of the kinetics for fluorescence changes and the directly measured binding of Ca^{2+} are consistent with Ca^{2+} binding to preexisting low-affinity sites on the cytoplasmic surface of SR vesicles (Guillain et al., 1980; Champeil et al., 1983; Dupont, 1984, 1985). The existence of such sites is not consistent with models in which the calcium binding sites initially face inward in the free enzyme ("E_2" or "E*") or ATP is required in order to induce a change from inward to cytoplasmic-facing sites (Carvalho et al., 1976; Inesi et al., 1980; Pick, 1981; Reynolds et al., 1985).

The observation of a burst in the time course for the formation of ATP-reactive enzyme upon addition of high concentrations of exterior calcium (Figures 1–3; Table I) provides direct evidence that free enzyme preincubated with EGTA binds exterior calcium very rapidly and is then able to react with ATP, as shown in Scheme II. This extends the conclusion that immediately upon addition of ATP to free enzyme there are relatively low-affinity calcium binding sites available on the cytoplasmic surface of the vesicle; two Ca^{2+} bind to these sites and allow phosphorylation by ATP with no induction period and a rate constant of 70 s^{-1}. This is the pathway for phosphorylation in the normal catalytic cycle of the enzyme under steady-state conditions (Stahl & Jencks, 1987). The rapid binding of two Ca^{2+} is not consistent with a "flickering gate" model in which opening of the gate is a kinetically significant step (Forbush, 1987).

The yield of 12% phosphoenzyme in the burst upon binding two Ca^{2+} to E is smaller than the yield of 17–20% phosphoenzyme from the species $E{\cdot}Ca_2{\cdot}$ATP upon the addition of EGTA (Stahl & Jencks, 1987). The difference suggests that the $E{\cdot}Ca_2{\cdot}$ATP intermediate may partition slightly differently between dissociation of Ca^{2+} and phosphorylation when it is formed from $E{\cdot}Ca_2$ and when it is formed from E. The yield of phosphoenzyme in the burst phase is much smaller than the 70% yield obtained from $^{c}E{\cdot}Ca_2$ with ATP plus EGTA because the dissociation of Ca^{2+} is much faster from $E{\cdot}Ca_2$ than from $^{c}E{\cdot}Ca_2$; the rate constant for dissociation of Ca^{2+} from $E{\cdot}Ca_2{\cdot}$ATP is approximately 280 s^{-1} (Stahl & Jencks, 1987).

The fact that the presence or absence of intravesicular Ca^{2+} has little or no effect on the burst size or the rate constant for

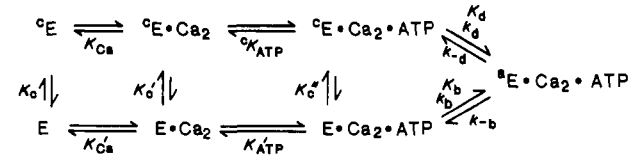
the formation of ATP-reactive enzyme after the addition of 200 μM Ca^{2+} (Figure 1) shows that 20 mM Ca^{2+} in loaded vesicles does not have a significant effect on Ca^{2+} binding to the outside of the vesicle or the development of ATP reactivity. It also has no effect on the 70-s^{-1} step that gives phosphorylation by ATP (Stahl & Jencks, 1987). There is evidence that an interior Ca^{2+} binding site exists in the unphosphorylated enzyme, which inhibits phosphorylation by inorganic phosphate and Mg^{2+} binding (Beil et al., 1977; Chaloub et al., 1979; Prager et al., 1979; Suko et al., 1981). Evidently, this is not the same site as that for binding of cytoplasmic Ca^{2+}.

Figure 2 shows that, within experimental error, the stoichiometry of 2 Ca/EP is maintained throughout the time course for the formation of ATP-reactive enzyme at high [Ca^{2+}] when the time courses for reactions of ^{45}Ca (◊) and [^{32}P]ATP (○) are compared. This shows that low-affinity binding sites are available for 2, not 1, Ca^{2+} ions on the exterior surface of the free enzyme. These data show that the second exterior site is available for Ca^{2+} binding at very short times after addition of the first Ca^{2+}, within 5 ms, and before the slow Ca^{2+}-dependent conformational change occurs. This pathway is consistent with the mechanism of Dupont and Leigh (1978); it differs from mechanisms in which a single Ca^{2+} ion binds before a conformational change (Inesi et al., 1980; Guillain et al., 1981; Dupont, 1982; Champeil et al., 1983; Tanford et al., 1987).

The Calcium-Dependent Conformational Change. The exponential time course for appearance of ATP-reactive enzyme following the burst must represent a conformational change of the calcium-bound enzyme, E·Ca_2, because the rate constant of $k_c' = 38$–40 s^{-1} is independent of calcium concentration in the range 120–300 μM (Figures 1 and 2). This conformational change converts the enzyme from E·Ca_2, which partitions to give 12% phosphoenzyme upon the addition of ATP plus EGTA, to ^cE·Ca_2, which partitions to 70% phosphoenzyme under the same conditions. The first-order course of the reactions, with no induction period, shows that there is no kinetically significant intermediate, such as ^cE·Ca_1, in this pathway; the reaction gives ^cE·Ca_2 directly as shown in Scheme II. The rate constant is consistent with a rough estimate of $k_c' \sim 30$ s^{-1} for the same step in the reaction cycle for steady-state hydrolysis of acetyl phosphate (Bodley & Jencks, 1987). This suggests that acetyl phosphate, which gives relatively slow phosphorylation and lacks the activating effect of ATP, reacts through the ^cE·Ca_2 pathway.

It is difficult to obtain a reliable estimate of the dissociation constant for Ca^{2+} from the low-affinity sites on E·Ca_2. The best estimate is obtained from the dependence on [Ca^{2+}] of the initial rates for the formation of ^cE·Ca_2 from E·Ca_2 (Figure 3), which give $K \simeq (20\ \mu M)^2$. This value is consistent with the estimated midpoint of the curve for the dependence on [Ca^{2+}] at $\sim$20 μM for the increase in the rate of the fluorescence change associated with Ca^{2+} binding that was observed by Champeil et al. (1980) under similar conditions, and the $K_{0.5}$ of 25 μM for the rate constants obtained by direct measurements of Ca^{2+} binding at −10 °C in 30% glycerol by Dupont et al. (1985). A value of $K_{0.5} \simeq 20$ μM is also consistent with the dependence of the magnitude of the initial burst on [Ca^{2+}] at 0–5 ms after Ca^{2+} addition (Table I). A rate constant of 280 s^{-1} has been estimated for the dissociation of Ca^{2+} from E·Ca_2·ATP, which is fast relative to the conformational change and phosphorylation with $k_b = 70$ s^{-1} (Stahl & Jencks, 1987); it is likely that dissociation of at least 1 Ca^{2+} from E·Ca_2 is also fast, so that this step is nearly at equilibrium.

Scheme V



The rate constant of $\sim$40 s^{-1} for the Ca^{2+}-dependent conformational change at saturating [Ca^{2+}] (Figures 1 and 2) is significantly larger than the rate constants of 5–15 s^{-1} observed for the change in intrinsic fluorescence associated with calcium binding to E under similar conditions (pH 6.8–7.0, 20–25 °C, 80–100 mM KCl, 5–10 mM Mg^{2+}) (Dupont & Leigh, 1978; Guillain et al., 1980; Fernandez-Belda et al., 1984). If this difference does not arise from a difference in the SR preparations, the change in fluorescence must reflect a change in enzyme conformation subsequent to Ca^{2+} binding and activation of the enzyme for phosphorylation by ATP. On the other hand, Guillain et al. (1981) have shown that at pH 6 in the absence of KCl the rate constant of 1.5 s^{-1} for the development of reactivity toward ATP in the presence of Ca^{2+} is consistent with the rate constant of 1.3 ± 0.4 s^{-1} for the fluorescence change under the same conditions, and Dupont (1984) has observed the same rate constant for Ca^{2+} binding and a change in fluorescence at pH 7.2. These differences suggest that it is desirable to calibrate the kinetics of fluorescence changes with a chemical assay before they are assigned to kinetically significant steps in the reaction cycle.

Differences between rate constants for changes of activity and of fluorescence suggest that a relatively small change in conformation occurs rapidly, which changes the enzyme activity in a metastable state, while a larger readjustment of conformation occurs more slowly as the enzyme relaxes to the thermodynamically stable conformation. A similar slow conformational relaxation may explain the slow change between 0.2 and 15 s from 62–65% to 70–72% phosphorylation in the partitioning of the enzyme between Ca^{2+} dissociation and phosphorylation after the addition of ATP and EGTA (Figure 7).

Estimates or limits for several additional rate and equilibrium constants in Scheme V may be obtained from the data. The absence of a burst in the time course for phosphorylation upon the addition of ATP plus Ca^{2+} to the free enzyme (Stahl & Jencks, 1987) or for the binding of 3.2 μM Ca^{2+} (Figure 4) shows that no detectable concentration of the hypothetical calcium-free, high-affinity species ^cE is present in the absence of calcium; if we assume that a burst of 5% would have been detected, the value of K_c is <0.05. The value of $K_c' = 35$, which was obtained from $K'_{Ca} = (20\ \mu M)^2$, the overall dissociation constant for Ca^{2+} of $K_{ov} = (3.4\ \mu M)^2$ (Petithory & Jencks, 1988), and the relationship $K_c' = K'_{Ca}/K_{ov}$, shows that the high-affinity conformation of the enzyme is strongly favored in the presence of calcium. The value of K_c'' is probably not grossly different from K_c' because the binding of ATP to ^cE·Ca_2 and to E is similar, with dissociation constants of 12 μM and 5 μM, respectively, and the first-order phosphorylation of ^cE·Ca_2·ATP with $k = 220$ s^{-1} gives no indication of the presence of any E·Ca_2·ATP, which phosphorylates more slowly with $k_b = 70$ s^{-1} (Stahl & Jencks, 1987; Petithory & Jencks, 1988; Lacapère & Guillain, 1988). Taking $K_c'' = 35$, the value of K_b is >50, from the relationship $K_b = K_c''K_d$ and the value of $K_d > 1.4$ (Stahl & Jencks, 1987). The value of k_{-b}, for the reversion of ^{a}E·Ca_2·ATP to E·Ca_2·ATP, is then <2 s^{-1}, from $k_{-b} = k_b/K_b$. One significant conclusion from this is that it is unlikely that the reverse reaction of ^{a}E·Ca_2·ATP occurs to

a significant extent through k_{-b} and the lower pathway of Scheme V. The value of k_{-d} is not known, but it is probably in the range 50–150 s^{-1} (Petithory & Jencks, 1986; Stahl & Jencks, 1987) so that the reverse reaction is expected to proceed preferentially to form the stable, high-affinity species $^cE{\cdot}Ca_2{\cdot}ATP$.

The "High-Affinity" Pathway for Calcium Binding. The binding and dissociation of low concentrations of Ca^{2+} can be described by the minimal mechanism and rate constants of Scheme IIIb. The rate constants of $k_{-2} = 60\ s^{-1}$ and $k_{-1} = 30\ s^{-1}$ for dissociation of the outer and inner Ca^{2+} ions, respectively, and the ratio $k_{-1}/k_2 = 0.7\ \mu M$ have been reported previously (Petithory & Jencks, 1988). These values give a second-order rate constant of $k_2 = 4 \times 10^7\ M^{-1}\ s^{-1}$ for binding of the second Ca^{2+} ion and an equilibrium constant of $k_{-2}/k_2 = 1.4 \times 10^{-6}$ M for dissociation of this Ca^{2+} from $^cE{\cdot}Ca_2$. The rate constant of $k_f = 8\ s^{-1}$ for calcium binding in the presence of 3.2 μM Ca^{2+} corresponds to an apparent second-order rate constant of approximately $3 \times 10^6\ M^{-1}\ s^{-1}$ for k_1; however, one value of k_f does not demonstrate that the reaction is second order. The overall equilibrium constant of $K_1K_2 = 1.2 \times 10^{-11}\ M^2$ (Petithory & Jencks, 1988) and $K_2 = 1.4 \times 10^{-6}$ M give $K_1 = 9 \times 10^{-6}$ M. The facile binding of low concentrations of Ca^{2+} to $^cE{\cdot}Ca_1$ to give $^cE{\cdot}Ca_2$, with $k_{-1}/k_2 = 0.7\ \mu M$, is responsible for inhibition of the dissociation or exchange of the inner Ca^{2+} of $^cE{\cdot}Ca_2$; it is also presumably responsible for the inhibition of the overall reverse reaction by very low concentrations of calcium (Barlogie et al., 1971; de Meis, 1976).

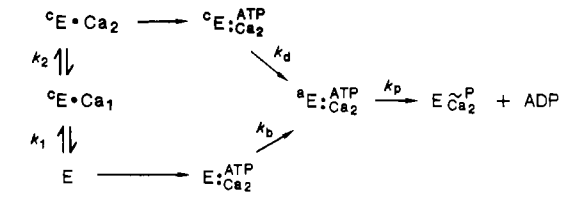
Scheme IIIb

$$E + Ca^{2+} \underset{k_{-1} = 30\ s^{-1}}{\overset{k_1 = 3 \times 10^6\ M^{-1}\ s^{-1}}{\rightleftharpoons}} {}^cE{\cdot}Ca_1 \underset{k_{-2} = 60\ s^{-1}}{\overset{k_2 = 4 \times 10^7\ M^{-1}\ s^{-1}}{\rightleftharpoons}} {}^cE{\cdot}Ca_2$$

The difference of ~10-fold between the rate constants for binding the first and second Ca^{2+} ions shows that binding of the first Ca^{2+} to give $^cE{\cdot}Ca_1$ is much slower than diffusion controlled. The slow rate is consistent with a conformational change associated with binding of the first Ca^{2+}. This conformational change is presumably responsible for the cooperativity that is observed for the binding of two Ca^{2+} (Inesi et al., 1980). In principle, the conformational change could be (1) concerted with Ca^{2+} binding, (2) a very rapid step after slow Ca^{2+} binding, or (3) a slow step after rapid binding of 1 Ca^{2+}. However, mechanisms 1 and 2 are not consistent with the observed slow conformational change after very fast binding of two Ca^{2+} (Figures 1 and 2), so that the data support mechanism 3. Rapid initial binding of one Ca^{2+} before a conformational change has been proposed previously by a number of workers (Inesi et al., 1980; Guillain et al., 1981; Dupont, 1982; Champeil et al., 1983; Tanford et al., 1987). This result is consistent with fast binding of the second Ca^{2+}, compared with the formation of $^cE{\cdot}Ca_1$, and with the ratio $k_{-1}/k_2 = 0.7\ \mu M$, which corresponds to 83% partitioning of the $^cE{\cdot}Ca_1$ intermediate to give $^cE{\cdot}Ca_2$ rather than E in the presence of 3.2 μM Ca. It should be noted that although this pathway occurs at low $[Ca^{2+}]$, the "high affinity" is a consequence of the relative rate constants and cooperativity; the initial binding of Ca^{2+} to E is not necessarily high affinity.

The value of k_1 can also be calculated from the independently determined values of $k_{-1} = 30\ s^{-1}$, $K_1K_2 = 1.2 \times 10^{-11}\ M^2$, and $K_2 = 1.4 \times 10^{-6}$ M (Petithory & Jencks, 1988). The resulting value of $k_1 = 3.5 \times 10^6\ M^{-1}\ s^{-1}$ gives a value of $k_f = 11\ s^{-1}$ in the presence of 3.2 μM Ca^{2+}. This is in reasonable agreement with the value of $k_f = 8\ s^{-1}$ obtained from the experiment shown in Figure 4, considering the number of rate and equilibrium constants that were used in the comparison. The result shows that there is internal consistency of the rate and equilibrium constants in Scheme IIIb. A recent report describes an observed rate constant of 7 s^{-1} for the directly measured binding of 5 μM Ca^{2+} to E, from which a similar second-order rate constant of $1.4 \times 10^6\ M^{-1}\ s^{-1}$ was estimated; however, other rate constants that were proposed for Ca^{2+} binding are different from those reported here (Inesi, 1987).

Scheme VI



Conformational Changes. One reason that we have questioned the appropriateness of the E_1–E_2 (or E–E*) model for the calcium ATPase is that these models describe a single conformational change, back and forth between E_1 and E_2, in the reaction cycle. In fact, there is evidence for three or four conformational changes just in the pathway for Ca^{2+} binding followed by ATP binding and phosphorylation, as well as a number of other conformational changes in the reaction cycle; there may be a conformational change in every step (Stahl & Jencks, 1987). Scheme VI shows several of these changes: (1) Binding of a single Ca^{2+} ion (k_1) gives a conformational change that increases the affinity for binding the second Ca^{2+} and is responsible for the cooperativity of Ca^{2+} binding. (2) Binding of the second Ca^{2+} (k_2) makes possible phosphorylation by ATP with an observed rate constant of $k_d = 220\ s^{-1}$. This could represent a conformational change; however, it would also be accounted for simply by a requirement that this Ca^{2+} must be bound before the next conformational change can occur. (3) The rate-limiting step for phosphorylation of $^cE{\cdot}Ca_2{\cdot}ATP$, with $k_d = 220\ s^{-1}$, is a conformational change to form catalytically active $^aE{\cdot}Ca_2{\cdot}ATP$ (Petithory & Jencks, 1986). (4) The phosphorylation step itself, which is too fast to measure ($k_p > 1000\ s^{-1}$), is coupled to the most important conformational change in the reaction cycle. It is in this step that the vectorial specificity for the dissociation of Ca^{2+} changes from the cytoplasmic side to the inside of the membrane (Sumida & Tonomura, 1974; Inesi et al., 1978; Sumida et al., 1978; Dupont, 1980; Petithory & Jencks, 1986).

While it is interesting to dissect these Ca^{2+}- and ATP-induced conformational changes into separate steps, they are combined into a single kinetically significant step under physiological conditions for turnover of the enzyme. In the presence of ordinary concentrations of ATP the reaction proceeds through the lower pathway of Scheme VI, with fast binding of both ATP and Ca^{2+} to E, followed by a first-order, rate-limiting conformational change with $k_b = 70\ s^{-1}$ that converts the enzyme to the active form for rapid phosphorylation (Stahl & Jencks, 1987).

All of this suggests that the enzyme is mobile and susceptible to a large number of conformational changes upon binding to different ligands. However, when both Ca^{2+} and ATP bind, several of these conformational changes can be combined into a single, first-order reaction.

The experiments described here and in the previous paper (Petithory & Jencks, 1988) show that there is no need to postulate several of the conformational changes that have been proposed previously. This work provides no evidence for a slow

conformational change that renders the enzyme ATP reactive either before the first Ca^{2+} has bound to the enzyme (Carvahlo et al., 1976; de Meis & Vianna, 1979) or after the first but before the second Ca^{2+} has bound (Coan et al., 1979; Inesi et al., 1980; Dupont, 1982; Champeil et al., 1983). There is also no evidence for conformational changes that change the orientation of the Ca^{2+} binding sites (de Meis & Vianna, 1979) or "uncover" cytoplasmic Ca^{2+} binding sites on the free enzyme (Coan et al., 1979; Champeil et al., 1983; Fernandez-Belda et al., 1984), and the mechanism of cooperativity for Ca^{2+} binding is different from that predicted from a conformational change between E_1 and E_2 before Ca^{2+} binding (Tanford et al., 1985).

Registry No. ATPase, 9000-83-3; Ca, 7440-70-2.

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