Analysis of Calcium Binding and Release by Canine Cardiac Relaxing System (Sarcoplasmic Reticulum)

THE USE OF SPECIFIC INHIBITORS TO CONSTRUCT A TWO-COMPONENT MODEL FOR CALCIUM BINDING AND TRANSPORT*

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SUMMARY

ATP induces calcium binding to a cardiac relaxing system preparation (sarcoplasmic reticulum; CRS) under specific conditions. This is followed by spontaneous calcium release, a thermodynamically and kinetically distinct process that represents a separate phase of an ATP-dependent binding-release cycle. Inhibitors of calcium binding studied include X537A, a broadly specific antibiotic ionophore, and arsenate. These inhibitors were significantly more potent on calcium uptake (accumulation in the presence of oxalate) than on binding (accumulation in the absence of oxalate). Inhibition decreased with decreasing magnesium concentration suggesting that the agents were acting primarily on a magnesium-dependent process.

Computer analysis of the kinetics of ATP-dependent calcium binding to CRS suggested that there are two distinct binding sites or components that differ in response to magnesium, pH, and in their sensitivity to the actions of inhibitors. Under conditions of optimal ATP and calcium concentrations, a continuous curve for calcium binding adhered to a pseudo-first order model for occupation of free calcium binding sites: $A + B \rightarrow A_{\text{calc}} + B_{\text{calc}}$. Site A required very low magnesium (40% of maximal binding at 9 \(\mu\text{M}\) magnesium), was ATP-specific and was less sensitive to the inhibitors of calcium binding, X537A ($K_i$, 10 to 15 \(\mu\text{M}\)) and arsenate (unaffected by concentrations up to 10 \(\text{mM}\)). Calcium was released spontaneously from Site A in the presence or absence of oxalate, therefore, this pool of calcium does not appear to be connected to an oxalate space (vesicular lumen). In contrast, Site B was related to calcium transport (i.e. uptake, see above) in that it required a relatively high concentration of magnesium ($K_m$, 2.7 to 4.7 \(\mu\text{M}\)) and was much more sensitive to the noncompetitive inhibitors, X537A ($K_i$, 3.7 \(\mu\text{M}\)) and arsenate ($K_i$, 1 to 2 \(\text{mM}\)). The predicted initial rates of calcium binding to Site B (rate = $k_B$) were similar to the average range of rates of calcium uptake in the presence of 5 \(\mu\text{M}\) oxalate. Nucleoside triphosphates other than ATP activated only Site B suboptimally (50 to 60%). When Site B was activated by nucleotides other than ATP, its sensitivity to pH, arsenate, and X537A was unchanged; however, the magnesium requirement of Site B disappeared. Nucleotides other than ATP did not activate the calcium-dependent ATPase and did not support continuous calcium uptake. Sodium azide specifically inhibited the rate of calcium release without affecting binding. All of the inhibitors were specific for the described individual phases and did not affect calcium-dependent ATPase.

The sarcoplasmic reticulum of the heart (cardiac relaxing system, "microsomal vesicles") is thought to be an important intracellular calcium-binding membrane system that modulates cardiac contraction and relaxation (1). There have been two general reactions described for calcium accumulation to this preparation: calcium "binding" and "uptake." The term uptake has been used to denote an ATP-dependent, continuous calcium accumulation presumably within sarcoplasmic reticulum vesicles that occurs in the presence of a calcium-precipitating anion (oxalate or phosphate); the term binding has been used to describe an ATP-dependent calcium accumulation process that occurs in the absence of these anions. Binding is a much more rapid process than uptake, reaching a maximum within 1
The relationship between these two processes has not been well delineated. It has been presumed by some investigators that they are both part of the same process, i.e. binding represents a steady state between active calcium accumulation and passive calcium efflux, and uptake involves a precipitation of accumulated calcium within the vesicle (product consumption) which prevents a steady state. In almost all experiments reported, binding and uptake generally change in similar directions. Therefore, a definitive separation of the two processes has been difficult. However, the energy of activation of uptake is considerably higher than that of binding (2), and uptake and binding have been reported to have different kinetics with respect to calcium concentration (3). These observations suggest that binding and uptake may be different processes. Moreover, recent work from this laboratory has demonstrated a difference in sensitivity of the two processes to the antibiotic ionophore inhibitors X537A (4, 5) and A23187 (5); uptake was completely inhibited at a concentration where binding was inhibited only 50 to 75%.

Using a spectrophotometric method that continuously monitors calcium accumulation, it is possible to measure the removal of free calcium from a reaction medium (6). In previous work, it has been shown that calcium removal induced by the addition of ATP is characterized by very rapid binding (7) followed by a separate, spontaneous calcium release phase. The latter has specific thermodynamic and kinetic characteristics (8) that differ from those calculated for the binding phase. The data suggested a saturable system of calcium binding sites that can be shown to behave independently, in that under specific conditions, some sites may be binding while other sites may be releasing calcium. In addition, it was demonstrated that the calcium-binding phase of the cycle behaved in a complex manner with respect to magnesium. It was found that double reciprocal kinetic plots describing the magnesium sensitivity of either total calcium binding or rate of calcium binding (measured as the amount accumulated during the initial 5-s period) revealed a hyperbolic relationship rather than a straight line (8). It was also observed that after calcium release had occurred, calcium binding could be restimulated upon the addition of ATP and that the percentage of calcium bound the second time (percentage restimulation) was inversely related to magnesium (e.g. percentage of restimulation was 50 to 75% at 10 mM and nearly 100% at 1 mM). These findings led us to examine the possibility that two distinct classes or sites of reactions existed, one requiring high magnesium concentration and not completely restimulable by ATP, and the other requiring much less magnesium and easily restimulable by the addition of ATP.

In the present study, we have attempted in several ways to examine further the possibility that there are two separate sites of calcium binding to cardiac relaxing system: (a) a computer analysis of the continuous calcium binding in terms of pseudo-first order kinetics of the disappearance of calcium binding sites; (b) the use of two inhibitors with differential effects on calcium binding and uptake, X537A, which has been previously reported (4, 5) from this laboratory to inhibit uptake to a greater degree than binding, and arsenate.

In addition, other characteristics of the binding-release cycle were examined, including nucleotide specificity and the possible relation of calcium binding and uptake to calcium-stimulated ATPase. Finally, the effect of adeno on spontaneous calcium release is reported and its possible significance discussed. The results of these experiments should be considered with the reservation that the "sarcoplasmic reticulum" preparation employed is not pure, although enzymatic activity appears to be limited to a calcium accumulation process.

**METHODS**

**Method of Isolation**—Cardiac relaxing system was isolated from mongrel dogs in a manner similar to that previously reported by Harigaya and Schwartz (2) using a bicarbonate-azide buffer. The isolation procedure was modified in that initial homogenization was accomplished with a larger polytron instrument (Brinkmann PT35) with the rheostat set at 50. Homogenization was carried out for 10 s and repeated twice. The homogenate was then centrifuged at 3,000 × g for 20 min and 8,700 × g for 20 min, and the pellets discarded. The supernatant was centrifuged at 100,000 × g for 30 min. The pellet was harvested and resuspended in 20 mM Tris-maleate and 0.6 mM potassium chloride in order to remove contractile protein contaminants and centrifuged at 100,000 × g. The final pellet was resuspended in 20 mM Tris-maleate (pH 6.8) and used within 12 hours of preparation. This procedure resulted in a yield of 0.7 to 1.0 mg per g wet weight heart, biochemical marker and morphological analyses revealed very little identifiable contamination with myofibrils or mitochondrial fragments. This, however, does not in any way imply that all of the membrane area is associated with the functional studies described.

**Method of Assay**—Calcium binding and uptake were measured by both a Millipore method (2) and an Amino-Chance dual wave length spectrophotometer (307, 542 nm) using the chelometric dye, murexide, as a calcium indicator to monitor calcium removal from the assay solution under the various conditions described for these experiments (2, 8). In all experiments that were analyzed kinetically, the ATP concentration was greater than that shown to stimulate maximal binding (greater than 0.1 mM) and the calcium concentration was also maintained greater than that shown to result in optimal binding (> 10 μM) until maximum binding was reached. Calcium and magnesium present in the reaction mixture, the reagents, and the amounts endogenously bound to the CRS were measured by atomic absorption spectrophotometry after LaCl-HCl extraction. The endogenous calcium in the CRS was 10 to 20 nmoles per mg; endogenous magnesium was 5 to 7 nmoles per mg. The reagent contamination was 9 to 11 μM calcium and 4 to 5 μM magnesium.

**ATPase Measurements**—ATPase was measured by phosphate liberation (9) and by a linked pyruvate kinase-lactate dehydrogenase method measuring absorbance changes at 340 nm (NADH oxidation) (10, 11). The results using these methods were comparable; both methods were used to ensure that observed changes resulted from effects on ATPase per se and not on the method of estimation.

**Computer Program and Statistics**—The continuous curve of calcium binding measured was assumed to represent the disappearance of available sites for calcium binding. Since the calcium concentration was kept greater than 10 μM throughout the calcium binding process, and the binding constant for CRS (2) is about 2 × 10^5 M^-1, total binding was presumed to be an indicator of total available binding sites under the assay conditions involved. At each 1-s interval, the amount of calcium bound was subtracted from the total binding to give the amount of remaining free, available sites at that point. Each experimental curve was repeated two to five times; the variation in the data, using identical conditions and the same preparation, was less than 2.5%. The experimental curves were fit with one (A =

The abbreviation used is: CRS, cardiac relaxing system.
Aoe$^{-kt}$ and two ($A + B = Aoe^{-kt} + Boe^{-kt}$) component models using a $\chi^2$ minimization program (12) (see "Appendix"). The fits of the two models were then compared using a $\chi^2$ (variance) ratio test (13). The two-component model was accepted if the $\chi^2$ for that fit was significantly ($p < 0.01$) less than the $\chi^2$ for the one-component fit. Usually, one of the two models fit the data much better than the other. Because of individual variations in the preparations, experiments presented will represent those done with a single preparation on a single day; however, all experiments were repeated three to five times with no appreciable quantitative or qualitative difference in the data or the results.

Calculations of ATP Species—The concentration of the various ATP species present in the reaction medium was calculated by solving the set of simultaneous equations generated by the various equilibrium constants involved and the stoichiometric identities. The stability constants used (14) were: $K_{MbATP} = 10^{4.24}$, $K_{CSATP} = 10^{6.53}$, $K_{bATP}^{-1} = 10^{6.53}$, and $K_{ATP}^{-1} = 10^{10.53}$.

Parameters Measured—The parameters measured, in addition to the computer calculations, are the same as those described in previous studies (4, 5), and are shown in Fig. 1, namely, total ATP-dependent calcium binding, initial 5-s calcium binding, and maximum calcium release rate. Calcium uptake measured in the presence of oxalate was measured as the linear rate of calcium accumulation that followed the "initial binding burst," recently described by this laboratory (4), and a slow phase of uptake, to be discussed further in this report.

RESULTS
Calcium Binding Inhibitors

Effect of Arsenate—Arsenate (AsO$_4^-$) is a known inhibitor of mitochondrial energy transduction. In spite of the absence of measurable mitochondrial contamination, arsenate did affect calcium binding and calcium uptake. Calcium binding was 30 to 40% of control in the presence of 5 mM arsenate, a concentration that completely inhibited calcium uptake (Fig. 2). The $I_{50}$ for arsenate for calcium uptake was about 1.75 mM compared to about 4 mM for calcium binding. Concentrations of ATP from 0.1 to 2.0 mM failed to overcome, or in any way influence, the amount of arsenate-induced inhibition of the amount of calcium binding in these experiments. Arsenate inhibited calcium binding when it was added before the initiation of the reaction with ATP, simultaneously with the initiation of the reaction with ATP or when it was added 5 s after the initiation of the binding reaction with ATP (data not shown).

Both calcium binding and uptake by the cardiac relaxing system have been shown to be sensitive to magnesium. Therefore, the effect of magnesium on the relative inhibition by arsenate of calcium binding was studied. As magnesium concentration was decreased, calcium binding decreased. The percentage of inhibition by a given concentration of arsenate, however, also decreased as magnesium concentration decreased (Fig. 3). At 10 mM arsenate, there was no significant magnesium dependence of calcium binding (Fig. 3A).

Effect of Ionophores—We have reported that the ionophores X537A (4, 5) and A23187 (5) inhibit calcium binding and calcium uptake.
Five consecutive experiments showing data fitted to one-versus two-component model

Assay medium: 3 ml, 30°C, 0.8 mg of CRS protein per ml, 40 mM Tris-maleate (pH 6.6), 0.25 mM ATP, 10 mM MgCl₂, 0.1 M KCl, and 50 μM CaCl₂. Each of five experiments demonstrates a significantly better fit for a two-component model (p < 0.01). df = degrees of freedom. Method of analysis is found in “Appendix.”

| Experiment | One component kA (s⁻¹) | One component Binding | χ² df |
|------------|-------------------------|-----------------------|-------|
| Experiment 1 | 0.77 | 51 | 43.25 |
| Experiment 2 | 0.775 | 53 | 48.14 |
| Experiment 3 | 0.566 | 51 | 46.60 |
| Experiment 4 | 0.016 | 47 | 78.96 |
| Experiment 5 | 0.014 | 57 | 448.01 |

Rationale for Data Analysis

These findings, coupled with our previous observations of a hyperbolic double reciprocal relationship of calcium binding versus magnesium concentration, suggested that X537A and arsenate might be preferentially inhibiting a component of the total calcium binding that (a) required millimolar magnesium concentrations; and (b) was linked to calcium uptake (transport to an oxalate space).

Analysis of Calcium Binding Curve

Initially, five calcium binding curves experimentally derived under identical conditions were analyzed by the procedure described under “Methods.” The results of an analysis of a two-component versus a one-component model and the actual values are shown in Table I. From the values of χ² it is apparent that the data do not fit a one-component model but are quite compatible with a two-component model. Analysis of experiments revealed that Site A at 10 mM magnesium represented from 20 to 40% of the total calcium binding sites and had a rate constant that ranged from 0.25 to 0.7 s⁻¹. Site B represented 60 to 80% of the sites and had a rate constant that varied from 0.04 to 0.08 s⁻¹. A three-component model (Table II) offered no better fit than for a two-component one. Therefore, the two-component model was adopted.

Effects of Magnesium—When the magnesium concentration was varied, it was found that the rate constants of both Sites A and B were not significantly changed. It was noted, however, that the computer-extrapolated intercept on the ordinate of the log plot, presumed to represent the initial amount of binding sites, yielded a linear double reciprocal relationship with respect to magnesium concentration for Site B (Fig. 5). The intercepts on the abscissae (Kₐ for magnesium) ranged from 2.7 to 4.7 mM. The intercept at Site A was not affected by alterations in magnesium concentration above 0.33 mM. Below this concentration, binding to Site A began to fall, but the data did not adhere to simple, double reciprocal kinetics when plotted as a function of...
Magnesium dependence of Site A and B. Analysis as under "Methods"; total binding sites represent the computer-extrapolated ordinate intercept of the log plot for each site. Reaction mixture as in Fig. 3 with magnesium varied between 1 to 10 mM.

**Table III**

Effect of restimulation on two proposed sites

|               | $k_A$ | $k_B$ | $k_A$ | $k_B$ |
|---------------|-------|-------|-------|-------|
| Control       | 0.25  | 18.3  | 0.04  | 45.6  |
| Restimulation | 0.26  | 17.4  | 0.04  | 13.7  |

Effect of pH—It was previously reported (8, 15) that calcium binding at various pH ion concentrations again revealed no significant difference in the rate constants for either Site A or Site B (Fig. 6). Site A was unaffected by pH change between 6.0 and 7.5; the total amount of Site B available for binding decreased steadily with increasing pH. In addition, increasing the initial pH or lowering magnesium, both of which reduced the number of Site B sites, markedly reduced the amounts of calcium released from CRS by a sudden alkalinization (15) after peak binding had occurred (data not shown). This further suggested that Site A was relatively insensitive to pH changes.

Calcium uptake (calcium accumulation in the presence of oxalate) exhibited a pH dependence similar to that recorded for Site B (Fig. 6). At pH 7.4, and in the presence of a concentration of 1 mM magnesium, spontaneous release of bound calcium (presumably to Site A) occurred in the presence of oxalate, again suggesting that upon inactivation of Site B, uptake into the oxalate space ceased (Fig. 7).
Effect of X537A and Arsenate—Analysis of the effects of X537A on calcium binding to the two Sites A and B revealed that the ionophore decreased the amount of binding to Site B (the $K_i$ was 3.7 $\mu$g per mg). This inhibition appeared to be noncompetitive (Fig. 8) with respect to magnesium. Further analysis revealed that Site A was relatively resistant to X537A ($K_i$ was 10 to 15 $\mu$g per mg of protein; data not shown). Lowering the magnesium again markedly attenuated the release induced by additions of X537A until the concentration of the ionophore was in excess of 25 $\mu$g per mg of protein (data not shown). This suggests that the sensitivity to X537A-induced release was greater at Site B. The $K_i$ for X537A for Site B correlated well with the 50% inhibition of calcium uptake of 4 $\mu$g per mg previously reported by this laboratory (1).

Arsenate was found to inhibit Site B ($K_i$, 1 to 2 mM) without affecting the rate constant of the remaining sites. Arsenate did not affect Site A in a range of concentrations as high as 10 mM. Similar to X537A, the inhibition was noncompetitive with respect to magnesium (Fig. 9). Analysis revealed that the per cent inhibition by various concentrations of arsenate for Site B and for uptake rate were not significantly different (Fig. 10).

Relation of Calcium Uptake to Calcium Binding Sites

The different properties for Sites A and B and for calcium uptake are summarized in Table IV. In contrast to Site A, Site B and calcium uptake are both equally sensitive to the inhibitors X537A and arsenate and both require relatively high magnesium. This suggests the possibility that Site B might be linked to calcium uptake into the CRS “lumen” (oxalate space), whereas Site A might be a “superficial” site not directly connected to the oxalate space.

The demonstration of spontaneous calcium release in the presence of 5 mM oxalate (Fig. 7) suggests that, at least under certain circumstances, Site A is not converted to a species in contact with the oxalate space. Fig. 7 also demonstrates a qualitatively typical calcium uptake curve, as previously reported (2, 4). There is a rapid initial burst of binding at a rate compatible with that of Site A. After a plateau, calcium uptake begins at an initially slowed rate which abruptly becomes linear until all calcium is released from the solution. This curve is typical of calcium uptake in the presence of sufficient ATP (0.5 mM or phosphoenolpyruvate-pyruvate kinase regenerating system or both) so that the nucleoside triphosphate was present for at least 4 min after the initiation of the reaction. It was considered probable that the initial binding might represent, predominantly, binding at the Site A “pool,” and that the initial slower phase of the uptake process might represent the net result of calcium uptake by a Site B pool and simultaneous Site A release. This was suggested by the temporal correlation of the slow phase of the uptake process with the release from Site A observed when Site B was inactivated by X537A (4) or by low magnesium and high pH (Fig. 7).

To test this hypothesis, a CRS protein concentration of 0.8 mg per ml, an ATP concentration of 0.25 mM and a magnesium concentration of 10 mM were used to study calcium uptake since this combination maintains an effective ATP concentration for only 2 to 5 min (8). It was found that 2 to 3 min were sufficient to remove all the calcium from the solution; however, a certain amount of calcium was released despite the presence of oxalate (upper trace, Fig. 11). This further suggested that at least some of the initial calcium taken up was bound to a site (presumably Site A) which could not be directly transported to the oxalate space. If ATP was doubled (0.5 mM) (lower trace, Fig. 11), an ATP regenerating system used or CRS protein concentration lowered so that ATP persisted for 4 to 5 min, calcium was retained as shown in the lower trace (Fig. 11). This suggested that in the presence of a persistent ATP concentration, the calcium released from Site A was taken up by Site B. This represents another example of the observation that upon Site B inactivation (X537A (4), ATP depletion (Fig. 11), pH 7.4 in the presence of 1 mM Mg$^{2+}$ (Fig. 7)), net calcium release occurs presumably from Site A in the presence of oxalate.

In previous work (4) we reported that once calcium had been taken up into the oxalate space (5 min) there was no induced release effected by X537A. Because of the observations reported here, we mapped the susceptibility to X537A (25 $\mu$g per mg) as a function of the time in the uptake reaction. We found that some calcium release could be induced (progressively de-
creasing quantities) by X537A in the presence of oxalate only during the period represented by the initial binding burst and slow phase of uptake. Once rapid linear uptake ensued, no significant release was induced by X537A (uptake, however, was completely inhibited). Thus, once calcium bound to Site A had been released and taken up into the oxalate space, it could not be released. The data, therefore, suggest that in the uptake process (transport?) an important component is an initial binding to a "superficial" site.

Azide

The total binding by a cardiac relaxing system is not affected by sodium azide in concentrations as high as 10 mM, and the time of onset of release is not significantly affected by this agent (Fig. 12). This further suggested that there was no significant mitochondrial contribution to calcium binding. The rate of release, however, was specifically inhibited by azide ($K_i$, 2.5 mM) in a concentration dependent manner. Because of the known high "energy of activation" of calcium release from CRS, the effect of temperature on azide-induced inhibition was studied. A Dixon plot of sodium azide at three temperatures demonstrated that the $K_i$ for azide inhibition was unchanged by temperature. The $Q_{10}$ for calcium release was also unchanged by sodium azide (2.4 to 2.6) (Fig. 13). Previous work from this laboratory revealed that the rate of release of calcium was affected by hydrogen ion concentration; present studies demonstrate that the rate of release variation with pH was unaffected by azide as well (Fig. 14). In addition, the release induced by abrupt changes in pH, as previously described by this laboratory (14), was unaffected by the presence of sodium azide (data not shown). Magnesium concentration between 1 to 10 mM did not significantly affect the inhibition of release effected by azide. In a previous work, it was demonstrated that magnesium concentration did not affect the release rate as a function of total calcium binding (8).

Effects of Inhibitors on Calcium-stimulated ATPase

None of the agents studied had any effect on calcium-stimulated ATPase (Table V). Sodium azide inhibited the basic rate of release of calcium was also unchanged by sodium azide (2.4 to 2.6) (Fig. 13). Previous work from this laboratory revealed that the rate of release of calcium was affected by hydrogen ion concentration; present studies demonstrate that the rate of release variation with pH was unaffected by azide as well (Fig. 14). In addition, the release induced by abrupt changes in pH, as previously described by this laboratory (14), was unaffected by the presence of sodium azide (data not shown). Magnesium concentration between 1 to 10 mM did not significantly affect the inhibition of release effected by azide. In a previous work, it was demonstrated that magnesium concentration did not affect the release rate as a function of total calcium binding (8).

Table V

| Experiment 1 | Basic | Calcium-stimulated |
|--------------|-------|---------------------|
| Control      | 25.8  | 9.8                 |
| AsO4 1 mM    | 26.0  | 9.7                 |
| AsO4 5 mM    | 28.4  | 9.5                 |
| AsO4 10 mM   | 26.4  | 9.9                 |
| X537A 3 pg/mg| 27.0  | 10.1                |
| X537A 30 pg/mg| 26.8 | 9.7                 |

| Experiment 2 | Basic | Calcium-stimulated |
|--------------|-------|---------------------|
| Control      | 26.0  | 7.7                 |
| Azide 0.5 mM | 23.4  | 7.8                 |
| Azide 1 mM   | 18.0  | 7.8                 |
| Azide 5 mM   | 10.1  | 6.8                 |

![Fig. 11](image1.png)

**Fig. 11**. Release of calcium in the presence of oxalate resulting from ATP depletion before completion of Site A release; 3 ml, 30°, 0.8 mg of CRS protein per ml; 40 mM Tris-maleate (pH 6.6), 10 mM MgCl₂, 0.1 M KCl, 50 μM CaCl₂, 0 mM oxalate, and ATP as shown. The absence of ATP was ascertained by adding additional calcium (not shown) and the failure of CRS to take it up as it would have been ATP present (4).

![Fig. 12](image2.png)

**Fig. 12 (left)**. Effect of NaN₃ on calcium binding release curve; 3 ml, 30°, 0.8 mg of CRS protein per ml, 40 mM Tris-maleate (pH 6.6), 10 mM MgCl₂, 0.1 M KCl, 50 μM CaCl₂. Azide as shown present from initiation of reaction. The absence of ATP was ascertained by adding additional calcium (not shown) and the failure of CRS to take it up as it would have been ATP present (4).

![Fig. 13](image3.png)

**Fig. 13 (center)**. Dixon plot of NaN₃ inhibition of $R_B/B$: effect of temperature. Reaction as in Fig. 12. $R_B = maximal rate of calcium release; B = total calcium binding. Temperature as shown.

**Fig. 14 (right)**. Effect of pH on NaN₃ inhibition of $R_B/B$. Reaction as in Fig. 12. pH as shown.
TABLE VI

Other nucleotides versus ATP-induced calcium binding

Reaction as in Table I except as shown. All nucleotides were in 0.25 mM. No changes occurred with higher concentrations of nucleoside triphosphate.

|       | $\chi^2$ | df | $A$     | $k_A$  | $B$     | $k_B$  |
|-------|----------|----|---------|--------|---------|--------|
| ATP   | 4.89     | 8  | 12.4 ± 4.0 | 0.39 ± 0.17 | 29.6 ± 4.10 | 0.067 ± 0.008 |
| ITP   | 22.23    | 10 | 20.3 ± 0.52 | 0.048 ± 0.003 |
| GTP   | 5.5      | 8  | 18.6 ± 0.5  | 0.058 ± 0.005 |
| UTP   | 5.3      | 7  | 13.8 ± 0.6  | 0.064 ± 0.008 |

Fig. 15. Effect of AsO$_4$ (above) and X537A (below) on GTP-dependent calcium binding. Reaction as in Figs. 4 and 5 except GTP was used (0.25 mM).

Magnesium ATPase (in the absence of significant calcium) in a concentration similar to its effective concentrations for inhibition of calcium release ($K_i$, 3 mM). The ionophores in high concentration also inhibited only the magnesium-ATPase and not the calcium-dependent ATPase, as previously reported (4, 5).

Effect of Nucleotides

It was reported from this laboratory that nucleotides other than ATP could activate calcium binding by cardiac relaxing system (2), although the specificity for ATP by cardiac relaxing system was greater than that reported for skeletal relaxing system (15-17). Calcium binding curves were analyzed for three nucleotides: the triphosphates of inosine, guanosine, and uridine. With ATP, the two components previously described were demonstrated; GTP, UTP, and ITP showed very good fits for a one-component model with no significant second-component activation. The rate constant of the single component resembled Site B (Table VI). The three nucleotides studied, therefore, seemed to effect an activation of Site B. The effects of the inhibitors X537A and arsenate (Fig. 15) are consistent with the hypothesis that Site B was the site being activated by nucleotides other than ATP. The effect of pH on calcium binding induced by GTP was similar to that shown previously in Fig. 6 for Site B.

Fig. 16. Effect of pH on GTP-dependent calcium binding. Reaction as in Fig. 6.

In addition, examination of the ability of ITP, GTP, and UTP to restimulate calcium binding was similar to that demonstrated for Site B, ranging from 30 to 36% of the first binding cycle. The rate of release as a function of total binding, $R(t)/B$, was unchanged by these nucleotides, as previously reported (8).

The effect of magnesium, however, on calcium binding induced by GTP or ITP revealed another complexity. In concentrations of magnesium from 0.33 to 10.0 mM, little if any magnesium sensitivity could be shown (data not shown). In addition, we again observed the previously reported failure of other nucleotides to sustain continuous calcium uptake after the initial binding "burst" (see Reference 2).

All nucleotides stimulated basic magnesium-nucleotidase in a manner similar to ATP (Table VII). However, calcium-stimulated nucleoside triphosphatase could only be demonstrated with ATP in the presence or absence of oxalate.

Effect of Magnesium on Calcium-stimulated ATPase

The role of calcium-stimulated ATPase in calcium binding and uptake is complicated; previous work from this laboratory suggests that although these processes may be related, they are not obligatorily identical. The effect of magnesium on calcium-stimulated ATPase is shown in Table VIII. The calcium-stimu-
TABLE VII
Calcium-stimulated and basic NTPase
Reactions were carried out at 30°C; 25 to 35 μg per ml; phosphate liberation was measured after 10 min (0). Assay media: 10 mM Tris-maleate (pH 6.8), 10 mM MgCl₂, 0.1 mM KCl, 50 μM CaCl₂, 1.0 mM nucleoside triphosphate, and 5 mM oxalate. The tubes used for basic ATPase contained 0.5 mM EGTA. They were subtracted from the total ATPase to derive the calcium-stimulated portion.

| NTPase            | μmoles/mg/hr | Calcium-stimulated NTPase |
|-------------------|--------------|---------------------------|
| ATP               | 19.9         | 7.1                       |
| GTP               | 18.6         |                           |
| UTP               | 17.9         |                           |
| ITP               | 17.2         |                           |

TABLE VIII
Effect of magnesium and ATP concentrations on calcium-stimulated ATPase
Reaction as in Table V.

| [Mg²⁺] | 1 mM ATP | 0.25 mM ATP | 0.1 mM ATP |
|--------|----------|-------------|------------|
| 10 mM  | 9.0      | 9.5         | 10.5       |
| 3 mM   | 7.5      | 9.0         | 12.0       |
| 1 mM   | 3.0      | 9.5         | 12.0       |
| 0.5 mM | 2.0      | 7.5         | 10.5       |

Fig. 17. Values for calcium-stimulated ATPase as a function of (HATP⁻³ + ATP⁻⁴). Data from Table 8.

Calcium-stimulated ATPase did not require magnesium over a range from 10 to 0.5 mM unless the ATP concentration was high. It would appear from the data shown in Table VIII that a ratio of magnesium to ATP of about 3:1 is required for optimal calcium-stimulated ATPase activity. One role of magnesium may be to reduce the free ATP concentration to a noninhibitory level. These phases or components may be distinct. The previous studies also revealed several other aspects. (a) The relationship of calcium binding to magnesium concentration is complex; double reciprocal plots of magnesium concentration versus total calcium binding or calcium binding rate result in a hyperbolic relationship rather than a straight line. (b) After calcium release, calcium binding can be restimulated by the addition of ATP. (c) The percentage of calcium bound the second time (percentage of restimulation) bears a direct and inverse relationship to magnesium concentration, i.e. the percentage of restimulation was 50 to 75% at 10 mM and nearly 100% at 1 mM magnesium. These findings led us to suggest, as one possibility, that two distinct classes of sites or components may be associated with a CRS preparation and both are ATP-dependent (8). One of these "sites" might require a high magnesium concentration and is not completely restimulated by a second addition of ATP, while the other site requires much less magnesium and is easily restimulated by ATP. In the present study, we have found that the inhibitors of calcium binding, arsenate, X357A and A23187 are only slightly effective at low magnesium concentrations. This implies that the inhibitors may be acting at some magnesium-dependent step in binding, or indeed, may be preferentially and directly interacting with the previously postulated magnesium-dependent site of calcium binding. Considering the latter postulate, we proposed that calcium uptake, which was dependent on millimolar magnesium concentrations and depressed to a greater degree by the inhibitors than total calcium binding, might in some way be linked to the magnesium-dependent site.

The data suggest that calcium binding to CRS is most compatible with a two-component system, which has previously been suggested by this laboratory (4, 5, 8). When calcium and ATP are kept at optimal concentrations, application of pseudo-first order kinetics reveals two components, one of which requires high magnesium (Kₘ 2.7 to 4.7 mM) and represents 60 to 80% of the total calcium binding sites. This component resembles calcium uptake in its sensitivity to magnesium and to the inhibitors X357A and arsenate and may be directly linked to calcium transport into the oxalate vesicles. If this is so, it would explain the greater sensitivity of calcium uptake to the inhibitors when compared to total calcium binding, which includes the binding to both Site A and Site B. Since Site A would be relatively resistant to these inhibitors at any given concentration, total binding would be less inhibited than the rate of uptake. It would appear that Site A requires little magnesium, is relatively resistant to arsenate, X357A, and pH, and is capable of releasing calcium even in the presence of oxalate. This suggests that either it is a distinct site unrelated to Site B or uptake, or that it functions as an activator or priming site for Site B.

The slow phase prior to the onset of linear calcium uptake coincides with the time of release of Site A calcium. Thus, during the time from 1 to 4 min when calcium is presumably being released from Site A, net calcium uptake is slowed because the release of calcium from Site A slows the net removal of calcium from the medium by Site B. If ATP is limiting so that it is removed before Site A releases completely, then the released calcium from Site A is not taken up by Site B, and net release actually ensues in the presence of oxalate. Net calcium release in the presence of uptake conditions can also be demonstrated by Site B inactivation with X357A, high pH, or low magnesium. The kinetic analysis reveals that neither magnesium, pH, nor the inhibitors alter the rate constants of calcium binding to the individual sites. Indeed, it appears that the major influence is...
on the number of individual sites able to be activated by ATP to bind calcium. Fig. 18 demonstrates a proposed model for ATP-dependent calcium binding to the two components. Site B apparently requires millimolar magnesium, and it is proposed that the association of magnesium with Site B results in a species that can be activated by ATP to bind calcium, which presumably can be, or is, subsequently "transported" into the tubular lumen. Activation of this site is potentiated by hydrogen ion and inhibited by low concentrations of X337A and arsenate. In the presence of ATP, the calcium transported to the tubular lumen is precipitated if oxalate is present, allowing a linear uptake process (product consumption). In contrast, Site A is not influenced by pH, arsenate, or low concentrations of X337A, requires very little magnesium, and is not directly in contact with the oxalate space. It, however, obligatorily requires ATP.

A proposed model for the other nucleotides is also shown in Fig. 18. There is no activation of a fast site compatible with Site A. Site B can be activated by GTP without apparent magnesium dependence within the range of 0.3 to 10 mM but is still sensitive to decreased hydrogen ion concentration, X337A, and arsenate. Again, after GTP mediates Site B activation and calcium binding, uptake does not occur into the tubular lumen to any significant degree, suggesting that ATP is specific also for the proposed transport process. This would imply that calcium uptake into the tubular lumen is characterized by an initial binding phase at Site B activated by ATP, GTP, ITP, or UTP and that a distinct transport process occurs subsequent to the initial binding and that transport specifically requires ATP.

It would seem, therefore, that Site A is selective for ATP and will not be activated by other nucleotides. This may account for the slower initial rates of calcium binding when activated by other nucleotides with various sarcoplasmic reticulum preparations from skeletal muscle, and to a much greater degree, in cardiac muscle, as previously described (2, 16-18). The calcium binding activation affected by the other nucleotides probably occurs at Site B in that it resembles Site B in rate constant, pH sensitivity, arsenate sensitivity, X337A sensitivity, and "restimulation." In addition, the amount of calcium binding activated as a single component is in excess of the total amount of Site A binding site observed in experiments with the same preparation. The apparent loss or reduction in magnesium requirement is not explainable at this time, but presumably magnesium must be important for ATP-specific activation.

It is attractive to relate the quantity of magnesium required for calcium binding to Site A to the ATP concentration which has a $K_m$ of 10 to 20 $\mu$M (8). Because of the requirement of an ATP regenerating system for magnesium, this analysis could not be done at very low magnesium concentrations. Attempts to relate the initial concentrations of magnesium-ATP, calcium-ATP, or magnesium ion concentrations suggest that the relationships between magnesium and Site A may be quite complex.

It is difficult to directly relate either of the sites for calcium accumulation to the calcium-stimulated ATPase, since the magnesium sensitivity of calcium-stimulated ATPase was dependent upon ATP concentration and required much lower magnesium than might be anticipated if it were directly related to Site B or a Site B calcium uptake interaction. However, the high magnesium requirement might only be related to the initial step of ATP activation in the presence of ATP (and not in the presence of GTP, ITP, and UTP), whereas calcium-stimulated ATPase was more closely related to the transport process per se, as suggested by numerous authors (19). This is further suggested by the fact that in cardiac tissue, the calcium transport process and calcium-stimulated ATPase are both highly specific in their requirement for ATP, whereas Site B initial activation can occur with other nucleotides. In skeletal muscle, the nucleotide specificity is much less, and again, is similar for transport and ATPase (18).

The relationship of Site A to a calcium-stimulated ATPase is also possible since they both specifically require ATP and need only very low magnesium concentrations. In addition, calcium-stimulated ATPase and Site A have in common the fact that they are both apparently resistant to X337A and arsenate. It is conceivable that the Site A calcium may be the calcium required to initially prime the ATPase-driven pump site that transports calcium bound to Site B. It has been demonstrated by Weber (20) and by Yamada et al. (21) that there is an initial rapid burst of calcium-stimulated ATPase which might actually be related to the initial rapid binding to Site A.

The stoichiometry of ATPase to calcium uptake was approximately 2:1 (i.e. 2 moles of calcium taken up per mole of calcium-stimulated ATP hydrolysis) as previously reported by others, further suggesting that calcium-stimulated ATPase provides a critical step in calcium transport. The finding of inhibition of uptake in the absence of inhibition of ATPase has been previously reported (4, 5, 22-24); the converse (inhibition of ATPase only) has not been reported. The findings in this study have already suggested that these inhibitors may act on the initial binding step and not on the transport process itself; implying that calcium-stimulated ATPase might not be affected since it is apparently more intimately related to the transport process rather than to the initial binding. These findings, therefore, do not "dissociate" calcium-stimulated ATPase from calcium binding or uptake but suggest that the relationship is complex and consists of several steps.

The mechanism of action of the inhibitors of binding is not known. The ionophores are known to induce release of previously bound calcium when added after calcium binding has occurred (4, 5) and presumably have an effect on calcium affinity. Arsenate, on the other hand, does not induce release when added after calcium binding has ensued, and its mechanism of action is unknown. It has been proposed that arsenate might uncouple energy transduction and inhibit ion transport in mitochondria (25). However, the concentration of arsenate necessary for inhibition of CRS is much lower than that required for mitochondrial (20 to 40 mM). In addition, there is no effect on either stimulation or on inhibition of ATPase which one might expect if arsenate was acting in such a fashion on CRS.

The effect of azide, a known mitochondrial inhibitor of energy transduction, is intriguing. In previous work from this laboratory, it was pointed out that the rate of calcium release was unaffected by either the presence of or concentration of ATP after Fig. 18. Proposed model for calcium binding and uptake with ATP and other nucleotides.
peak binding has occurred. It was suggested, therefore, that the high energy of activation of calcium release must be supplied from some membrane energy transduction step, as yet undefined (8). Although the mechanism of action of sodium azide on calcium release is unknown, it may function by interfering with this energy transduction. It is also of interest that sodium azide inhibits magnesium-ATPase in concentrations similar to that required to inhibit calcium release; however, other inhibitors of magnesium-ATPase such as valinomycin and oligomycin do not inhibit either calcium binding or calcium release despite the fact that they are equally potent in inhibiting magnesium-ATPase.

The differential effects of these inhibitors coupled with previous results suggest that ATP induces a complex calcium binding release cycle with distinct calcium binding and release phases. The data further suggest that binding may take place at two classes of calcium binding sites or components that may or may not be interrelated. The data do not exclude the possibility of a conversion of one site or component to another "energetic" state. On the other hand, the data do suggest that the actual transport of calcium to the oxalate space (vesicular lumen) may occur as a separate component with separate control aspects. Recent descriptions have been reported of several purified proteins from sarcoplasmic reticulum fragments, two of which bind calcium with different but high affinity ("extrinsic or soluble and loosely bound proteins"), and the third which contains the intrinsic protein; Site B may be part of the intrinsic protein network. Recent work by Racker (29) has demonstrated that the calcium pump ATPase (molecular weight ~102,000), a proteolipid (so-called "intrinsic or insoluble and tightly bound proteins"), and is similar to the "M" band protein described by Martonosi (26-28). Site A may represent binding to an extrinsic protein; Site B may be part of the intrinsic protein network. Recent work by Racker (29) has demonstrated that the latter can be returned to an artificial phospholipid environment and form vesicles which possess calcium transport and calcium-ATPase activity and are sensitive to A857A and A23187.

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APPENDIX

Explanation of $\chi^2$ Minimization

Assuming that the experimental data are normally distributed about the predicted values, then the best method for estimating the parameters requires minimizing the function

$$\chi^2 = \sum_{i=1}^{N} \frac{[y(x_i) - f(a_1, \ldots, a_N; x_i)]^2}{\sigma_i}$$

(1)

where $y(x_i)$ = experimental result at $x_i$; $f(a_1, \ldots, a_N; x_i)$ = the theoretical function used to predict the true value at $x_i$; $a_k (k = 1, NV)$ = the parameters to be estimated; $\sigma_i$ = the standard deviation of the experimental results about the true value at $x_i$; $NV$ = the number of experimental points; $NV$ = the number of variable parameters. To minimize the function $\chi^2$, the $NV$ simultaneous equations

$$\frac{\delta\chi^2}{\delta a_k} = 0 \quad k = 1, NV$$

must be solved. The computed program was written to solve Equation 2 for the $a_k$ values. Since Equation 2 is seldom solvable in closed form, the program utilizes the iterative Newton-Raphson method (12) to determine the $a_k$ values.

If the assumption of normality is valid, then the program also calculated the errors associated with each of the $a_k$ estimates. Also, since the function $\chi^2$ is $\chi^2$ distributed, the value of the function, using the estimated parameters, indicated how well the experimental results fit the theoretical predictions.

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