Persechini, A., & Kretsinger, R. H. (1988) *J. Biol. Chem. 263*, 12175.
Persechini, A., Blumenthal, D. K., Jarett, H. W., Klee, C. B., Hardy, D. O., & Kretsinger, R. H. (1989) *J. Biol. Chem.* (in press).
Porod, G. (1951) *Kolloid-Z. 124*, 83.
Putkey, J. A., Draetta, G. F., Slaughter, G. R., Klee, C. B., Stull, J. T., & Means, A. R. (1986) *J. Biol. Chem. 261*, 9896.
Putkey, J. A., Ono, T., VanBerkum, M. F. A., & Means, A. R. (1988) *J. Biol. Chem. 263*, 11242.
Seeger, P. A., & Pynn, R. (1986) *Nucl. Instrum. Methods Phys. Res., Sect. A 424*, 115.
Seeger, P. A., Williams, A., & Trewhella, J. (1987) in *Proceedings of the International Collaboration on Advanced Neutron Sources-IX* (Atchison, F., & Fischer, W., Eds.) SIN Report ISBN 3-907990-01-4, p 437, Swiss Institute for Nuclear Research, Villigen, Switzerland.
Seeger, P. A., Hjelm, R. P., & Nutter, M. J. (1989) *Mol. Cryst. Liq. Cryst.* (in press).
Seeholzer, S. H., Cohn, M., Putkey, J. A., Means, A. R., & Crespi, H. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3634.
Small, E., & Anderson, S. R. (1988) *Biochemistry 27*, 419.
Stull, J. T. (1988) in *Molecular Aspects of Cellular Regulation* (Cohen, P., Klee, C. B., Eds.) Vol. 5, pp 91-119, Elsevier, Amsterdam.
Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078.
Wylie, D. C., & Vanaman, T. C. (1988) in *Moleclar Aspects of Cellular Regulation* (Cohen, P., Klee, C. B., Eds.) Vol. 5, pp 1-15, Elsevier, Amsterdam.

Postulated Role of Calsequestrin in the Regulation of Calcium Release from Sarcoplasmic Reticulum†

Noriaki Ikemoto,*,‡,§ Michel Ronjat,‡,∥ László G. Mészáros,‡,⊥ and Makoto Koshita‡,#

Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Received November 22, 1988; Revised Manuscript Received May 2, 1989

ABSTRACT: Ca^{2+} release from heavy sarcoplasmic reticulum (SR) vesicles was induced by 2 mM caffeine, and the amount (A) and the rate constant (k) of Ca^{2+} release were investigated as a function of the extent of Ca^{2+} loading. Under both passive and active loading conditions, the A value increased monotonically in parallel to Ca^{2+} loading. On the other hand, k sharply increased at partial Ca^{2+} loading, and upon further loading, it decreased to a lower level. Since most of the intravesicular calcium appears to be bound to calsequestrin both under passive and under active loading conditions, these results suggest that the kinetic properties of induced Ca^{2+} release show significant variation depending upon how much calcium has been bound to calsequestrin at the time of the induction of Ca^{2+} release. An SR membrane segment consisting of the junctional face membrane (jfm) and attached calsequestrin (jfm–calsequestrin complex) was prepared. The covalently reacting thiol-specific conformational probe *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) was incorporated into several proteins of the jfm, but not into calsequestrin. The fluorescence intensity of DACM increased with Ca^{2+}. Upon dissociation of calsequestrin from the jfm by salt treatment, the DACM fluorescence change was abolished, while upon reassociation of calsequestrin by dilution of the salt it was partially restored. These results suggest that the events occurring in the jfm proteins are mediated via the attached calsequestrin rather than by a direct effect of Ca^{2+} on the jfm proteins. We propose that the [Ca^{2+}]-dependent conformational changes of calsequestrin affect the jfm proteins and in turn regulate the Ca^{2+} channel functions.

Calsequestrin is one of the major protein components of the sarcoplasmic reticulum (SR),[1] but its physiological role has not yet been thoroughly understood. During the nearly 2 decades since its discovery (MacLennan & Wong, 1971; Ikemoto et al., 1971), extensive studies have been carried out on its physicochemical properties (MacLennan et al., 1983; Campbell, 1986). The isolated calsequestrin molecule in solution is presumably in a monomeric form (Ikemoto et al., 1974; Hymel et al., 1984) with an asymmetric structure (Cozens & Reithmeier, 1984). The isolated protein is highly acidic (MacLennan & Wong, 1971; Ikemoto et al., 1974; MacLennan et al., 1983; Campbell, 1986; Fliegel et al., 1987; Scott et al., 1988) and has characteristic Ca^{2+} binding properties (MacLennan & Wong, 1971; Meissner et al., 1973; Ikemoto et al., 1974; Slupsky et al., 1987) with a large capacity

† This work was supported by Grant AR-16922 from the National Institutes of Health and by a grant from the Muscular Dystrophy Association of America.

* Address correspondence to this author at the Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114.
‡ Boston Biomedical Research Institute.
§ Harvard Medical School.
∥ Permanent address: Laboratoire de Biophysique Moleculaire et Cellulaire, Centre d'Etudes Nucleaires, Grenoble 38041, France.
⊥ Present address: Second Institute of Biochemistry, Semmelweis University Medical School, Budapest, Hungary.
Present address: Department of Physiology, Nagoya City University Medical School, Nagoya, Japan.

[1] Abbreviations: DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; jfm, junctional face membrane; MES, 2-(*N*-morpholino)ethanesulfonic acid; SR, sarcoplasmic reticulum; T-tubule, transverse tubular system.

0006-2960/89/0428-6764$01.50/0

(about 40 mol/mol) and a relatively low affinity ($K_{assoc} = 10^3$ M^{-1}). Dramatic [Ca^{2+}]-dependent changes of various properties of isolated calsequestrin, such as tryptophan fluorescence (Ikemoto et al., 1974; Slupsky et al., 1987), circular dichroism (Ikemoto et al., 1974; Ostwald et al., 1974; Aaron et al., 1984; Slupsky et al., 1987), Raman (Williams & Beeler, 1986) and ^1H nuclear magnetic resonance (Aaron et al., 1984), and the proteolytic digestion pattern (Ohnishi & Reithmeier, 1987), suggest that upon Ca^{2+} binding the protein undergoes extensive changes in its conformation. The complete primary structure of both the skeletal muscle (Fliegel et al., 1987) and cardiac muscle (Scott et al., 1988) forms of calsequestrin has recently been reported.

Earlier electron microscopic studies (Meissner, 1975) demonstrated that calsequestrin is localized in the vesicles derived from the terminal cisternal region of SR. More recently, some intriguing structural features have been revealed, such as a meshlike assembly of the calsequestrin molecules (Saito et al., 1984) and a tight linkage of calsequestrin with the terminal cisternal SR membrane (Franzini-Armstrong et al., 1987), especially with the region containing the junctional face membrane [jfm (Costello et al., 1986)]. Little or no portion of calsequestrin is accessible at the cytoplasmic surface of the SR membrane (Michalak et al., 1980; Hidalgo & Ikemoto, 1977); any portion of it does not seem to penetrate the membrane (Volpe et al., 1987). Thus, calsequestrin seems to be anchored to the internal surface of the membrane presumably via integral proteins of the jfm. The protein constituent of the feet with a molecular weight of approximately 400K is one of the major protein components of the jfm (Costello et al., 1986). This protein, when incorporated into planar lipid bilayers, behaves in several ways as a Ca^{2+} channel (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988; Smith et al., 1988). These facts have suggested the hypothesis that calsequestrin may interact directly or indirectly with the Ca^{2+} channels and regulate Ca^{2+} release functions.

We have found that kinetic properties of Ca^{2+} release from the SR vesicles are altered with the extent of Ca bound to the internal Ca^{2+} binding sites, which is chiefly accounted for by calsequestrin, under both passive and active loading conditions. Thus, upon induction of Ca^{2+} release from SR when Ca^{2+} binding to calsequestrin is partial, the induced release is characterized by a smaller amount of Ca^{2+} released with a higher rate constant. On the other hand, when more Ca is bound to calsequestrin, the induced Ca^{2+} release is characterized by a larger amount of Ca^{2+} released with a lower rate constant. Using an SR membrane segment consisting of the junctional face membrane (jfm) and attached calsequestrin, we demonstrate here that the fluorescence intensity of the conformational probe DACM attached to several jfm proteins including the 400-kDa foot protein increased with Ca^{2+}. The response of these proteins to Ca^{2+} is abolished upon dissociation of calsequestrin, while upon reassociation of calsequestrin it is restored. This suggests that Ca^{2+}-dependent conformational changes of calsequestrin are transmitted to the jfm and regulate the jfm proteins. We propose that this mechanism plays a major role in the regulation by calsequestrin of the Ca^{2+} release function revealed in this study.

Experimental Procedures

Preparation of SR. The heavy fraction of SR was prepared from rabbit leg and back muscles (fast twitch muscle) by differential centrifugation as described previously (Kim et al., 1983). Briefly, the muscle homogenates were centrifuged at 10000*g* for 3 min in a GSA rotor (Sorvall). The supernatant fraction was filtered through eight layers of cheesecloth and then Whatman filter papers (no. 2) and centrifuged at 17000*g* for 30 min. The sedimented fraction was washed with 0.15 M KCl, and 20 mM MES (pH 6.8) by repeating two cycles of homogenization and sedimentation at 17000*g*.

Preparation of Calsequestrin. Calsequestrin was purified by solubilizing the heavy fraction of SR with Triton X-100 followed by Ca^{2+} precipitation and DEAE-cellulose column chromatography as described previously (Ikemoto et al., 1972).

Preparation of Junctional Face Membrane with or without Attached Calsequestrin. For preparation of the jfm, the heavy SR vesicles (4.0 mg mL^{-1}) suspended in a 3.0-mL solution of 0.3 M sucrose, a standard set of proteolytic enzyme inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 10 μg mL^{-1} aprotinin, 0.8 μg mL^{-1} antipain, and 2 μg mL^{-1} soybean trypsin inhibitor), and 20 mM MES (pH 6.8) were solubilized by adding 8.0 mg mL^{-1} Triton X-100 (2:1 Triton:SR protein ratio, w/w) at 0 °C. The insoluble fraction, which contained the jfm and the attached calsequestrin (designated as jfm–calsequestrin complex), was collected by centrifugation in a TL-100.3 rotor in a Beckman TL-100 table-top ultracentrifuge at 90000 rpm for 5–15 min. To dissociate the attached calsequestrin from the jfm, the fraction containing the jfm–calsequestrin complex (2.4–2.8 mg) was homogenized in 0.6 mL volume of a solution of 0.5 M NaCl, proteolytic enzyme inhibitors (see above), with 0.24–0.28 mg mL^{-1} cholate or without cholate, and 20 mM MES (pH 6.8) and centrifuged as above. The resultant sedimentable fraction contained the calsequestrin-depleted jfm, and the supernatant contained the dissociated calsequestrin. To reconstitute the jfm–calsequestrin complex, the jfm–calsequestrin complex (2.4–2.8 mg) was homogenized in 0.6 mL volume of a solution of 0.5 M NaCl, with or without cholate, proteolytic enzyme inhibitors, and 20 mM MES (pH 6.8) to first dissociate calsequestrin from the jfm. Then the NaCl concentration of the mixture of the jfm and dissociated calsequestrin was reduced to 0.1 M by diluting with a 0.3 M sucrose solution containing proteolytic enzyme inhibitors. The reconstituted jfm–calsequestrin complex was collected by sedimentation.

Stopped-Flow Measurements of Ca^{2+} Uptake by, and Release from, SR Vesicles. In order to establish a linear signal/Ca^{2+} relationship during the Ca^{2+} uptake and release assays, the following calibration procedures were carried out. Increasing concentrations of EGTA were added stepwise to a solution containing 0.15 M KCl, 9 μM arsenazo III, and 1.2 mg mL^{-1} SR that were permealized by treatment with 0.5 mg mL^{-1} Triton X-100, 3 mM MgATP, and 20 mM MES, pH 6.8 (the conditions equivalent to those during the Ca^{2+} uptake reaction; see below). The difference in the absorbance of arsenazo III at 650 and 680 nm ($A_d = A_{650} - A_{680}$) was determined with a dual-beam spectrophotometer as described previously (Ikemoto et al., 1984). Because of the Ca^{2+} contamination in the SR preparation used (e.g., 100–150 nmol/mg of SR protein), upon initial additions of EGTA there was little or no change in A_d (phase i). With more $[EGTA]_{added}$, A_d decreased linearly as a function of $[EGTA]_{added}$ (phase ii). Upon further additions of EGTA, the $A_d/[EGTA]_{added}$ response decreased again (phase iii), due to a buffering action of an EGTA–Ca mixture in the range of $[Ca]_{added}/[EGTA]_{added} \leq 1$. To assure a linear response of arsenazo III to $[Ca^{2+}]_o$ throughout the Ca^{2+} uptake and release reactions, therefore, the amount of EGTA required to eliminate phase i (see above) was added before the Ca^{2+} uptake reaction was started. The total amount of Ca present in the SR preparation was determined from the intercept of the lines fitted to the A_d values of phases ii and iii. The $A_d/[Ca^{2+}]$ relationship,

which was used for calculation of the amounts of transported or released Ca^{2+}, was obtained from the slope of the A_d versus $[EGTA]_{added}$ plot. We found that the size of the initial rapid phase of Ca^{2+} uptake increased by preincubation of SR (e.g., >10 min) to remove contaminating Ca^{2+}; but incubation of SR with arsenazo III for a prolonged time (e.g., >15 min with 9 μM arsenazo III) inhibited Ca^{2+} release: hence, in the stopped-flow experiments, arsenazo III was added at the time when the Ca^{2+} uptake reaction was initiated (see below).

For the Ca^{2+} uptake assay, SR was diluted to a final concentration of 1.5 mg mL^{-1} in a solution containing 0.15 M KCl, 20 mM MES (pH 6.8), and sufficient amounts of EGTA to eliminate the excess Ca^{2+} determined in the above titration experiments (solution A_1; A, B, and C imply the solutions to be loaded in cylinders A, B, and C, respectively). After incubation for at least 15 min (to reduce the internally bound [Ca]), the Ca^{2+} uptake reaction was initiated by mixing 80 μL of solution A_1 and 20 μL of solution B_1 (0.15 M KCl, 45 μM arsenazo III, 15 mM $MgCl_2$, 15 mM ATP, and 20 mM MES, pH 6.8) by driving cylinder A and cylinder B of a three-cylinder stopped-flow apparatus (BioLogic SFM-3) (stepping-motor driving time = 40 ms). Changes in the A_d of arsenazo III were monitored with the dual-wavelength spectrophotometer system (Ikemoto et al., 1984).

For the induction of Ca^{2+} release at various times during the Ca^{2+} uptake reaction, Ca^{2+} uptake was initiated by mixing 40 μL of solution A_1 and 10 μL of solution B_1 as above, and at various times after the addition of ATP (t_{ATP}), Ca^{2+} release was induced by mixing 1 part of the A_1 + B_1 Ca^{2+} uptake reaction mixture with 1 part of solution C_1 (0.15 M KCl, 9 μM arsenazo III, 4 mM caffeine, and 20 mM MES, pH 6.8). The resultant changes in A_d were monitored as described above. For calibration of $[Ca^{2+}]$, a solution with the same composition as the above Ca^{2+} uptake reaction mixture (A_1 + B_1) was prepared, to which 0.25–0.5 mg mL^{-1} Triton X-100 and increasing concentrations of EGTA were added, and the A_d values were plotted as a function of $[EGTA]_{added}$ as described above.

The above three-cylinder stopped-flow system was also used for the assay of Ca^{2+} release at different levels of passive loading. Heavy SR (2 mg mL^{-1}) was incubated in solution A_2 containing 0.15 M KCl, 20 mM MES (pH 6.8), and various amounts of total Ca^{2+} for 5–6 h at 22 °C. To achieve lower levels of loading (e.g., <0.1 mM), excess amounts of Ca^{2+} present were chelated by EGTA added to solution A_2. To start Ca^{2+} release, 10 μL of solution A_2 was mixed first with 90 μL of solution B_2 containing 0.15 M KCl, 20 mM MES (pH 6.8), and various concentrations of EGTA by driving cylinder A and cylinder B (the final concentrations of total Ca^{2+} and added EGTA after mixing were made equal). At this point, the $[Ca^{2+}]_o$ was about 10 μM. Because of low concentrations of Ca–EGTA after mixing (viz., ≤0.2 mM), there was no change in the pH. After 50 ms, 1 part of the A_2 + B_2 mixture was mixed with 1 part of solution C_2 (0.15 M KCl, 4 mM caffeine, 20 μM $CaCl_2$, 18 μM arsenazo III, and 20 mM MES, pH 6.8). The time course of Ca^{2+} release was determined by spectrophotometric monitoring of A_d, and the $A_d/[Ca^{2+}]$ relationship was determined as described above.

Assay of Ca^{2+} Binding to Intravesicular Binding Sites. The heavy SR vesicles (0.1–0.2 mg mL^{-1}) were incubated in 0.15 M KCl/20 mM MES (pH 6.8) at various concentrations of $CaCl_2$ (0.05–20 mM Ca^{2+}) containing $^{45}Ca^{2+}$ (0.25–1 μCi mL^{-1}) for 60 min at 22 °C. A 0.1-mL portion of the solution was filtered through the Millipore filter (0.45 μm) and washed with 2 × 2.5 mL of a solution containing 0.15 M KCl, 20 mM MES (pH 6.8), 1 mM EGTA (to remove the externally bound ^{45}Ca), and 5 μM ruthenium red (to prevent a leak of the internally loaded ^{45}Ca). The filter was air-dried, and the amount of ^{45}Ca trapped within the vesicles was determined by scintillation counting. The amount of Ca bound to the intravesicular binding sites was calculated by subtracting the amount equivalent to the intravesicular free $[Ca^{2+}]$ from the total amount of ^{45}Ca trapped in the vesicles. The amount equivalent to the internal free $[Ca^{2+}]$ was calculated from the Ca^{2+} concentration used for passive loading and the volume of the SR vesicles. The volume of the vesicles was determined according to the [^{14}C]inulin equilibration method (Kasai, 1980). The vesicular volume of our heavy SR fraction was 4.2 ± 0.27 μL/mg of SR protein, and remained constant in the $[Ca^{2+}]$ range of 0–10 mM.

Spectrometric Studies of Ca^{2+}-Induced Conformational Changes of Purified Calsequestrin. For measurements of both circular dichroism and tryptophan fluorescence, purified calsequestrin (see above) was dissolved in a solution containing 0.15 M KCl/20 mM MES (pH 6.8) at a protein concentration of 0.1 mg mL^{-1}. To titrate Ca^{2+}-dependent spectrometric changes, increasing amounts of Ca^{2+} were added in a sequential fashion. For the circular dichroism measurements, the above titration was carried out in a cell with a path length of 2 mm, and the spectrum (185–250 nm) was recorded at each step of the Ca^{2+} increment in an Aviv circular dichroism spectropolarimeter (Model 160 DS). The mean residue weight ellipticity ($-\theta$) at 222 nm was plotted as a function of added $[Ca^{2+}]$ [cf. Ikemoto et al. (1974)]. For the tryptophan fluorescence measurements, the above Ca^{2+} titration was carried out in a cell with a path length of 1 cm, and the fluorescence emission intensity at 340 nm (excitation at 280 nm) was recorded at each step of the Ca^{2+} increment using a SPEX fluorolog fluorometer.

RESULTS

Regulation of Ca^{2+} Release from SR Vesicles as a Function of Internally Bound Calcium Concentration. After equilibration of heavy SR vesicles with various concentrations of Ca^{2+}, Ca^{2+} release was induced by the addition of 2 mM caffeine at $[Ca^{2+}]_o \simeq 10$ μM. The time course of Ca^{2+} release could be fitted with the monoexponential equation $A_0 + A(1 - e^{-kt})$. The amount of Ca^{2+} released (A) and the rate constant of Ca^{2+} release (k) are plotted as a function of the levels of Ca^{2+} loading at the time of induction of Ca^{2+} release ($[Ca^{2+}]_i$, Figure 1A). As seen, the amount of Ca^{2+} released (A) is a monotonic function of $[Ca^{2+}]_i$; it levels off at $[Ca^{2+}]_i \simeq 2$ mM. The rate constant of Ca^{2+} release (k), on the other hand, manifests interesting multiphasic changes with $[Ca^{2+}]_i$. It sharply increases in the low $[Ca^{2+}]_i$ range (≤0.2 mM), reaches a peak, and then decreases to a plateau.

The amounts of intravesicular Ca were determined for various levels of $[Ca^{2+}]_i$, and in Figure 1B, bound [Ca] and total free $[Ca^{2+}]$, in nanomoles per milligram of SR, are plotted against $[Ca^{2+}]_i$. The amounts of Ca^{2+} released (A) shown in Figure 1A were replotted in Figure 1B to facilitate comparison. It is interesting that in the range of $[Ca^{2+}]_i \leq 2$ mM, where the major changes in Ca^{2+} release occur (see above), almost all (≥90%) intravesicular Ca is bound to the internal binding sites. In this range of $[Ca^{2+}]_i$, almost all internal Ca^{2+} binding is accounted for by the Ca^{2+} binding to calsequestrin (the concentration of the calsequestrin Ca^{2+} binding sites within the luminal space is presumably very high), suggesting that the $[Ca^{2+}]_i$-dependent alteration in the kinetic properties is in fact correlated with Ca^{2+} binding to calsequestrin. Upon further increase of $[Ca^{2+}]_i$, however, the free

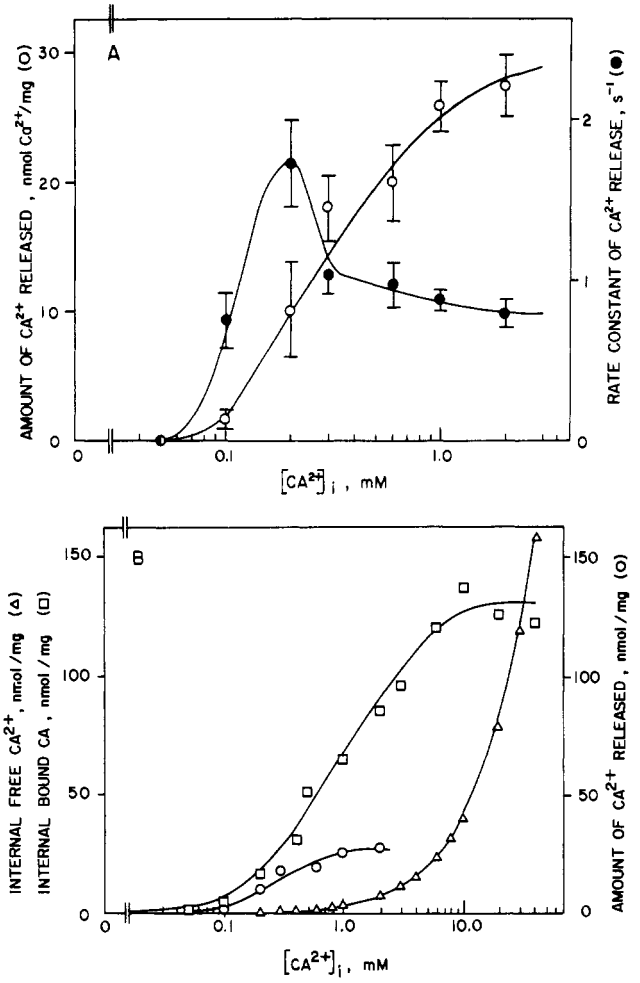

FIGURE 1: Caffeine-induced release of passively loaded Ca^{2+} from SR vesicles as a function of the intravesicular $[Ca^{2+}]$. Heavy SR vesicles were passively loaded by equilibration with various concentrations of Ca^{2+}, and caffeine-induced Ca^{2+} release was carried out in a three-cylinder stopped-flow system as described under Experimental Procedures. The parameters characterizing the kinetics of Ca^{2+} release—the amount of Ca^{2+} released (A) and the rate constant of Ca^{2+} release (k)—were determined by a computer fitting of an exponential, $A_0 + A(1 - e^{-kt})$, to the time course of Ca^{2+} release. Each point was obtained from averaging the data originating from three experiments. The bar indicates the standard deviation. (B) Internal bound [Ca] and internal free $[Ca^{2+}]$ as a function of $[Ca^{2+}]_i$. Heavy SR vesicles were passively loaded with various amounts of $^{45}Ca^{2+}$ as done in the experiments of (A), and the amounts of ^{45}Ca bound to the intravesicular binding sites and of the internal free Ca^{2+} were determined by Millipore filtration as described under Experimental Procedures. The Ca^{2+} release data (A) shown in (A) were replotted to facilitate comparison.

$[Ca^{2+}]_i$:[bound Ca] ratio sharply increases, although the bound [Ca] increases steadily up to $[Ca^{2+}]_i \simeq 10$ mM.

We also investigated the relation between the Ca^{2+} release kinetics and the extent of internal Ca^{2+} binding under active loading conditions (Figures 2 and 3). The Ca^{2+} uptake reaction was initiated by mixing the heavy SR vesicles with MgATP. At various times after the addition of MgATP, Ca^{2+} release was triggered by the addition of caffeine. Figure 2 depicts a family of the Ca^{2+} release time courses obtained at different times of Ca^{2+} uptake (t_{ATP}). If added very early during Ca^{2+} uptake (e.g., $t_{ATP} = 0.2$ s), caffeine induces no Ca^{2+} release. The addition of caffeine at $t_{ATP} = 0.5$–2 s led to a small but rapid Ca^{2+} release followed by reaccumulation of Ca^{2+}. The amount of Ca^{2+} released increased with t_{ATP}, but there was little further increase in the amount of Ca^{2+} released after 10 s.

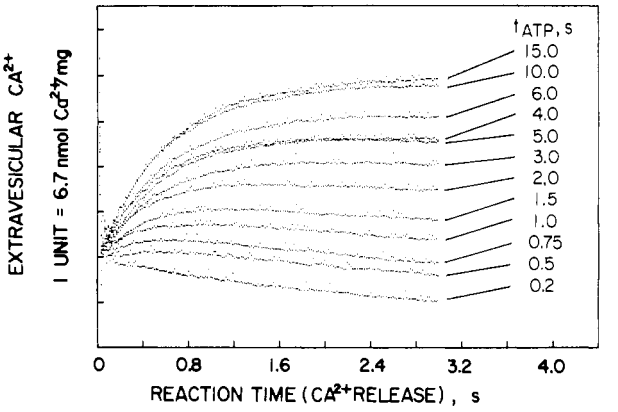

FIGURE 2: Time course of Ca^{2+} release induced by the addition of caffeine at various times (t_{ATP}) during the Ca^{2+} uptake reaction. The active Ca^{2+} uptake reaction was initiated by mixing the heavy SR vesicles (1.2 mg mL^{-1} in a final concentration) with MgATP (3 mM in a final concentration) by driving the first two cylinders of a three-cylinder stopped-flow apparatus. At various times during the uptake reaction (t_{ATP}), Ca^{2+} release was induced by adding caffeine using the third cylinder. Then, the time courses of Ca^{2+} release were monitored by following the changes in the difference absorbance of arsenazo III at 650 and 680 nm. For further details, see Experimental Procedures.

Various parameters expressing the kinetic characteristics of Ca^{2+} release, i.e., the amount (A), the rate constant (k), and the initial rate (Ak), were calculated from each set of the Ca^{2+} release time courses shown in Figure 2 as described in the legend to Figure 3, and plotted as a function of t_{ATP} in Figure 3A. The amount (A) and the initial rate (Ak) of Ca^{2+} release increased sharply in the initial 2–3 s of Ca^{2+} uptake, approximately paralleling the initial rapid Ca^{2+} uptake (Figure 3B); this was followed by a slower increase as the Ca^{2+} uptake also became slower. On the other hand, the rate constant of Ca^{2+} release (k) shows much higher values when Ca^{2+} release is induced when Ca^{2+} accumulation is only partial ($t_{ATP} < 1$ s), while it becomes significantly lower when release is induced at higher levels of Ca^{2+} accumulation ($t_{ATP} \geq 6$ s, Figure 1B). The initial rate (Ak) increased sharply in the initial phase with the increase in the rate constant (k), and subsequently increased slowly approximately in parallel to the increase in the amount of Ca^{2+} release (A). Almost all the transported Ca^{2+} is presumably bound to calsequestrin under such conditions ($\leq$50 nmol of Ca/mg of SR) as deduced from the experiments shown in Figure 1B. Thus, it appears that the same type of mechanism of regulation of Ca^{2+} release as seen in the passive loading experiments, viz., monotonic increase in the amount of Ca^{2+} released and multiphasic changes in the rate constant of Ca^{2+} release with an increase in the amount of Ca bound to the internal binding sites, presumably to calsequestrin, operates also during the active transport process.

Interactions of Calsequestrin with the Junctional Face Membrane. An insoluble fraction of heavy SR after treatment with Triton X-100 at a 2:1 detergent:SR protein ratio contains a membrane segment derived from the region of the terminal cisternal SR that is facing the T-tubule membrane, i.e., the jfm (Costello et al., 1986). This jfm preparation contains a considerable amount of calsequestrin associated with the jfm segments [designated as the jfm–calsequestrin complex (Figure 4A lane 1)]. Homogenization of the jfm–calsequestrin complex with 0.5 M NaCl in the presence of low concentrations of cholate (the presence of cholate was not essential for dissociation) led to a selective removal of calsequestrin from the jfm, and upon centrifugation, the sedimentable fraction contained jfm that was virtually devoid of calsequestrin, designated as calsequestrin-depleted jfm (Figure 4A, lane 2). Upon re-

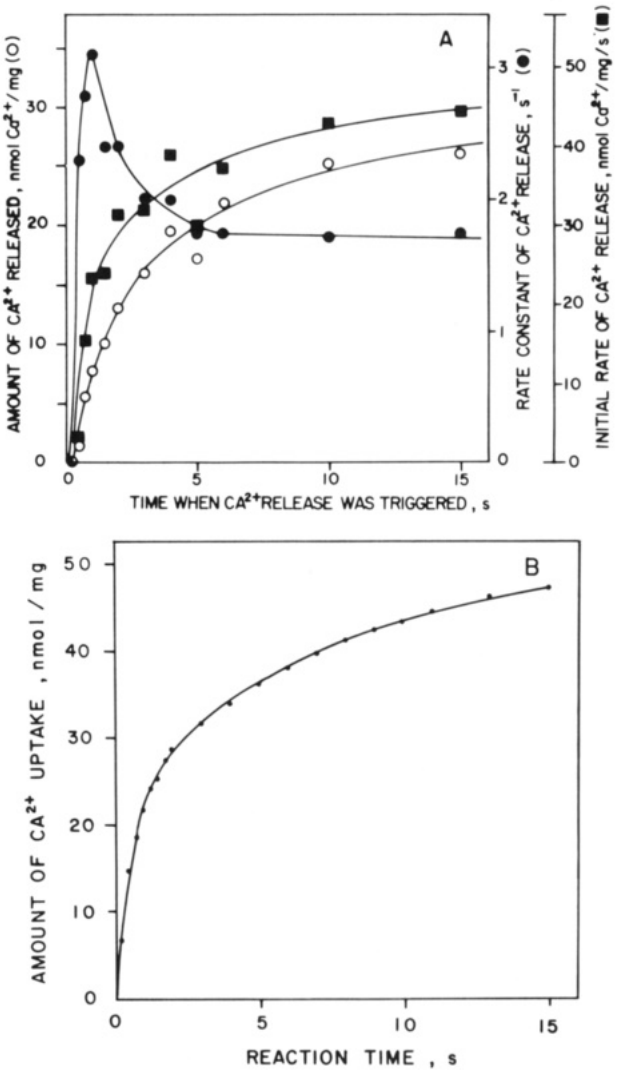

FIGURE 3: Various kinetic constants (the amount, the rate constant, and the initial rate) of Ca^{2+} release as a function of the time of the Ca^{2+} uptake reaction when Ca^{2+} release was triggered (A) in reference to the time course of Ca^{2+} uptake (B). (A) The parameters expressing the kinetic characteristics of Ca^{2+} release induced at various t_{ATP}—the amount (A), the rate constant (k), and the initial rate (Ak)—were calculated by computer fitting of an exponential, $A_o + A(1 - e^{-kt})$, to the release time courses ($t_{ATP} > 3.0$ s) shown in Figure 2. For the time courses of release induced at $t_{ATP} < 3.0$ s, where Ca^{2+} release was followed by Ca^{2+} reuptake, these constants were calculated by fitting two exponentials, $y_r + y_u$, where y_r is an exponential representing the release component (see above) and y_u represents the reuptake component ($y_u = A_{u0} + A_u e^{-k_u t}$). (B) The Ca^{2+} uptake reaction was carried out by mixing cylinder A and cylinder B solutions, and the time course was monitored by following the changes in A_d of arsenazo III at 650 and 680 nm as described in the legend to Figure 2. The Ca^{2+} uptake time course was determined spectrophotometrically as described under Experimental Procedures.

duction of the NaCl concentration from 0.5 to 0.1 M by dilution of the mixture of the calsequestrin-depleted jfm and the dissociated calsequestrin, the sedimentable fraction contained the reconstituted jfm–calsequestrin complex (Figure 4A, lane 3). When the heavy SR vesicles were labeled with the covalently reacting thiol-specific fluorescence probe *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM), and then these three types of jfm preparations were prepared, the major sites of DACM labeling are the 400-kDa foot protein and proteins of M_r 29K–32K. The fluorescence labeling patterns of the jfm–calsequestrin complex (Figure 4B, lane 1), of the calsequestrin-depleted jfm (Figure 4B, lane 2), and of the reconstituted jfm–calsequestrin complex (Figure

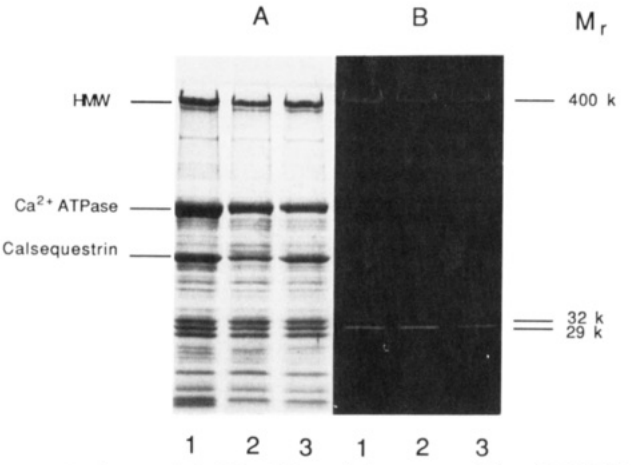

FIGURE 4: Coomassie brilliant blue staining pattern (A) and DACM fluorescence labeling pattern (B) of electrophoretically separated protein components of the jfm–calsequestrin complex (lane 1 of panel A and B), the calsequestrin-depleted jfm (lane 2 of panels A and B), and the reconstituted jfm–calsequestrin complex (lane 3 of panels A and B), which were prepared from the heavy SR labeled with the covalently reactive thiol-specific fluorescence probe *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM). Heavy SR (4 mg mL^{-1}) reacted with 1 μM DACM in a solution containing 0.3 M sucrose, proteolytic enzyme inhibitors, and 20 mM MES (pH 6.8) at 22 °C for 2 min. The reaction was stopped by adding 1 mM DTT, and the DACM-labeled SR vesicles were centrifuged in a Beckman bench-top ultracentrifuge to remove unreacted probe. To prepare the jfm–calsequestrin complex, the DACM-labeled SR (the final protein concentration = 4.0 mg mL^{-1}) was suspended in a 1.0-mL solution of 0.3 M sucrose, the standard set of proteolytic enzyme inhibitors (see Experimental Procedures), and 20 mM MES (pH 6.8) and solubilized by adding 8.0 mg mL^{-1} Triton X-100 at 0 °C (2:1 Triton X-100:SR ratio, w/w). The jfm–calsequestrin complex was collected by centrifugation and homogenized in 1.0 mL of 0.3 M sucrose solution described above. To prepare the calsequestrin-depleted jfm, the above sedimented fraction containing the jfm–calsequestrin complex (originated from 4 mg of DACM-labeled SR) was homogenized in a 0.6-mL volume of a solution of 0.5 M NaCl, proteolytic enzyme inhibitors, 0.25 mg mL^{-1} cholate, and 20 mM MES (pH 6.8) and centrifuged as above. The calsequestrin-depleted jfm collected in the sedimentable fraction was homogenized in a 1.0-mL volume of the 0.3 M sucrose solution described above. To prepare the reconstituted jfm–calsequestrin complex, the jfm–calsequestrin complex (originated from 4 mg of DACM-labeled SR) was homogenized in 0.6 mL volume of a solution of 0.5 M NaCl, proteolytic enzyme inhibitors, and 20 mM MES (pH 6.8) to first dissociate calsequestrin from the jfm. The NaCl concentration was reduced to 0.1 M by diluting the above solution with the 0.3 M sucrose solution containing proteolytic enzyme inhibitors. The reconstituted jfm–calsequestrin complex was collected in the sedimentable fraction after rapid centrifugation (TL-100.3 rotor in a Beckman TL-100 table-top ultracentrifuge at 90000 rpm for 5 min). Twenty microliters of each sample was placed in each well of a gradient (5–15% polyacrylamide) slab gel for electrophoretic separation of the protein components according to Laemmli (1970). The gel was first subjected to fluorescence photography (B) and subsequently stained with Coomassie brilliant blue R (A).

4B, lane 3) are identical. No DACM incorporation took place into calsequestrin, since skeletal muscle calsequestrin has no thiol group (Ikemoto et al., 1974; Fliegel et al., 1987).

Figure 5[2] shows that the fluorescence intensity of the labeled DACM increased with an increase of $[Ca^{2+}]$ in the range of 0.1–1.0 mM if calsequestrin is attached to the jfm. In the calsequestrin-depleted jfm, however, there is virtually no $[Ca^{2+}]$-dependent increase, but rather decreases in the higher $[Ca^{2+}]$ range. Upon reassociation of calsequestrin with the

[2] Differences in the F/F_0 values before and after calsequestrin reassociation (Figure 5) are statistically significant with at least 95% confidence in the higher $[Ca^{2+}]$ range (≥0.5 mM) on the basis of t distribution.

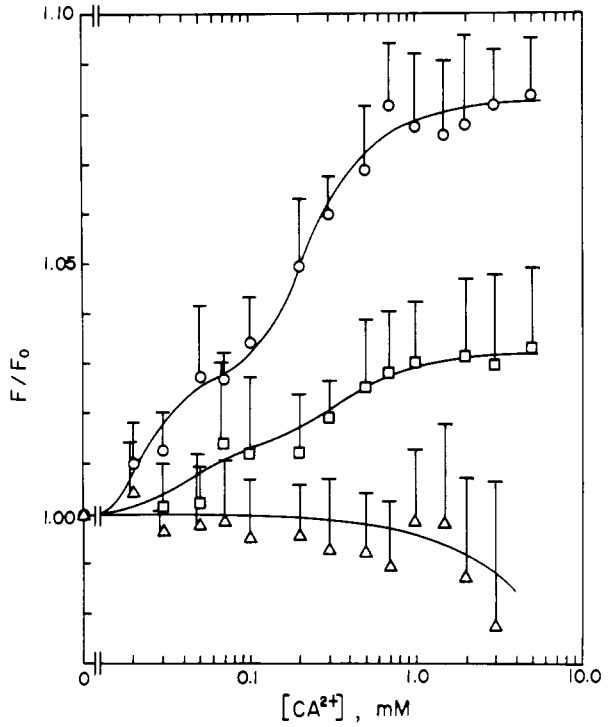

FIGURE 5: [Ca^{2+}]-dependent changes of the fluorescence intensity of DACM incorporated into the jfm moiety: the jfm–calsequestrin complex (○), the calsequestrin-depleted jfm (△), and the reconstituted jfm–calsequestrin complex (□). The three types of DACM-labeled samples were prepared as described in the legend to Figure 4. The samples (0.25–0.35 mg mL^{-1}) were suspended in 0.3 M sucrose, proteolytic inhibitors, and 20 mM MES (pH 6.8) by homogenization, and increasing amounts of Ca^{2+} were added in a sequential fashion. The intensity of DACM fluorescence (excitation at 391 nm and emission at 464 nm) was recorded in a SPEX fluorolog fluorometer as a function of the total amount of Ca^{2+} added. The changes in the fluorescence intensity were expressed as a fractional value, F/F_0, where F is the fluorescence intensity with various amounts of added Ca^{2+} and F_0 the fluorescence intensity in the absence of added Ca^{2+}. Each point represents the mean ± the population standard deviation for data obtained from seven measurements.

jfm as described in the legend to Figure 4, the [Ca^{2+}]-dependent DACM fluorescence increase was partially restored. These results suggest that dissociation of calsequestrin rather than the procedures used for calsequestrin dissociation is the major cause of the loss of the DACM fluorescence response in the calsequestrin-depleted jfm. These results further suggest that conformational states of the jfm proteins are controlled via the attached calsequestrin in a [Ca^{2+}]-dependent fashion.

Ca^{2+}-Induced Conformational Changes of Purified Calsequestrin. In order to examine the above proposals that the conformational states of the jfm proteins as well as the kinetic properties of induced Ca^{2+} release are regulated by calsequestrin, we investigated Ca^{2+}-induced conformational changes of calsequestrin by monitoring the changes in circular dichroism and tryptophan fluorescence as described previously (Ikemoto et al., 1974). As seen from the comparison of Figure 6 with Figure 5, the Ca^{2+} dependence of the fluorescence intensity of DACM attached to the jfm proteins (Figure 5) shows striking similarity to that of both the circular dichroism and tryptophan fluorescence, especially to the biphasic increase in the ellipticity at 222 nm (Figure 6). The Ca^{2+} dependence profile of the increase in the amount of Ca^{2+} released (A) shown in Figure 1 also shows a close parallelism to the Ca^{2+}-dependent conformational changes of calsequestrin (Figure 6). Furthermore, as seen from comparison of Figure 5 with Figure 1A, the biphasic changes in the rate constant of Ca^{2+} release (Figure 1A) also coincide with the biphasic

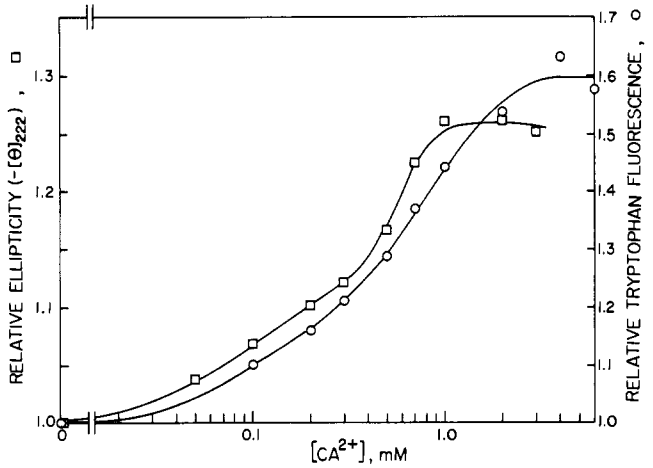

FIGURE 6: [Ca^{2+}]-dependent conformational changes of purified calsequestrin as determined by changes in circular dichroism and tryptophan fluorescence. Increasing amounts of Ca^{2+} were added in a sequential fashion to a solution containing 0.1 mg mL^{-1} calsequestrin, 0.15 M KCl, and 20 mM MES (pH 6.8), and circular dichroism and tryptophan fluorescence were determined as described under Experimental Procedures. The changes in the fluorescence intensity and in the mean residue weight ellipticity were expressed as a relative value (the determined value with various amounts of added Ca^{2+}/the value with no added Ca^{2+}). Each data point was obtained from averaging the data originating from three different measurements.

increase of both DACM fluorescence (Figure 5) and ellipticity (Figure 6). We propose that Ca^{2+}-induced conformational changes of calsequestrin affect the states of the jfm proteins, and in turn regulate Ca^{2+} release kinetics.

Discussion

It is widely accepted that the major functional role of calsequestrin is to buffer transported Ca^{2+} by serving as an intravesicular sink and thus to facilitate active transport (MacLennan & Wong, 1971; Ikemoto et al., 1974; MacLennan et al., 1983; Martonosi, 1984; Campbell, 1986). If this were the sole function of this protein, it is difficult to see why calsequestrin is localized in the limited region of the SR. As a matter of fact, calsequestrin appears to be tightly linked with the jfm as evidenced by electron microscopically discernible connections (Saito et al., 1984; Franzini-Armstrong, 1987) and by the fact that calsequestrin can bind with some protein constituents of the junctional face membrane, such as a 26-kDa protein (Mitchell et al., 1988) and the 400-kDa foot protein (Kawamoto et al., 1986). Since the 400-kDa foot protein performs some functions attributable to the Ca^{2+} release channel (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988; Smith et al., 1988), it is tempting to speculate that calsequestrin may play an active role in the Ca^{2+} release mechanism.

One of the most important aspects in the present study is the finding that under both passive and active loading conditions there is a close correlation between the Ca^{2+} release kinetics and the initial amount of Ca bound to internal binding sites (viz., the amount of Ca that has been bound at the time when Ca^{2+} release is induced). Thus, induction of Ca^{2+} release at up to ~10% saturation of internal Ca^{2+} binding sites leads to release of smaller amounts of Ca^{2+} with higher rate constants, while induction at higher levels of internal Ca^{2+} binding (up to ~70% saturation) leads to release of larger amounts of Ca^{2+} with lower rate constants.

In the $[Ca^{2+}]_i$ range where major changes in Ca^{2+} release occur (0.05–2.0 mM), almost all the internal Ca^{2+} binding is accounted for by binding to calsequestrin. Therefore, the close parallelism between the Ca^{2+} release kinetics and the amount

of the internal Ca^{2+} binding described above suggests that there is an intimate correlation between the kinetic properties of induced release and the extent of Ca^{2+} binding to calsequestrin.

As shown here, the fluorescence intensities of the conformational probe DACM attached to several proteins of the jfm including the 400-kDa foot protein and the 29–32-kDa proteins increase with an increase of $[Ca^{2+}]$. The Ca^{2+}-induced DACM fluorescence increase is abolished upon dissociation of calsequestrin from the jfm, while reassociation of calsequestrin with the jfm restores the Ca^{2+} sensitivity of DACM fluorescence. The DACM fluorescence changes do not represent conformational changes of calsequestrin per se, since calsequestrin has no fluorescence label. They do not represent a direct effect of Ca^{2+} on the jfm proteins either, since dissociation of calsequestrin reversibly blocks them. Thus, these results indicate that the changes in the jfm proteins monitored by the DACM probe are mediated by the Ca^{2+}-induced conformational changes of the attached calsequestrin. It is interesting that the calsequestrin-mediated changes in the jfm proteins as well as changes in the ellipticity of calsequestrin at 222 nm occur in two phases. These phases correlate well with the biphasic changes in the rate constant of release (k) shown in Figure 1A. Taking these results together, we propose that calsequestrin undergoes conformational changes upon Ca^{2+} binding; the information is transmitted to the channel-containing protein located in the jfm such as the 400-kDa foot protein, and in turn regulates the kinetic properties of induced Ca^{2+} release.

The protein components of the jfm of M_r 29K–32K as well as the 400-kDa protein were labeled rather intensely with DACM, suggesting that these proteins may also be involved in the observed fluorescence changes. In fact, our previous studies have shown that the fluorescence intensity of DACM incorporated into SR, whose primary incorporation site detected in the previous studies is the 32-kDa protein, shows rapid decrease in parallel to Ca^{2+} release from the SR vesicles (Morii et al., 1986). During the course of the present DACM incorporation studies, however, we have noticed that the content of the ~30-kDa polypeptides (doublets or sometimes triplets) in the jfm varied depending on the preparation, often in an inversely proportionate fashion to the amount of the 400-kDa polypeptide. Thus, it may well be that the 29–32-kDa DACM-labeled proteins represent proteolytic degradation products of the 400-kDa foot protein. If this is the case, the Ca^{2+}-dependent DACM fluorescence change shown in the present study primarily represents the events occurring in the 400-kDa channel-containing protein. However, according to a recent report (Mitchell et al., 1988), cardiac calsequestrin interacts with the jfm via a 26-kDa protein component in a $[Ca^{2+}]$-dependent fashion. Futhermore, earlier studies (Kawamoto et al., 1986) suggested that the 400-kDa foot protein binds not only with calsequestrin but also with several SR proteins. Thus, it might be possible that two or more proteins of the jfm are involved in the structural and functional interactions between the jfm and calsequestrin. It remains to be investigated whether the channel functions are regulated by direct interactions of calsequestrin with the 400-kDa protein or mediated indirectly via the other proteins.

In conclusion, the present results suggest that Ca^{2+}-dependent conformational changes of calsequestrin are transmitted to the jfm proteins including the 400-kDa channel-containing protein and other small molecular weight proteins regulating the Ca^{2+} release function. In further support of the concept that calsequestrin plays a critical role in the Ca^{2+} release function, we have recently found that Ca^{2+} release is abolished by selective depletion of calsequestrin but restored upon its recombination with the membrane (Ronjat & Ikemoto, 1989).

ACKNOWLEDGMENTS

We thank Drs. John Gergely and Terrence L. Scott for their comments on the manuscript. Adel Tarcsafalvi provided excellent technical assistance.

Registry No. Ca, 7440-70-2.

REFERENCES

Aaron, F.-M. B., Oikawa, K., Reithmeier, R. A. F., & Sykes, B. D. (1984) *J. Biol. Chem. 259*, 11876–11881.

Campbell, K. P. (1986) in *Sarcoplasmic Reticulum in Muscle Physiology* (Entman, M. L., & Van Winkle, W. B., Eds.) Vol. I, pp 65–99, CRC Press, Boca Raton, FL.

Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A., & Fleischer, S. (1986) *J. Cell Biol. 103*, 741–753.

Cozens, B., & Reithmeier, R. A. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1167–1171.

Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A. F., & MacLennan, D. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1167–1171.

Franzini-Armstrong, C., Kenney, L. J., & Varriano-Marston, E. (1987) *J. Cell Biol. 105*, 49–56.

Hidalgo, C., & Ikemoto, N. (1977) *J. Biol. Chem. 252*, 8446–8454.

Hymel, L., Maurer, A., Berenski, C., Yung, C. Y., & Fleischer, S. (1984) *J. Biol. Chem. 259*, 4890–4895.

Hymel, L., Inui, M., Fleischer, S., & Schindler, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 441–445.

Ikemoto, N., Bhatnagar, G. M., & Gergely, J. (1971) *Biochem. Biophys. Res. Commun. 44*, 1510–1517.

Ikemoto, N., Bhatnagar, G. M., Nagy, B., & Gergely, J. (1972) *J. Biol. Chem. 247*, 7835–7837.

Ikemoto, N., Nagy, B., Bhatnagar, G. M., & Gergely, J. (1974) *J. Biol. Chem. 249*, 2357–2365.

Ikemoto, N., Antoniu, B., & Kim, D. H. (1984) *J. Biol. Chem. 259*, 13151–13158.

Imagawa, T., Smith, J. S., Coronado, R., & Campbell, K. P. (1987) *J. Biol. Chem. 262*, 16636–16643.

Kasai, M. (1980) *J. Biochem. 88*, 1081–1085.

Kawamoto, R. M., Bunschwig, J.-P., Kim, K. C., & Caswell, A. H. (1986) *J. Cell Biol. 103*, 1405–1415.

Kim, D. H., Ohnishi, S. T., & Ikemoto, N. (1983) *J. Biol. Chem. 258*, 9662–9668.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q.-Y., & Meissner, G. (1988) *Nature 28*, 315–319.

MacLennan, D. H., & Wong, P. T. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1231–1235.

MacLennan, D. H., Campbell, K. P., & Reithmeier, R. A. F. (1983) in *Calcium and Cell Function* (Cheung, W., Ed.) Vol. 4, pp 151–173, Academic Press, New York.

Martonosi, A. N. (1984) *Physiol. Rev. 64*, 1240–1320.

Meissner, G. (1975) *Biochim. Biophys. Acta 389*, 51–68.

Meissner, G., Conner, G. E., & Fleischer, S. (1973) *Biochim. Biophys. Acta 298*, 246–269.

Michalak, M., Campbell, K. P., & MacLennan, D. H. (1980) *J. Biol. Chem. 255*, 1317–1326.

Mitchell, R. D., Simmerman, H. K. B., & Jones, L. R. (1988) *J. Biol. Chem. 263*, 1376–1381.

Morii, M., Danko, S., Kim, D. H., & Ikemoto, N. (1986) *J. Biol. Chem. 261*, 2343–2348.

Ohnishi, M., & Reithmeier, R. A. F. (1987) *Biochemistry 26*, 7458–7465.

Ostwald, R. J., MacLennan, D. H., & Dorrington, K. J. (1974) *J. Biol. Chem. 249*, 5867–5871.
Ronjat, M., & Ikemoto, N. (1989) *Biophys. J. 55*, 13a.
Saito, A., Seiler, S., Chu, A., & Fleischer, S. (1984) *J. Cell Biol. 99*, 875–885.
Scott, B. T., Simmerman, H. K. B., Collins, J. H., Nadal-Ginard, B., & Jones, L. R. (1988) *J. Biol. Chem. 263*, 8958–8964.
Slupsky, J. R., Ohnishi, M., Carpenter, M. R., & Reithmeier, R. A. F. (1987) *Biochemistry 26*, 6539–6544.
Smith, J. S., Imagawa, T., Ma, J., Fill, M., Campbell, K. P., & Coronado, R. (1988) *J. Gen. Physiol. 92*, 1–26.
Volpe, P., Cutweniger, H. E., & Montecucco, C. (1987) *Arch. Biochem. Biophys. 253*, 138–145.
Williams, R. W., & Beeler, T. J. (1986) *J. Biol. Chem. 261*, 12408–12413.

Competitive Binding of ATP and the Fluorescent Substrate Analogue 2′,3′-*O*-(2,4,6-Trinitrophenylcyclohexadienylidine)adenosine 5′-Triphosphate to the Gastric H^+,K^+-ATPase: Evidence for Two Classes of Nucleotide Sites†

Larry D. Faller

Center for Ulcer Research and Education, Department of Medicine, University of California at Los Angeles School of Medicine and Veterans Administration Hospital Center, Los Angeles, California 90073

Received September 13, 1988; Revised Manuscript Received January 13, 1989

ABSTRACT: ATP and the fluorescent substrate analogue TNP-ATP bind competitively to the gastric H,K-ATPase. Substrate and product completely reverse the fluorescence enhancement caused by TNP-ATP binding to the enzyme. The fluorophore is displaced monophasically from apoenzyme. However, ATP displaces TNP-ATP from the Mg^{2+}-quenched state in two steps of equal amplitude. The midpoints of the titrations differ by more than 2 orders of magnitude. The estimated substrate constants are in reasonable agreement with published Michaelis constants. TNP-ATP is not a substrate for the H,K-ATPase. The fluorophore prevents phosphorylation by ATP and competitively inhibits the K^+-stimulated pNPPase and ATPase activities of the enzyme. K_i is approximately the same for both hydrolytic activities and consistent with the K_d of TNP-ATP measured directly. K_m for pNPP is 1.48 ± 0.15 mM. Two Michaelis constants are required to fit the ATPase data: K_{m1} = 0.10 ± 0.01 mM and K_{m2} = 0.26 ± 0.05 mM.

The gastric proton pump is classified as an E_1E_2-type ATPase. The distinguishing characteristic of this class of active transport enzyme is the formation of a covalent phosphoenzyme intermediate. Another common feature of the calcium (Inesi et al., 1967; Yamamato & Tonomura, 1967), sodium (Kanazawa et al., 1970), and proton (Wallmark et al., 1980) pumps is increased catalytic efficiency at higher ATP concentrations. Early attempts to explain the latter phenomenon postulated two nucleotide sites, usually on interacting protomers of a dimeric structure. More recently it has been shown that the E_1 and E_2 conformers, which were originally proposed to explain catalysis–transport coupling and the physical translocation of ions, can also explain the substrate dependence of the hydrolysis reaction if the affinity of the enzyme for ATP is different in the E_1 and E_2 conformations (Smith et al., 1980). A critical issue in deciding between two-site mechanisms and one-site, two-state models is the number of nucleotide sites.

One approach to this problem is the use of fluorescent substrate analogues to count the number of nucleotide sites. TNP-ATP[1] is a fluorescent analogue of ATP that has been used extensively to investigate both the sodium (Moczydlowski & Fortes, 1981a,b) and calcium (Dupont et al., 1982, 1985; Watanabe & Inesi, 1982; Dupont & Pougeois, 1983; Nakamoto & Inesi, 1984; Bishop et al., 1984, 1987; Berman, 1986) pumps. Sartor showed the feasibility of using TNP-ATP to probe nucleotide sites on the H,K-ATPase also and reported preliminary evidence for only one class of TNP-ATP site in the absence of divalent cations, but both biphasic TNP-ATP binding and displacement by nucleotides in the presence of Mg^{2+} (Sartor et al., 1982). A reinvestigation of TNP-ATP binding to the H,K-ATPase has shown that Mg^{2+} causes a slow change in the conformation of the substrate analogue–enzyme complex without affecting the fluorophore dissociation constant significantly, or the number of binding sites (Faller, 1989). The number of TNP-ATP sites (N) was counted and found to be twice the stoichiometry of phosphoenzyme formation (E–P), suggesting that there may be two classes of nucleotide sites. The competition experiments reported in this paper provide additional support for a two-site interpretation of the N/E–P ratio.

†This work was supported by National Science Foundation Grant DMB 83-09756 and U.S. Public Health Service Grants DK 36873 and section C2 of program project AM 17328 (George Sachs overall principal investigator).

[1] Abbreviations: H,K-ATPase, Mg^+-dependent, H^+-transporting, and K^+-stimulated ATPase (EC 3.6.1.3); Na,K-ATPase, Mg^{2+}-dependent and Na^+- and K^+-stimulated ATPase; Ca-ATPase, Ca^{2+}- and Mg^{2+}-dependent ATPase; SR, sarcoplasmic reticulum; TNP-AXP, 2′,3′-*O*-(2,4,6-trinitrophenylcyclohexadienylidine)ATP or -ADP; AMP-PNP, adenyl-5′-yl imidodiphosphate; pNPP, *p*-nitrophenyl phosphate; $H_2VO_4^-$, vanadate ion; FITC, fluorescein 5′-isothiocyanate; eosin Y, 2′,4′,5′,7′-tetrabromofluorescein; omeprazole, 5-methoxy-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl}-1*H*-benzimidazole.

0006-2960/89/0428-6771$01.50/0