Mechanism of Allosteric Regulation of the Ca,Mg-ATPase of Sarcoplasmic Reticulum: Studies with 5′-Adenylyl Methylenediphosphate†

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ABSTRACT: Four mechanisms for the allosteric regulation of the calcium and magnesium ion activated adenosinetriphosphatase (Ca,Mg-ATPase) of sarcoplasmic reticulum were examined. Negative cooperativity in substrate binding was not supported by ^{3}H-labeled 5′-adenylyl methylenediphosphate (AMPPCP) binding, which was best fit by a single class of sites. Although calcium had no effect on the absence of cooperativity, it did increase the affinity of the enzyme for AMPPCP. Allosteric regulation via an effector site for AMPPCP or ATP on the same ATPase chain was eliminated by the stoichiometry of ATP and AMPPCP binding, 1 mol of site per mole of enzyme. The possibility that AMPPCP acts at an effector site was eliminated by showing that it competitively inhibits the rate of phosphoenzyme formation. Allosteric regulation of kinetics via site–site interaction in an oligomer was eliminated by showing that the inhibition of ATPase activity by fluorescein isothiocyanate is linearly dependent upon its incorporation into the sarcoplasmic reticulum. The fourth mechanism considered was stimulation of ATPase activity by the binding of ATP or AMPPCP at the active site after departure of ADP but before the departure of inorganic phosphate. This hypothesis was supported by site stoichiometry and by the observation that AMPPCP or ATP stimulates v/EP, the rate of ATP hydrolysis for a given level of phosphoenzyme. Computer simulation of this branched monomeric model could duplicate all experimental observations made with AMPPCP and ATP as allosteric regulators. The condition that the affinity of ATP binding to the enzyme be reduced when it is phosphorylated, which is required by the computer model, was confirmed experimentally.

Double-reciprocal plots of the dependence of the ATPase activity of SR[1] on ATP concentration, at low (0.75–50 μM) ATP concentrations, are nonlinear (Yamamoto & Tonomura, 1967; Kanazawa et al., 1971; Panet et al., 1971; Vianna, 1975; Yates & Duance, 1976; Neet & Green, 1977; Møller et al., 1980). The downward deflection observed in these plots is typical of negative cooperativity (Levitsky, 1978) and has been attributed to site–site interactions by Møller et al. (1980). The step or steps in the hydrolytic mechanism that are modified by active-site interactions have not, however, been identified. Levitsky & Koshland (1976) have noted that "in all cases studied the negative cooperativity observed in kinetic activity is paralleled by negative cooperativity in substrate binding".

Negative cooperativity in substrate binding has not, however, been observed. Meissner (1973) and Dupont (1977) obtained linear Scatchard plots of ATP binding to SR when calcium was omitted. Calcium could not be included during the binding studies because formation of EP depresses ATP binding (Meissner, 1973), and ATP is hydrolyzed during the binding assay. TNP-ATP, a hydrolyzable analogue of ATP, has also been used to follow substrate binding. In the absence of calcium, cooperativity is not seen in TNP-ATP binding (Dupont et al., 1982). In the presence of calcium, Scatchard plots of TNP-ATP binding are concave upward, indicating negative cooperativity (Watanabe & Inesi, 1982). Whether this is due to the presence of calcium or the formation of phosphoenzyme cannot be decided. Pang & Briggs (1977) found that Scatchard plots of the binding of the nonmetabolizable analogue of ATP, AMPPCP, to SR in the absence of calcium indicated that the binding was, like the binding of ATP and TNP-ATP, linear.

Since micromolar concentrations of calcium produce effects on the conformation of the Ca,Mg-ATPase (Dupont, 1977; Dupont & Leigh, 1978; Inesi et al., 1980; Ikemoto et al., 1978; Nakamura et al., 1979), it is conceivable that negative cooperativity in ATP binding will only be observed in the presence of calcium. In the report that follows we have used AMPPCP to determine if calcium induces negative cooperativity in the binding of this analogue to SR. The advantages of a nonmetabolizable analogue are 2-fold: the analogue will not phosphorylate the enzyme, and it will not be destroyed during the experiment. Before proceeding to the binding studies, we present evidence that AMPPCP binds to the active site.

In the course of these studies four mechanisms for the allosteric regulation of ATPase activity by ATP were considered: negative cooperativity in substrate binding, kinetic effects due to substrate binding at an effector site on the same ATPase molecule, kinetic effects due to the interaction of active sites in an oligomeric complex, and kinetic effects due to the binding of ATP to the active site after departure of ADP but before departure of inorganic phosphate.

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[1] Abbreviations: AMPPCP, 5′-adenylyl methylenediphosphate; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); ATPase, adenosinetriphosphatase; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EP, phosphorylated enzyme; FITC, fluorescein isothiocyanate; HPLC, high-pressure liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P_i, inorganic phosphate; PCA, perchloric acid; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; TCA, trichloroacetic acid; TLC, thin-layer chromatography; TNP-ATP, trinitrophenyl-ATP; Tris, tris(hydroxymethyl)aminomethane; V_i, initial rate of EP formation; v/EP, ATPase rate divided by phosphoenzyme concentration.

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Materials and Methods

Membrane Preparations. SR was prepared from rabbit back and hind legs essentially as described by Meissner (1975) for light SR. The vesicular material that sedimented out of a 31.5% sucrose, 1 M KCl, and 20 mM MOPS, pH 7.0, solution at 27000*g* in 1 h was discarded, and the material sedimented out of a 1:1 dilution of this solution with 20 mM MOPS in 1 h at 50000*g* was collected and stored at −70 °C in 40% sucrose and 20 mM MOPS. Seventy-two percent of this protein was judged to be Ca,Mg-ATPase by HPLC (Barrabin et al., 1984). These preparations transported Ca at 12–20 μmol mg^{-1} min^{-1} from a solution of the following composition: 100 mM KCl, 20 mM MOPS, 5 mM $MgCl_2$, 5 mM ATP, 5 mM oxalate, and 0.2 mM $CaCl_2$, pH 7.0, at 37 °C.

Kinetic Studies. (*A*) *ATPase.* SR (1–4 μg) was incubated in 0.20 mL of a solution containing 100 mM KCl, 20 mM MOPS, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 200 nmol of A23187 (mg of protein)$^{-1}$, with the designated concentrations of [γ-^{32}P]ATP and AMPPCP for 1–5 min at 10 °C. ATPase activity was quenched with 0.6 M perchloric acid, and the liberated phosphate was extracted as the molybdate complex into butyl acetate (Wahler & Wollenberger, 1958). The ATPase rate was calculated from the linear least-squares fit of four data points.

(*B*) V_f. Initial rates of EP formation were measured with a Durrum Multimix-133 at 10 °C. Syringe A contained 100 mM NaCl, 20 mM MOPS, pH 7.0, 2 mM $MgCl_2$, 0.10 mM $CaCl_2$, 100 nmol of A23187 (mg of protein)$^{-1}$, and 0.5 mg of protein mL^{-1}. Syringe B contained the same salt solution except that A23187 and protein were replaced by various concentrations of [γ-^{32}P]ATP and AMPPCP. The instrument was operated at 90 lb in.$^{-2}$. The reaction was quenched, syringe C, with 1.2 M PCA. EP concentration was measured as described below. The values of V_f were determined from the linear least-squares fit of 12 observations between 15 and 60 ms (0.5, 1, 2, 3 μM ATP) or of 8 observations between 15 and 30 ms (6 and 9 μM ATP).

(*C*) v/EP. The steady-state ATPase rate divided by the level of phosphoenzyme was determined under the conditions for the measurement of ATPase activity. For the AMPPCP studies the ATP concentration was 100 μM and the protein concentration was 0.02 mg mL^{-1}. Aliquots of the reaction mixture were quenched with 10% TCA at 0.15, 0.30, 0.45, and 0.60 min, and P_i was determined by spotting 1-μL aliquots on TLC plates as described below.

(*D*) *EP.* Phosphoenzyme was isolated by two methods. For the determination of V_f, the initial rate of formation of EP, the PCA-quenched reaction mixture was collected by low-speed centrifugation and resuspended in cold 5% TCA containing 20 mM KH_2PO_4. The precipitated sarcoplasmic reticulum was collected on a glass filter, washed with 20 mL of resuspension solution, and counted in a liquid scintillation spectrometer. The reported EP values are the experimentally obtained values minus the background values obtained by quenching the reaction mixture before the addition of [γ-^{32}P]ATP. These background values were less than 0.01 nmol (mg of protein)$^{-1}$.

The method used to measure EP in the determination of the effect of EP on ATP binding was as follows. Seven milliliters of a solution containing 0.56 mg of SR protein was incubated at 0 °C. A 4-mL fraction was taken to measure ATP binding. After 0.3 min, the time required to filter the 4-mL fraction, 2 mL of the incubation mixture was mixed with 2 mL of ice-cold 20% TCA and 20 mM P_i solution and filtered through a glass filter and two Millipore filters (HAWP, 0.45 μm). The filters were washed twice with 5 and 3 mL of 10% TCA and 10 mM P_i. It was assumed that all of the protein was collected on the glass filter plus the first Millipore filter. The amount of nonspecific binding was given by the second Millipore filter. Comparison of this method with conventional centrifugation methods gave excellent results.

FITC Incorporation. SR was treated with FITC essentially as described by Mitchinson et al. (1982) at 1 mg of SR mL^{-1} in 100 mM NaCl, 40 mM Tris, pH 8.0, and 0–10 μM FITC. The solutions were incubated at room temperature for 10 min, and the reaction was stopped by the addition of 10 mM dithiothreitol. The unbound FITC was separated on a Sephadex G-50 column (0.5 × 15 cm). SR was then assayed immediately for ATPase activity by the coupled enzyme method of Warren et al. (1974) at 5 mM ATP. The amount of FITC bound was determined by measuring the absorbance at 494 nm in 1% SDS and 0.1 N NaOH; an extinction coefficient of 80000 M^{-1} was used.

Nucleotide Binding. (*A*) [^{3}*H*]*AMPPCP Binding.* [^{3}H]-AMPPCP was custom prepared for us and purified as described below. Binding was measured essentially as described by Dupont and co-workers (Dupont, 1977; Dupont et al., 1982). Sarcoplasmic reticulum protein (200 μg) in 200 μL of 100 mM KCl, 20 mM MOPS, pH 7.0, 4 μM A23187, 0.1 mM $CaCl_2$ or 5 mM EGTA, and the designated concentrations of AMPPCP was incubated at 10 °C for 30 s and then rapidly separated from the incubation solution by filtration through Millipore filters (HAWP, 0.45 μm). The concentration of AMPPCP was determined by using a molar absorption coefficient of 15400 M^{-1} cm^{-1} at 259 nm. The values of n, the concentration of sites, and k, the formation constant for that site, were initially estimated from Scatchard plots (Scatchard, 1949) and then refined by the program LIGAND (Munson & Rodbard, 1980).

(*B*) *ATP Binding.* ATP binding was measured as described by Dupont et al. (1982) by using [γ-^{32}P]ATP and [α-^{32}P]ATP as substrates at 0 °C. [α-^{32}P]ATP and 20 mM EGTA were used to obtain binding in the absence of EP. [α-^{32}P]ATP was used to determine the concentration of ATP in the bath and ATP bound to the phosphorylated enzyme. [γ-^{32}P]ATP and 0.1 mM $CaCl_2$ were used to determine the level of phosphorylation of the enzyme and the amount of ATP bound to the phosphorylated enzyme. A portion (see below) of this mixture (4 mL) was filtered (Dupont et al., 1982), and the ^{32}P on the filter gave bound ATP plus EP. To obtain EP, a second, 2-mL, portion of the mixture was quenched with TCA, and EP was determined as described above. The fraction of ATP hydrolyzed during filtration was determined by TLC as described below. Although all experiments were carried out at 0 °C, hydrolysis of ATP was detected when Ca was added. At initial concentrations of 10, 30, and 90 μM, the fraction hydrolyzed was 0.28, 0.085, and 0.055, respectively. Since the composition of the bound nucleotide might not be the same as the bath composition, the fraction of ATP and ADP in the bound nucleotide was determined by TLC chromatography (see below) in those baths where hydrolysis was expected. Bound [α-^{32}P]ATP was distinguished from bound [α-^{32}P]ADP by addition of EDTA (20 mM final concentration) after 0.3 min of incubation, by filtering the incubation medium, and then by washing the filter with 1.0 mL of a solution of identical composition as the bath. Ninety percent of the ^{32}P on the filter was removed by this step.

Miscellaneous Methods. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin

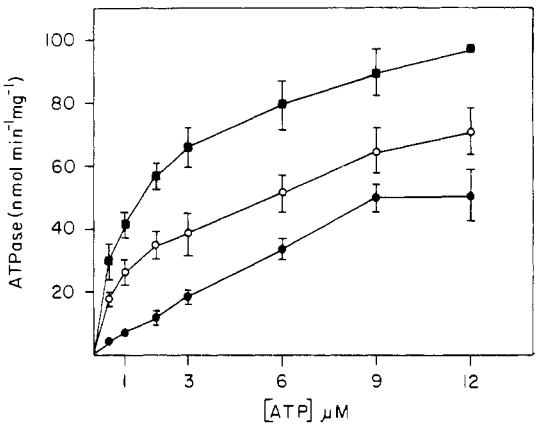

FIGURE 1: Effect of AMPPCP on ATPase rate. The initial velocity of phosphate liberation was measured at the indicated ATP concentrations in a solution of 100 mM KCl, 20 mM MOPS, pH 7.0, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 200 nmol of A23187 (mg of protein)$^{-1}$, and 0.010–0.020 mg of SR mL^{-1} at 10 °C in the presence of 0 (○), 0.1 (■), and 2.4 mM (●) AMPPCP. The progress of the reaction was followed by extraction of the [^{32}P]P_i liberated from [γ-^{32}P]ATP as the molybdate complex into butyl acetate as described under Materials and Methods. The data points represent the mean ± SE for 6–12 determinations.

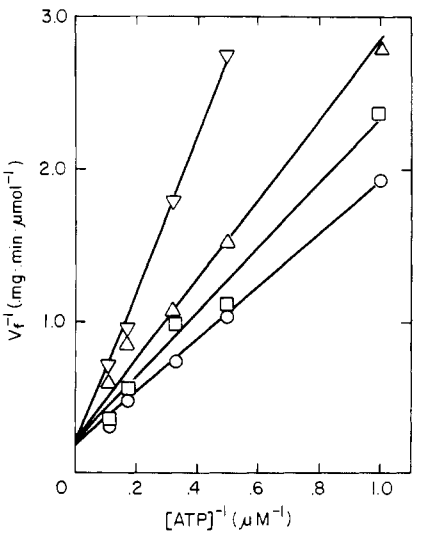

FIGURE 2: Effect of AMPPCP on V_f. The initial velocity of EP formation (V_f) was measured with a Durrum Multimix-133 at the indicated concentrations of ATP. The conditions were the same as in Figure 1 except that the protein concentration was 0.25 mg mL^{-1} and AMPPCP concentrations were 0 (○), 25 (□), 50 (△), and 100 μM (▽). The PCA-precipitated SR was collected on a glass filter, and V_f was estimated from the dependence of EP on time between 15 and 60 ms as described under Materials and Methods.

as standard. [^{3}H]AMPPCP was custom tritiated by Amersham-Searle using AMPPCP obtained from Sigma Chemical Co. We isolated [^{3}H]AMPPCP from the crude mixture by chromatography on DEAE-Sephacel columns with a linear gradient (0.025–1.0 M) of triethylammonium carbonate, pH 7.4. The final product had the same ultraviolet absorption spectrum as authentic AMPPCP, and more than 99% of the ^{3}H compound comigrated with AMPPCP on thin-layer chromatograms developed in two solvent systems. [γ-^{32}P]ATP was separated from ^{32}P and [α-^{32}P]ATP was separated from [α-^{32}P]ADP by chromatography on TLC plates. Aliquots (1 μL) were spotted on TLC plates (PEI cellulose F, Brinkman) with carriers ATP, ADP, and AMP, developed with 2 N formic acid and 0.5 M LiCl, and dried. The areas corresponding to ATP, ADP, or P_i were cut out and counted by liquid scintillation. [γ-^{32}P]ATP was obtained from New England Nuclear Corp. The calcium ionophore A23187 was obtained from Calbiochem. All other chemicals were reagent grade.

RESULTS

Site of Action of AMPPCP. It was essential for the binding studies that follow to determine if, at the concentrations of AMPPCP studied, AMPPCP binds exclusively to the active site. The observation by Pang & Briggs (1977) that ATP competitively displaces AMPPCP from sarcoplasmic reticulum supports but does not prove this site as the site of action of AMPPCP. The observation that AMPPCP can increase the rate of ATP hydrolysis (Yates & Duance, 1976; Taylor & Hattan, 1979) does not appear to support the exclusive binding of AMPPCP to the active site. These observations required resolution.

The data presented in Figure 1 show that 100 μM AMPPCP stimulates and 2.4 mM AMPPCP inhibits ATPase activity. This inhibitory effect of 2.4 mM AMPPCP does not directly contradict the study of Taylor & Hattan (1979), as reported in their Figure 5, because they did not test the effect of AMPPCP at ATP concentrations as low as those shown in Figure 1. Low concentrations of ATP were used in this study because negative cooperativity in ATPase activity is clearly demonstrated at these concentrations. The first step in the analysis of the mechanism of action of AMPPCP was to determine if it inhibits the rate of EP formation as required if it competes with ATP for the active site. The data plotted in double-reciprocal form in Figure 2 show that 25, 50, and 100 μM AMPPCP inhibit the rate of EP formation. These data appear linear (Figure 2), intersect at a common point on the ordinate, and indicate that over a limited range of ATP concentrations AMPPCP competitively depresses V_f. This conclusion is in agreement with that of Shigekawa & Kanazawa (1982), who have also reported that AMPPCP competes with ATP for the active site.

At this point we need to explain how AMPPCP can simultaneously inhibit the rate of EP formation (Figure 2) and stimulate the overall rate of ATP hydrolysis (Figure 1). The explanation that we propose depends upon two facts: (1) the rate of formation of phosphoenzyme exceeds the rate of phosphoenzyme hydrolysis (Briggs et al., 1978; Kanazawa et al., 1971; Froehlich & Taylor, 1975), and (2) AMPPCP can accelerate the rate of phosphoenzyme hydrolysis (Shigekawa et al., 1978). The hypothesis we propose is that AMPPCP at moderate concentrations (e.g., 25–100 μM) stimulates the overall rate of ATP hydrolysis by binding to the active site of the phosphoenzyme after ADP departure but before EP hydrolysis (McIntosh & Boyer, 1983). The consequence of this binding is the stimulation of EP hydrolysis. A moderate reduction in the rate of EP formation, as produced by competition of AMPPCP for the active site, would not significantly reduce EP levels since the rate of EP formation is greater than the rate of EP hydrolysis. Moderate stimulation of EP hydrolysis by AMPPCP, for the same reason, would not greatly lower EP but would increase ATPase activity.

Two predictions of the hypothesis given above are that the EP level will not be greatly depressed by low concentrations of AMPPCP and that v/EP, a measure of the rate of EP hydrolysis, will be increased. The data presented in Figure 3 sustain these predictions. The level of EP declines with the increase in AMPPCP concentration, and the velocity of ATP hydrolysis increases so that the ratio of v/EP is increased. The data plotted in Figure 4 show that ATP, like AMPPCP, is capable of increasing v/EP and that the pattern of increase is like that of AMPPCP. Thus, the mechanism of stimulation

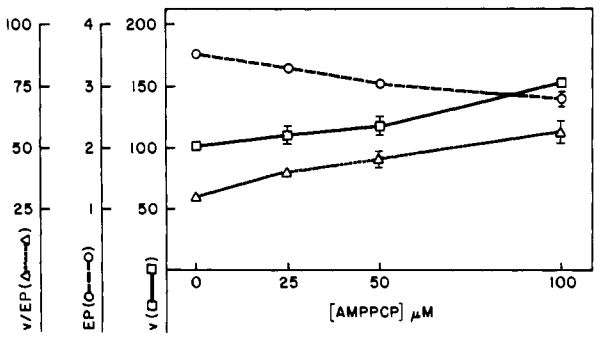

FIGURE 3: Effect of AMPPCP on v/EP. The initial rate of $[^{32}P]P_i$ formation and the level of EP were measured in the same reaction bath under the conditions described in Figure 1 at 100 μM [γ-^{32}P]ATP and 0.020 mg of protein mL^{-1}. Aliquots of the reaction mixture were quenched with 10% TCA, and 2 μL of the quenched sample was spotted on TLC plates for the determination of the rate of $[^{32}P]P_i$ liberation as described under Materials and Methods. The phosphoenzyme was recovered from the remainder of the aliquot on glass filters as described under Materials and Methods. The data points are the mean ± SE for four to eight determinations.

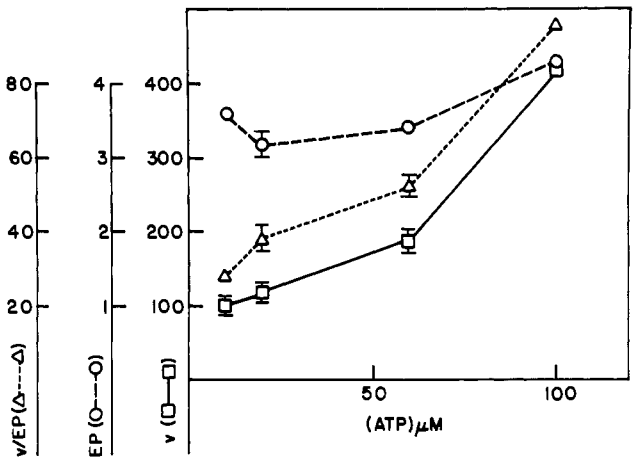

FIGURE 4: Effect of ATP on v/EP. The initial rate of $[^{32}P]P_i$ formation and the level of EP were measured as described in Figure 3 at the indicated concentrations of [γ-^{32}P]ATP. The data points represent the mean ± SE for three to eight determinations.

of ATPase activity by AMPPCP and ATP appears to be the same and to not involve an effector site.

Negative Cooperativity of Substrate Binding. The data that have been presented support the assertion that AMPPCP competes with ATP for the active site. We are ready to determine if the apparent cooperativity observed in the kinetics of ATP hydrolysis arises from negative cooperativity in the binding of ATP to the enzyme. AMPPCP binding to sarcoplasmic reticulum was studied in the presence of 0.1 mM calcium and in the absence of calcium (5 mM EGTA). The data obtained from these studies are shown in panels A and B, respectively, of Figure 5. The AMPPCP binding data that are plotted in Figure 5A were fit with the curve shown in Figure 5A by assuming 7.0 nmol of a single class of sites (n) (mg of protein)$^{-1}$ with a dissociation constant k_D of 44 μM. The program LIGAND (Munson & Rodbard, 1980) gave a value of 40.3 ± 3.3 μM for the dissociation constant and a value of 6.92 ± 0.2 nmol mg^{-1} for the maximum number of sites. With LIGAND, attempts were made to fit the data to two classes of binding sites having similar binding constants. None of these attempts produced acceptable fits to the data. Two additional tests for cooperativity (Levitsky, 1978) were applied to the data. A double-reciprocal plot of bound AMPPCP vs. free AMPPCP gave 7.03 nmol mg^{-1} as the maximum number of sites and a dissociation constant of 47 μM with a correlation

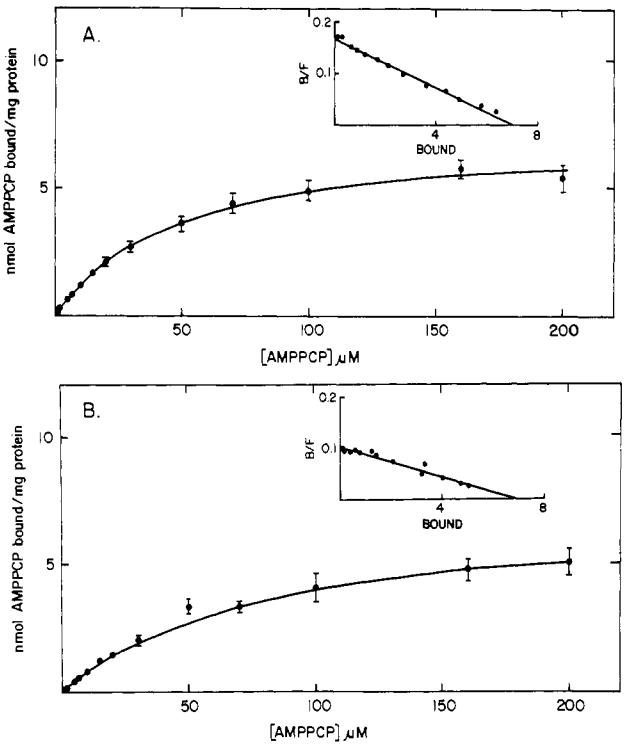

FIGURE 5: Binding of [^{3}H]AMPPCP to SR. The amount of [^{3}H]-AMPPCP bound was determined under the same conditions as for Figure 1 at 1.0 mg of protein mL^{-1} in the presence of 0.1 mM $CaCl_2$ (A) or 5 mM EGTA (B). Bound [^{3}H]AMPPCP was separated from unbound by filtration, and the fraction bound was determined from the amount of radioactivity remaining on the filter as described under Materials and Methods. The values of n and K were estimated as described under Materials and Methods. The data points are the mean ± SE for 6–10 duplicate determinations.

coefficient of 0.999. A Hill plot of this data gave a Hill coefficient of 1.0 and a correlation coefficient of 0.998. All of these tests show that, in the presence of calcium, AMPPCP binds to the Ca,Mg-ATPase in a simple noncooperative fashion. The data plotted in Figure 5B show the binding of AMPPCP to the Ca,Mg-ATPase in the absence of calcium. The curve shown in Figure 5B and the Scatchard plot shown in the insert to Figure 5B were fit by using a dissociation constant of 77 μM and 7 nmol mg^{-1} for the maximum number of sites. The fitting routine of LIGAND gave values of 69.66 ± 5.7 μM and 7.1 ± 0.4 nmol mg^{-1} ($P > 0.05$) for the dissociation constant and the maximum number of sites, respectively, in the presence of 1 mM EGTA. A double-reciprocal plot of the data gave a dissociation constant of 96 μM and 8.06 nmol mg^{-1} for the maximum number of sites with a correlation coefficient of 0.999. A Hill plot of the data gave a Hill coefficient of 1.02 and a correlation coefficient of 0.999. Calcium did not induce cooperative binding. It did increase the affinity of the Ca,Mg-ATPase for AMPPCP. The negative cooperativity observed in the kinetics of ATP hydrolysis thus cannot be explained by negative cooperativity in the binding of the substrate ATP.

The Oligomeric Basis for Negative Cooperativity. Negative cooperativity could arise from active-site interaction in an oligomer, the effect being to increase the rate of some kinetic step(s) beyond substrate binding. To test this hypothesis, the active sites were titrated with FITC and the effect thereof on ATPase activity was determined. As the fraction of active sites reacted with FITC increases, the chance of site–site interaction decreases and thus the chance of allosteric regulation by this mechanism is decreased (Mitchinson et al., 1982; Anderson et al., 1982). The data plotted in Figure 6 do not indicate

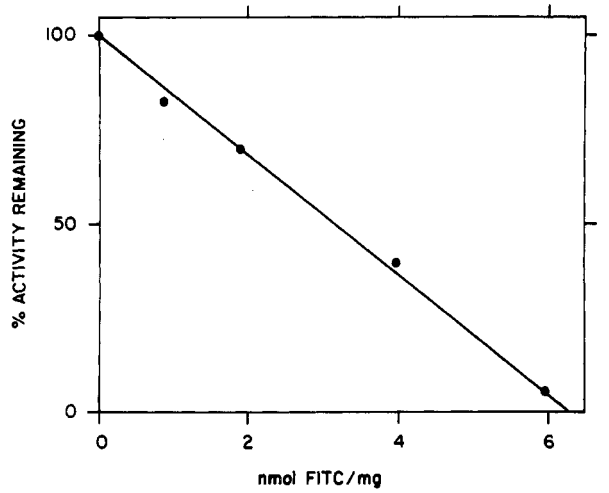

FIGURE 6: Effect of FITC on Ca-dependent ATPase activity. ATPase activity was measured under the conditions listed in Figure 1 by using the coupled enzyme system of Warren et al. (1974). FITC was incorporated as described by Mitchinson et al. (1982).

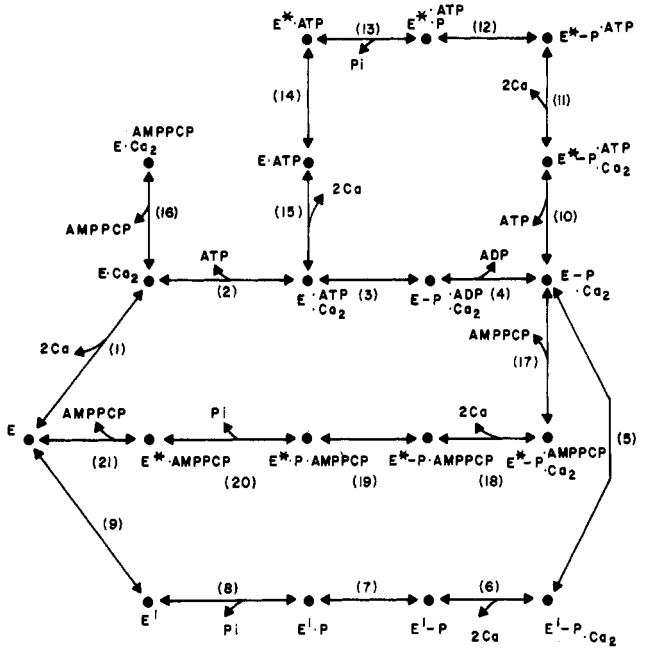

FIGURE 7: Branched Ca,Mg-ATPase cycle. In this reaction scheme AMPPCP or ATP binds to EP after ADP departs and stimulates the rate at which the process EP ↔ E + P occurs.

departure of ATPase activity from a linear dependence on the fraction of sites not reacted with FITC and therefore do not support the hypothesis that allosteric regulation arises from site–site interactions.

Does Allosteric Regulation Involve an Effector Site? ATP and AMPPCP could bind to a site in addition to the catalytic site and stimulate EP hydrolysis. The number of sites titrated by FITC, 6.2 nmol mg^{-1}, closely approximates the number of AMPPCP sites (Figure 5). These SR preparations were found to be 72% ATPase by HPLC, and the molecular weight of the enzyme is 115000. Therefore, 6–7 nmol of ATP sites (mg of protein)$^{-1}$ is 1 mol of sites per mole of ATPase. These results exclude the existence of a high-affinity ATP or AMPPCP binding site in addition to the catalytic site.

The Branched Pathway. ATP and/or AMPPCP could activate ATPase activity by binding to the active site after ADP has departed but before P_i has departed. This reaction mechanism is shown in Figure 7 and is the one we used, in a general way, to argue that AMPPCP binds to an active site

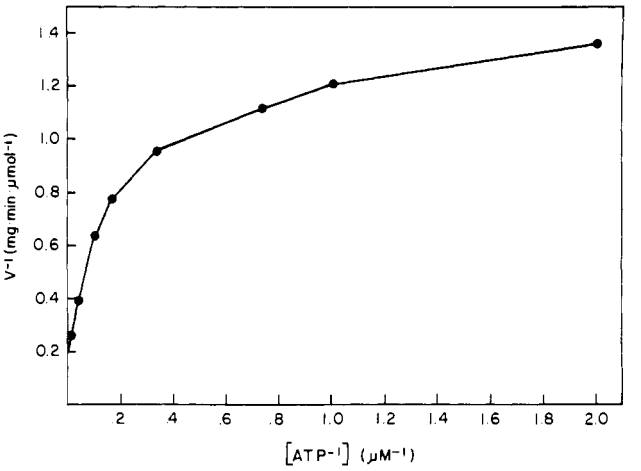

FIGURE 8: Double-reciprocal plot of ATPase activity vs. AMPPCP concentration from computer simulation. The data plotted are the steady-state rates of phosphate liberation produced by the SPICE2 simulation listed in the Appendix.

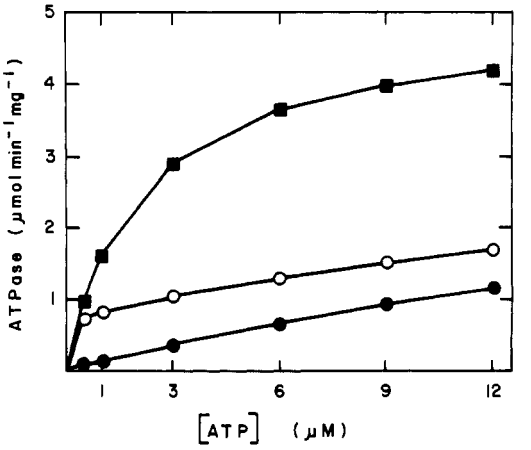

FIGURE 9: Computer simulation of the effect of AMPPCP on the ATPase rate. The data plotted show the steady-state rates of phosphate liberation produced by the SPICE2 program listed in the Appendix: (○) 0 AMPPCP control; (■) 100 μM AMPPCP; (●) 1.2 mM AMPPCP.

rather than an effector site. If this model is to be tenable, it must be shown that a single-site model can account for (1) the complex kinetics of ATP hydrolysis, (2) the stimulation of v/EP by ATP and AMPPCP, and (3) the inhibition of V_f by AMPPCP. A computer-generated double-reciprocal plot of ATPase activity is shown in Figure 8. The details of the simulation by SPICE2 are given in the Appendix. No attempt was made to adjust the kinetic parameters to reproduce the data exactly since a unique fit is not possible given the lack of precise values for all kinetic parameters at 10 °C. Nevertheless, a comparison between the theoretical results and the experimental results is of interest. The theoretical curve deflects downward at high ATP concentrations as does a plot of the data (data plot not shown). The computer-generated curves in Figure 9 show that AMPPCP will produce both stimulation and inhibition of the overall rate of ATP hydrolysis. The pattern of stimulation by 100 μM AMPPCP and inhibition by 2.4 mM AMPPCP is similar to the data shown in Figure 1. In order to obtain both stimulation and inhibition of the overall rate, the binding constant for AMPPCP had to be less than the binding constant for ATP. The constants used (40 μM for AMPPCP and 1 μM for ATP) were in good agreement with the data of Figure 5 for AMPPCP and with that of Dupont et al. (1982) for ATP. The double-reciprocal plots of the effect of AMPPCP on V_f (Figure 10) clearly

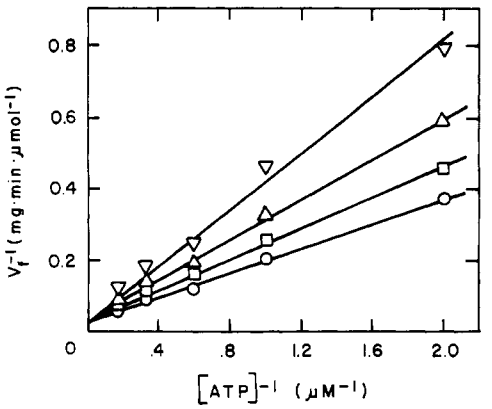

FIGURE 10: Computer simulation of the effect of AMPPCP on V_f. The data plotted were estimated from the time course of EP formation produced from the SPICE2 program listed in the Appendix: (○) 0 AMPPCP control; (□) 25 μM AMPPCP; (△) 50 μM AMPPCP; (▽) 100 μM AMPPCP.

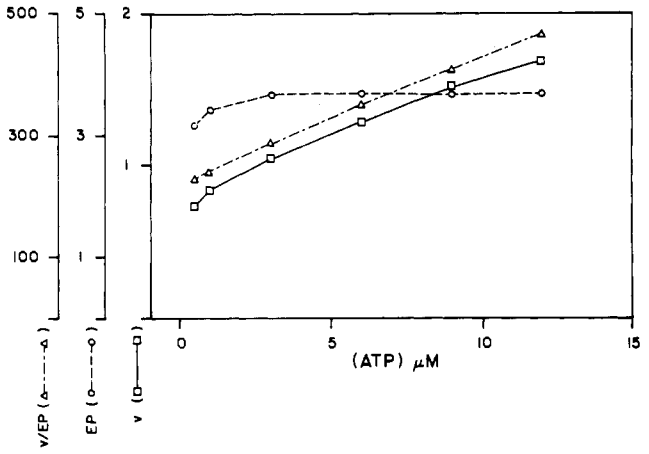

FIGURE 11: Computer simulation of the effect of ATP on v/EP. The data were produced from the SPICE2 program listed in the Appendix. The units for velocity are μmol min^{-1} mg^{-1}, those for EP are nmol mg^{-1}, and those for v/EP are min^{-1}.

demonstrate that the model predicts the inhibition of V_f to be in the same range of AMPPCP concentrations as those that stimulate ATPase activity. The amount of inhibition produced by AMPPCP in the simulation is similar to that produced by AMPPCP experimentally (Figure 2).

Since the kinetic scheme shown in Figure 6 produces stimulation of the ATPase rate by ATP via an acceleration of EP hydrolysis, the computer simulation should show an increase in the parameter v/EP with ATP concentration similar to that shown in Figure 4. The data plotted in Figure 11 show that our proposed kinetic scheme will produce an increase in v/EP of about 2-fold. This is similar to the 3-fold increase shown in Figure 4.

The kinetic model (Figure 7) requires that ATP or AMPPCP bind to the active site before P_i has departed. To test this requirement, we determined the effect of EP on ATP binding. Enzyme phosphorylation was measured with [γ-^{32}P]ATP, and ATP binding was measured with [γ-^{32}P]ATP and [α-^{32}P]ATP as described under Materials and Methods. The concentrations of ATP chosen for these studies are those that are on the approximately linear portion of the binding curve (Guillain et al., 1984). The results, reported in Figure 12, show that phosphorylation of the enzyme reduces ATP binding. The bound nucleotide levels reported when the enzyme was phosphorylated are an average of the data obtained with [γ-^{32}P]- and [α-^{32}P]ATP. The phosphoenzyme levels observed at the initial ATP concentrations of 10, 30, and 90

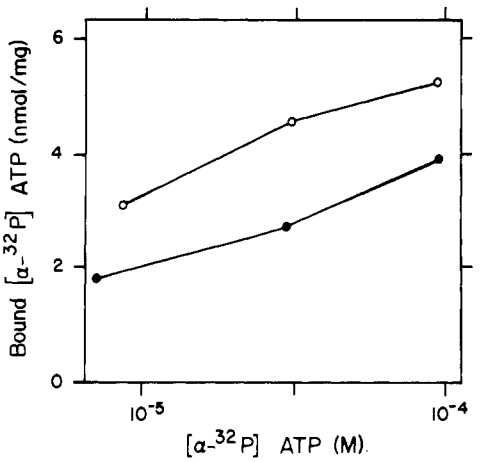

FIGURE 12: Effect of enzyme phosphorylation on ATP binding. Bath composition: 100 mM KCl, 0.1 mM $MgCl_2$, 20 mM MOPS, 1 μM A23187, pH 7.0, and 0.1 mg of SR mL^{-1}, 0 °C. For other details see Materials and Methods and Results. (○) Mean of six observations of [α-^{32}P]ATP binding in the presence of 20 mM EGTA; (●) mean of six observations of [α-^{32}P]ATP binding and six observations of [γ-^{32}P]ATP binding in the presence of 0.1 mM $CaCl_2$. Standard errors of the mean are not shown because they are below the size of the symbols.

μM were 3.4 ± 0.3, 2.9 ± 0.3, and 3.9 ± 0.1 μmol mg^{-1}, respectively. These approached the value of EP(max) (4.1 μmol mg^{-1}), EP at 5 mM $CaCl_2$, observed with this SR preparation. The ATP content of the bound nucleotide observed when EP was present was checked by elution of the bound α-^{32}P nucleotide and subsequent TLC. These analyses showed 82, 80, and 93% ATP in the bound nucleotide. The fractional ATP compositions of the baths used to measure ATP binding were 0.72, 0.92, and 0.94, respectively. Except for the 10 μM bath, where the bound ATP was a greater fraction than that represented by the bath composition, the bound nucleotide composition closely approximated the bath composition. The rightward shift in ATP binding caused by phosphorylation of the enzyme was equal to about a 5-fold reduction in affinity.

DISCUSSION

Pang & Briggs (1977) proposed the use of AMPPCP as an analogue of ATP in studies with SR because ATP competitively displaced AMPPCP. However, subsequent observations by Dupont (1977) and by Taylor & Hatan (1979) that AMPPCP stimulates ATPase activity led these authors to propose that AMPPCP binds to an effector site rather than the active site. Since it was possible that ATP was displacing AMPPCP from an effector site in the study by Pang and Briggs, it was essential to determine where AMPPCP is bound. The observation that AMPPCP competitively inhibits EP formation (Figure 2) makes one site of action the active site. The nearly 1 to 1 relation between the number of AMPPCP binding sites, 7 nmol mg^{-1} (Figure 5), and FITC binding sites, 6.2 nmol mg^{-1} (Figure 6), indicates that there is only 1 mol of AMPPCP site per mole of enzyme. The value of EP(max), 4.1 nmol mg^{-1}, observed with the SR used in the experiment reported in Figure 12, is significantly less than the values for FITC sites and AMPPCP sites. Since only one peptide can be isolated from SR labeled with FITC (Mitchinson et al., 1982) and it is well established that FITC acts at the active site (Mitchinson et al., 1982; Anderson et al., 1982; Pick, 1981), it is most likely that there is some denatured Ca,Mg-ATPase in the isolated SR. The presence of denatured enzyme in SR preparations has been reported by Thorley-Lawson & Green (1973).

Table I: Rate Constants for Network Simulation of ATPase Activity of Sarcoplasmic Reticulum

step[a]	k_f	k_r	reference
1	4.3×10^{12} M^{-2} s^{-1}	15 s^{-1}	Pickart & Jencks, 1984
2	3.7×10^{7} M^{-1} s^{-1}	37 s^{-1}	Dupont, 1977[b]
3	600 s^{-1}	1250 s^{-1}	Pickart & Jencks, 1984
4	3800 s^{-1}	6×10^{6} M^{-1} s^{-1}	Pickart & Jencks, 1984
5	25 s^{-1}	50 s^{-1}	Inesi et al., 1982
6	17 s^{-1}	6×10^{6} M^{-1} s^{-1}	Pickart & Jencks, 1984
7	17 s^{-1}	30 s^{-1}	Inesi et al., 1982[b]
8	1×10^{3} s^{-1}	10^{5} M^{-1} s^{-1}	Inesi et al., 1982[b]
9	100 s^{-1}	100 s^{-1}	
10	2×10^{6} M^{-1} s^{-1}	100 s^{-1}	
11	68 s^{-1}	3×10^{7} M^{-2} s^{-1}	
12	60 s^{-1}	90 s^{-1}	
13	1×10^{3} s^{-1}	10^{5} M^{-1} s^{-1}	
14	200 s^{-1}	20 s^{-1}	
15	2.4×10^{12} M^{-2} s^{-1}	37 s^{-1}	
16	2.4×10^{6} M^{-1} s^{-1}	100 s^{-1}	
17	2.4×10^{6} M^{-1} s^{-1}	100 s^{-1}	
18	68 s^{-1}	3×10^{7} M^{-2} s^{-1}	
19	85 s^{-1}	30 s^{-1}	
20	1×10^{3} s^{-1}	10^{5} M^{-1} s^{-1}	
21	400 s^{-1}	20 s^{-1}	

[a] Reaction identified in Figure 7. [b] Rate constants estimated from equilibrium constants.

AMPPCP was used to probe the mechanism of allosteric regulation because it is not metabolized and can be used to determine if negative cooperativity occurs when substrate is bound (Levitsky & Koshland, 1976). The data in Figure 5 clearly indicate that negative cooperativity in substrate binding does not occur whether calcium is present or not. Pick (1981) obtained the same result using AMPPNP as an ATP analogue. The 8-fold increase in AMPPNP affinity observed with Ca by Pick (1981) was, however, considerably greater than the 2-fold increase in affinity observed with AMPPCP (Figure 5).

Allosteric regulation could take place by the binding of ATP at an active site in an oligomeric complex. The abundant evidence for an oligomeric structure has recently been reviewed by Ikemoto (1982) and Møller et al. (1982). It is possible of course that, although the structure exists, the proposed coupling does not. Although nonhyperbolic kinetics of ATP hydrolysis disappears upon conversion of the enzyme to a monomer (Dean & Tanford, 1978; Møller et al., 1980), it is possible that detergent treatment alters the kinetics of ATP hydrolysis in addition to disrupting protein–protein interactions. In studies on the amount of phosphoenzyme formed in monomeric and oligomeric ATPase, Kosk-Kosicka et al. (1983) found no new phosphorylation sites exposed in the monomeric enzyme. The enzyme does not, therefore, show half-of-the-sites reactivity. The most compelling argument against the involvement of site–site interactions in an oligomeric complex in allosteric regulation of ATPase activity is the failure of FITC to inhibit negative cooperativity (Anderson et al., 1982) and the success of FITC in inhibiting ATPase activity in a linear fashion (Figure 6) (Mitchinson et al., 1982).

Three allosteric mechanisms, activation of an effector site, negative cooperativity in substrate binding, and site–site interaction in an oligomeric complex, have thus been eliminated. The fourth possible mechanism is a branched pathway. This type of mechanism has been proposed by Moczydlowski & Fortes (1981) for the Na,K-ATPase and has been considered by Neet & Green (1977) and by McIntosh & Boyer (1983) for the Ca,Mg-ATPase. The scheme we propose requires that ATP and ATP analogues be bound to EP during the catalytic cycle and that EP bind ATP with a reduced affinity. The data in Figure 12 show that ATP can bind to EP and that the affinity of EP for ATP is lower than the affinity of the free enzyme for ATP. Meissner (1973) has also reported that the formation of EP lowers the affinity of SR for ATP. There are other reports that nucleotides or their analogues can bind to SR when the enzyme is turning over, i.e., when EP is present (Coan et al., 1979; Nakamoto & Inesi, 1984). It should be noted, however, that Guillain et al. (1984) have reached the opposite conclusion for SR phosphorylated at pH 6.0 by inorganic phosphate. Their data do not, however, show that ATP binding is completely blocked; rather they found ATP binding to be depressed.

Computer simulation of the kinetic scheme diagrammed in Figure 7 using only a single ATPase chain is capable of producing all the kinetic effects observed with ATP and AMPPCP. The rate constants used were taken from Inesi et al. (1982) and Pickart & Jencks (1984). Values for the unknown constants were chosen so that the model produced reasonable rates under the conditions of the simulation. The available rate constants were obtained at higher temperatures than those used in the present study so that the rates produced by the computer program were higher than the actual data. In order for the model to produce a downward deflection in the double-reciprocal plot of the dependence of ATPase activity on ATP, the affinity of the phosphoenzyme for ATP had to be less than the affinity of the free enzyme for ATP. Likewise, in order for the model to reproduce the stimulation of the overall rate by AMPPCP, we had to choose an affinity for AMPPCP lower than the affinity for ATP. We were able to obtain a reasonable reproduction of the data using dissociation constants of 1 μM and 40 μM for ATP and 40 μM for AMPPCP, respectively. These values agree well with that found by Dupont et al. (1982) for ATP and with those in the present work (Figure 5A) for AMPPCP. The model also requires that ADP depart before ATP or AMPPCP is bound. The observations by Makinose & Boll (1979) that the enzyme has very low affinity for MgADP may explain why ADP departs once EP is formed. The results of the simulation were qualitatively similar to the actual rates in all cases: the ATPase kinetics were nonhyperbolic, AMPPCP stimulated the overall ATPase rate at 100 μM and inhibited the rate at 1200 μM, and AMPPCP inhibited the initial rate of phosphoenzyme formation at all concentrations.

Appendix

Computer simulations of the effect of ATP and AMPPCP on the Ca-ATPase activity of SR were carried out by using the electronic network simulation program SPICE2. The method has been described previously (Feher & Briggs, 1982; Thakker et al., 1982) and has been shown to provide useful insight into a variety of physiological and biochemical problems [see Mikulecky (1983) for a review]. The rate constants used in the simulation appear in Table I and were taken from the literature where possible.

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