The Calmodulin-Activated Form of the Ca²⁺-Pumping ATPase of the Cardiac Sarcolemmal Membrane Produces Ca²⁺ Gradients with a Thermodynamic Efficiency of 100%

Deborah A. Dixon[1] and Duncan H. Haynes[1,2]

Abstract

The thermodynamic efficiency of the calmodulin-activated form of the Ca²⁺-pumping ATPase of the bovine cardiac sarcolemma (SL) was evaluated in sealed vesicles under reversible conditions. The free internal Ca²⁺ concentration ([Ca²⁺]ᵢ) established in the SL vesicle lumen by action of the ATPase was determined as a function of the $[ATP]/([ADP][P_i])$ ratio for the following experimental conditions: 250 mM sucrose, 100 mM KCl, 0.1 mM Mg²⁺, 25 mM HEPES, 25 mM Tris, pH 7.40, at 37°C, $[Ca^{2+}]_o \approx 50$ nM (1 mM Ca/EGTA buffer), 0.75 mM Mg-ATP, 0.1 mM P$_i$, variable [ADP]. Under these conditions, with the pump working near its K_m of 64 nM, the $[Ca^{2+}]_i$ achieved was $\leqslant 18$ mM, decreasing with increasing [ADP] for $[ADP] \geqslant 0.84$ mM. A plot of the square of the $[Ca^{2+}]_i/[Ca^{2+}]_o$ ratio against $[ATP]/([ADP][P_i])$ gave a straight line with a slope of 1.5×10^7 M. This was in agreement, within the experimental error, with the equilibrium constant for ATP hydrolysis under these conditions (1.09×10^7 M). These results demonstrate (1) tight coupling between Ca²⁺ transport and ATP hydrolysis with a stoichiometry of 2 Ca²⁺ moved per ATP split and (2) a low degree of passive leakage. Analysis at low [ADP] (< 0.83 mM) showed the unexpected result that ADP increases the rate of the *forward* reaction of the pump. The maximal effect on the initial rate is a 96 ± 5% increase, with an EC₅₀ of approximately 0.4 mM (ADP). Similar but lesser stimulation was observed with CDP. The implications of the above results for the energetics of the pump and for its physiological function in the beating heart are discussed.

Key Words: Ca²⁺ transport; heart; sarcolemma; Ca²⁺-pumping ATPase; ion transport; phosphate potential; bioenergetics; ion gradient; chlorotetracycline.

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[1]Department of Pharmacology, University of Miami School of Medicine, P.O. Box 016-189, Miami, Florida 33101.
[2]To whom correspondence should be addressed.

Introduction

Calcium extrusion from cardiac cells of beating heart is accomplished by a
Na/Ca^{2+} exchanger (Reeves and Sutko, 1979) and by a Ca^{2+}-ATPase (Caroni
and Carafoli, 1981a,b). The Na^+/Ca^{2+} exchanger has a stoichiometry of
three Na^+ per Ca^{2+} (Pitts, 1979), has a high maximal velocity (V_{max}),[3] and has
a K_m for Ca^{2+} in the range of 10–40 μM (Reeves and Sutko, 1979; Philipson
et al., 1982) which can be reduced to approximately 2 μM by regulatory
phosphorylation Caroni and Carafoli, 1983). It is thought to make the
greatest contribution to Ca^{2+} removal in the systolic phase (Carafoli, 1984).
The Ca^{2+}-ATPase has a lower V_{max} and has a much higher affinity for Ca^{2+}.
Its K_m for Ca^{2+} is 10 or 1.8 μM in the unactivated state. By the combined
action of regulatory phosphorylation and calmodulin (CAM) the K_m for
Ca^{2+} can be reduced to 300 $\pm$ 200 nM or 64 $\pm$ 1.4 nM (Caroni and Carafoli,
1981a or Dixon and Haynes, 1989a, respectively). The Ca^{2+}-ATPase is
thought to make the greater contribution in the later stages of Ca^{2+} removal
and to be responsible for setting diastolic levels of 100 nM or less (Carafoli,
1984; Dixon and Haynes, 1989a). The Ca^{2+}-pumping ATPase can be studied
conveniently in sealed vesicles prepared from cardiac sarcolemma (SL,
Reeves and Sutko, 1979). The Na^+/Ca^{2+} exhanger, which is colocalized with
it in the SL vesicles, can be rendered inoperative by omission of Na^+ from
the medium. In the present study, the ability of the Ca^{2+}-pumping ATPase
to form Ca^{2+} gradients will be analyzed at various levels of thermodynamic
driving force, set by the $[ATP]([ADP][P_i])$ ratio.

Mechanism of the Cardiac SL Ca^{2+} Pump

The cardiac SL Ca^{2+}-ATPase shares many features with the Ca^{2+}-Mg^{2+}-
ATPase of rabbit skeletal sarcoplasmic reticulum (SR). The latter is an
extremely well-studied example of a transport ATPase (Hasselbach and
Makinose, 1963; Weber et al., 1966; Kanazawa et al., 1971; MacLennan and
Holland, 1975; Inesi, 1972; Froehlich and Taylor, 1975, 1966; de Meis and
Vianna, 1979; Haynes, 1983; Haynes and Mandveno, 1987) and it is useful
to compare the two enzymes. Unlike the skeletal SR enzyme, the cardiac SL

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[3] Abbreviations: V_{max}, maximal velocity at saturating $[Ca^{2+}]$; CAM, calmodulin; SL, sarcolem-
ma; SR, sarcoplasmic reticulum; $cAMP$ PK, $cAMP$-dependent protein kinase; n, stoichiometry
between Ca^{2+} moved and ATP split; $[Ca^{2+}]_i$, concentration of free Ca^{2+} in the SL lumen;
P_i, inorganic phosphate; HEPES, 4-(2-hydroxy)-1-piperazineethanesulfonic acid; Tris,
tris(hydroxy)aminomethane; K_{eq}, equilibrium constant for ATP hydrolysis or for transport
reaction; CTC, chlorotetracycline; k_{leak}, first-order rate constant for passive leakage of Ca^{2+}
from lumenal space (min^{-1}); V_0, maximal rate of Ca^{2+} uptake observed in progress curve for
a defined set of experimental conditions.

enzyme can exist in four states of activation: (a) unactivated, (b) cAMP-dependent protein kinase (cAMP PK) activated, (c) CAM-activated, and (d) CAM-activated plus cAMP-PK-activated. The cAMP PK activations were accomplished using its catalytic subunit (C-subunit). The external Ca²⁺ concentration ($[Ca^{2+}]_o$) dependence of forms (a) and (b) gave Hill coefficients of 1.6 ± 0.3 and 1.7 ± 0.1 with the low affinity noted above. The Hill coefficients are consistent with a basic mechanism whereby two Ca²⁺ are moved per transport event (Dixon and Haynes, 1989a). With the two CAM-activated forms (c) and (d), the Hill coefficients were two units higher due to the influence of the Ca²⁺-to-CAM binding reaction and the high degree of activation observed with Ca_n-CAM binding. The analysis of the present study is made on the CAM-activated form (c) of the enzyme. Also relevant to the present study is the finding that the CAM-activated form is further activated by ADP in the millimolar concentration range (Dixon and Haynes, 1989a).

Like the skeletal SR, the cardiac SL pump can create a large Ca²⁺ gradient: The CAM activated form, working at $[Ca^{2+}]_0$ values near its K_m, can create a maximum gradients of 4.2×10^5 (Dixon and Haynes, 1989a; compare with Haynes and Mandveno, 1983). The skeletal SR Ca²⁺ pump was subjected to a thermodynamic analysis which showed that the Ca²⁺ gradient produced by the pump at defined $[ATP]/([ADP][P_i])$ ratios is equal to the theoretical value derived from the pump stoichiometry ($2Ca^{2+}/ATP$ or $n = 2$) and from the equilibrium constant for ATP hydrolysis (Trevorrow and Haynes, 1984). This was proof of intrinsically tight coupling between ATP hydrolysis and Ca²⁺ transport and of 100% thermodynamic efficiency of the pump. Fagan and Dewey (1985) arrived at the same conclusion from an analysis of the kinetics of the forward and reverse reactions of the SR pump reconstituted in phospholipid vesicles. The present study applies the previous method (Trevorrow and Haynes, 1984) to define the stoichiometry and thermodynamic efficiency intrinsic to the bovine cardiac SL pump.

Theoretical Basis of Analysis of Pump Thermodynamics

As was developed for the skeletal SR pump, a stoichiometic equation for tightly coupled transport with $n = 2$ will be specified by

$$2Ca_o^{2+} + ATP \xrightarrow{K_{eq}} 2Ca_i^{2+} + ADP + P_i \tag{1}$$
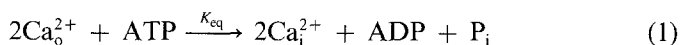
where the subscripts o and i refer to the outside and inside, respectively, of the SL vesicle. For a net reaction to occur, the above reaction must be compensated by movement of four electronic charges per transport

event and ATP split. This can be accomplished by countermovement of H^+,

$$4H_i^+ \longleftrightarrow 4H_o^+ \tag{2}$$

by countermovement of alkali cations (M^+),

$$4M_i^+ \longleftrightarrow 4M_o^+ \tag{3}$$

or by comovement of anions (A^-) such as Cl^-,

$$4Cl_o^- \longleftrightarrow 4Cl_i^- \tag{4}$$

or by a combination of all three processes. In principle, the counter- or cotransport reactions could occur on the pump or could occur as parallel processes mediated by ion channels. Our studies of the cardiac SL pump suggest that countertransport of H^+ by the pump is intrinsic to its mechanism (Dixon and Haynes, 1989a). The initial rate of the pump is not affected by changes in K^+ or Cl^- concentration (Dixon and Haynes, 1989a), is not affected by imposed positive or negative membrane potential (Dixon and Haynes, 1989b), but does require an adequate buffer capacity in the SL lumen (Dixon and Haynes, 1989b). In the present study, conditions were chosen to eliminate trans-membrane gradients of H^+ or Cl^- (or K^+), such that the thermodynamic analysis is independent of which counter- or cotransport mechanism is operative. Elimination of these gradients also serves to further eliminate membrane potential as a possible factor.

For the case where the above conditions are fulfilled and the vesicles do not have an appreciable Ca^{2+} leak, the following relationship will hold for an equilibrium established by the pump:

$$\frac{[Ca^{2+}]_i^2}{[Ca^{2+}]_o^2} = K_{eq} \frac{[ATP]}{[ADP][P_i]} \tag{5}$$

where K_{eq} is the equilibrium constant for ATP hydrolysis. It predicts proportionality between the *second power* of $[Ca^{2+}]_i$ and the quantity on the right for constant $[Ca^{2+}]_o$. In a study of rabbit skeletal SR (Trevorrow and Haynes, 1984), we determined equilibrium values of $[Ca^{2+}]_i$ at fixed $[Ca^{2+}]_o$, [ATP], and $[P_i]$ and variable [ADP], and showed that Eq. (5) was obeyed for finite values of the latter. The value of K_{eq} obtained (9.02×10^6 M) was identical to the equilibrium constant for ATP hydrolysis under these experimental conditions (0.1 mM Mg^{2+}, pH 7.0, 30°C), indicating that the enzyme is 100% efficient under the conditions tested. In the present communication, the Ca^{2+}-pumping ATPase of bovine cardiac sarcolemma (SL) will be put to the same test.

Since it has been suggested that the SL pump may have a stoichiometry of only one Ca^{2+} per ATP split (Caroni *et al.*, 1983), we will also consider the process

$$Ca_o^{2+} + ATP \xrightleftharpoons{K_{eq}} Ca_i^{2+} + ADP + P_i \tag{6}$$
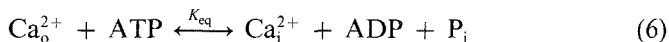
and its correlate equilibrium expression

$$\frac{[Ca^{2+}]_i}{[Ca^{2+}]_o} = K_{eq} \frac{[ATP]}{[ADP][P_i]} \tag{7}$$

This equation predicts proportionality between the *first power* of $[Ca^{2+}]_i$ and the right-hand quantity for fixed $[Ca^{2+}]_o$. It also predicts much larger gradients than Eq. (5).

Materials and Methods

Sarcolemmal (SL) Vesicles

Sarcolemmal vesicles were prepared from bovine left ventricles as described previously (Dixon *et al.*, 1984; Dixon and Haynes, 1989a). The vesicles were kept frozen in liquid N_2 and were thawed before use. The reader is also referred to the above-cited papers for information on quality control and for quantitation of active uptake, as sketched below.

Measurement of Active Uptake

Facile and detailed study of the pump function is made possible through the use of the fluorescence of chlorotetracycline (CTC; cf. Caswell and Hutchinson, 1971; Caswell, 1972; Caswell and Warren 1972) which serves as a continuous monitor of the free Ca^{2+} concentration in the vesicle lumen ($[Ca^{2+}]_i$) during the transport process (Dixon *et al.*, 1984). This quantity can be equally well described by the terms "ionized calcium" or "calcium activity" which are conventions of the physiological and chemical literature, respectively. No Debye-Huckel corrections were made for ionic strength. The methodology for quantitative use of CTC to measure $[Ca^{2+}]_i$, including calibration of its fluorescence vs. $[Ca^{2+}]_i$ relationship in SL vesicles, its application to active transport, and correction for vesicle sidedness have been described in detail (Dixon *et al.*, 1984; Dixon and Haynes, 1989a). The relationship between $[Ca^{2+}]_i$ and the total (free plus bound) Ca^{2+} measured by 45-Ca^{2+} has been described previously (Dixon *et al.*, 1984; Dixon and Haynes, 1989a,b).

Active uptake was examined in cardiac SL vesicles (30 µg/ml) in the presence of saturating calmodulin (1 µM). The uptake medium contained

250 mM sucrose, 100 mM KCl, 0.1 mM Mg^{2+}, 0.10 mM P_i, 1×10^{-5} M CTC, 25 mM HEPES, and 25 mM Tris (HCl salt), pH 7.40, at 37°C. The pH adjustments were made with KOH or HCl. The concentration of free Ca^{2+} was set at 50 nM, chosen to be close to the pump's K_m of 64 nM (Dixon and Haynes, 1989a), using an EGTA buffer ($[EGTA]_T = 1$ mM). The total Ca^{2+} to be added ($[Ca^{2+}]_T$) was determined by computer-assisted calculations of the $[Ca^{2+}]_o$ vs. $[Ca^{2+}]_T/[EGTA]_T$ relationship for the $[Mg^{2+}]$, pH, and temperature of our experiment using the intrinsic binding constants and enthalpy values tabulated by Martell and Smith (1974). To this medium was added varying amounts of ADP to give the desired $ATP/(ADP \cdot P_i)$. Mg^{2+} was added in a molar ratio to ADP of 0.057 to maintain constant free Mg^{2+}.

Activate uptake was initiated by the addition of 0.75 mM MgATP. Time-resolved CTC fluorescence was converted into $[Ca^{2+}]_i$ by use of calibration data obtained from vesicles passively loaded with a wide range of $[Ca^{2+}]_i$ values as described previouly (Dixon et al., 1984). The initial calibration encompassed both the right-side-out (RSO) and inside-out (ISO vesicles), whereas only the latter contributes to transport.

The relative proportions of these two forms were determined to be 47 and 40%, respectively, by the ouabain/digoxin binding method (Jones et al., 1980; Brandt, 1985) as described earlier (Dixon and Haynes, 1989a), and a factor of $(40\% + 47\%)/40\%$ was used in the calculation of the $[Ca^{2+}]_i$ values for the ISO vesicles.

Maintenance of Balance of Other Ions

As mentioned above, our evidence indicates that the pump countertransports H^+. Experiments are designed to minimize imbalance of H^+ by including 25 mM HEPES and 25 mM Tris. The latter is membrane permeant and can replenish H^+ by a mechanism involving comovement of Cl^- (Haynes, 1982). The maximal $[Ca^{2+}]_i$ value achieved by active transport under the present conditions was 18 mM. Thus the counter- or comovement of $\leqslant 36$ mM monovalent ions is required. Previous analysis showed that Cl^- and K^+ equilibrate across the membrane with $t_{1/2}$ values of 34 and 136 sec, respectively (Dixon and Haynes, 1989b). At our chosen external [KCl] (100 mM) the $[Cl^-]_i/[Cl^-]_o$ or $[K^+]_o/[K^+]_i$ ratios would be not greater than 1.36. The ratios would probably be much closer to 1.18 since the imbalance would be shared by both the anion and the cation.

Maximal Value of $[Ca^{2+}]_i$: The Kinetically-Controlled vs. Equilibrium-Controlled Cases

As we have shown previously with skeletal SR, the maximal uptake achieved in the plateau phase of the progress curve can be determined either

by kinetic or equilibrium properties depending on experimental design (compare Dixon *et al.*, 1982 and Trevorrow and Haynes, 1984). In the absence of ADP and P_i, the final $[Ca^{2+}]_i$ is determined not by thermodynamic properties of the pump, but as the balance achieved between its uptake rate and the rate of passive leakage. As described for the SR (Haynes and Mandveno, 1983), steady state is reached as the accumulated Ca^{2+} slows the net rate of the pump (trans-inhibition) and increases the rate of leakage (mM/min, given by $k_{leak} \cdot [Ca^{2+}]_i$). With both the SR and the SL vesicles, k_{leak} has a low value, and very large values of $[Ca^{2+}]_i$ are observed. Under this condition the total system is thermodynamically irreversible and $[Ca^{2+}]_i$ can be considered to be under "kinetic control."

When ADP and P_i are present, the back reaction of the pump is enabled. When the ADP and P_i concentrations are raised, the rate of the back reaction will become comparable to $k_{leak} \cdot [Ca^{2+}]_i$ and will eventually exceed it. The steady-state maximal values of $[Ca^{2+}]_i$ will then be lowered and will reflect the true equilibrium set by the pump (i.e., the properties of the pump operating under *reversible* conditions). Under this condition the $[Ca^{2+}]_i$ is under "equilibrium control."

For the analysis to correctly determine the $[Ca^{2+}]_i^2/[Ca^{2+}]_o^2$ vs. $[ATP]/([ADP][P_i])$ relationship intrinsic to the pump equilibrium, the reverse rate of the pump must be many times larger than the rate of passive leakage. This can be substantiated by direct measurement of the rate of passive leakage and comparison with the rate of pump reversal measured directly or inferred. Reference to Fig. 3 of Dixon and Haynes (1989a) describes the behavior of pump and leak for the CAM-activated pump operating at 70 nM external Ca^{2+} (near its K_m) in the absence of added ADP or P_i. For $[Ca^{2+}]_i \leqslant 18$ mM (condition for present study), the forward rate of the pump is $\geqslant 12$ times the leak rate and the vesicles continue to take up Ca^{2+} (Fig. 3 and Table 2 of Dixon and Haynes, 1989a.[4] In the presence of ADP and P_i (Fig. 1), the pump reversal operates at a rate comparable to the forward rate, and the former determines the $[Ca^{2+}]_i$ value established. The rate of leakage is $\leqslant 1/11$ of the reversal rate. Increasing the ADP concentration increases the rate of the back reaction of the pump and decreases the maximal values of $[Ca^{2+}]_i$ achieved.

The transport behavior observed when the enzyme was in the *unactivated* form proved to be kinetically controlled. The rate of the unactivated enzyme is ca. 1/9 that of the CAM-activated form (Dixon and Haynes, 1989a). The performance of the unactivated pump was so degraded by the

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[4]The final $[Ca^{2+}]_i$ achieved is approximately 46 mM. This value is the result of progressive attenuation of the rate of the pump by trans-inhibition as the internal Ca^{2+} is raised from 20 mM to its final value ($IC_{50} = 32$ mM) and of progressive increase in the rate (mM/min) of passive leakage which is proportional to $[Ca^{2+}]_i$.
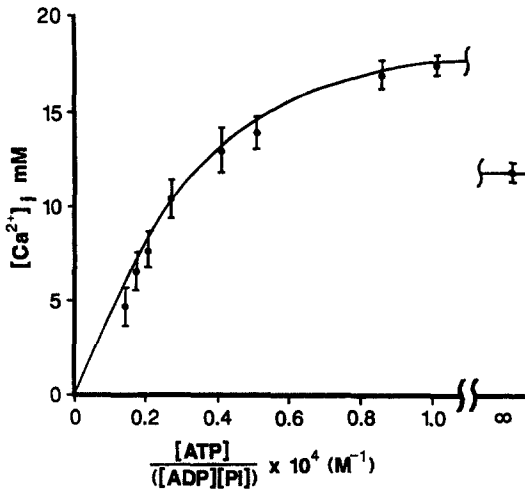
Fig. 1. Dependence of the maximal internal free Ca^{2+} concentration on the $[ATP]/([ADP] \cdot [P_i])$ ratio. Bovine cardiac sarcolemma ($30\,\mu g/ml$) was preincubated with calmodulin at ($1\,\mu M$) in the medium described in Materials and Methods ($[Ca^{2+}]_0 = 50\,nM$). The uptake was initiated by adding, as a single aliquot, $0.75\,mM$ Mg-ATP, $0.1\,mM$ P_i (K^+ salt), and a variable concentration of ADP. The aliquot contained extra Mg^{2+} in a constant ratio of 0.057 to the ADP in order not to disturb the free Mg^{2+} concentration ($0.1\,mM$) of the medium. The progress curve of fluorescence increase was recorded after the addition of the aliquot. Maximal $[Ca^{2+}]_i$ was determined from the plateau level in the active uptake progress curve. The presented data are the average ($\pm$ SD) of three separate determinations on two preparations. For selected points on the left portion of the figure, the experiments were repeated with the Mg-ATP (and ADP) concentration doubled, and no difference in $[Ca^{2+}]_i$ was found (data not shown).

passive leakage that it was not possible to deduce its *intrinsic* ability to make a Ca^{2+} gradient.

Calculation of K_{eq}

The theoretical value of K_{eq} is calculated from the free energy of ATP hydrolysis for the conditions appropriate to the experiment. The free energy of hydrolysis of ATP at $25°C$, pH 7.4, in the presence of $0.10\,mM$ Mg^{2+} is $-9.4\,kcal/mol$ (Alberty, 1968; Shikama and Nakamura, 1973), giving $K_{eq25} = 7.84 \times 10^6\,M$. This value was corrected to $37°C$ using the enthalpy value of $-4.9\,kcal/mol$ given by Phillips *et al.* (1969) for $0.1\,mM$ Mg^{2+}, $0.1\,M$ ionic strength, and pH 7.5. We arrive at $K_{eq37} = 1.09 \times 10^7\,M$.

Results

As noted earlier, the Ca^{2+} pump of cardiac SL is stimulated by ADP in the millimolar range (Dixon and Haynes, 1989a). Since this affects the range

Table I.   Effect of [ADP] or Nucleotide on Rate and Maximal $[Ca^{2+}]_i$ for ATP-Driven Uptake under the Experimental Conditions of This Study[a]

Concentration of modifier (mM)	V_0 (mM/min)	V_0 ratio	$[Ca^{2+}]_{i,max}$ (mM)	Ratio
0.00 mM ADP	0.97	(1.0)	13.0	(1.0)
0.37 mM ADP	1.38	1.42	16.5	1.27
0.75 mM ADP	1.86	1.92	18.0	1.38
0.83 mM ADP	1.91	1.96	17.6	1.35
0.75 mM CDP	1.35	1.39	16.3	1.25
0.75 mM CTP	0.95	0.98	12.8	0.98
0.75 mM GTP	0.98	1.01	13.1	1.00
0.75 mM ITP	0.97	1.00	13.0	1.00

[a] The table presents values for V_0 (maximal rate observed in progress curve) and maximal $[Ca^{2+}]_i$ observed in the plateau phase of the uptake. SL vesicles were preincubated for ca. 2 min in the standard medium, pH 7.4, 37°C, with CAM, Ca^{2+}/EGTA buffer, 0.1 mM Mg, 0.1 mM P_i, 10 μM CTC, and the indicated modifier. When the modifier was ADP, extra Mg^{2+} was added in a molar ratio of 0.057. When the modifier was CTP, GTP, or ITP, extra Mg^{2+} was added in a molar ratio of 1.0. The uptake reaction was initiated by adding 0.75 mM Mg-ATP. The data presented are a composite of three experiments. the experimental uncertainties of the relative and absolute values are ca. 5 and 12%, respectively.

of applicability of Eq. (5) [or Eq. (7)], the size of the effect was also determined for the experimental conditions of the present study ($[Ca^{2+}]_o = 50$ nM, $[P_i] = 0.1$ mM). Table I shows that 0.75 mM ADP increases the maximal rate observed in the initial phase of uptake (V_0) by 98% and increases the maximal $[Ca^{2+}]_i$ by 38%. This is in qualitative agreement with our observations for the pump operating in the irreversible or kinetically controlled mode at saturating $[Ca^{2+}]_o$ (Table 2 of Dixon and Haynes, 1989a). Table I shows that 0.75 mM ADP gives a near-maximal stimulatory effect under our conditions. Data to be given below show that ADP concentrations greater than 0.83 mM produce a decline in the maximal $[Ca^{2+}]_i$ values. This is the expected effect of ADP and P_i serving as substrates for the reverse reaction of the pump.

    Table I shows that CDP can also produce stimulation. However, CTP, GTP, and ITP do not give stimulation. Thus, the stimulatory effect can be seen with nucleotide diphosphates but not nucleotide triphosphates.

    The dependence of free internal calcium concentration generated by the pump as a function of ATP/(ADP · P_i) is depicted in Fig. 1. The concentrations of ATP and P_i were held constant at 0.75 and 0.1 mM, respectively, and the [ATP]/([ADP][P_i]) ratio was decreased by increasing [ADP]. Figure 1 shows that the $[Ca^{2+}]_i$ decreases as the [ATP]/([ADP][P_i]) ratio is decreased from $1.0 \times 10^4 M^{-1}$ to $0.14 \times 10^4 M^{-1}$. This corresponds to [ATP]/[ADP] ratios between 1.0 and 0.14 or [ADP] values between 0.75 and 5.4 mM, respectively. For [ADP] > 0.75 or 0.83 mM, the above-noted ADP stimulation

The y-axis is labeled $\dfrac{[Ca^{2+}]_i^2}{[Ca^{2+}]_o^2} \times 10^{11}$ with values 0, 0.5, 1.0, 1.5.

The x-axis is labeled $\dfrac{[ATP]}{([ADP][Pi])} \times 10^4 \ (M^{-1})$ with values 0, 0.2, 0.4, 0.6, 0.8, 1.0.
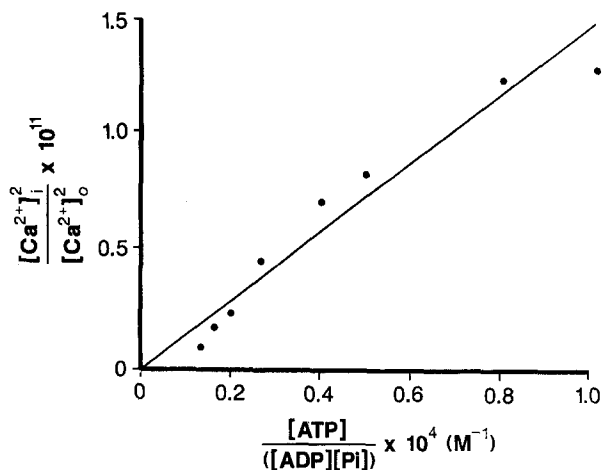
Fig. 2. Plot of the data of Fig. 1 according to Eq. (5).

of the forward reaction is maximally developed, and further increases in [ADP] increase the rate of the reverse reaction. The information given in the Materials and Methods section under "control for passive leakage" shows that the data for $[Ca^{2+}]_i \leqslant 18\,mM$ and $[ATP]/([ADP][P_i]) \leqslant 1.0 \times 10^4\,M$ are appropriate for the condition of equilibrium set by the pump. The data are thus analyzed in terms of Eqs. (7) and (5).

It is noted that the 1 : 1 stoichiometry model of Eq. (7) predicts a linear relationship of Fig. 1, with a slope equal to the equilibrium constant for ATP hydrolysis. Inspection of Fig. 1 shows that the linear relationship does not obtain; the discussion below will show that the slope in Fig. 1 is orders of magnitude below that predicted for a 1 : 1 stoichiometry.

Figure 2 is a plot of the data of Fig. 1 according to Eq. (5) and the assumption of 2 : 1 stoichiometry. The *second power* of $[Ca^{2+}]_i$ is plotted against and a straight line is observed. A slope (K_{eq}) of $1.5 \times 10^7\,M$ is obtained. This value is close to the slope of $1.1 \times 10^7\,M$ expected from the equilibrium constant for ATP hydrolysis appropriate to these conditions.

Discussion

The most important finding of the present study is that the dependence of $[Ca^{2+}]_i$ on $[ATP]/([ADP][P_i])$ supports a mechanism of Eq. (5) by which the CAM-activated pump transports 2 Ca^{2+} transported per ATP split in a tightly coupled process and that the data are in gross disagreement with the

assumption of a 1 : 1 stoichiometry for this state of the enzyme. The latter point will be considered first.

Evidence against a 1 : 1 Stoichiometry for the CAM-Activated Enzyme

As noted, earlier, if a 1 : 1 stoichiometry were operative, a linear relationship should have been observed in Fig. 1, contrary to our observations. Although it could be argued that the data for $[Ca^{2+}]_i \leqslant 13.5 \, \text{mM}$ and $[ATP]/([ADP][P_i]) \leqslant 0.4 \times 10^4 \, \text{M}^{-1}$ represent a linear dependence, the K_{eq} value which would be derived from the slope defined by those data points would be only 80 M. If this represented a true K_{eq}, it would be five orders of magnitude lower than the equilibrium constant for ATP hydrolysis $(1.1 \times 10^7 \, \text{M})$, requiring an explanation for this sizable discrepancy.[5] Nor can it be argued that this sizable discrepancy is due to degradation of the performance of the pump by passive leakage and inhibition by Ca^{2+} in the lumen. We have shown that the IC_{50} for inhibition by internal Ca^{2+} is 32 mM (Dixon and Haynes, 1989a) and that the degree of inhibition at $[Ca^{2+}]_i$ is negligible (Fig. 3 of that paper). Thus the present results rule out a 1 : 1 stoichiometry for the CAM-activated enzyme in our preparation.

Although it would have been interesting to obtain thermodynamic results for the *unactivated* enzyme in our preparation, this was not possible. The rate of Ca^{2+} transport by unactivated enzyme was so slow that its Ca^{2+} accumulation was seriously degraded by passive leakage at high $[Ca^{2+}]_i$. In the absence of ADP and P_i, the unactivated enzyme produces $[Ca^{2+}]_i/[Ca^{2+}]_o$ ratios of 4.8×10^3, compared to 4.0×10^5 for the CAM-activated enzyme (Table 1 and Fig. 4 of Dixon and Haynes, 1989a). Thus, analysis as in Figs. 1 and 2 would be inappropriate. It is appropriate to point out that our finding a Hill coefficient of 1.6 ± 0.1 from the Ca^{2+} dependence of the unactivated forms argues against a mechanism in which only one Ca^{2+} is moved per transport event (Dixon and Haynes, 1989a).

In apparent contrast are the findings of Caroni *et al.* (1983) for cardiac SL Ca^{2+} pump reconstituted into liposomes. They reported an approximate stoichiometry of 1 Ca^{2+}/ATP for the reconstituted enzyme in the absence of added CAM. Further characterization of the purified enzyme would be clearly useful for understanding the action of the pump in the SL membrane.

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[5]Under the assumption of 100% efficiency, the model of Eq. (7) predicts that $[Ca^{2+}]_i$ achieved $[ATP]/([ADP][P_i]) = 0.4 \times 10^4 \, \text{M}^{-1}$ should be $4.4 \times 10^3 \, \text{M}$. While it would obviously not be fair to consider failure to achieve the same as evidence against 1 : 1 stoichiometry, there is no reason why the pump, working at this stoichiometry, should not have been able to generate $[Ca^{2+}]_i$ values of 46 mM (Table 2 of Dixon and Haynes, 1989a) at all of the $[ATP]/([ADP][P_i])$ ratios of the present study.

Evidence for a 2:1 Stoichiometry

The good agreement between the data as plotted in Fig. 2 and the predictions of Eq. (5) provide evidence that the CAM-activated pump in the SL membrane transports 2 Ca^{2+} per ATP split in a tightly coupled reaction. This is in agreement with the basal mechanism of Ca^{2+} handling as revealed by the Hill coefficients for the unactivated and cAMP-PK-activated forms of the pump (1.6 ± 0.1 and 1.7 ± 0.1, respectively).[6] These values are within the range of values reported for the skeletal SR pump (Inesi *et al.*, 1980; Riviero and Vianna, 1978; Haynes and Mandveno, 1983; Haynes, 1983) for which a stoichiometry of 2:1 is generally accepted.

Experimental Uncertainty in E_{eq}

As noted earlier, the agreement between the slope of Fig. 2 (1.5 × 10⁷ M) and the equilibrium constant for ATP hydrolysis (1.1 × 10⁷) was good but not perfect. It is thus appropriate to consider that the various sources of experimental error could cause the experimental value of K_{eq} to exceed its theoretical value by 36%. The calculations depend on basic elements: (1) the equilibrium constant for hydrolysis of ATP, (2) the Ca:EGTA dissociation constant used to calculate $[Ca^{2+}]_o$, (3) the passive calibration procedure for CTC, and (4) the determination of the relative population of vesicles of inside-out orientation. Elements (1) and (2) are considered to represent true thermodynamic constants which are subject to minimal error. Sample-to-sample and run-to-run variation in calibrations and progress curves for active uptake (fluorescence vs. time) did not vary more than 6%. The standard deviations shown in Fig. 1 (⩽ 12.5%) reflect this and the day-to-day variation observed. Thus Element (3) can contribute as much as 12.5% error in $[Ca^{2+}]_i$ or 26% error in K_{eq}. Element (4) could be the source of additional variation. An absolute variation in the percent of the population in the ISO orientation of 3.3% would result in a 7% uncertainty in $[Ca^{2+}]_i$, a 14% uncertainty in $[Ca^{2+}]_i/[Ca^{2+}]_o$, and a 30% uncertainty in K_{eq}. Taken together, the experimental uncertainty in Elements (3) and (4) is sufficient to explain the difference between the experimental and theoretical value of K_{eq}.

Energetics of the Cardiac SL and Skeletal SR Pumps

The present findings allow a more detailed consideration of differences in how these two transport ATPases partition their energy between the

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[6] As noted earlier, the Hill coefficients of the CAM-activated forms of the SL pump are approximately 2 units higher due to the Ca^{2+} dependence of Ca_n-CAM formation and the high degree of activation produced by binding of those species.

various steps in their cycles. As an initial step, we tabulated the kinetic constants for the two pumps (Dixon and Haynes, 1989a). Compared with the SR pump, the SL pump binds ATP more weakly, binds external Ca^{2+} with nearly equal affinity, and dissociates ADP and transported Ca^{2+} more strongly (Dixon and Haynes, 1989a). Since the overall equilibrium constants for the two pumps are equal, we can deduce that the mathematical product of the equilibrium constants for the processes of dephosphorylation, phosphate dissociation, and the remaining processes of the SL pump are ca. 31 times smaller for the SR pump. The SR pump has been subjected to the extensive analysis of phosphoenzyme behavior ("high energy" form: Yamada and Tonomura, 1972; Froehlich and Taylor, 1975, 1976; "low energy form": Masuda and de Meis, 1973; cf. Haynes, 1983b). The SL pump is present at much lower concentration in its host membrane, and phosphoenzyme analysis has not yet been performed. It is of interest that the SL pump is more sensitive than the SR pump to the inhibitory effects of vanadate (a phosphate substitute). The k_i values for the SL pumps are approximately $1\,\mu$M whereas those for SR pumps are in the range of 10–$100\,\mu$M (Wang et al., 1979; Niggli et al., 1981; Caroni and Carafoli, 1981). An important question is whether this difference will also be found for cytoplasmic P$_i$. The answer will have implications for the action of these two pumps in their respective muscle under conditions of metabolic stress.

Stimulatory Effect of ADP

The stimulatory effect of nucleotide diphosphates (ADP and CDP) appears to be unique to the cardiac SL ATPase. No stimulation was observed with CTP, GTP, or ITP (or ATP). This is in distinction to the behavior of the skeletal and cardiac SR pumps which are stimulated by ATP. The stimulatory effect of ADP on the SL pump could be useful in rescuing a heart cell in metabolic extremis. For a cell with high [Ca^{2+}]$_{cyt}$, low [ATP], and high [ADP], removal of cytoplasmic Ca^{2+} by extrusion would be of higher priority than removal by sequestration into the SR from which it could be released, requiring it to be handled once more. It would thus be reasonable for the SL pump to be activated by ADP and for the SR pump to be similarly activated by ATP.

Implications for the Beating Heart

Analysis of our previous data for the cardiac SL pump operating under irreversible conditions (zero ADP and P$_i$) showed that the pump is easily able to subserve its physiological function of lowering [Ca^{2+}]$_{cyt}$ to 100 nM (or lower) against an external Ca^{2+} concentration of about 2 mM (Dixon and Haynes, 1989a). It is of interest to consider the size of the gradients which the

pump is required to produce in the "healthy" case in which the pump would operate irreversibly. Since the diastolic $[Ca^{2+}]_{cyt}$ is comparable to or larger than the K_m of the CAM-activated pump and the systolic $[Ca^{2+}]_{cyt}$ is much larger, it is probable that the pump operates at ca. 100% of its V_{max} most of the time and that its rate decreases to 50% of V_{max} only at the end of each beat. Since the Na^+/Ca^{2+} exchanger is thought to remove most of the Ca^{2+} during systole, the relative importance of the SL pump would be measured by its relative contribution to the total rate of removal. As $[Ca^{2+}]_{cyt}$ is decreased to diastolic levels, the exchanger, which has a higher K_m, will make increasingly smaller contributions and the status of the SL pump becomes more important.

The present data are totally consistent with the pump operating under conditions corresponding to healthy heart ([ADP]/[ATP] < 1, [P_i] ⩽ 1 mM). In fact, the ADP and P_i concentrations could rise to much higher values and still allow the pump to continue operating. Our analysis of the pump operating under irreversible conditions suggests that it operates in an electrically neutral charge-compensated mechanism. The initial rate of the pump is insensitive to imposed membrane potential ($+71$ or -67 MV; Dixon and Haynes, 1989b). Momentarily neglecting H^+ gradients, using Eq. (5) with $K_{eq} = 1.5 \times 10^7$ M, taking the plasma Ca^{2+} concentration as 2 mM, and choosing $[Ca^{2+}]_{cyt} = 64$ nM (its K_m; Dixon and Haynes, 1989a), we calculate the equilibrium value of the $[ATP]/([ADP][P_i])$ ratio to be 65.1. The pump could thus function for ratios of 65.1 or larger. This means that for $[P_i] = 1$ mM, the pump could continue operating until [ADP] became 15.4 times as large as [ATP]. Alternatively, for $[P_i] = 5$ mM, the pump could continue operating until [ADP] becomes 3.1 times as large as [ATP]. Thus, the pump has sufficient bioenergetic reserve to operate efficiently for all but very ischemic conditions.

The above analysis assumes the absence of gradients of counter- or coaccumulated ions. We have given evidence for H^+ being a countertransported ion (Dixon and Haynes, 1989a). Under normal physiological conditions the small trans-membrane pH gradient (cytoplasm slightly acid relative to plasma) would not be sufficient to raise the equilibrium value of $[Ca^{2+}]_{cyt}$ to 64 nM. Therefore, the H^+ countertransport mechanism is not bioenergetically unfavorable to operation of the pump under normal conditions. Under ischemic conditions, acidification of the cytoplasm might assume importance comparable to the [ADP] and [P_i]. The pH dependence of the pump will be the subject of a future publication.

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