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Ca^{2+} Pumping ATPase of Cardiac Sarcolemma is Insensitive to Membrane Potential Produced by K^+ and Cl^- Gradients but Requires a Source of Counter-Transportable H^+

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Summary. The sensitivity of the Ca^{2+} pumping ATPase of bovine cardiac sarcolemma (SL) to changes in membrane potential was studied in a preparation of sealed SL vesicles. Membrane potential was imposed by preincubating the vesicles in media of defined ion composition (K^+, Cl^-, choline$^+$ and gluconate$^-$) and diluting into media of differing ion composition. The durations of the ion gradients and relative ion permeabilities were determined in separate experiments by the dependence of the half time for net K^+ (or choline$^+$) movement coupled with these anions (Cl^- or gluconate$^-$), registered by the fluorescence of 1-anilino-8-naphthalene sulfonate (Chiu, V.C.K., Haynes, D.H. 1980. *J. Membrane Biol.* **56**:203–218). Relative permeabilities were: 1.0, K^+; $\geq$ 10.0, 1 μM valinomycin-K^+; 4.0, Cl^-; 0.66, choline$^+$; 0.38, gluconate$^-$. Durations of the gradients ranged between 17 sec (KCl, valinomycin) to 195 sec (K^+-gluconate$^-$). In separate experiments, active Ca^{2+} uptake was monitored using chlorotetracycline (CTC) fluorescence, a technique validated by 45-Ca^{2+} measurements (Dixon, D., Brandt, N., Haynes, D.H. 1984. *J. Biol. Chem.* **259**:13737–13741). Active Ca^{2+} uptake was initiated in the presence of monovalent ion gradients. The values of the membrane potentials (E_m) imposed by the monovalent ion gradients were calculated using the ion concentrations, their relative permeabilities and the Goldman-Hodgkin-Katz equation. No effect of membrane potential on transport rate was observed ($\leq$4%, for 5–7% SD) for imposed potentials as extreme as $\geq$+71 and $\leq$−67 mV. Formal analysis shows that the above observations are not compatible with models in which the Ca^{2+} pumping ATPase functions in an electrogenic or charge-uncompensated fashion. Further experimentation showed that the pump rate is slowed when uptake is measured at less-than-adequate concentrations of buffer (5 *vs.* 25 mM HEPES/Tris). This, together with further control experiments using nigericin and FCCP, gave evidence that the pump requires a source of counter-transportable H^+ in the vesicle lumen. The above experimentation also underlines the need for control of internal pH to obviate erroneous interpretation of ion perturbation experiments. The results are compared with results obtained with the Ca^{2+} ATPase pump of skeletal sarcoplasmic reticulum.

Key Words Ca^{2+} pumping ATPase · transport ATPase · ion transport · membrane potential · heart · sarcolemma · ionophore

Introduction

Calcium extrusion from cardiac cells of beating heart is accomplished by a Na^+/Ca^{2+} exchanger (Reeves & Sutko, 1979) and by a Ca^{2+}-ATPase (Caroni & Carafoli, 1981*a*,*b*). The action of these two systems is important for removal of Ca^{2+} from the cytoplasm and relaxation of tension from beat to beat in the working heart. The Na^+/Ca^{2+} exchanger (Reuter & Seitz, 1968) has been studied in sealed vesicles prepared from cardiac sarcolemma (SL, Reeves & Sutko, 1979). It has a stoichiometry of three Na^+ per Ca^{2+} (Pitts, 1979). The exchanger has a K_m for Ca^{2+} in the range of 10–40 μM (Reeves & Stuko, 1979; Bers, Philipson & Nishimoto, 1980, Philipson, Bersohn & Nishimoto, 1982) which can be reduced to approximately 2 μM by regulatory phosphorylation (Caroni & Carafoli, 1983). The Ca^{2+}-ATPase, the subject of this communication, is colocalized with the exchanger in the isolated SL vesicles. It has a Hill coefficient of 1.6 in the unactivated state, consistent with the movement of 2 Ca^{2+} per transport event (*cf.* Dixon, 1987; Dixon & Haynes, 1989). It has a K_m for Ca^{2+} of 10 or 1.8 μM, which can be reduced to 300 ± 200 or 64 ± 1.4 nM by regulatory phosphorylation and the action of calmodulin (Caroni & Carafoli, 1981*a*, or Dixon & Haynes, 1989, respectively). Carafoli (1984) reviewed the available information on the two extrusion systems and concluded that the Na^+/Ca^{2+} exchanger predominates in the initial phases of Ca^{2+} removal and that the Ca^{2+}-ATPase becomes more important in determining the final diastolic Ca^{2+} level.

Due to its 3 Na^+/Ca^{2+} stoichiometry and attendant charge imbalance, the exchanger can be driven

by and influenced by membrane potential (E_m).[1] The implications of this for the behavior of the exchanger during the transition from systole to diastole in the beating heart are generally appreciated. On the other hand, it is not possible to make similar predictions for the Ca^{2+} pumping ATPase because the literature lacks definitive in vitro studies addressing the E_m dependence of the pump. If the pump moved 2 Ca^{2+} per cycle in an electrogenic (charge uncompensated) manner, then one would expect that it would be less able to export Ca^{2+} during the diastolic phase because it would have to pump Ca^{2+} against a membrane potential. On the other hand, if the pump worked in a charge-compensated manner, counter-transporting H^+ or K^+, a membrane potential dependence would not necessarily be expected but the pump kinetics would be greatly influenced by the absolute concentrations and availability of these ions. The present study, which is part of general study of Ca^{2+}-ATPase pump mechanism (Dixon, 1987; Dixon & Haynes, 1989), addresses this question using sealed sarcolemmal (SL) vesicles.

The present study will show that imposed membrane potential has no effect on the rate of the Ca^{2+} pump. The authors used a similar approach with the well-characterized Ca^{2+}-Mg^{2+}-ATPase of skeletal sarcoplasmic reticulum (SR) and also found no evidence for a membrane potential dependence of the transport rate or electrogenic transport (Chiu & Haynes, 1980*a*,*b*; Haynes, 1982). This conclusion is at odds with the mechanistic conclusions of a number of SR studies which we have critiqued previously (Chiu & Haynes, 1980*a*,*b*; Haynes, 1982). It can be succinctly and correctly stated that no published study of the SR enzyme has shown membrane potential to increase the Ca^{2+} transport rate by more than 85% or decrease the rate by more than 4%. The authors consider these effects to be too small to constitute evidence for an electrogenic mechanism. The authors' analysis of these studies, and more recent studies is given in Appendix A. A single study reporting electrogenic behavior based on a ca. 20% effect of membrane potential on the SL pump (Kuwayama, 1988) is discussed in Appendix B.

In the present study of the SL Ca^{2+} pump, the E_m is manipulated during transport. Control of E_m is based on determination of the intrinsic permeabilities of the SL membrane, imposition of monovalent ion gradients and use of the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949). This method of E_m manipulation has been used in SL vesicles by a number of investigators and has been supported by studies with E_m-sensitive fluorescent dyes. For example, Bartschat, Cyr and Lindenmayer (1980) and Schilling et al. (1984) used the E_m-sensitive fluorescent dye (DiS-C_3-(5)) to measure membrane potentials generated by manipulations of the internal and external KCl concentrations with and without valinomycin. Bartschat et al. (1980) recorded a fluorescence decrease reflecting an inside negative membrane potential upon dilution of vesicles loaded with 150 mM KCl into a medium containing 147.5 mM choline Cl plus 2.5 mM KCl. In absence of valinomycin the dye response took ca. 2 min to develop, but was stable for at least several minutes thereafter (*cf.* Fig. 1, Bartschalt et al., 1980). This indicated that the K^+ conductance was the dominant conductance of the membrane and that the K^+ gradient was maintained for at least several minutes. Addition of 0.3 μM valinomycin after establishment of the gradient and fluorescence response caused a further decrease in signal (more negative membrane potential) which was expressed instantaneously. The valinomycin-induced decrease was less stable, reversing at a rate of ca. 15% per minute. Schilling et al. (1984) obtained similar results. Manipulations of KCl in the presence of valinomycin have also been used in the study of the Na^+/Ca^{2+} exchanger (Kadoma et al., 1982).

[1] Abbreviations used in this paper are as follows: E_m, membrane potential; SL, sarcolemma; SR, sarcoplasmic reticulum; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; CTC, chlorotetracycline; V_0, maximum rate of Ca^{2+} uptake in the initial phases of uptake (*cf.* Materials and Methods, Chlorotetracycline Method); ANS^-, 1-anilino-8-napthalene sulfonate; val, valinomycin; ψ_o, membrane *surface* potential; M^+, monovalent cation; A^-, anion; V_{max}, maximal value of V_0 at saturating Ca^{2+} concentration; K_m, Ca^{2+} concentration for half maximal V_0; FCCP, carbonyl cyandide *p*-trifluoromethoxyphenylhydrazone; *T*, translocator or Ca^{2+} binding site; $[Ca^{2+}]_o$, $[Ca^{2+}]_i$, Ca^{2+} concentrations outside and inside SL vesicles; $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{pl}$ denote Ca^{2+} concentrations in cytoplasmic and plasma compartments in diastolic phase (this publication only); BLM, black lipid membrane; A23187, Ca^{2+} ionophore.

Materials and Methods

Standard Buffer

Standard buffer was prepared by adding to a concentrated HEPES solution a stoichiometric amount of Tris. The final pH was adjusted to 7.4 at 37°C with HCl or KOH. This buffer, diluted to 25 mM HEPES (and Tris), was used in preparation of SL vesicles and as the medium for most experiments. It contained approximately 5 mM Cl^- and less than 3 mM K^+. Tris is a penetrating buffer which can cross the membrane in both the unprotonated form and in the protonated form as an ion pair with Cl^- (Haynes, 1982). It thus has the ability to deliver HCl to an alkalinized vesicle interior.

Sarcolemmal (SL) Vesicles

Sarcolemmal vesicles were prepared from bovine left ventricles as described previously (Dixon, Brandt & Haynes, 1984; Dixon & Haynes, 1989). 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 quantitation of active uptake.

ANS^- Method of Determination of Monovalent Cation and Anion Permeabilities

The electrically active permeabilities (both relative and absolute values) of the SL vesicles to K^+ and Cl^- and other monovalent ions were deduced from observations of the rate of equilibration of cation (M^+) and anion (A^-) pairs across the membrane. The response of the fluorescent probe 1-anilino-8- naphthalene sulfonate (ANS^-) to the $[K^+]_o/[K^+]_i$ (outside/inside) ratio is a convenient means of monitoring these ion equilibration reactions (Chiu & Haynes, 1980*a*). In the presence of valinomycin (Val) and K^+ it crosses the membrane rapidly as a Val-K^+-ANS^- ion pair (Haynes & Simkowitz, 1977) and is thus sensitive to imbalances in the K^+ concentration across the membrane (Chiu & Haynes, 1980*a*; Haynes, 1982). The anionic probe is also responsive to changes in *surface* potential (Chiu et al., 1980*a*). The amount of ANS^- bound to the inside surface is given by:

$$[ANS^-]_{b,i} = \exp(e\psi_{o,i}/kT)\frac{[K^+]_o}{[K^+]_i}[ANS^-]_o \quad (1)$$

where $[ANS^-]_{b,i}$ is the concentration of ANS^- bound on the inside surface, $[ANS^-]_o$, $[K^+]_i$ and $[K^+]_o$ are the internal and external K^+ concentrations, respectively, and where $\exp(e\psi_{o,i}/kT)$ is the function of electrostatic surface potential $\psi_{o,i}$ of the inside surface (Chiu et al., 1980*a*). ANS^- fluorescence does not respond to membrane potential (E_m), but through the coupling described in Eq. (1) it can give the *appearance* of doing so for the special case where E_m is "clamped" by valinomycin and a K^+ gradient (Haynes & Simkowitz, 1977; or *see* comments of Chiu et al., 1980*b*, on experimentation of Zimniak & Racker, 1978).

The dependence of Eq. (1) has been used to determine the rates of passive monovalent cation (M^+) and anion (A^-) equilibration across skeletal SR membrane (Chiu & Haynes, 1980; Haynes, 1982). The dependence in Eq. (1) is useful because KCl jumps from 10 to 100 mM have a larger effect on $[K^+]_o/[K^+]_i$ than on $\exp(e\psi_{o,i}/kT)$. Rates of K^+ equilibration across the membrane in conjunction with anions (A^-) was measured in $K^+ - A^-$ jump experiments. In these experiments, a rapid initial rise in fluorescence was observed, corresponding to increased ANS^- binding to the inner surface as a consequence of the $[K^+]_o/[K^+]_i$ ratio. This is followed by a decrease in ANS^- fluorescence and binding to the inner surface as $[K^+]_i$ increases due to the influx of $K^+ + A^-$. The half time for the decrease is the measure of the half time of the $K^+ + A^-$ permeability (Haynes, 1982) and has been shown to be in agreement with measurements of the same process using light scattering to read out osmotic shrinkage and reswelling (Kometani & Kasai, 1978). Spot checks in the present (SL) system showed that the $K^+ - A^-$ jumps gave 90° light-scattering changes at 600 nm with kinetics similar to those observed for ANS^-.

In the present work, SL vesicles (30 μg protein/ml) were equilibrated with ANS^- (30 μM) in sucrose (250 mM), Tris 25 mM, HEPES 25 mM, pH 7.4 and 0.1 mM $MgCl_2$ at 37°C. When indicated (*cf.* Table 1), valinomycin was added to the preincubation medium to increase the electrically active K^+ permeability. When sufficient valinomycin is added, the rate of A^- permeation becomes rate limiting to the overall process of $K^+ - A^-$ equilibration observed. The salts to be used in the test jump were added to a final concentration of 50 mM. Time-resolved fluorescence change in ANS^- was monitored at excitation and emission wavelengths 368 and 420 nm, respectively. The electrically active permeabilities of the M^+ and A^- species (P_M and P_A, respectively) were calculated as described in the Results section.

Membrane Potential Perturbations

Concentration jumps of several combinations of K^+ (± valinomycin, choline$^+$, Cl^- and gluconate$^-$ were made and the expected E_m value was calculated from the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$E_m = 61.5 \log \frac{\sum P_M[M^+]_o + P_A[A]_i}{\sum P_M[M^+]_i + P_A[A]_o}. \quad (2)$$

The use of manipulations of KCl concentration in the presence of valinomycin to produce membrane potentials in cardiac SL vesicles has been described earlier (Bartschat et al., 1980; Schilling et al., 1984; Kadoma et al., 1982; Introduction, this communication). Estimation of E_m durations and relevant experimental details are given in the Results section.

Chlorotetracycline Method

The application of the chlorotetracycline (CTC) method to the quantitative determination of the free-Ca^{2+} concentration in the SL lumen ($[Ca^{2+}]_i$), its agreement with $^{43}Ca^{2+}$ measurements, and the measurement of the maximal rate of uptake in the initial phases of uptake (V_0) are as described previously (Dixon et al., 1984; Dixon & Haynes, 1989). Of particular relevance to the present study is the fact that CTC crosses the membrane in the neutral form and that its Ca^{2+} response is thus expected to be unperturbed by E_m (Millman et al., 1980). This expectation is supported by experimentation described herein (*cf.* Figs. 3 and 4). Passive calibrations of the fluorescence response *vs.* $[Ca^{2+}]_i$ were made for all ionic compositions tested (*cf.* Table 2) and applied to the calculation of rates. The effect of ionic composition of the fluorescence response was less than 5% of the measured values. In the ion perturbation experiments described in Table 2, *relative* rates of transport were determined as the rate of CTC fluorescence change observed in the first 20 sec of active uptake. In the experimentation described in Table 3, actual rates (V_0) of active transport were calculated from the fluorescence progress curve at 2 min $\leq t \leq$ 3 min. Values of V_0 are given in terms of free-Ca^{2+} concentration ($[Ca^{2+}]_i$) within the volume of the inside-out-vesicles (mM/min) using the fraction inside-out of 39% determined in these studies (Dixon, 1987; Dixon & Haynes, 1989). The pump rates can also be expressed in terms of nmol free Ca^{2+} per mg protein concentration (nmol/(mg · min)) using measured trapped volume of 2.2 μl/mg (Dixon & Haynes, 1989). Thus a rate of 10 mM/sec corresponds to 22 nmol/(mg · min).

It is appropriate at this point to make a further observation on the methodology. As reported previously, CTC fluorescence shows strict proportionality to $[Ca^{2+}]_i$ for values between 0 and 50 mM (Dixon et al., 1984). Measurements with 45-Ca^{2+} show that the total Ca^{2+} ($[Ca^{2+}]_T$) in the vesicle lumen is the sum of the

Table 1. Rate of cation (M^+) and anion (A^-) equilibration across the cardiac SL membrane measured by the rate of decrease after an $M^+ + A^-$ jump

$M^+ + A^-$ pair jumped: M^+	A^-	$t_{1/2}$ for ANS^- Fl decrease (sec)[a]	Rate-limiting ion	$t_{1/2}$ Limiting (sec)[b]	Relative permeability
K^+	Gluconate$^-$	195	Gluconate$^-$	356	0.38
Choline$^+$	Cl^-	120[c]	Choline$^+$	206	0.66
K^+	Cl^-	85	K^+	136	(1.0)
K^+	Cl^- + 0.1 μM val	17	Cl^-	34	4.0
K^+	Cl^- + 1.0 μM val	17	Cl^-	34	(4.0)
			0.1 μM val-K^+	$\leq$136[d]	$\geq$1.0
			1.0 μM val-K^+		$\geq$10.0[e]

[a] Standard deviations of the $t_{1/2}$ values were approximately 17% of the measured values.
[b] Determined as $t_{1/2(M+A)} = (1/2)(t_{1/2(M)} + t_{1/2(A)})$, derived from the Nernst relationship (Kometani & Kasai, 1978). The individual ion permeabilities are proportional to the $1/t_{1/2(M)}$ or $1/t_{1/2(A)}$ values. The value of $t_{1/2(M+A)}$ is the measured net rate of a pair, such as KCl.
[c] In this case (only), the ANS^- fluorescent response was based on a valinomycin-independent coupling of choline$^+$ and ANS^-, such that [choline$^+$] substitutes for [K^+] in Eq. (1).
[d] The estimate of valinomycin-assisted K^+ permeability is a lower limit, since raising the valinomycin concentration from 0.1 to 1.0 μM did not give a further decrease in the $t_{1/2}$ for KCl permeation.
[e] Contribution assumed to be proportial to valinomycin concentration.

free Ca^{2+} ($[Ca^{2+}]_i$), and the amount bound to a saturable Ca^{2+} binding in the lumen with a maximal capacity equilvalent to 10 mM Ca^{2+} and a *Kd* of 2.5 mM. Both calibration curves (Dixon et al., 1984) and calculations (Eq. 3, Methods, Dixon & Haynes, 1989) show that $[Ca^{2+}]_i$ approximates $[Ca^{2+}]_T$ for $[Ca^{2+}]_i \geq 10$ mM. The V_0 values presented in this study are calculated for this condition and are measures of the absolute rate of the pump. On the other hand, during the first 20 sec of the reaction (data of Table 2), $[Ca^{2+}]_i = 0.45\ [Ca^{2+}]_T$ and corrections must be applied to obtain absolute rates (*cf.* Fig. 3, Dixon & Haynes, 1989). The rate data of Table 2 are presented as relative values.

Results

Assessment of Passive Ion Permeabilities

The passive cation and anion permeabilities of the cardiac SL vesicles were determined using the ANS^- method (*cf.* Materials and Methods; Haynes & Chiu, 1980; Haynes, 1982). The method measures the time required for K^+ to come into equilibrium after a concentration jump of a K^+ salt. This process requires compensating movement of anions and is rate limited by the slower partner. The K^+ permeability can be increased by the addition of valinomycin. Table 1 gives results for the SL vesicles of our preparation. The approximate half time for K^+-gluconate$^-$ copermeation is 195 sec. Comparison with the half time for KCl (85 sec) shows that the gluconate$^-$ permeation was rate limiting when paired with K^+. Similar reasoning shows that choline$^+$ permeation is slower than Cl^- permeation. The comparisons also show that gluconate$^-$ permeability is lower than Cl^- permeability, in agreement with the findings of Kadoma et al. (1982). Table 1 shows that 0.1 μM valinomycin, which gives electrogenic K^+ permeability to membranes (Harris & Pressman, 1967), increases the rate of KCl permeation. Increasing the valinomycin concentration to 1.0 μM did not further increase the rate of KCl permeability. This suggests that the intrinsic Cl^- permeability was greater than the intrinsic K^+ permeability in the vesicles of our preparation.[2] The results show that 0.1 μM valinomycin is sufficient to make K^+ the dominant permeability of the system. Table 1 gives estimates of the relative permeabilities of the cations and anions of this study, calculated by the method of Kometani and Kasai (1978) using the half times ($t_{1/2}$) of the ANS^- response. These relationships are illustrated in Fig. 1.

Lack of Effect of K^+ and Cl^- Gradients and Imposed Membrane Potential on Ca^{2+} Transport Rate

The effects of ion gradients were tested on the Ca^{2+} pump working in the calmodulin-activated state

[2] This result is surprising since the Cl^-/K^+ permeability and conductance ratios for intact ventricular muscle are 0.11 and 0.17, respectively (Fozzard & Lee, 1976). However, the process of isolation of the membrane from the cytoplasm may change these permeabilities. Schilling et al. (1984) reported a P_{Cl}/P_K ratio of 0–0.134 for their isolated vesicles, but noted that many preparations failed to show the polarization response in the absence of valinomycin. Most important is the fact that valinomycin made K^+ the dominant permeability in all systems studied (Bartschat et al., 1980; Schilling et al., 1984; present study).

Table 2. Lack of effect of ion substitutions and membrane potential perturbations on Ca^{2+} transport rate

Incubation medium	→Dilution half life (sec)	Gradient (mV)	(E_m)[a] (%control)[b]	Uptake rate
A. $Choline^+$ Cl^-	$Choline^+$ Cl^-	—	zero 0	(100)
B. $Choline^+$ Cl^-	KCl + val	17	pos. (≥+30)	100 ± 7
C. $Choline^+$ Cl^-	K^+ $gluconate^-$ + val	120	pos. (≥+71)	102 ± 6
D. (Buffer only)	(buffer only)	—	zero 0	(100)
E. (Buffer only)	KCl + val	17	pos. (≥+26)	100 ± 7
F. (Buffer only)	K^+ $gluconate^-$ + val	195	pos. (≥+71)	104 ± 7
G. KCl	KCl	—	zero 0	(100)
H. KCl	$Choline^+$ Cl^- + val	17	neg. (≤−30)	95 ± 7
I. K^+ $gluconate^-$	K^+ gluconate	—	zero 0	(100)
J. K^+ $gluconate^-$	KCl + val	17	~ zero (−7 −0)	100 ± 7
K. K^+ $gluconate^-$	KCl	≥34	neg. (−31)	101 ± 6
L. K^+ $gluconate^-$	$Choline^+$ Cl^- + val	120	neg. (≤−67)	99 ± 5

All media contained 0.5 M sucrose, 0.1 mM $MgCl_2$, 25 mM HEPES and 25 mM Tris, pH 7.4, 10 μM CTC, 1 μM calmodulin and Ca/EGTA buffer adjusted to give $[Ca^{2+}]_o$ = 70 nM. (The sucrose concentration was increased over its normal value (0.25 M) to provide additional osmotic buffering.) The cations and anions indicated in the table were at 50 mM concentration. Ionic substitutions were made by dilutions (30-fold) as described in the Results section. The dilution medium also contained 2 mM MgATP, which initiates active transport at the same instant that the external ionic medium is changed, and 1 μM valinomycin (val) added immediately before the dilution. Gradient half lives were taken from Table 1 using the $t_{1/2}$ of the fastest copermeating pair or ion, as applicable. The rates of Ca^{2+} transport, measured by the CTC signal, were taken in the first 20 sec and were referred to the respective control values (100) given above them. Experimental and control progress curves were generally observed to be superimposable over the first 3 min of reaction.

[a] Calculated from Eq. (2), the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949), using P_M and P_A values from Table 1. The buffer contained 7 mM Cl^- and 3 mM K^+.

[b] The presented rate data are the average of at least three values (±SD) normalized to the control (no perturbation) data. The control values (V_0) varied from 4.6 to 5.1 mM/min (*cf.* Table 3, Dixon & Haynes, 1989).

with $[Ca^{2+}]$ = 70 nM, near its K_m of 64 nM (Dixon & Haynes, 1989). Figure 2 is a schematic illustrating the use of the ion substitution paradigm (*cf.* Bartschat et al., 1980; Schilling et al., 1984) to impose negative or positive membrane potentials. Active uptake against a positive (inside) membrane potential was tested by preincubating cardiac SL vesicles in a low K^+ medium and introducing the vesicles into a high-K^+ medium at the same instant when active uptake is initiated. Likewise, a high-to-low K^+ perturbation produces a negative (inside) membrane potential. A series of experiments is described in Table 2, which gives the ion substitutions made, the lifetime of the gradient, the sign and magnitude of the E_m and the rate of the active Ca^{2+} uptake measured in the first 20 sec.

Rows A-C of Table 2 illustrate experiments giving positive E_m. Vesicles were loaded with 50 mM $choline^+$-Cl^- in a medium containing all factors required for active uptake except ATP. They were then diluted 30-fold into a medium containing either $choline^+$-Cl^- (control), KCl or K^+-$gluconate^-$ and Mg-ATP to initiate active uptake. Valinomycin was added to the dilution medium immediately before

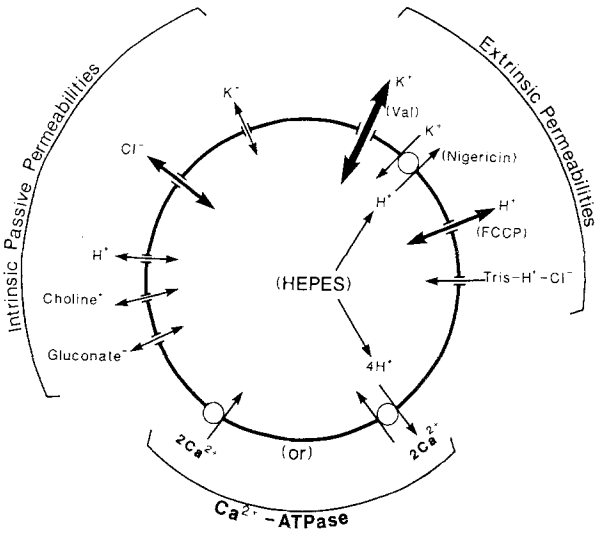

Fig. 1. The intrinsic ion permeabilities of the SL membrane and its modification by ionophores. The thickness of the arrows indicate the relative magnitude. Symbols involving circles indicate carriers (electrogenic or exchange). Valinomycin and FCCP are indicated as simple arrows for clarity although they actually function as mobile (electrically active) carriers. The remaining figures will show only those mechanisms relevant to the experiment under consideration

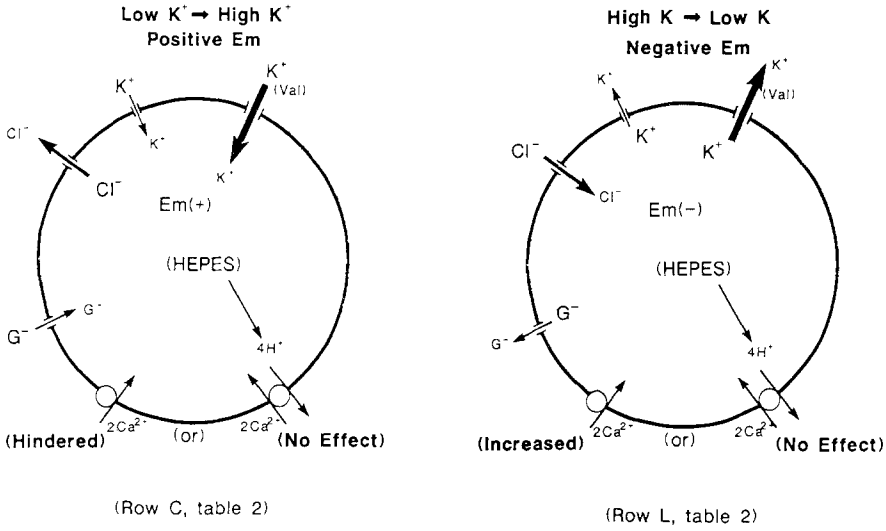

Fig. 2. Expected effect of ionic manipulations on membrane potential and Ca^{2+} transport. The figure depicts the gradients and directions of ion movement at the instant of dilution for the experiments of rows C and L of Table 2

the dilution. The K^+ for choline$^+$ substitution is expected to generate an inside postive potential. The initial rates of active Ca^{2+} uptake were determined using chlorotetracycline (CTC) fluorescence. Table 2 shows that the KCl for choline$^+$ Cl^- substitution does not alter the initial rate from control (choline$^+$ Cl^-) values (row B *vs.* A). The K^+ gradient and valinomycin are expected to generate a positive (inside) potential. In this case, the half life of the gradient was 17 sec, a time comparable to that of the rate measurement.

Row C shows the lack of effect of an inwardly directed K^+ gradient and outwardly directed Cl^- gradient in the presence of impermeable counterions (gluconate$^-$ and choline$^+$, respectively). In this case, the half life of the K^+ (and Cl^-) gradient is expected to be 120 sec, the equilibration time of choline$^+$ in concert with Cl^-. The half life of the gradient is six times longer than the duration of the Ca^{2+} transport rate measurement. Large positive membrane potentials are expected from the effects of the K^+ and Cl^- gradients. Active Ca^{2+} uptake at the control rate was observed, leading to the conclusion that positive membrane potential is without effect on the pump. These observations are repeated in the absence of choline in rows D-F.

Table 2 shows that a negative membrane potential does not increase the rate of the pump (row H *vs.* G and rows K and L *vs.* I). In the Discussion section, we will show that these observations are at odds with an electrogenic model of Ca^{2+} pump function. As an aid to evaluation of these noneffects, the membrane potentials were estimated using the Goldman-Hodgkin-Katz equation and the permeability data of Table 1. Table 2 shows that the imposed membrane potentials, conservatively estimated, varied between +71 and −67 mV (rows C and K, respectively). It is possible that these two extremes of imposed potential could have been as large as +104 and −104 mV (the Nernstian limit) since our experimentation gave only the lower limit of the valinomycin contribution to K^+ permeability. It is further noted that the gradients supporting these extreme E_m values have half lives of ca. 120 sec, or six times the duration of the Ca^{2+} uptake rate measurement. Also, the control experiments and consistent lack of an E_m effect on Ca^{2+} rate for a large number of ion combinations make it unlikely that a true E_m effect could have been obscured by some other effect of ionic conditions. The insensitivity of the pump to monovalent cations has already been presented (Dixon & Haynes, 1989).

Requirement of H^+ Buffer in Lumen or H^+ Permeability for Maximal Rates of Transport

A nonelectrogenic mechanism requires that cations be counter-transported or that anions be cotransported by the pump. In a previous communication (Dixon & Haynes, 1989), we have shown that the pump rate is indifferent to the presence or absence of 160 mM KCl when the medium contains 25 mM HEPES/Tris. This was taken as evidence that K^+ is not counter-transported and that Cl^- is not cotransported on the pump. Table 3 gives evidence that there must be a source of exportable H^+ in the lumen for the pump to operate at maximal rates. Rows A and I show that maximal rates are observed in the presence of 25 mM HEPES/Tris and that under this condition, the uptake reaction is indifferent to the presence or absence of 250 mM KCl, in agreement with our previous findings. Rows A-D and I-L

Table 3. Requirement of exportable H^+ in the vesicle lumen for maximal transport rate

0.5 M Sucrose or 0.25 M KCl	Concentration of HEPES/Tris (mM)	Ionophore or protonophore:	Rate: V_0 (mM/min)
A. Sucrose	25	None	4.7 ± 0.3
B. Sucrose	25	Nigericin	4.9 ± 0.4
C. Sucrose	25	FCCP	5.1 ± 0.6
D. Sucrose	25	Valinomycin	4.7 ± 0.5
E. Sucrose	5	None	0.9 ± 0.4
F. Sucrose	5	Nigericin	1.0 ± 0.4
G. Sucrose	5	FCCP	3.6 ± 0.7
H. Sucrose	5	Valinomycin	0.9 ± 0.3
I. KCl	25	None	5.0 ± 0.2
J. KCl	25	Nigericin	5.3 ± 0.6
K. KCl	25	FCCP	4.7 ± 0.7
L. KCl	25	Valinomycin	4.8 ± 0.6
M. KCl	5	None	1.1 ± 0.3
N. KCl	5	Nigericin	4.3 ± 0.4
O. KCl	5	FCCP	4.1 ± 0.6
P. KCl	5	Valinomycin	1.0 ± 0.2

Ionophore/protonophore concentrations were 1 μM nigericin, 10 μM FCCP and 1 μM valinomycin. Other experimental conditions were identical to those of the legend of Table 2, with the exception of 0.5 M sucrose *or* 0.25 M KCl and 5 or 25 mM HEPES/Tris and the time of the rate measurements (2 min ≤ t ≤ 3 min).

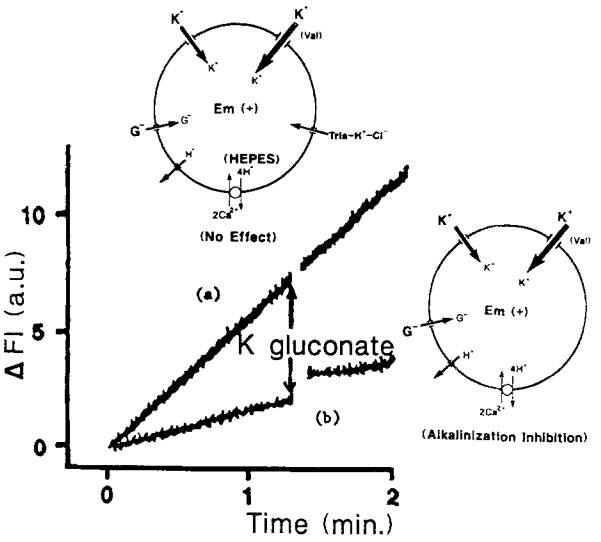

Fig. 3. Effect of K^+-gluconate$^-$ jump in the presence of valinomycin on the progress curve of the active transport reaction. The SL vesicles were preincubated in a medium identical to that described in the legend of Table 2 except that valinomycin was absent and the HEPES/Tris concentrations were varied: curve *a*, 25 mM; curve *b*, 5 mM. The medium did not contain K^+, Cl^-, choline$^+$ or gluconate$^-$. Active uptake was initiated at (t = 0) by dilution into the otherwise identical medium containing 2 mM Mg-ATP as described in the Table legend. K^+-gluconate$^-$ was added to a final concentration of 25 mM at the point indicated. Valinomycin (1 μM) was also added at this point. The schematics interpret the results in terms of the charge-compensated (Ca^{2+}/H^+) model of Ca^{2+} pump function

also show that in the presence of 25 mM HEPES/Tris the uptake reaction is indifferent to the presence or absence of nigericin, FCCP or valinomycin. Table 3 also shows that when the HEPES/Tris concentration is only 5 mM, low rates of transport are observed (row E *vs.* A; row M *vs.* I). These low rates cannot be improved by the addition of 250 mM KCl (row M *vs.* E). The above observations show that K^+ and Cl^- can be eliminated from the medium without affecting the transport rate. Thus K^+ and Cl^- are not transported ions or cofactors. The observations also show that H^+ is an essential part of the transport mechanism. For optimal rates, an adequate combination of buffer capacity in the vesicle lumen (HEPES + Tris) and permeable buffer (Tris, *cf.* Haynes, 1982) must be present. When a source of internal H^+ is absent, the pump becomes inhibited.

The remaining data in Table 3 show how the ionophores and protonophores can be used to relieve the inhibition seen at low buffer concentration. Table 3 shows that nigericin can relieve the inhibition when KCl is present (row N *vs.* M), but not when KCl is absent (row F *vs.* E). This is expected for the known K^+/H^+ exchange activity of this ionophore which can deliver H^+ to the vesicle lumen (*cf.* Fig. 1). Table 3 also shows that FCCP can relieve the inhibition (row O *vs.* M). This protonophore supplies electrically active H^+ permeability. Together with the existing K^+ and Cl^- permeabilities (Fig. 1; Table 1), this is sufficient to deliver H^+ to the vesicle lumen. Table 3 also shows that valinomycin, which supplies electrically active K^+ permeability (only), does not relieve the inhibition.

Additional Tests for Electrogenic Pump Mechanism

Since the CTC technique allows continuous monitoring of the transport process, we devised experiments to test the effect of imposition of a membrane potential while the transport process was in mid-reaction ($[Ca^{2+}]_i$ = 10 mM). This is a final test for electrogenicity and an additional control against the possibility that true changes in pump activity were being masked by changes in low-affinity binding activity in the lumen. Figure 3 (curve *a*) shows the effect of a K^+-gluconate$^-$ jump in the presence of valinomycin during transport. At high buffer concentration, maximal rates are observed which are not attenuated by the K^+ jump designed to give a positive membrane potential. This is in agreement with the observations of Table 2 (row F. *vs.* D) and

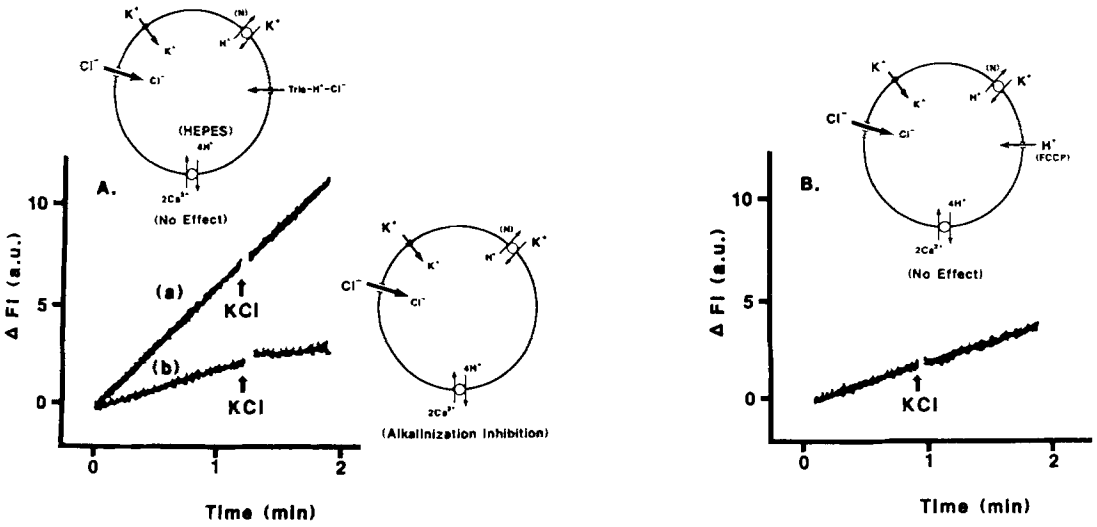

Fig. 4. Inhibition of pump at low buffer concentration by K^+ + nigericin-induced alkalinization. Active uptake was initiated as in Fig. 3 in the absence of valinomycin but in the presence of 1 μM nigericin. At the point indicated, KCl was added to a final concentration of 20 mM. (*A*) curve *a*: The buffer was 25 mM HEPES/Tris; curve *b*: The buffer was 5 mM HEPES/Tris. (*B*) The buffer was 5 mM HEPES/Tris and the medium also included 10 μM FCCP. The schematics interpret the results in terms of the charge-compensated (Ca^{2+}/H^+) model of Ca^{2+} pump function

with the indifference of the pump to the presence of K^+. The absence of a discontinuity in the trace can be taken as additional proof that the CTC response to $[Ca^{2+}]_i$ is not affected by changes in E_m. The above experimentation shows that the pump is not sensitive to E_m but does require a source of counter-transportable H^+.

Effect of Ion Gradients at Low Buffer Concentration

A corollary to the above is that M^+ and A^- gradients imposed at low buffer concentrations can perturb the internal pH, thereby affecting pump performance. The additional experimentation presented in Fig. 3, shows that a K^+ gluconate$^-$ gradient at low internal buffer capacity can have this *indirect* effect on the pump. This is illustrated by curve *b* and the accompanying schematic. At low buffer concentration the K^+ gradient is expected to produce an oppositely directed gradient (alkalinization), to the extent that H^+ is permeable. Figure 3 also shows that the K^+ jump results in a rapid increase in CTC fluorescence attributable to an increase in CTC responsiveness to internal Ca^{2+} upon alkalinization of the lumen (Millman et al., 1980; Haynes, 1982).[3] After this rapid change, the rate of the Ca^{2+}-transport-associated fluorescence increase is slower. This is also the result of internal alkalinization, which would be expected to slow the rate of a pump which expels H^+. The above-described effects are due to H^+ movement rather than membrane potential since they are not observed at high buffer concentration.

In Fig. 4, the internal alkalinization effect was produced and studied more directly using nigericin, which catalyzes H^+ for K^+ exchange, and an inwardly directed K^+ gradient. Valinomycin was omitted and a KCl jump was substituted for the K^+-gluconate$^-$ jump, to minimize membrane potentials generated by the ion gradient. Figure 4*A*, curve *b*, shows that in the presence of nigericin at a low buffer concentration (only), a K^+ (Cl^-) jump also produces an instantaneous jump in fluorescence similar to that of Fig. 3 (curve *b*). Following this, a decreased rate is observed. Both of these effects are expected for alkalinization (curve *b*, Fig. 4*A*). Curve *a* of Fig. 4*A* shows that the effect is absent at high buffer concentration. Figure 4*B* shows that the alkalinization effect of the K^+ jump in the presence of nigericin at low buffer concentration is abolished by FCCP, which can deliver H^+ to the vesicle lumen. The above experiments underline the need for control of internal pH during ion perturbation experiments. In the absence of such knowledge or control, pH effects could be falsely interpreted as membrane potential effects.

[3] For example, alkalinization from pH 7.4 can increase the percent of the anionic form of uncomplexed aqueous CTC species from 80% to approximately 100%, thereby increasing the apparent Ca^{2+} affinity by 25%. Alkalinization can also increase the negative surface charge intrinsic to the membrane, thereby increasing the amount of Ca-CTC complex binding to the surface without requiring an increase in the free internal Ca^{2+}. Therefore, to maintain CTC fluorescence as a pure indicator of free internal Ca^{2+}, pH variations in the lumen must be suppressed by adequate buffering and use of permeable buffers (Millman et al., 1980; Haynes, 1982).

Table 4. Expected properties for electrogenic and electrically compensated mechanisms of Ca^{2+}-ATPase function for selected forms[a] of the translocator (T)

Model number	Overall mechanism	Charges of translocator (T)				E_m-sens. step?
		T forward form	T-Ca_2 forward form	T or T-H_4 return form		
1.	Electrogenic	0	+4	(T)	0	Forward crossing
2.	Electrogenic	−4	0	(T)	−4	Return crossing
3.	Charge-compensated	0	+4	($T - H_4$)	+4	Forward & return
4.	Charge-compensated (preferred model)	−4	0	($T - H_4$	0	None

[a] Only the limiting cases are considered.

Discussion

The most important finding of the present study is that the cardiac SL Ca^{2+} pump does not respond to imposed membrane potential. We conclude from this that the pump is not significantly electrogenic. Kuwayama (1988) came to the opposite conclusion based on ≤20% effects of valinomycin on the rate of the same pump. We believe that the effects reported by him are too small to form the basis of a quantitative argument for an electrogenic mechanism. Our analysis is given in greater detail in Appendix B. The lack of a significant E_m dependence of the pump rate is of considerable mechanistic and physiological consequence, which will be discussed in the next subsection.

The second most important finding of the present study is that the pump requires a source of internal buffer capacity or diffusible buffer. A third finding is that the pump is slowed by conditions which alkalinize the lumen of the vesicle. The above suggests that H^+ is a counter-transported species. Further evidence for this, is found in the pH dependence of the V_{max} and the K_m for Ca^{2+} (Dixon, 1987; and *in preparation*). In this regard, it is of interest that Smallwood et al. (1983) have given evidence that the plasmalemmal Ca^{2+} pump of the erythrocyte counter-transports H^+.

Mechanistic Implications of the Lack of E_m Dependence

The demonstrated insensitivity of the rate of the pump to positive or negative membrane potential shows that the pump does not have a rate-limiting step whose equilibrium or kinetics is influenced by membrane potential. Our experimentation was for $[Ca^{2+}]_o$ values near the K_m, allowing for the expression of both K_m and V_{max} effects, had they been present. Computer-assisted kinetic analysis of the skeletal SR Ca^{2+} pump has shown the expression of maximal velocity requires that the enzyme be distributed between the many states of its cycle (Haynes & Mandveno, 1987) In fact, a number of states are rate limiting; increasing or decreasing their rate constants increases or decreases the V_{max} (Haynes & Mandveno, 1987). It is thus unlikely that the cardiac SL Ca^{2+} pump could be intrinsically electrogenic (engaging in charge-uncompensated Ca^{2+} transport) without having at least a small dependence on membrane potential. In order to approach the electrogenicity question in a more formalistic manner, we will consider two limiting cases for the electrogenic model and two limiting cases for the charge-compensated model (Table 4). For the *electrogenic model*: (i) If the translocator (T) or Ca^{2+} binding site is uncharged, the forward crossing (inward reorienting) complex would have a +4 charge and would be electrogenic and voltage sensitive. (ii) If the translocator bore a −4 charge, then the unladen return would be voltage sensitive. The *charge-compensated* model can also be analyzed under these two limiting assumptions: (iii) If the translocator were neutral, both its forward and return-crossing complexes would be +4 charged and voltage sensitive. The pump would show voltage sensitivity, but of an indeterminate direction. (iv) If the translocator bore a −4 charge, but crossed (or reoriented) only in the fully charge-compensated form, no voltage sensitivity would be observed.[4]

[4] The model considers only the limiting cases with regard to the charge of T. The term "crossing" is considered operationally identical to "reorientation" of the binding site. In either case, electrical work (equal to the product of the charge of the complex and the voltage difference of the two aqueous phases) must be done to accomplish the process. Since our experiments were done at $[Ca^{2+}]$ near the K_m of the enzyme, membrane potential sensitive changes in affinity, had they been present, would also have been encompassed. In considering the electrical work involved in net transport, the return of the binding site must also be taken into account. None of these requirements cannot be obviated by use of the term "conformational change". In the same sense, studies of active Ca^{2+} transport which depict the net process as simply $Ca^{2+}_{out} \rightarrow Ca^{2+}_{in}$ must be considered incompetent from the electrochemical point of view.

Table 5. Calculated equilibrium value of $[Ca^{2+}]_{cyt}$ as a function of the number of uncompensated charges per transport event (n) for selected conditions

n value	[ATP]/[ADP]	$[P_i]$ (mM)	E_m (mV)	Descriptor	$[Ca^{2+}]_{cyt}$ (nM)
4.0	10.0	0.1	−61.5	Normal	211
3.0	10.0	0.1	−61.5	Normal	67
2.0	10.0	0.1	−61.5	Normal	21
1.7	10.0	0.1	−61.5	Normal	15
1.7	10.0	0.1	−78.0	Normal	25
4.0	1.0	0.1	−61.5	Ischemic	666
2.0	1.0	0.1	−61.5	Ischemic	211
1.0	1.0	1.0	−61.5	Ischemic	67

Calculated for a stoichiometry of 2 Ca^{2+}/ATP according to Eq. (2) using $K_{eq} = 9.02 \times 10^6$ M (*cf.* Trevorrow & Haynes, 1984) and taking $[Ca^{2+}]_{pl} = 2$ mM.

We prefer model 4 because it predicts the absence of membrane potential effects and the presence of pH effects.

Implications for Ca^{2+} Pump Function in the Beating Heart

The Ca^{2+} pump is considered to make its greatest contribution relative to the Na^+/Ca^{2+} exchanger in the diastolic phase in which the membrane is repolarizing (Carafoli, 1984). An electrogenic mechanism would require the pump to expel Ca^{2+} against both its gradient and the electrical force of the membrane potential. A charge-compensated or ion-exchanging Ca^{2+} pump would be spared this second impediment to transport. Furthermore, mathematical and thermodynamic analysis places substantial limits on how large the degree of uncompensation of the charge can be. Given a tightly coupled electrogenic pump with a stoichiometry of 2 Ca^{2+} moved per transport event (Dixon & Haynes, 1989) and ATP split, the stoichiometric equation for the "half-cell" is:

$$2\ Ca_o^{2+} + ATP \leftrightarrow 2\ Ca_i^{2+} + ADP + P_i \qquad (3)$$

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 counter-movement of cations (M^+)

$$4\ M_i^+ \leftrightarrow 4\ M_o^+ \qquad (4)$$

or by co-movement of anions (A^-)

$$4\ Cl_o^- \leftrightarrow 4\ Cl_i^- \qquad (5)$$

or by any suitable combination of the two. In order to consider the plausibility of electrogenic models in a most general way, we will define n as the number of uncompensated charges moved by each pump event. Thus a tightly coupled pump which counter-transports 1 H^+ would have $n = 3$, with the processes of Eqs. (4) and (5) giving the remainder of the charge compensation and having stoichiometries, which add up to 3. Applying this to the heart cell for the case that the Ca^{2+} pumping ATPase was the sole determinant of the Ca^{2+} concentration in the diastolic phase ($[Ca^{2+}]_{cyt}$) gives:

$$[Ca^{2+}]_{cyt} = \frac{[Ca^{2+}]_{pl}}{((K_{eq} * [ATP]/([ADP] * [P_i])) * (10 ** (n * E_m/61.5\,mV)))^{1/2}} \qquad (6)$$

where * represents multiplication, ** represents exponentation, where the subscripts cyt and pl refer to the cytoplasm and plasma, where K_{eq} is the equilibrium constant for ATP hydrolysis, E_m is the membrane potential and where 61.5 mV is the value of RT/F appropriate to 37°C. Table 5 gives equilibrium values of $[Ca^{2+}]_{cyt}$ calculated for a physiological $[Ca^{2+}]_o$ and for selected values of [ATP]/[ADP], $[P_i]$ and E_m and for various degrees of electrogenicity ($1.0 \leq n \leq 4.0$). The calculations show that thermodynamics does put serious restrictions on the value of n. Table 5 also shows that $n \leq 3.0$ is required for the pump to be able to reduce $[Ca^{2+}]_{cyt}$ to 67 nM under conditions appropriate to the diastolic phase normal heart (reasonable adenylate energy charge and $E_m = -61.5$). If the pump is to function under conditions appropriate to initial ischemia (elevated ADP and P_i, membrane still polarized), n can be no larger than 1.0. These calculations are considered for illustrative purposes only; the reader may wish to perform calculations using other values. The calculations do put severe restrictions on the degree of charge imbalance which would be permitted with the pump working at a stoichiometry of 2 near its K_m of 64 nM (Dixon & Haynes, 1989). The restrictions are probably more severe than calculated since it is improbable that a process as important as Ca^{2+} extrusion would be driven by a reaction close to equilibrium.

It must be noted that the thermodynamic restrictions calculated above would become completely inoperative if the pump worked with a Ca^{2+}/ATP stoichiometry of 1.0. Caroni et al. (1983) reported "an approximate stoichiometry to ATP of 1" with reconstituted cardiac SL Ca^{2+} pump. Their experiment (*cf.* Fig. 4, Caroni et al., 1983) was based on a comparison of the rate of Ca^{2+} uptake (monitored by a Ca^{2+}-specific electrode) with the rate of H^+ production (monitored by a pH electrode), assuming a ratio of 0.7 H^+ produced per

ATP split. Due to the broad implications of the reported stoichiometry, the present authors have evaluated the experiment depicted in Fig. 4 of Caroni et al. (1983) in attempt to determine whether a stoichiometry of 2 is ruled out by that experiment. While the present authors felt themselves unable to make accurate rate evaluations from the slopes of that figure, they were able to calculate that between the instant of ATP addition and A23187 addition, 34.5 nmol of Ca^{2+} were removed from the medium and 11.6 nmol of H^+ appeared in the medium. Using the 0.7 H^+/ATP ratio, this would correspond to a Ca^{2+}/ATP ratio of 2.0. It would thus seem that additional information or experimentation would be necessary to exclude a stoichiometry of 2. Indirect evidence for a stoichiometry of 2 lies in the Hill coefficient of the rate *vs.* $[Ca^{2+}]_o$ characteristic discussed earlier in this communication and in the dependence of the Ca^{2+} gradient on the [ATP]/([ADP] * [P_i]) ratio (Dixon, 1987, Chap. 4). The latter, which demonstrates conformance with Eq. (6) for variation of [ADP], will be the subject of a future publication.

In conclusion, the present study has shown that the Ca^{2+} pumping ATPase of cardiac sarcolemma is indifferent to membrane potential and appears to use H^+ as a counter-transported ion.

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Appendix A

Critical Review of Reports of Electrogenic Behavior of the Ca^{2+} Pump of Skeletal Sarcoplasmic Reticulum

The claims of demonstration of electrogenic behavior of the Ca^{2+}-Mg^{2+}-ATPase of skeletal SR have been made by a number of authors. The studies can be conveniently classified by strategy and host membrane: (*i*) E_m clamp of Ca^{2+} pump reconstituted in phospholipid vesicles, (*ii*) E_m "clamp" of Ca^{2+} pump in SR, (*iii*) measurement of E_m produced by the Ca^{2+} pump in SR and (*iv*) measurement of current produced by Ca^{2+} pump in SR added to black lipid membrane. Experiments of types *i* and *ii* can be summarized as follows: Imposition of E_m values as negative as -83 mV has never been shown to increase the rate of the pump more than 85%; imposition of E_m values as positive as $+93$ mV has never been shown to decrease the rate of the pump more than 4%. We consider these effects to be too small to be supportive of an electrogenic mechanism. Experiments of type iii give a maximal pump-generated E_m of $+10$ mV. Experiments of type iv can be summarized as follows: Ca^{2+} transport in SR vesicles apposed to black lipid membranes (BLM) in the presence of Ca^{2+} ionophores and protonophores can result in a trans-BLM current less than 1% of that expected from the estimated surface coverage and expected Ca^{2+} transport activity. All four types of claims for electrogenic behavior of the pump are considered below.

Transport and Effect of E_m "Clamp" of Ca^{2+} Pump Reconstituted in Phospholipid Vesicles

As will be described below, the SR has intrinsic cation and anion channels which must be taken into account in setting E_m. A potentially simpler approach is the purification and reconstitution of the pump in phospholipid vesicles devoid of these channels. Thus if the pump worked by an obligatory electrogenic (charge-unbalanced) mechanism, its activity would be completely abolished when reconstituted into phospholipid vesicles, known to have low K^+ and Cl^- permeability. This expectation is derived from the fact, well known to electrochemists and electro-

physiologists, that large membrane potentials can be created by capacitative separation of chemically insignificant quantities of electrolyte (e.g., Ca^{2+}). Values of biomembrane capacitance are well known, and Faraday's Law and the charge/voltage/capacitance relationship are too fundamental to warrant review in these pages. Zimniak and Racker (1978) reported a reconstitution in which the pump functioned at the rate of 164–229 nmol Ca^{2+} uptake/mg/min, sustained for at least 2 min. Although not stated by those authors, this observation leads to the conclusion that either (*i*) the pump must have been operating nonelectrogenically or (*ii*) appreciable K^+ or anion permeabilities were present, allowing the pump to operate electrogenically. The authors proposed that the pump was operating electrogenically but did not give any information on the K^+ or Cl^- permeability of their vesicles, nor did they offer any explanation as to which counter- or co-ions were thought to move in order to allow *any* movement of Ca^{2+}.

Instead, they reported a small (≤85%) enhancement effect of 2 μM valinomycin on the rate and treated this as evidence for electrogenicity. The authors showed that the ratio of rates $^+$valinomycin/$^-$valinomycin varied between 1.85 and 0.96 for variation of $[K^+]_o/[K^+]_i$ ratio between 0.038 and 38.5, respectively. Assuming a Nernstian relationship (without experimental support), they converted the $[K^+]_o/[K^+]_i$ into E_m values and replotted their data in the form of a valinomycin stimulation *vs.* "ΔE_m val" with values ranging between −83 and +93 mV. The ultimate step of their analysis was the statement that "the potential difference which did not affect the rate of Ca^{2+} transport, +60 ± 10 mV (positive inside) was considered to reflect the membrane potential established by an electrogenic . . . Ca^{2+} uptake . . . ." The implication of this reasoning is that at the $[K^+]_o/[K^+]_i$ corresponding to the "membrane potential" of +60 mV, the rate of the pump is zero. Reference to the last two rows of Table 3 of Zimniak and Racker (1978) shows that they report absolute rates in the range of 187–205 nmol Ca^{2+}/mg/min, showing that this is clearly not the case. These logical inconsistencies render all of the authors' mechanistic arguments untenable. The same can be said of the authors' use of ANS^- as a "probe for membrane potential" (Zimniak & Racker, 1978) using procedures and assumptions which were clearly contraindicated by mechanistic studies in phospholipid vesicles (Haynes & Simkowitz, 1977).

Our critique of the above-described experiments was published (Chiu & Haynes, 1980*b*), was made known to the authors in direct correspondence, and remains unanswered. We feel compelled to repeat these criticisms in greater detail because the "valinomycin ratio experiment" has been treated as a paradigm by other workers (*cf.* discussion of Kuwayama (1988) in Appendix B).

E_m Clamp of Ca^{2+} Pump in Isolated SR

The remainder of the experimentation involving E_m and transport sought to either impose an E_m or to measure an E_m generated by the pump. Both approaches required a knowledge of the intrinsic monovalent cation and anion permeability. McKinley and Meissner (1977, 1978) showed that isolated SR consists of two fractions with respect of alkali cation (M^+) permeability, one containing a channel (Type I) and one with the channel absent (Type II). Research by those authors showed that Type I and Type II comprise two-thirds and one-third of the vesicles, respectively. Further research showed that Type I vesicles have the rank order of electrically active permeability $Cl^- > K^+$, $Na^+ > H^+$ (Kometani & Kasai, 1978; Chiu & Haynes, 1980*a*). Our analysis showed Type II vesicles have electrically active permeability only for H^+ (Haynes, 1982). Experimentation concerning the electrogenicity question has been directed at both fractions.

In an experimental approach similar to that of the present study, we showed that an inwardly facing KCl gradient which was expected to generate a negative E_m did not alter the rate of Ca^{2+} transport in Type I vesicles (Chiu & Haynes, 1980*b*). We took this as evidence against an electrogenic mechanism, since it is unlikely that the pump would be unaffected by membrane potential if any of the transmembrane steps in its mechanism involved the movement of uncompensated charge. Beeler (1980) showed that pump rates observed immediately after a Na^+ for K^+ substitution are 78% greater than those observed when K^+ is kept constant (Fig. 4, Beeler, 1980). He interpreted this result as evidence that a negative E_m increases the pump rate. Ion substitutions designed to give positive E_m values were without effect on the pump rate. Using K^+-gluconate$^-$ for $Tris^+$-gluconate$^-$ substitutions, Meissner (1981) showed a small (50%) increase in rate from an outwardly directed 10-fold K^+ gradient ($E_m = -55$ mV) and small unspecified decrease (estimated as 30%) in rate for an inwardly directed 10-fold K^+ gradient (Fig. 4 in Meissner, 1981). We consider these effects to be too small to be offered in support of an electrogenic mechanism. The experiment presented in Fig. 4 of Meissner (1981) confirmed our finding that the presence of K^+ in the lumen is necessary for maximal rates (Chiu & Haynes, 1980*b*), an observation which we considered adequately explained in terms of the K^+ counter-transport model of the pump first proposed by Kanazawa et al. (1971). Later the observations of Chiesi and Inesi (1980) of H^+ expulsion during Ca^{2+} transport, our observations of the effects of permeant *vs.* impermeant buffers (Haynes, 1982) and the effect of pH on the pump rate (Haynes & Mandveno, 1983) lead us to include H^+ counter-transport in the model.

Other experimentation on the electrogenicity question involved protocols designed to elicit such behavior in Type II vesicles. Meissner (1981) calculated that 0.06–0.75 86-Rb^+ or 22-Na^+ are removed from Type II vesicles per Ca^{2+} accumulated in a Tris-containing medium under the assumption that Ca^{2+} transport in these vesicles was fully active (*cf.* Table IV of Meissner, 1981). From the fact that the above stoichiometries were less than 2 and based on cyanine dye and K^+ or H^+ gradient experimentation, he concluded that the pump was electrogenic. However, he stated that his experimentation did not rule out a directly coupled electrically neutral $2H^+/Ca^{2+}$ exchange. Our experimentation showed that the contribution of Type II vesicles to Ca^{2+} transport (Chiu & Haynes, 1980*b*; Haynes, 1982) is masked, a circumstance suggesting that the above stoichiometries might be revised upwards. The above-cited studies showed that active Ca^{2+} uptake by Type II vesicles could be restored by use of ionophores which conferred a net KCl permeability to the membrane. This was achieved by (*i*) valinomycin at high concentrations at which the ionophore gave net KCl transport by an ion pair mechanism (Chiu & Haynes, 1980*b*) and by (*ii*) the combination of nigericin, which gives K^+/H^+ exchange, and Tris, which transports $H^+ + Cl^-$ by an ion pair mechanism (Haynes, 1982). Analysis showed agreement with models in which the Ca^{2+}-Mg^{2+} ATPase functions as Ca^{2+}/cation exchanger and were in disagreement with models in which the enzyme functions electrogenically.

More recently Morimoto and Kasai (1986) isolated a fraction of vesicles devoid of M^+ and A^- permeability (Type II) and reported that valinomycin unmasked Ca^{2+} transport in this fraction. The present authors take this as a confirmation of their

conclusions described above (Chiu & Haynes, 1980*a*,*b*). Morinoto and Kasai (1986) used the valinomycin to conclude that the pump is electrogenic. We disagree with this conclusion because the effect can also be explained by the ionophore replenishing K^+ as KCl by the ion pair mechanism described above. We further note that the study of Morimoto and Kasai (1986) failed to define the mechanism(s) by which charge was balanced to enable net transport and did not consider H^+ as a counter-transported or reactive species.

Measurement of E_m Produced by the Ca^{2+} Pump in SR

The membrane potential-sensitive cyanine dyes respond to the changes in the Ca^{2+} concentration in the SR lumen (Beeler & Martonosi, 1979; Russell, Beeler & Martonosi, 1979*a*,*b*; Beeler, Russell & Martonosi, 1979). This makes it difficult to detect membrane potential generated by the Ca^{2+} pump by means of these dyes. Beeler, Farmen and Martonosi (1981) produced an extensive study focused on this problem and showed that Nile Blue A has less interference from Ca^{2+} than the cyanine and oxanol dyes. Active Ca^{2+} uptake did not produce absorbance change with this potential-sensitive dye (Fig. 7 of Beeler et al., 1981, and authors' discussion thereof), which can be taken to mean that no pump-driven potential was produced (present authors' interpretation). Active transport caused changes in the absorbance of Di-I-C^i (5) (cyanine) and oxanol VI (Fig. 14 of Beeler et al., 1981). The kinetics of these changes could be slowed somewhat by valinomycin. From this observation Beeler et al. (1981) estimated that the pump generated a membrane potential of about +10 mV in Type II vesicles (*only, cf.* p. 134 of Beeler et al., 1981) during the initial phase of transport, a result which they believed consistent with an electrogenic mechanism of pump function. The present authors believe that a much larger potential would have been generated by a pump operating in Type II vesicles by true electrogenic mechanism in the ionic milieu of that experiment. The present authors hasten to point out that a 10 mV E_m could result from a 10**(10/61.5) = 1.45-fold imbalance in any ion in the system, including H^+ which they believe to be unbalanced in Type II vesicles in the presence of external Ca^{2+} and ATP (Haynes, 1982).

Measurement of Current Produced by Ca^{2+} Pump in SR Added to Black Lipid Membranes

Hartung et al. (1987) presented experiments combining the black lipid membrane (BLM) and SR vesicles, which they interpreted as evidence for the transport event creating a net positive potential in the SR lumen. SR was preincubated with BLM and the vesicle and BLM membranes were shown to be closely apposed but not fused. Initiation of transport in the presence of A23187 and the protonophore 1799 resulted in slow sustained potentials. According to the author's interpretation, the A23187 served to relieve inhibition of the pump by accumulated Ca^{2+} and the function of the protonophore was to deliver positive charge across the two apposed membranes to allow for measurement of current with the macroscopic electrodes. Currents were sustained for ca. 45 sec and were closely associated with the transport process. The authors interpreted this as evidence for intrinsic electrogenicity of the pump.

The present authors believe that these experimental results have alternative interpretations. Major difficulties in interpretation arise from the fact that the SR vesicles were not fused with the BLM but were merely apposed to it, leading to complicated circuits for both the preferred and nonpreferred mechanism. For example, it is possible that the A23187 causes the transported Ca^{2+} to be lost by Ca^{2+}/H^+ exchange which results in increased H^+ concentration in the SR lumen and that the protonophore translates this into a trans-BLM potential and current. This behavior could be the indirect effect of Ca^{2+} by a counter-transporting pump and the paper does not give sufficient information to dismiss the above mechanism. The above alternative would be ruled out by chosing valinomycin as a charge carrier. Hartung et al. (1989) does describe some experimentation using valinomycin in asolectin-reconstituted vesicles, but the measured currents were only 4% of those measured with SR. No information on the reversal potential was given.

Hartung et al. (1987) also describe a "capacitative current" which does not depend on A23187 or protonophore. However, this movement was not considered to be the result of a complete cycle of the pump. Thus, the existence of a capacitative current cannot be taken as evidence for electrogenicity of the total pump cycle. Also, there are many reactions which can produce transient E_m's and capacitative charge movement without having net movement of ions across the bilayer. For example, MacDonald and Bangham (1972) showed that binding of Ca^{2+} to one side of the membrane can set up a capacitive displacement which will be sensed by as a potential difference between macroscopic electrodes in the respective bulk solutions.

The above problems would have been obviated, had the SR vesicles been fused with the BLM membrane. Fusion is technically possible and had, in fact, been performed to characterize the SR K^+ channel in terms of numbers of ions actually moved (Miller, 1978). In the study of Hartung et al. (1987) the weakness of coupling of the trans-BLM current to the Ca^{2+} transport can be readily appreciated by reference to the last paragraph of p. 216 of that paper. It is stated that if 5% of the bilayer were covered by vesicles working at their normal rate, a current of 1,000 nA/cm^2 would have been seen. This can be compared with the $\leq$10 nA/cm^2 actually seen. We believe that the observed currents are an indirect consequence of the pump activity.

Appendix B.

Critical Review of Report of Electrogenicity of Ca^{2+} Pump of Cardiac Sarcolemma

Kuwayama (1988) reported that valinomycin addition increases the rate of active Ca^{2+} accumulation in cardiac SL by 20% under conditions designed to give E_m values of ca. −100 mV and has no effect for conditions designed to give E_m values of ca. +100 mV. The analysis, which bore great similarity to that of Zimniak and Racker (1978), was based on the *ratio* of Ca^{2+} uptake rates in valinomycin-treated to untreated SL vesicles in the presence of ion gradients. The valinomycin ratio was studied with inwardly and outwardly directed KCl gradients calculated to give membrane potentials of ca. −120 to ca. +120 mV. The valinomycin ratio varied between ca. 1.2 and 1.0 (respectively) over this range (*cf.* Fig. 4, Kuwayama, 1988), suggesting a *very small* acceleration of rate for negative E_m. Furthermore, we consider the val-

inomycin *ratio* to be an inappropriate measure for evaluating membrane potential effects on pump rate. It begs the question of what ions were moving in the absence of valinomycin. Kuwayama's (1988) use of the valinomycin carries the implicit assumption that no membrane potential is developed by the KCl gradients, which is in contradiction to the findings of Bartschat et al. (1980), Schilling et al. (1984) and the present study. If the Goldman-Hodgkin-Katz equation can be used to calculate E_m in the presence of valinomycin, it should also be used to calculate E_m in the absence of valinomycin; the change in transport rate should be compared to the change in E_m due to the valinomycin addition.

If it is argued that there is insufficient information on E_m in the absence of valinomycin, then it is still possible to consider the data in the presence of valinomycin and the *absolute rate* of Ca^{2+} transport. If the absolute rates are considered, the direction of the E_m effect, such as it is, is reversed! Figure 1 of Kuwayama (1988) shows that higher absolute rates are observed for positive E_m (43 nmol/mg/5 min, panel *A*) than for negative E_m (32 nmol/mg/5 min).

Kuwayama (1988) presented data giving larger $^{+}$valinomycin/$^{-}$valinomycin rate ratios in the presence of tetraethylammonium or Ba^{2+}. These cations increased the range of variation of the ratio to 1.4–0.6 and 1.9–0.6 (respectively) for $-100\ \text{mV} \leq E_m \leq +100\ \text{mV}$. We question what particular "electrical" properties of tetraethylammonium or Ba^{2+} would be responsible for these modest effects. In written communication in response to the above, Dr. Kuwayama made the following points: (*i*) That his studies were performed on fresh samples. (*ii*) That freezing and storage at —80°C increases the passive permeability of the vesicles to K^+, Li^+ and Cl^-. (*iii*) That the pump is sensitive to monovalent cations. (*iv*) That the pump is activated more strongly by K^+ than by Li^+ and that the difference in absolute rates in Fig. 1*a* and 2*b* of his paper is a result of this. (*v*) That he believes that it would be impossible to distinguish a membrane potential effect from a monovalent cation effect using the absolute rates.