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DETERMINANTS OF CALCIUM LOADING AT STEADY STATE IN SARCOPLASMIC RETICULUM

JOSEPH J. FEHER and F. NORMAN BRIGGS

Department of Physiology, Medical College of Virginia, Richmond, VA 23298 (U.S.A.)

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The determinants of steady-state calcium loading by sarcoplasmic reticulum vesicles were evaluated by measuring the contribution of different pathways of calcium flux to the total calcium flux at steady state. The diffusional passive pathway was least significant at all calcium loads studied. Diffusional passive calcium flux was evaluated by a number of methods which gave comparable results and support its designation as passive and diffusional. These methods included (a) flux measurements with the simple pump-leak system which pertains when acetyl phosphate is used to load the vesicles; (b) flux measurements made after quenching the pump with EGTA; (c) flux measurements made after quenching the pump with glucose plus hexokinase; and (d) evaluation of the effect of pump activity on the efflux of mannitol. The calcium efflux not accounted for by the diffusional pathway was assigned to non-diffusional pathways. Efflux through the non-diffusional pathways required ATP, ADP and extravesicular Ca^{2+}. The ADP-dependent, phosphoenzyme-independent pathway described by Beirao and DeMeis (Biochim. Biophys. Acta (1976) 433, 520–530) was not significantly involved in efflux. We propose that the level of calcium loading achieved at steady state is determined by the levels of the intermediates of the calcium pump which are established at this pseudo-equilibrium condition, these levels being determined by the concentrations of intravesicular and extravesicular calcium ($[Ca^{2+}]_i$ and $[Ca^{2+}]_o$), ATP and ADP. The different levels of calcium loading achieved by skeletal and cardiac sarcoplasmic reticulum are attributed to different nucleotide and calcium kinetics in these two types of sarcoplasmic reticulum and possibly to different intravesicular volumes. Differences in diffusional permeability are not responsible for differences in calcium loading.

Introduction

An important physiological function of the sarcoplasmic reticulum is to serve as a source of calcium to activate the contractile elements of muscle and to sequester activator calcium and thereby cause relaxation of muscle [1–3]. Most of the work on sarcoplasmic reticulum has been performed using fragmented sarcoplasmic reticulum isolated by differential or equilibrium density centrifugation. The fragments of skeletal sarcoplasmic reticulum appear to form right-side-out vesicles which consist of a number of phospholipid classes and a number of proteins [4]. When placed in a medium containing Ca^{2+}, ATP and Mg^{2+}, sarcoplasmic reticulum vesicles accumulate calcium and split ATP until a steady state is reached. It is known that calcium taken up by the vesicles represents active transport into an enclosed vesicular space because the accumulated calcium is rapidly released upon addition of calcium ionophores [5–

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

7]. In this communication we have sought to identify the pathways of calcium flux which are important determinants of the level of steady-state calcium uptake.

Early experiments [8–10] concerning attainment of steady state showed that calcium influx and ATPase activity were inhibited by accumulated calcium [8] and that calcium efflux was decreased by the concurrent reduction in outside calcium [9–10]. Later, Katz and collaborators [6,11,12] found that calcium efflux at steady state in anion-loaded skeletal sarcoplasmic reticulum depended upon the ratio $[Ca^{2+}]_i/[Ca^{2+}]_o$. Because efflux tended to depend on $[Ca^{2+}]_o$ in the same way as ATPase activity, they concluded that the $(Ca^{2+} + Mg^{2+})$-ATPase provided a major pathway for calcium flux. Similar results on the effect of $[Ca^{2+}]_o$ on calcium efflux have been obtained in cardiac sarcoplasmic reticulum [13,14]. All of these later studies [6,11–14] measured calcium efflux as calcium influx at steady-state calcium uptake. Since influx must equal efflux during steady state and since influx is virtually totally depenent upon the operation of the pump, it follows that efflux must depend upon the same factors as influx and therefore must depend upon $[Ca^{2+}]_o$. One cannot conclude from this observation that efflux is via the pump and for the same reason dependence of efflux on $[Ca^{2+}]_i/[Ca^{2+}]_o$ does not establish a role for the pump.

Despite the objections to the methods previously used, the conclusion that calcium efflux at steady state is mediated largely by the pump may be valid. It has been known for some time that at steady state there is a rapid calcium-calcium exchange [15] and rapid ATP-ADP exchange [16]. It is also known that calcium efflux can be coupled to ATP synthesis from ADP and P_i in both skeletal [17,18] and cardiac [19,20] sarcoplasmic reticulum. There are a number of postulated mechanisms for the CA^{2+}-ATPase of sarcoplasmic reticulum [21–23]. Each of these mechanisms invokes transitions between intermediates of the calcium pump and these transitions usually involve binding or desorption of ligands including outside and inside calcium. At steady-state calcium uptake, it is expected that there will be a 'pseudo-equilibrium' [24] between the intermediate states of the enzyme. Since some transitions between intermediates correspond to calcium influx and the reverse transitions correspond to calcium efflux, it is expected that the pump will participate in calcium efflux. Hara and Kasai [25] considered the rate of calcium accumulation to be given as

$$d[Ca^{2+}]_i/dt = \left([Ca^{2+}]_o V/(K_o + [Ca^{2+}]_o)\right) - \left([Ca^{2+}]_i V/(K_i + [Ca^{2+}]_i)\right) - K([Ca^{2+}]_i - [Ca^{2+}]_o) \quad (1)$$

where the first term represents pump-mediated calcium influx, the second term is pump-mediated calcium efflux and the third term represents the balance of two unidirectional diffusional fluxes. Although Hara and Kasai measured net diffusional efflux after adding EGTA to vesicles at steady-state calcium uptake, no values were obtained for the pump-mediated fluxes.

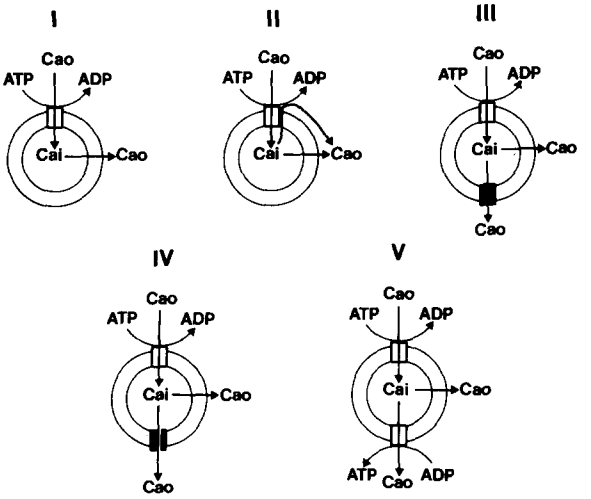

Fig. 1. Models of the possible origin of calcium fluxes at steady state. Model I is a pump-leak model in which calcium influx mediated by the calcium pump is balanced by an equal passive leak. Model II is a variation of the pump-leak model in which the passive leak is accelerated by the running pump. Model III postulates that calcium efflux can occur through two routes: a passive pathway and carrier-mediated facilitated diffusion where the carrier is distinct from the calcium pump. Model IV is similar to Model III except a gated calcium channel, rather than a carrier, is postulated to provide a pathway for calcium efflux. Model V postulates that the calcium pump may mediate both influx and efflux of calcium, their difference being the passive flux. Cao and Cai, extravesicular and intravesicular calcium, respectively.

Thus, a number of investigators have measured total calcium efflux at steady state, but no quantitative assessment has been made of the role of the various possible routes of efflux. These possble pathways for calcium efflux include passive diffusion, carrier-mediated facilitated diffusion, diffusion through gated channels, and pump-mediated transport. Models including these pathways are shown in Fig. 1. Possible mechanisms of calcium efflux mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase have been identified by other investigators. These mechanisms include: calcium flux via an ADP-activated, phosphoenzyme-independent exchange [26]; calcium flux via a phosphoenzyme-dependent pathway not linked to nucleotide flux [23]; and calcium flux via a phosphoenzyme-dependent pathway linked to nucleotide exchange [22]. Our goal has been to determine the quantitative significance of these pathways when calcium uptake reached steady state. In this paper we have partitioned calcium efflux into a diffusional route and non-diffusional routes. The dependence of efflux on ADP, ATP and $[Ca^{2+}]_o$ suggests that a major pathway of efflux is the partial reversal of the ATPase from ADP and phosphoenzyme. A preliminary account of this partitioning of calcium efflux has appeared [27].

Methods

Sarcoplasmic reticulum preparation

Skeletal sarcoplasmic reticulum was isolated from rabbit back muscle by a modification of the methods of Harigaya and Schwartz [28] in which 10 mM imidazole replaced 10 mM sodium bicarbonate, actomyosin was solubilized with 1 M KCl, 10 mM imidazole and the final pellet was suspended in 30% sucrose, 20 mM Tris-HCl, pH 7.0. Oxalate-supported calcium uptake by this mixed skeletal sarcoplasmic reticulum preparation was assayed as described previously [29] and was found to average 8.9 ± 1.3 (S.E.) $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at 37°C. Ca^{2+}-dependent ATPase activity was measured colorimetrically from the same reaction bath used to measure calcium uptake [29] and averaged 6.64 ± 0.67 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The overall apparent coupling ratio averaged 1.34 mol Ca/mol ATP.

Cardiac sarcoplasmic reticulum was isolated from minced canine ventricular myocardium by a modification of a previous method [29], as follows. The tissue was homogenized (1 g tissue/3 volumes 10 mM imidazole buffer, pH 7.0) for 1 min at 4°C in a Sorvall Omnimixer and the resulting homogenate was centrifuged at $4000 \times g$ for 20 min. The pellet was rehomogenized in 3 volumes imidazole buffer and again spun at $4000 \times g$ for 20 min. The combined supernatants were filtered through four layers of cheesecloth and centrifuged at $10000 \times g$ for 15 min. The supernatant was then filtered through eight layers of cheesecloth and centrifuged at $31000 \times g$ for 2 h. The pellets from this spin were rehomogenized in a Potter-Elvehjem homogenizer with Teflon pestle in 1 M KCl, 10 mM imidazole buffer adjusted to 32% sucrose, and centrifuged at $54000 \times g$ overnight. The supernatants were pooled, diluted with an equal volume of cold (4°C) imidazole buffer and centrifuged at $54000 \times g$ for 1 h. The resulting pellet was rehomogenized in 30% sucrose, 20 mM Tris-HCl, pH 7.0 and kept at −20°C. The oxalate-supported calcium uptake rate for the cardiac sarcoplasmic reticulum preparations reported herein averaged 2.04 ± 0.05 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and Ca^{2+}-dependent ATPase activity averaged 3.07 ± 0.32 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The overall apparent coupling ratio averaged 0.68 mol Ca/mol ATP. These specific activities of cardiac sarcoplasmic reticulum compare favorably with those of other investigators [30,31]. The lower apparent coupling ratio of cardiac sarcoplasmic reticulum compared to skeletal sarcoplasmic reticulum is a common observation [32,33].

ATPase activity

ATPase activity was determined from the rate of $^{32}P_i$ release from [γ-^{32}P]ATP as previously described [29].

Steady-state calcium uptake

It is assumed in all experiments that the total calcium in the reaction bath is distributed among four compartments. These are Ca_o, the calcium in solution outside the vesicles; Ca_{bo}, the calcium bound to the outside of the vesicles; Ca_{bi}, calcium bound to intravesicular binding sites; and Ca_i, calcium free within the intravesicular space. Thus the total calcium in the bath is

$$Ca_T = Ca_o + Ca_{bo} + Ca_{bi} + Ca_i \quad (2)$$

and the calcium associated with the sarcoplasmic reticulum is

$$Ca_t = Ca_{bo} + Ca_{bi} + Ca_i \quad (3)$$

The total calcium in the reaction bath was measured by atomic absorption spectrophotometry following wet ashing of the reaction bath including sarcoplasmic reticulum. The total calcium associated with the sarcoplasmic reticulum was obtained by Millipore filtration and was calculated from the total ^{45}Ca in the reaction bath and the ^{45}Ca in the filtrates of the reaction bath.

Diffusional passive calcium efflux (J_p)

Diffusional calcium efflux was meaured after steady-state calcium uptake was reached by quenching pump-mediated calcium fluxes and observing net release of calcium by Millipore filtration. Quenching of the pump-mediated fluxes was produced in two ways: by addition of EGTA to a final concentration of 2.5 mM or by addition of glucose (12.5 mM final concentration) and hexokinase (Sigma Chem. Co., Type V, 0.06 mg · ml^{-1} final concentration). The diffusional efflux was calculated from the slope and initial values of the first-order efflux curves according to

$$J_p = (Ca_t - Ca_{bo})\, d\frac{\ln (Ca_t - Ca_{bo})}{dt} \quad (4)$$

From Eqn. 3 this becomes

$$J_p = d\frac{(Ca_i + Ca_{bi})}{dt} \quad (5)$$

The slope of the first-order efflux curve was determined by linear regression on six points obtained within the first 0.6 min after EGTA quench. The initial value ($Ca_t - Ca_{bo}$) was determined by extrapolating the first-order efflux curve to the time of addition of EGTA quench. This method is illustrated in Fig. 2B.

Unidirectional calcium influx (J_f)

The total calcium influx at steady state, J_f, was estimated from the time-dependent distribution of ^{45}Ca following a pulse of ^{45}Ca added to the pool of calcium outside the vesicles. In this calcium exchange reaction, steady-state calcium uptake was achieved in a reaction bath identical to that used to measure steady-state uptake except that ^{45}Ca was omitted. After steady-state was reached, a pulse of ^{45}Ca was added and aliquots of the bath were filtered for estimation of ^{45}Ca in the filtrates. J_f was calculated, as follows:

Let A^* be the amount of ^{45}Ca in the outside calcium pool of size A (expressed in nmol · mg^{-1}), and B^* be the amount of ^{45}Ca in the inside calcium pool of size B. We write

$$\frac{dA^*}{dt} = J_f\frac{A^*}{A} - J_f\frac{B^*}{B} \quad (6)$$

and

$$A_0^* = A^* + B^* \quad (7)$$

where A_0^* is the total ^{45}Ca in the pulse label and J_f is the total unidirectional flux at steady state in nmol · min^{-1} · mg^{-1}. Substitution, separation of variables and integration allow us to derive

$$\frac{A^*}{A} - \frac{B^*}{B} = \frac{A_0^*}{A} e^{\frac{A+B}{AB}J_f t} \quad (8)$$

This equation describes the relaxation of the differences in specific activity of pools A and B. Using values of A and B determined experimentally, we calculated J_f from the slope of the logarithm of $(A^*/A) - (B^*/B)$ against time. This method is illustrated in Fig. 2.

Results

When cardiac sarcoplasmic reticulum vesicles were placed in a medium containing Ca^{2+}, ATP and Mg^{2+}, they accumulated calcium until a steady state was reached (Fig. 3). Different initial values of outside free calcium were used to produce the different steady states. It is important to note that under the conditions used steady state persists long enough to allow measurement of calcium influx and efflux. Our goal was to identify the pathways for these fluxes and quantitate flux through each pathway.

Validation of methods for diffusional efflux

In all of the models we have considered (see Fig. 1), one component of efflux is passive diffu-

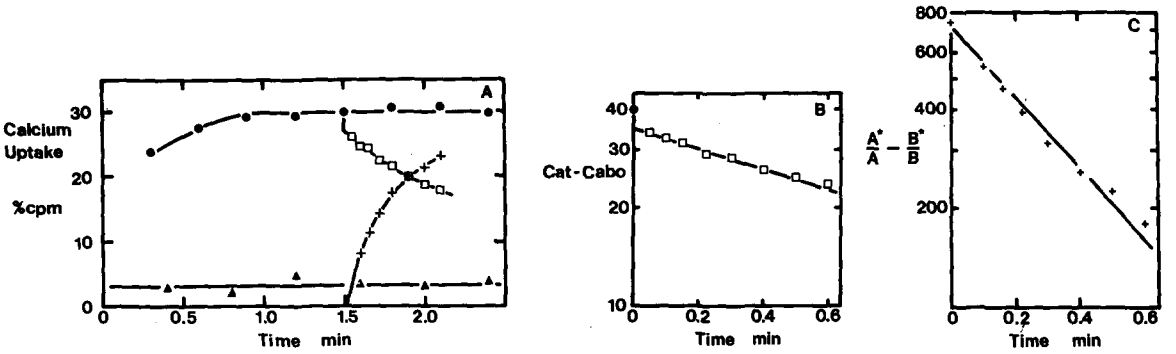

Fig. 2. Method for determining passive efflux and total influx. ^{45}Ca distribution between cardiac sarcoplasmic reticulum and extravesicular free calcium were determined by Millipore filtration. Total calcium uptake was determined by adding ^{45}Ca with unlabeled calcium at the start of the reaction (●). In a separate bath, the binding of calcium to outside sites on the vesicles was determined by replacing ATP with 400 μM β,γ-methylene ATP (▲). The passive efflux was estimated by the net efflux after adding 2.5 mM EGTA to vesicles at steady state (□). The first-order plot of efflux (B) was linear and the value of calcium uptake extrapolated to the time of addition of EGTA gave an estimate of total intravesicular calcium. Calcium uptake in B is given in nmol·mg^{-1} and time refers to the time addition of EGTA quench. Total calcium influx was estimated by adding ^{45}Ca to vesicles at steady-state uptake in a bath initially containing no ^{45}Ca (+). The plot of the rate of equilibration of specific activity (C) gives the influx as a function of its slope. The values of the specific activity are in cpm mg·nmol^{-1}.

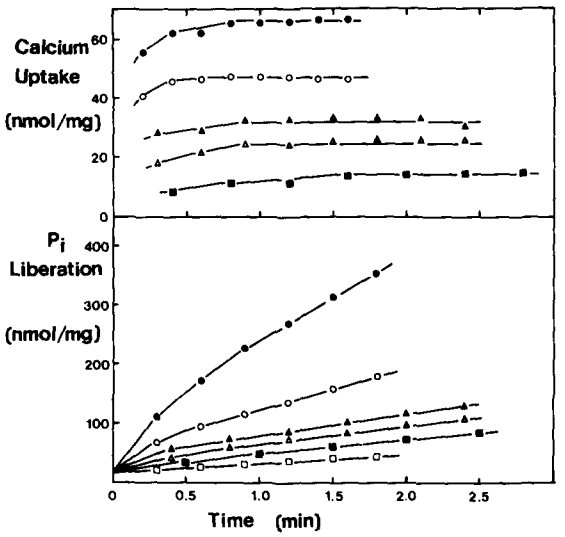

Fig. 3. Steady-state calcium uptake and ATP hydrolysis in cardiac sarcoplasmic reticulum vesicles. Calcium uptake (A) was estimated by Millipore filtration (0.45 μm) of aliquots from a reaction bath at at 27°C containing 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM NaN_3, 2.1 mM $MgCl_2$, 100 μM ATP, 11.0 μM total calcium, 0.112 mg/ml cardiac sarcoplasmic reticulum protein and containing 0 μM EGTA (○); 4 μM EGTA (●); 8 μM EGTA (△); 12 μM EGTA (▲); or 20 μM EGTA (■). ATP hydrolysis (B) was measured by the rate of $^{32}P_i$ liberation from [γ-^{32}P]ATP under the same conditions as in A except 2.5 mM EGTA (□) was also used.

sion. What we required was a measure of calcium flux through this pathway during the steady state when the pump was running. We first approached this problem by measuring the initial efflux after stopping the pump. We then asked two questions: (a) Is the initial efflux after stopping the pump a diffusional flux? (b) Is this initial efflux (taken to be a diffusional flux) referable to the steady state?

If the initial efflux obtained after quenching the pump is a diffusional flux, then it should not depend on the quench method, it should show diffusion kinetics and it should not be due to activity of the (Ca^{2+} + Mg^{2+})-ATPase; that is, the quench methods should in fact quench pump-mediated calcium fluxes. We have used two quench methods. Addition of EGTA quenches the pump by lowering activator calcium, and glucose plus hexokinases quenches the pump by converting substrate ATP to ADP and glucose 6-phosphate. The efflux curves obtained with these two quench methods were not identical. In the case of EGTA quench, there was an initial rapid drop in calcium associated with the sarcoplasmic reticulum followed by a slower and eventually complete release of calcium (Fig. 4). This initial drop was not seen when glucose plus hexokinase was used to quench

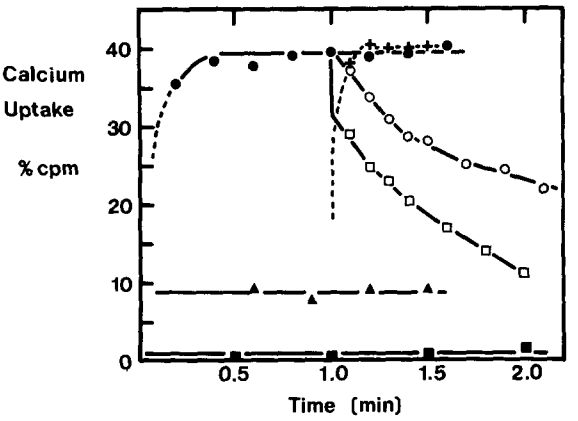

Fig. 4. Comparison of extravesicular calcium binding and calcium efflux with EGTA or glucose plus hexokinase quench. ^{45}Ca distribution was determined by Millipore filtration from the usual reaction bath (see Fig. 3) containing 11.3 μM total calcium and 0.108 mg/ml cardiac sarcoplasmic reticulum protein. Calcium uptake is given as % cpm in order to show data on calcium exchange after attainment of steady state. Control uptake (●) values were the same as those found upon complete ^{45}Ca equilibration added at 1.0 min (+). In this case calcium exchange was too poorly resolved to allow quantitation. Calcium uptake was completely prevented by 2.5 mM EGTA (■). Significant calcium binding occurred when ATP was replaced by β,γ-methylene ATP (▲). Net passive calcium efflux was initiated by adding 2.5 mM EGTA (□) or glucose plus hexokinase (○) after achieving steady-state uptake.

the pump and it was equal to the amount of calcium bound outside (Ca_{bo}, in nmol · mg^{-1}) when ATP was replaced with an equal concentration of the non-hydrolyzable analogue, β,γ-methylene ATP (Fig. 4) or when the accumulated calcium was released with 1 μM A23187 (data not shown). Thus, the initial drop in Ca_t, the total calcium associated with the sarcoplasmic reticulum, upon addition of EGTA was taken to represent desorption of calcium bound to the outside of the vesicles rather than an initial rapid efflux. Following desorption of the outside bound calcium, calcium efflux with the EGTA quench was identical to efflux when glucose and hexokinase were used as quenching agents (Table I).

Passive diffusion should obey Fick's first law:

$$J_p = K([Ca^{2+}]_i - [Ca^{2+}]_o) \quad (9)$$

where J_p is the diffusional flux, K is the permeability, $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ are the inside and outside concentrations of calcium, respectively. Since under the conditions of steady-state uptake $[Ca^{2+}]_o$ is very much less than $[Ca^{2+}]_i$, the contribution of $[Ca^{2+}]_o$ to the net passive diffusional flux is small and can be ignored. The data plotted in Fig. 5

TABLE I

COMPARISON OF CALCIUM INFLUX AND PASSIVE CALCIUM EFFLUX IN CARDIAC SARCOPLASMIC RETICULUM

Steady-state calcium uptake was attained in a reaction bath at 27°C containing 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM NaN_3, 100 μM ATP, 2.1 mM $MgCl_2$, 11.0 μM total calcium, 0.112 mg protein per ml and 0 to 20 μM EGTA. The initial passive efflux was calculated from the linear regression of $\ln(Ca_t - Ca_{bo})$ against time for the first 0.6 min after addition of 2.5 mM EGTA or 12.5 mM glucose plus 0.6 mg hexokinase per ml. Calcium influx was obtained as described in Methods. The free $[Ca^{2+}]_o$ was calculated according to published methods [37].

EGTA (μM)	Steady-state $[Ca^{2+}]_o$ (μM)	Steady-state ($Ca_t - Ca_{bo}$) (nmol · mg^{-1})	Calcium influx (J_f) (nmol · min^{-1} · mg^{-1})	Passive calcium efflux (J_p) (nmol · min^{-1} · mg^{-1})	
				EGTA quench	Glucose + hexokinase quench
0	3.5	60	453	47	46
4	2.3	47	129	37	32
8	1.3	27	73	16	21
12	0.7	22	34	13	13
20	0.3	14	18	6	7

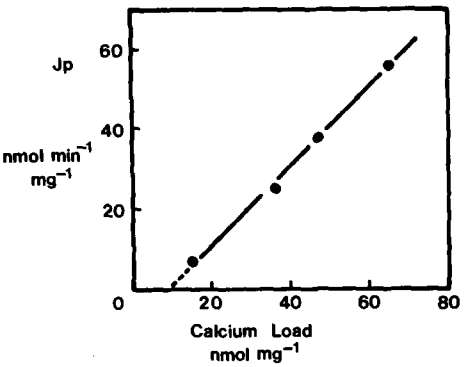

Fig. 5. Relation between passive efflux (J_p) and total intravesicular calcium load. Vesicles were actively loaded at 27°C in a medium containing 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM NaN_3, 400 μM ATP, 2.4 mM $MgCl_2$, 4 μM added Ca, and 0.090 mg/ml cardiac sarcoplasmic reticulum protein. The steady-state load was varied by adding 0, 5, 10 or 20 μM EGTA. Passive efflux was obtained from the first-order efflux curve, as described in Methods.

shows that the initial efflux obtained after addition of EGTA obeys diffusion kinetics as seen by the linear relation between efflux and intravesicular calcium load. This linear relation must become non-linear at lower loads because the linear relation does not extrapolate to zero efflux at zero load. We attribute this to the presence of intravesicular bound calcium (Ca_{bi}) that contributes to the total calcium load but not to the driving force for passive diffusion.

The efflux obtained after EGTA quench was unaffected by an ATP-regenerating system (Fig. 6A) and was not accompanied by a significant

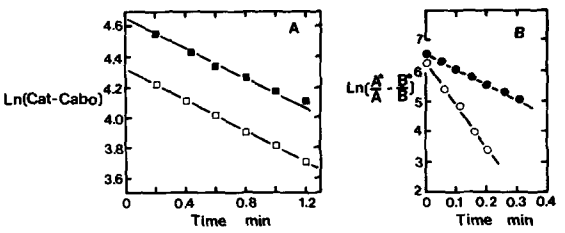

Fig. 6. Effect of an ATP-regenerating system on passive efflux and total influx. The usual reaction mixture was used (Fig. 3) except that total calcium was 9.4 μM and 0.045 mg/ml cardiac sacroplasmic reticulum protein was used. Plots are those described in Fig. 2. Control (□, ○); with 1 mM phospho*enol*-pyruvate and 0.05 mg/ml pyruvate kinase (■, ●). The results were not altered by doubling the concentrations of the regenerating system.

ADP → ATP flux (data not shown). These results and those presented above strongly suggest that the efflux observed upon addition of EGTA or glucose plus hexokinase is a diffusional efflux

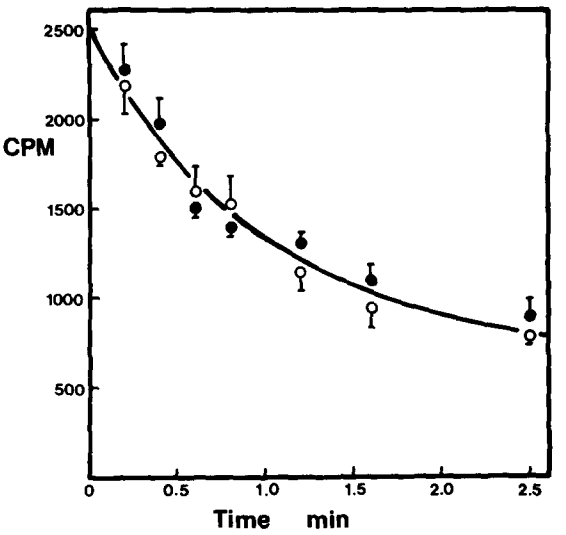

Fig. 7. Comparison of passive efflux with the pump running (●) and the pump stopped (○). Cardiac sarcoplasmic reticulum from three separate preparations were pooled and suspended in 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM NaN_3 and 15% (w/w) sucrose. The mixture was centrifuged at 100000 × *g* for 1 h and the resulting pellet was resuspended in 2 ml load medium containing 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM NaN_3 and 10% surcrose. Final protein concentration was 15.9 mg/ml. The vesicles were passively loaded with [^{14}C]mannitol (1.25 Ci/mmol) by adding a small volume of concentrated [^{14}C]mannitol to an aliquot of the cardiac sarcoplasmic reticulum. Loading was performed at 4°C and the final mannitol concentration was 7.1 mM. After loading for 3 h, the vesicles were diluted 100-fold in a diluting medium (27°C) identical to the loading medium except it contained no mannitol without (○) or with substrates for the Ca^{2+} pump (●); 1 mM ATP, 3 mM Mg^{2+} and 20 μM Ca^{2+}). Aliquots (1 ml) of the diluted cardiac sarcoplasmic reticulum were filtered through Millipore filter (0.045 μm) and washed with 1 ml diluting medium. The filters were then counted by liquid scintillation spectrometry. Counting the filters introduces considerable error due to variation in the amount of diluting medium retained by the filters. The values shown represent the mean ± S.E. for three separate experiments. The ATPase activity at 27°C of the diluted vesicles were determined in a separate, identical experiment in which phosphate liberation from ATP was assayed colorimetrically [42] from aliquots of filtrates from the diluted cardiac sarcoplasmic reticulum and found to be 0.53 μmol·min^{-1}·mg^{-1}. Oxalate supported calcium uptake and ATPase activity of control and loaded vesicles were measured as described previously [29] and it was found that loading did not affect either activity.

which is not due to nucleotide-dependent activity of the $(Ca^{2+} + Mg^{2+})$-ATPase.

Because the diffusional efflux was obtained by procedures which destroyed steady state, it was necessary to establish that the measured diffusional efflux was referable to the steady-state condition. Time-resolved X-ray diffraction studies have shown that during catalytic turnover the Ca^{2+}-ATPase undergoes conformational changes that might affect lipid mobility in the vicinity of the pump [34]. Thus turnover of the pump might affect the diffusional permeability and make it impossible to measure diffusional flux by quenching the pump. To test the hypothesis that turnover of the pump increases diffusional permeability, we observed the efflux of [^{14}C]mannitol from passively loaded vesicles diluted into media containing no substrate or containing ATP, Mg^{2+} and Ca^{2+}. Diffusional calcium flux could not be directly studied because any increase in calcium efflux caused by the activated pump could not be clearly ascribed to a diffusional process. The results (Fig. 7) clearly indicate that mannitol permeability is not affected by activation of the calcium pump. The permeability of [^{14}C]choline was similarly unaffected by activation of the pump (data not shown).

Evaluation of the pump-leak model of steady state

In the pump-leak model of calcium uptake (Fig. 1), steady state is achieved when the inwardly directed calcium transport by the pump is balanced by an outwardly directed diffusional leak. This model can be directly tested by measuring calcium influx and diffusional efflux to determine if they are equal. The unidirectional calcium influx, J_f, and diffusional passive efflux, J_p, were determined in cardiac and skeletal sarcoplasmic reticulum for various levels of steady-state uptake obtained by including low levels of EGTA in the reaction bath. Representative results, shown in Table I and Table II, indicate that J_p is usually much less than J_f when ATP is used as substrate. Since the J_p shown in Tables I and II is the diffusional efflux referable to the steady state, it follows that the simple pump-leak model does not adequately describe steady-state conditions for an ATP-driven system.

As further documentation of the inadequacy of the pump-leak model, we measured calcium influx and diffusional efflux in the presence and absence of an ATP-regenerating system. When the exhaustion of ATP and accumulation of ADP is prevented by an ATP-regenerating system, the steady-state level of calcium uptake is increased [10,35]. If the pump-leak model correctly describes the sarcoplasmic reticulum, then at higher levels of steady-state uptake the diffusional efflux should be increased. Since at steady-state calcium efflux is equal to calcium influx, it follows that calcium influx should also be increased. However, calcium influx at steady state was decreased in the presence of an ATP-regenerating system (Fig. 6 and Table III) as noted by Waas and Hasselbach [36].

TABLE II

COMPARISON OF CALCIUM INFLUX AND PASSIVE CALCIUM EFFLUX IN SKELETAL SARCOPLASMIC RETICULUM

Measurement of calcium influx and passive calcium efflux was as described in Table I except total calcium was 15.5 μM and 0.052 mg protein per ml was used.

EGTA (μM)	Steady-state $[Ca^{2+}]_o$ (μM)	Steady-state $(Ca_t - Ca_{bo})$ (nmol·mg^{-1})	Calcium influx (J_f) (nmol·min^{-1}·mg^{-1})	Passive calcium efflux (J_p) (nmol·min^{-1}·mg^{-1})
0	9.1	100	1370	31
8	2.6	91	1200	28
16	0.7	92	610	24
24	0.3	84	430	23
32	0.2	74	280	15

TABLE III

EFFECT OF AN ATP-REGENERATING SYSTEM ON CALCIUM INFLUX AND PASSIVE CALCIUM EFFLUX

Steady-state calcium uptake was attained in a reaction bath at 27°C identical to that described in Table I except protein concentrations were 0.045 mg cardiac sarcoplasmic reticulum protein per ml and 0.05 mg protein per ml and total calcium were 9.4 μM and 8.5 μM for experiments A and B, respectively. Regenerating system, when added, was 1 mM phospho*enol*pyruvate and 0.05 mg pyruvate kinase per ml reaction bath. Calcium influx and passive efflux were estimated according to the methods illustrated in Fig. 2. The competency of the regenerating system was established by adding [^{3}H]ADP (10 μCi/ml) to the reaction bath and determining the radioactivity in the ATP and ADP fraction separated by thin-layer chromatography [43].

	Steady-state $[Ca^{2+}]_o$ (μM)	Steady-state ($Ca_t - Ca_{bo}$) (nmol·mg^{-1})	Calcium influx (J_f) (nmol·mg^{-1}·min^{-1})	Passive calcium efflux (J_p) (nmol·min^{-1}·mg^{-1})
Experiment A				
Control	4.8	86	729	42
+ regenerating system	3.7	117	246	51
Experiment B				
Control	4.7	66	337	22
+ regenerating system	3.2	85	129	30

This experiment clearly indicates that the pump-leak model is insufficient to describe steady state when ATP is the energy source for calcium uptake.

Evaluation of some non-diffusional pathways of calcium efflux

Since diffusional efflux is much less than calcium influx at steady state (Tables I and II), it follows that there is some non-diffusional efflux which, together with the diffusional efflux, balances calcium influx. Beirao and DeMeis [26] suggest that the calcium pump can act as an exchange carrier. The calcium exchange they describe de-

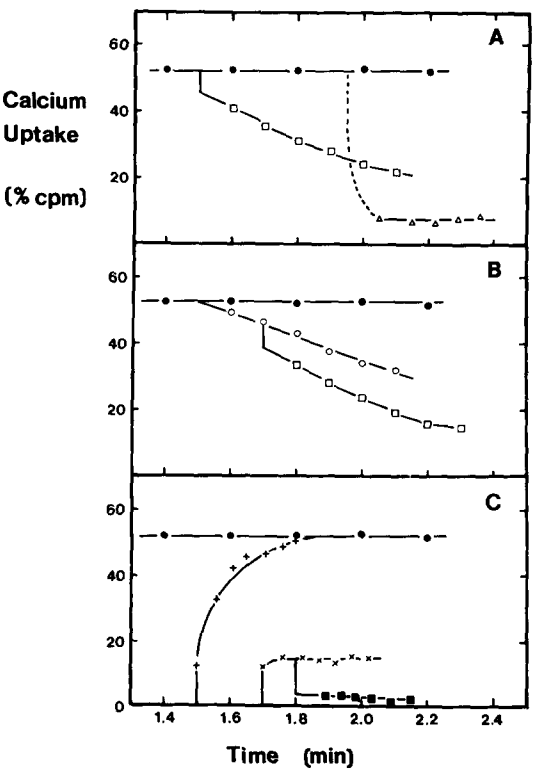

Fig. 8. Test of ADP-activated calcium exchange. The basic reaction bath was described in Fig. 3 except total calcium was 11.2 μM and 0.125 mg/ml cardiac sarcoplasmic reticulum protein was used. Control (●) shows calcium uptake at steady state. Panel A: outside bound calcium at steady state was estimated by extrapolation of the first-order efflux caused by 2.5 mM EGTA quench at 1.5 min. (□) and by adding 1 μM A23187 to dissipate the calcium gradient (△). Panel B: outside bound calcium after glucose plus hexokinase quench was estimated by adding glucose plus hexokinase at 1.5 min and 2.5 mM EGTA at 1.7 min. Extrapolation of the first-order efflux after EGTA to 1.7 min gave the intravesicular calcium at that time. Panel C: calcium exchange was measured by adding tracer ^{45}Ca after steady state was reached in a reaction bath initially containing no ^{45}Ca (+). In a separate reaction, ATPase activity was quenched by glucose and hexokinase added at 1.5 min and calcium influx was estimated subsequently by adding tracer Ca at 1.7 min (×). To test whether apparent calcium uptake was intravesicular, in a third reaction glucose plus hexokinase was added at 1.6 min, ^{45}Ca at 1.7 min and 2.5 mM EGTA was added at 1.8 min (■). Nearly all the ^{45}Ca taken up after glucose plus hexokinase quench was rapidly removed by EGTA. The first point in each influx curve (+, ×) represents the expected calcium binding to extravesicular calcium-binding sites.

TABLE IV

COMPARISON OF CALCIUM INFLUX AND PASSIVE CALCIUM EFFLUX IN VESICLES LOADED WITH ATP AND ACETYL PHOSPHATE

Steady-state calcium uptake was attained in a reaction bath at 27°C containing 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM NaN_3, 3.0 mM $MgCl_2$, 4 μM added calcium, 0.125 and 0.112 mg cardiac sarcoplasmic reticulum protein per ml for experiments A and B, respectively, and 1 mM ATP or 1 mM acetyl phosphate. Calcium influx and passive efflux were determined as described in Table I.

Substrate	Steady-state ($Ca_t - Ca_{bo}$) ($nmol \cdot mg^{-1}$)	Calcium influx (J_f) ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Passive calcium efflux (J_p) ($nmol \cdot min^{-1} \cdot mg^{-1}$)
Experiment A			
ATP	46	191	29
Acetyl phosphate	25	14	13
Experiment B			
ATP	38	225	43
Acetyl phosphate	26	27	39

pends upon ADP and extravesicular calcium but not phosphoenzyme. To determine whether this pathway was operating under our conditions, we quenched the pump and raised the level of ADP by adding glucose plus hexokinase to a reaction bath (Fig. 8) and then added a pulse of ^{45}Ca (Fig. 8C) to measure calcium-calcium exchange. The only calcium exchange observed was attributable to exchange with outside bound calcium and was readily removed by EGTA (Fig. 8). Thus, the calcium exchange pathway described by Beirao and DeMeis is not a quantitatively important pathway in cardiac sarcoplasmic reticulum.

A second pathway of calcium-flux mediated by the pump is dependent on phosphoenzyme but not ADP [23]. To evaluate this pathway, we measured diffusional efflux and calcium influx when ATP was replaced with acetyl phosphate. It is known that acetyl phosphate will support calcium uptake [38,39] and that acetate is quite ineffective in the reverse reaction [39]. The data (Table IV) show that calcium influx was equal to the diffusional calcium efflux when acetyl phosphate was used to support calcium uptake. This is consistent with the pump-leak model of calcium uptake. Since levels of phosphoenzyme with acetyl phosphate should have been equivalent to those observed with ATP [38,39], it follows that there is no significant phosphoenzyme-dependent, ADP-independent calcium pathway in cardiac sarcoplasmic reticulum. The identity of J_f and J_p when acetyl phosphate was used to support calcium uptake provides further support for the validity of our methods of evaluating diffusional efflux at steady state.

Discussion

Calcium uptake reaches steady state when calcium influx equals calcium efflux. This state is achieved by a reduction in influx when $[Ca^{2+}]_o$ decreases and by an increase in efflux as the sarcoplasmic reticulum accumulates calcium. Thus at steady state

$$J_f = J_e \tag{10}$$

where J_f is the total calcium influx and J_e is the total efflux. We have been able to partition J_e into a diffusional component and a non-diffusional component and we have investigated the pathways contributing to the non-diffusional component of efflux.

To partition efflux into diffusional and non-diffusional pathways, it was necessary to devise some method for evaluating diffusional efflux during active pumping. We argue that it is possible to evaluate the diffusional component of efflux at steady state by determining the initial net efflux after quenching ATPase activity. We have conducted a number of experiments that indicate this method measures a diffusional flux that is referable to the steady state. First, initial net efflux measured after quenching the ATPase with EGTA

shows diffusion kinetics provided a correction is made for intravesicular calcium binding (Fig. 5). This suggests the method measures a diffusional flux which is not carrier-mediated [40]. Second, since net efflux was similar when EGTA or glucose plus hexokinase were used as quench agents (Table I), it appears that the diffusional efflux is insensitive to changes in $[Ca^{2+}]_o$ over the range of calcium concentrations investigated. Because the permeability was unaffected by an ATP-regenerating system (Fig. 6 and Table III), the diffusional efflux also does not require ADP. This suggests that the reversal of the $(Ca^{2+} + Mg^{2+})$-ATPase does not contribute significantly to the diffusional efflux. Third, the similarity of J_p and J_f when acetyl phosphate replaced ATP (Table IV) strongly supports the validity of the quench method for evaluating diffusional efflux at steady state. Fourth, because the diffusional efflux of mannitol was unaffected by activation of the $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 7), we propose that diffusional efflux measured with the pump stopped is referable to the steady state when the pump is running.

Determination of J_p and J_f in skeletal and cardiac sarcoplasmic reticulum allowed an evaluation of the pump-leak model of calcium uptake with ATP as substrate. Since J_p was much less than J_f (Table I and II) it follows that the pump-leak model was insufficient to describe steady state. From Eqn. 10 we write

$$J_f = J_e \approx J_p + J_r \tag{11}$$

The total efflux can be partitioned into a diffusional component, J_p, and some other non-diffusional component(s), J_r. Our goal was to identify the pathways for J_r.

Four possible pathways for J_r were considered, as shown in Fig. 1. These were: (a) additional diffusional flux around the running pump; (b) a carrier for calcium distinct from the $(Ca^{2+} + Mg^{2+})$-ATPase; (c) a gated calcium channel; and (d) the $(Ca^{2+} + Mg^{2+})$-ATPase. The first of these, an additional diffusional flux around the running pump, simply re-states the hypothesis that diffusional flux measured when the pump is stopped is not referable to the steady state when the pump is running. We eliminated this possibility by showing that mannitol permeability was unaffected by activation of the pump. We eliminated a carrier for calcium and a gated calcium channel based on the requirements for optimal J_r.

Steady state occurs when influx balances efflux as described by Eqn. 10. Thus the total efflux at the steady-states given in Table I is the total influx, J_f. Addition of EGTA lowers $[Ca^{2+}]_o$ and causes a marked drop in the calcium efflux from that present at steady state to the diffusional efflux present at steady state. Similarly, addition of glucose and hexokinase, which removes ATP, also reduces efflux from J_e to J_p. Thus from Eqn. 11 J_r requires both extravesicular Ca^{2+} and ATP. Addition of an ATP-regenerating system also causes a reduction in the steady-state J_e (Fig. 6 and Table III). Since J_p is actually elevated in the presence of the regenerating system, because of the increased intravesicular load, it follows from Eqn. 11 that J_r also requires ADP for maximal activity. This is further born out when acetyl phosphate replaced ATP (Table IV) when J_r was close to zero.

From the above arguments it appears that J_r requires extravesicular Ca^{2+}, ATP and ADP for maximal activity. Although our data do not rule out the possibility that a gated calcium channel or carrier (other than the pump) might require all these substances, we consider it unlikely. However, calcium fluxes mediated by some operation of the $(Ca^{2+} + Mg^{2+})$-ATPase would be expected to have the oberved requirements.

Fig. 9 shows some possible pathways for calcium flux mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase. The ADP-dependent, phosphoenzyme-independent pathway (Fig. 9A and Ref. 26) was found to be insignificant because no calcium exchange occurred after addition of glucose and hexokinase (Fig. 8). The phosphoenzyme-dependent, ADP-independent pathway (Fig. 9B and Ref. 23) was also found to be quantitatively insignificant because efflux was due only to diffusion when acetyl phosphate was used as a substrate for the formation of phosphoenzyme (Table IV). Thus the calcium efflux not accounted for by J_p is most likely due to ADP-dependent reversal of the $(Ca^{2+} + Mg^{2+})$-ATPase.

One pathway for ADP-dependent reversal of the $(Ca^{2+} + Mg^{2+})$-ATPase is the complete reversal from ADP and P_i. This pathway is expected to

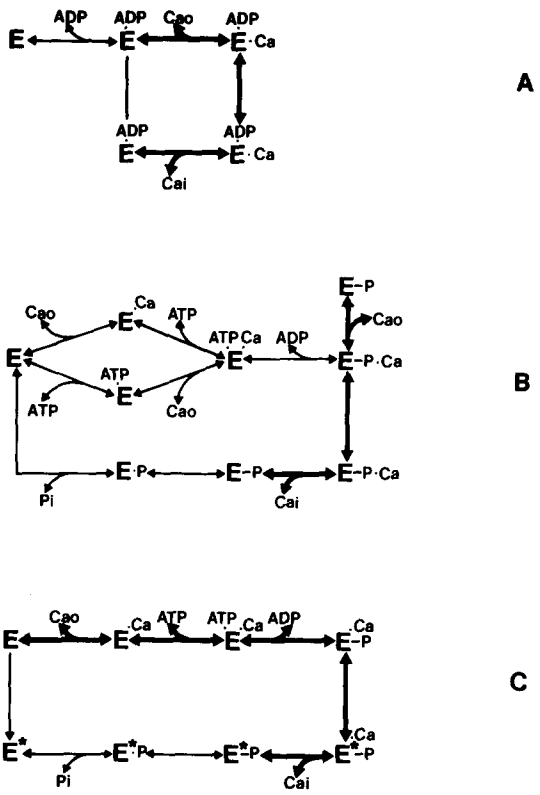

Fig. 9. Possible mechanisms of calcium flux by the $(Ca^{2+} + Mg^{2+})$-ATPase. Calcium flux is shown by transitions between enzyme states as indicated by the heavy lines. (A) Calcium flux may occur via an ADP-activated, phosphoenzyme (EP)-independent exchange [26], (B) calcium efflux might occur via an EP-dependent pathway not linked to nucleotide flux [23]; (C) calcium efflux might occur via an EP-dependent pathway linked to nucleotide exchange.

be trivial under our conditions because this reversal is inhibited by μM levels of extravesicular Ca^{2+} and ATP and requires mM levels of orthophosphate for maximal activity [41]. In an unpublished observation we found the rate of conversion of $^{32}P_i$ to [γ-^{32}P] ATP to be trivial under our conditions.

The most important pathway for calcium efflux mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase should be the partial reversal of the ATPase from ADP and phosphoenzyme [25]. The flux of nucleotide through this pathway has been evaluated as the rate of ATP-ADP exchange [16,44]. Although the calcium flux through this pathway has never been evaluated directly, several reports have evaluated total efflux [6,11–14,36,44]. In the one study in which calcium fluxes and ATP-ADP exchange were measured simultaneously [44] ATP-ADP exchange was faster than calcium efflux. Comparison of the calcium exchange data of Waas and Hasselbach [36] with the nucleotide exchange data of Ronzani et al. [24] further confirm this relation between nucleotide flux and calcium flux. We attempted to shut down this pathway by reducing ADP concentration with an ATP-regenerating system (Table III). The effect was a 60% reduction in efflux and an increase in the intravesicular calcium load. In spite of the 60% reduction in efflux, a large non-diffusional flux remained. This could be due to low levels of ADP or to the operation of another pathway. Measurement of residual ADP levels by thin-layer chromatography [43] indicated that ADP levels were about 1 μM after adding phospho*enol*pyruvate and pyruvate kinase. It is not possible to conclude from this experiment that 60% of the efflux at steady state is through an ADP-dependent pathway and the remaining 40% through some other pathway. If ADP is tightly bound, as suggested by Pang and Briggs [45] and Aderem et al. [46], then calcium exchange could take place between EP-ADP and EP-ATP. An essentially equivalent possibility exists if ADP dissociates from the enzyme late in the reaction sequence. This would permit calcium-calcium exchange in the absence of exchange between free ADP and ATP. If two calcium ions are carried by the enzyme it is also possible that one is released before the release of ADP while the other is released after. These possibilities cannot be distinguished by the experiments reported here.

The results of this study bear on the issue of the relative ability of cardiac and skeletal sarcoplasmic reticulum to accumulate calcium. Previous studies have suggested that the lower oxalate-supported calcium uptake rate by cardiac sarcoplasmic reticulum when compared to that of skeletal sarcoplasmic reticulum reflects a lower density of calcium pumps and a lower affinity for calcium [47]. Our results suggest that the lower density of calcium pump units is not an important consideration in determining the level of steady-state calcium uptake in the absence of calcium-precipitating anions because the parallel passive pathway carries negligible flux.

If steady-state calcium uptake is determined

almost entirely by the kinetic properties of the calcium pump, then differences in calcium uptake by cardiac and skeletal sarcoplasmic reticulum should be explained by differences in kinetic properties of their calcium pumps. Shigekawa et al. [47] found an apparent K_m for extravesicular Ca^{2+} of 1.3 μM for skeletal sarcoplasmic reticulum and 4.7 μM for cardiac sarcoplasmic reticulum. These values were obtained from the effect of extravesicular Ca^{2+} on ATPase activity at 0°C in the absence of calcium-precipitating anions and thus were obtained at steady-state filling. It is expected that there are additional differences in the kinetic properties of calcium pumps from cardiac and skeletal sarcoplasmic reticulum.

In addition to the pump and passive efflux, the enclosed volume and affinity of internal calcium-binding sites can also influence the level of steady-state calcium uptake when expressed as nmol/mg. In unpublished studies, we have found that skeletal sarcoplasmic reticulum contains more intravesicular calcium-binding sites than cardiac sarcoplasmic reticulum. From the mannitol load experiment reported herein, we calculated that the enclosed volume of cardiac sarcoplasmic reticulum was about 1.4 μl/mg. This value is less than that usually given for skeletal sarcoplasmic reticulum vesicles (48–54] but some investigators have obtained a similar value for the enclosed volume of skeletal sarcoplasmic reticulum [52]. Because of the uncertainties in the intravesicular volume and intravesicular calcium-binding properties, it is not yet possible to determine the intravesicular free calcium concentration or the relative importance of intravesicular volume and binding sites in determining the level of steady-state calcium uptake.

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