J. Membrane Biol. 56, 203–218 (1980)

The Journal of
Membrane Biology

Rapid Kinetic Study of the Passive Permeability of a Ca^{2+}-ATPase Rich Fraction of the Sarcoplasmic Reticulum

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Summary. A method was developed for the study of divalent cation transport events on the time scale of 20 msec or longer. Passive Ca^{2+} equilibration across the membranes of the Ca^{2+}-ATPase rich fraction of sarcoplasmic reticulum (SR) was studied. The method makes use of the divalent cation sensitivity of the surface binding of the fluorescent probe 1-anilino-8-naphthalenesulfonate (ANS^-). Binding to the inside and outside surfaces is distinguished in fluorescent stopped-flow experiments. The surface binding reactions of the probe are faster than the time resolution of the instrument (ca. 3 msec), while binding reactions requiring transport across the membrane could be resolved. In Ca^{2+} influx experiments, the time course of fluorescent enhancement was monitored following a Ca^{2+} jump. The kinetics of Ca^{2+} efflux were studied by pre-equilibrating Ca^{2+} across the membrane, removing the external Ca^{2+} with an EGTA jump, and observing the time course of the fluorescence decrease. Rapid transport of ANS^- (coupled to K^+) was ensured by the addition of valinomycin. Two processes of Ca^{2+} influx were observed: (i) a rapid process with small fluorescent amplitudes and a $t_{1/2}$ of 40–60 msec and (ii) a slow process with a large amplitude and a $t_{1/2}$ of 70–100 sec. The rates and extents of the two phases were quantitated in terms of the rates and extents of change in the Ca^{2+} concentration in the SR lumen. The slow phase accounted for a larger change, in the internal free Ca^{2+} concentration than did the first phase. For the influx of 10 mM Ca^{2+}, the rapid phase raises the internal Ca^{2+} concentration to ca. 1 mM within its apparent $t_{1/2}$ of 20 msec. The slow phase brings about an increase of the internal Ca^{2+} concentration to 4 mM within its apparent $t_{1/2}$ of 90 sec. The two phases have average rates of increase of internal free Ca^{2+} concentration, Δ $[Ca]_i$/sec of ca. 50 mM/sec and ca. 0.02 mM/sec, respectively. The Ca^{2+} influx rates increased with increasing KCl concentration and with increasing external Ca^{2+} concentration.

Two phases of Ca^{2+} efflux were observed. The amplitudes and rates were analyzed and the fast phase was shown to account for more Ca^{2+} movement than the slow phase. The rate of the fast phase was greatly increased by increasing the K^+ concentration. The rate of the slow phase efflux decreased with increasing Ca^{2+} concentration in the external medium. The concentration for half-maximal inhibition was 4 μM, a value close to the dissociation constant of the high affinity site on the Ca^{2+}-ATPase.

The above constitutes a body of circumstantial evidence that the passive Ca^{2+} permeability observed is mediated by the Ca^{2+}-ATPase, acting as a Ca^{2+} for 2 K^+ exchanger. The fast phases are explained as a partial turnover of the pump in the steady state. The slow rate is explained by a preference of the ion binding translocator site of the carrier for an outward orientation.

The ANS^- technique was applied to the monovalent cation permeability of the Ca^{2+}-ATPase rich SR and the results of other studies were corroborated and extended. The interaction of valinomycin with intrinsic permeability mechanisms of the SR was considered.

The present communication reports the use of 1-anilino-8-naphthalenesulfonate (ANS^-) in the study of the passive permeability of ATPase-rich sarcoplasmic reticulum (SR) to monovalent and divalent cations and to Cl^-. The question which is addressed is the origin of these permeabilities. Two limiting cases can be considered: (i) The passive permeability is due to proteins other than the ATPase pump. In this case the permeability will operate independently of the pump. It could contribute to the overall mechanism by acting in parallel to the pump or by "shorting out" the pump, with the choice between these possibilities dependent on the nature of the passive permeability and pump mechanisms. (ii) Alternatively, the passive permeability could be due to partial reactions of the pump which do not require ATP. In this case,

0022-2631/80/0056-0203 $03.20

the passive permeability might not be displayed under active transport conditions since ATP binding and enzyme phosphorylation would affect the conformational equilibria of the enzyme and would shift its distribution between its several states. These problems are studied in the present communication using the stopped-flow rapid kinetic methods with ANS^- as an indicator of internal ion concentrations. Evidence will be presented that the passive permeability of K^+ (Cl^-) does not depend on the ATPase while the passive Ca^{2+} permeability is the result of the 2 K^+/Ca^{2+} exchange function of the pump.

The recent literature provides a great amount of information relevant to these questions. McKinley and Meissner (1977, 1978) have shown that all fractions of SR are heterogeneous with respect to monovalent cation permeability. Their experiments were conducted with isotope and millipore filtration methods. Analysis of semi-log plots and the comparison of trapped volume measurements made with impermeable substances showed that $^2/_3$ of the SR vesicles are highly permeable with half-times of flux less than the filtration time (20 sec) while the remaining $^1/_3$ are slowly permeable with half-times of ca. 2 min. The authors suggested that a channel was responsible for the monovalent cation permeability and that $^2/_3$ of the SR vesicles contain this channel. Support for these observations and conclusions is given by the recent work of Kometani and Kasai (1978). These workers studied the permeation of monovalent cation-anion pairs by using light scattering changes to monitor SR volume changes in stopped-flow rapid mixing experiments. The time constant (τ) for KCl movement was found to be ca. 10 sec. The progress of the light scattering change associated with KCl movement had a nonexponential time course, an observation which the authors attributed to the heterogeneity of the SR with respect to permeability. The τ value showed small variation with the choice of anion but large variation with the choice of cation, from which it was concluded that the cation permeability was rate-limiting. Valinomycin was found to increase the rate of KCl permeation, but the rate approached a maximal value at high concentrations. The explanation of this result was that valinomycin contributed to the electrically-active K^+ permeability[1] such that the electrically-active anion permeability became rate limiting for the overall KCl transport reaction. The analysis of data in which valinomycin, the cations, and the anions were manipulated showed that the (electrically-active) anion permeability is higher than the monovalent cation permeability, with the order of decreasing permeability being $Cl^- \gg Rb^+ > K^+ > Na^+ > Li^+$. The Cl^- permeability was shown to be 50 times larger than the K^+ permeability.

Several studies have shown that the SR has an appreciable passive Ca^{2+} permeability. Inesi (1979) has studied the passive flux of $^{45}Ca^{2+}$ across the SR in a medium containing 100 mM KCl and 5 mM $MgCl_2$ and found that the process had an exponential time-course for submillimolar Ca^{2+} concentrations. He reported a permeability constant of 1.5×10^{-8} cm sec^{-1} for 37 °C. Jilka, Martonosi and Tillack (1975) studied the passive Ca^{2+} permeability of SR vesicles and vesicles containing purified Ca^{2+}-ATPase. The efflux of 1 mM Ca^{2+} was measured at 25 °C over a time range of minutes using the millipore filtration technique. Two explanations were considered for the passive permeability of Ca^{2+}: (i) That the permeability was due to the effect of the ATPase on bilayer structure and (ii) that the permeability was due to the effect of the ATPase acting as a carrier. They concluded that the permeability could not be accounted for by the ATPase acting as a carrier since they observed a linear dependence of the initial rate of Ca^{2+} efflux on the internal Ca^{2+} concentration in the 10^{-6} to 10^{-2} M range. The linearity indicates that the process was not mediated by a site which is saturable in this concentration range. A second criterion for the lack of carrier mediation was the absence of competition from other divalent cations. The study did show that the efflux rate was decreased when the external Ca^{2+} concentration was elevated in the 10^{-9} to 10^{-2} M range. A substantial effect was observed at 10^{-5} M external Ca^{2+}. This would be an expected result if the exchange were mediated by a carrier with a dissociation constant on the outside surface in the range of 10^{-6} to 10^{-5} M.

We have suggested that the passive Ca^{2+} permeability of the SR is due to the action of the ATPase pump (Chiu & Haynes, 1977; Haynes & Chiu, 1977, 1978). Our initial studies were carried out on unfractionated SR using spectroscopic indicators of external free Ca^{2+} (Chiu & Haynes, 1977). We showed that 10^{-4} M Ca^{2+} crosses the membrane and binds to the calsequestrin-type acidic binding proteins (MacLennan & Holland, 1975) with a half-time of 2.2 sec. Information about the heterogeneity of the SR with respect to the acidic binding proteins (Meissner, 1975) indicates that this half-time is characteristic of Ca^{2+} transport across the calsequestrin-rich SR. The ATPase-rich SR has little calsequestrin and thus made little contribution to the observed behavior. This illus-

[1] Electrically-active permeability is defined here as a unitary mechanism by which the ion is translocated alone such that its movement translocates its charge across the membrane. This permeability is as defined by the Goldman equation (Goldman, 1948) and is in contrast to ion-ion exchange diffusion or cotransport mechanisms in that membrane potentials can be generated and sensed.

trates a serious disadvantage of external equeous indicators: They indicate the Ca^{2+} lost to the external medium, but the change produced by the passive equilibration of ions into an internal aqueous compartment is not sufficiently large for quantitative study. Our desire to study the passive behavior of the SR and the Ca^{2+}-ATPase in the subsecond time range required the consideration of membrane-bound indicators. Membrane-active indicators include the fluorescent lanthanides (Rübsamen et al., 1976; Morris & Schober, 1977), chlorotetracycline (Caswell & Warren, 1972) and ANS^- (Haynes & Simkowitz, 1977; Haynes & Chiu, 1977, 1978). Use of the fluorescent lanthanides in measuring Ca^{2+} transport is precluded because they inhibit the pump process which we wish to study. Chlorotetracycline (CTC) cannot be used to study the rapid kinetics of Ca^{2+} accumulation because its transport across the membrane is too slow (Millman, Caswell & Haynes, 1980). This leaves 1-anilino-8-naphthalene-sulfonate (ANS^-) as the candidate of choice. Vanderkooi and Martonosi (1971), Haselbach and Heimberg (1970) and Ueno and Sekin (1978) have shown that increases in ANS^- fluorescence accompany active transport of Ca^{2+}. A quantitative basis for understanding this effect is given by our work of the response of ANS^- binding and fluorescence to ionic screening and binding reactions in phospholipid vesicles (Haynes, 1974) and in SR (Chiu et al., 1980).

In the third paper of this series (Chiu & Haynes, 1980) we show that the ANS^- response to active transport is due to the increase of ANS^- binding to the inside surface of the membrane due to the increased internal Ca^{2+} concentration. Our use of ANS^- as a method is based on our previous observations of its behavior in phospholipid vesicles (Haynes & Staerk, 1974; Haynes, 1974; Haynes & Simkowitz, 1977) and in the SR (Haynes & Chiu, 1977, 1978; Chiu et al., 1980). The method is based on the observation that the degree of binding of the probe to the membrane surface increases with increasing cation concentration in the medium. This effect is due to the increase in the "screening" of the negative charges on the membrane surface allowing more ANS^- to bind. Thus, for low SR and ANS^- concentrations, the ANS^- binding is given by

$$[ANS]_{b,i} = K\,[ANS]_i \exp(e\psi_{o,i}/kT) \tag{1}$$

where the subscripts b and i indicate the bound and free aqueous concentrations in the SR lumen, where K is a constant proportional to the binding constant, and where $\exp(e\psi_{oi}/kT)$ is the function of electrostatic surface potential $\psi_{o,i}$. This equation states that the degree of binding on the inside surface of the SR is proportional to its internal concentration and to the exponential function on surface potential. The latter increases in value towards unity as the concentration of mono- and divalent cations in the SR lumen is increased. As shown in the previous paper (Chiu et al., 1980), the "K_m" for the monovalent cation effect or $\exp(e\psi_{o,i}/kT)$ is ca. 100 mM while the value for the divalent cations is ca. 1 mM. Thus, the internal Ca^{2+} concentration can be measured at constant internal K^+ concentration if the value of $[ANS]_i$ is known. We have shown that ANS^- crosses the membrane as an ion pair with monovalent cations (M^+) and that the rate of crossing is catalyzed by valinomycin. The overall transport mechanism is thus

$$M_o^+ + ANS_o \underset{\text{Valinomycin}}{\overset{Keq}{\rightleftharpoons}} M_i^+ + ANS_i \tag{2}$$

where the o and i subscripts refer to the external and internal aqueous phases, respectively. This reaction sets up the following equilibrium

$$Keq = \frac{[ANS]_i\,[M^+]_i}{[ANS]_o\,[M^+]_o} = 1 \tag{3}$$

within the half-time for ANS permeation (ca. 8 sec and 20 msec in the absence and presence of valinomycin, respectively). Since the reaction transports zero net change, neither the rate nor the equilibrium responds to membrane potential[2]. This is the fastest transmembrane reaction involving ANS^- and the concentration $[ANS]_i$ will always be in equilibrium with it under our experimental conditions for times greater than 20 msec in the presence of catalytic concentrations of valinomycin. The combimation of Eqs. (1) and (3) gives

$$[ANS]_{b,i} = K \exp(e\psi_{o,i}/kT)\frac{[M^+]_o}{[M^+]_i}\,[ANS]_o \tag{4}$$

which shows that the degree of binding of ANS^- on the inside surface after this equilibrium is set up is proportional to the exponential function of surface potential and to the ratio of the monovalent cation concentrations outside and inside. In the Ca^{2+} permeation experiments of this study conditions are cho-

[2] ANS^- transport and rate of crossing the membrane would respond to the membrane potential only if ANS^- were permeable as a charged species. We consider this to be unlikely for the following reasons: (i) we have conducted experiments (Haynes & Simkowitz, 1977) which show that the rate of ANS^- permeation does not respond to attempts to generate a membrane potential in lipid vesicles, and (ii) the conductance increment of ANS^- in black lipid membranes has been shown to be small compared to that of ionophores and K^+ (*cf.* Fig. 6, McLaughlin et al., 1971). If the transmembrane distribution of ANS^- were to respond to membrane potential, then its electrophoretic permeability would have to be substantially greater than that of Eq. (2); otherwise, the effect would be continuously "shorted out."

sen such that the latter ratio is kept equal to unity. Experiments to measure the rate of KCl permeation are also possible since $\exp(e\psi_{o,i}/kT)$ and $[M^+]_o/[M^+]_i$ have quantitatively different dependencies on $[M^+]$. The information contained in Eq. (4) puts us in a good position to make quantitative use of the ANS^- fluorescence increase associated with passive transport or with active transport of Ca^{2+} into SR vesicles. The dependence of $\exp(e\psi_{o,i}/kT)$ on the internal Ca^{2+} concentration is determined as in the previous publication (Chiu et al., 1980) in rapid mixing experiments. The inside and outside ANS^- fluorescent contributions, due to binding to the outside and inside surfaces, are determined from the amplitudes of the instantaneous and slow ($t_{1/2}$ greater or equal to 20 msec) phases, respectively. The experimental design in the present study consists of rapidly mixing Ca^{2+}, Mg^{2+}, or K^+ with SR vesicles and monitoring the influx of these ions by following the changes in the ANS^- signal. The amplitude of the fluorescent change and the $t_{1/2}$ for its occurrence are the experimental parameters measured. For Ca^{2+} and Mg^{2+}, these are not direct measures of the internal Ca^{2+} concentration or its rate of influx, owing to the nonlinear relationship between fluorescent amplitude and Ca^{2+} concentration (Chiu et al., 1980). The nonlinear relationship results in half-maximal fluorescent amplitudes being seen at internal Ca^{2+} concentrations well below those corresponding to 50% equalization of the inside to outside Ca^{2+} concentration ratio. The extents and rates of Ca^{2+} movement are evaluated using the amplitude and $t_{1/2}$ data together with calibration graphs of the type presented in Fig. 7 of the previous paper (Chiu et al., 1980).

Subsecond time resolution of the transport events is guaranteed by the inclusion of valinomycin as a catalyst of ANS^- permeation. The possibility that the added valinomycin is perturbing or coupling to the existing permeabilities is assessed in experiments in which the valinomycin concentration is varied or omitted. Preliminary reports of the method and results have been made (Haynes & Chiu, 1977, 1978).

Materials and Methods

Methods and experimental procedures are as described in the previous communication (Chiu et al., 1980). Every process attributed to divalent cation transport was proven to be such by control experiments in which the process was accelerated by the addition of the Ca^{2+} ionophore X537A. Estimation of the changes in internal Ca^{2+} concentration from the amplitudes of kinetic phases is made using fluorescence *vs.* Ca^{2+} concentration curves of the type given in Fig. 7 of the previous paper (Chiu et al., 1980). Specifically, the total change in fluorescent amplitude observed after the Ca^{2+} or Mg^{2+} jump was scaled to a calibration graph of the type given in Fig. 7 of Chiu et al. (1980), determined at the appropriate monovalent cation concentration. The concentrations of internal Ca^{2+} achieved at $t=t_{1/2}$ for the appropriate phases were read off the graph. Average rates for each of the phases were determined by dividing the amount of Ca^{2+} moved in the $t_{1/2}$ by the value of $t_{1/2}$.

The possibility that the ANS^- signal was reading out changes specific to the environment of the Ca^{2+}-ATPase protein was ruled out by quantum transfer experiments as described in the previous paper (Chiu et al., 1980). A prototype of each major category of experiment was carried out using excitation at the tryptophan wavelength (280 nm), and the results were compared with those from direct excitation of the ANS^-. No significant differences in $t_{1/2}$ or relative amplitude values were observed. Additional control experiments showed that none of our experimental manipulations resulted in changes in tryptophan fluorescence greater than 5%.

The lumenal volume of the ATPase-rich fraction was determined by measurement of trapped ^{14}C sucrose. SR was incubated overnight in a buffer solution containing 0.25 M sucrose and 10 mM HEPES buffer, pH 7.2, 10 mM KCl (100 μg SR protein/50 μl), the sample was applied to a prewetted 0.42 μm millipore filter and washed with 2 ml cold solution. The radioactivity was quantitated by liquid scintillation counting. The chemical purity of the ^{14}C-sucrose (New England Nuclear, NEC-100×, Lot 1252-023) was given as >97%. A high degree of purity was detemined in our paper chromatography experiments. Over 95% of the radioactive material migrated as a single peak with an R_f value of ca. 0.29 in an *n*-butanol/ethanol/water (50:32:18) system. A trapped volume of 1.3±0.2 (SD) μl/mg Lowery protein was determined under a variety of conditions of temperature and ionic composition of the medium This value is substantially lower than the value of 5 μl/mg reported by Duggan and Martonosi (1970) for unfractionated SR and is lower than the choline space 5.8 μl/mg determined by McKinley and Meissner (1978) for the ATPase-rich fraction of SR. However, our value was obtained consistently with a number of preparations; the discrepancy could have been the result of a smaller diameter vesicle in our preparation.

Results

Divalent Cation Influx Kinetics

Rapid mixing of ANS^- with SR vesicles in the absence of Ca^{2+} results in an instantaneous ($t_{1/2}<$ 3 msec) fluorescence increase followed by a slow increase with an exponential time course and a $t_{1/2}$ of 8 sec. A typical experiment was shown in the previous paper. If both ANS^- and Ca^{2+} are rapidly mixed, increases in the amplitudes are observed due to the Ca^{2+} effect and a third process is observed.

Figure 1 shows the result of an experiment in the mixing configuration (SR) *vs.* ($ANS^- + Ca^{2+}$). In this case, three kinetic phases are observed: (i) the instantaneous phase ($t_{1/2} \leqq 3$ msec), (ii) the slow phase ($t_{1/2}$ =8 sec) and (iii) a still slower phase ($t_{1/2}=120$ sec). The 120-sec phase arises from the increase in ANS^- binding to the inside surface which results from movement of Ca^{2+} into the aqueous interior. This conclusion is supported by the observation that the 120-sec phase is not seen in the absence of divalent cations,

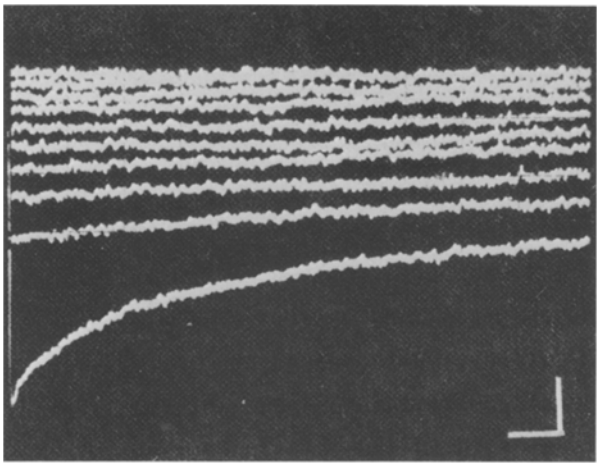

Fig. 1. Stopped-flow trace showing the influence of externally-added Ca^{2+} on the kinetics of ANS^- permeation through SR vesicle membranes. The vertical axis represents fluorescence (1.0 V/division). The horizontal axis represents time (5 sec/division with repetitive sweeps). Syringe *A* contained 0.2 mg/ml SR protein and medium. Syringe *B* contained 3×10^{-5} M ANS^-, 15 mM $CaCl_2$ and medium. The basic medium was 10 mM histidine, pH 7.2, 10 mM KCl and 0.6 M sucrose

Table 1. Dependence of $t_{1/2}$ and amplitude of ANS^- permeation on added calcium [a]

$[Ca^{2+}]_{added}$	A_o (volts)	$A_{i,a}$ (volts)	$t_{1/2,a}$ (sec)	$A_{i,b}$ (volts)	$t_{1/2,b}$ (sec)
0.0[b]	3.2	1.0	8	0.0	–
2.0×10^{-5} M	4.3	1.6	8	0.3	120
2.0×10^{-4} M	5.8	1.5	8	0.6	120
2.0×10^{-3} M	13.3	2.0	8	1.1	120
8.0×10^{-3} M	14.2	2.3	8	2.4	120
3.0×10^{-2} M	19.6	2.6	8	3.2	120
6.0×10^{-2} M	21.8	2.8	8	3.5	120

[a] Experimental conditions are given in Fig. 1. The results ($t_{1/2}$ and relative amplitudes) do not depend on the ANS^- concentration.

[b] 2×10^{-4} M EGTA added.

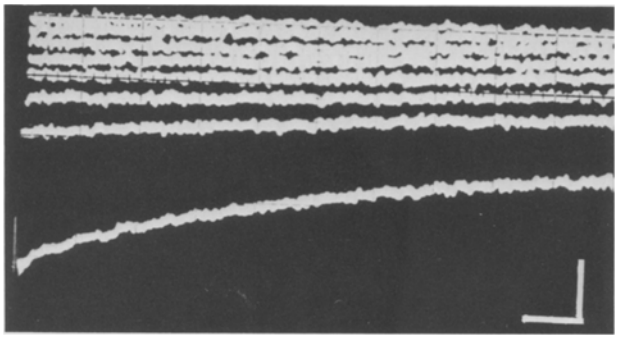

Fig. 2. Stopped-flow trace showing fluorescence increase accompanying passive Ca^{2+} influx to the lumen of light density SR vesicles. The mixing configuration was (0.2 mg/ml SR protein, 3×10^{-5} M ANS^-, 10 mM K^+, buffer) *vs.* (3×10^{-5} M ANS^-, 10 mM K^+, 15 mM Ca^{2+}, buffer). The buffer contains 0.6 M sucrose, 5 mM histidine, pH 7.2. The temperature is 25 °C. The vertical axis is 0.2 V/division and the horizontal axis is 5 sec/division. Multiple sweeps of the experiment were performed in the absence of valinomycin

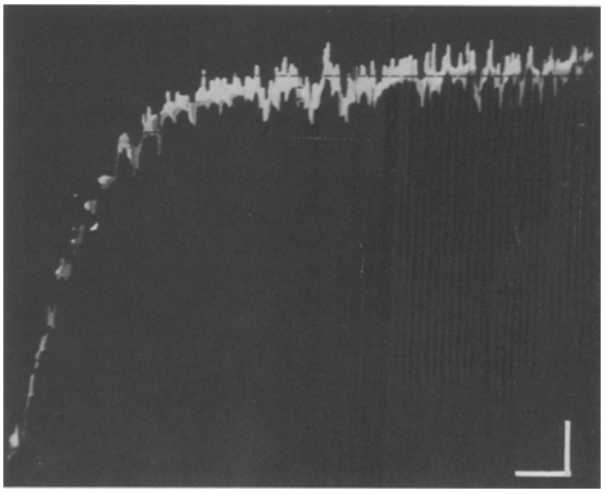

Fig. 3. Oscillograph trace showing kinetics of valinomycin + K^+-assisted permeation of ANS^-. The mixing configuration is 0.2 mg/ml (SR + 6 μM valinomycin + buffer) *vs.* (3×10^{-5} M ANS^- + buffer). The buffer solution contained 0.25 M sucrose, 10 mM KCl and 10 mM imidazole, pH 7.0

that its amplitude increases with increasing M^{2+} concentration, and that its $t_{1/2}$ is decreased by the addition of the Ca^{2+} ionophore X537A.

Table 1 shows the dependence of the $t_{1/2}$ and amplitude values on the added Ca^{2+} concentration. The $t_{1/2}$ values are essentially invariant with Ca^{2+} concentration while the amplitudes of all three phases increase with increasing Ca^{2+} concentration. An increase in amplitude of a kinetic phase represents an increase in the Ca^{2+} concentration responsible for that phase. Thus Ca^{2+} appears in the SR lumen in two kinetic processes, one having a $t_{1/2}$ of 8 sec or less and one having a $t_{1/2}$ of 120 sec. Figure 2 shows the result of a Ca^{2+} jump experiment performed under similar conditions. Two phases of Ca^{2+}-induced fluorescence increase are resolved with $t_{1/2}$ values identical to those of Fig. 1.

From the comparison of the experiments of Figs. 1 and 2 it is clear that the kinetics of Ca^{2+} movement for times less than 8 sec are obscured by the slow movement of ANS^- across the membrane, a process necessary for the change in Ca^{2+} concentration to be measured. In order for the Ca^{2+} transport kinetics to be resolved, the rate of ANS^- transport must be increased. As described in the introduction, this can be accomplished by the addition of valinomycin. Figure 3 shows the kinetics of the progress curve for ANS^- equilibration in the presence of valinomycin.

Table 2. Kinetics of Ca^{2+} Movement indicated by ANS^-

Expt.	Mixing configuration	$t_{1/2,a}$	$A_{i,a}$	$t_{1/2,b}$	$A_{i,b}$
1	(SR) *vs.* (30 mM Ca^{2+})	8 sec	1.0	100 sec	5.0
2	(SR + $^1/_2$ val)[a] *vs.* (30 mM Ca^{2+})	45 msec	0.9	120 sec	5.7
3	(SR + val) *vs.* (30 mM Ca^{2+})	47 msec	1.0	120 sec	6.0
4	(SR + val) *vs.* (60 mM Ca^{2+})	21 msec	1.1	70 sec	6.5
5	(SR + val, no K^+) *vs.* (30 mM Ca^{2+}, no K^+)	–	–	55 sec	5.0
6	(SR + 15 mM Ca^{2+} + val, no ANS^-) (15 mM Ca^{2+} + 2 × ANS^-[b])	24 msec	6.0	–	–

In all cases, both reservoirs contained 3.3×10^{-5} M ANS^-, 0.6 M sucrose, 5 mM histidine buffer, pH 7.2, and 10 mM KCl unless otherwise indicated. Val denotes 1.4×10^{-5} M valinomycin. $T = 23$ °C. The maximal experimental uncertainty is ±10% for A values and 15% for $t_{1/2}$ values.

[a] 7×10^{-6} M valinomycin.

[b] 6.6×10^{-5} M ANS^-.

The 8-sec half-time becomes 20 msec. In the experiments to follow, Ca^{2+} movement was studied in Ca^{2+}-jump experiments performed in the presence of valinomycin and K^+. Table 2 shows the results of a series of experiments of this type. Comparison of Expt. 6 with the experiment of Fig. 1 demonstrates that the $t_{1/2}$ for ANS^- equilibration is 24 msec. Comparison of Expts. 1, 2, and 3 indicates that the initial phase of fluorescence increase associated with Ca^{2+} influx has a $t_{1/2}$ of 47 msec or less. This process disappears when K^+ is omitted from the medium (Expt. 5). This behavior is expected if K^+ must diffuse out for Ca^{2+} to diffuse in. Experiment 5 also proves that the 47-msec reaction is not an artifact due to the presence of valinomycin in the membrane. Comparison of Expts. 2, 3, and 6 indicates that the measured $t_{1/2}$ values must be characteristic of the kinetics of the Ca^{2+} permeability system, and that the rate of ANS^- movement is not rate-limiting. Table 2 also shows that the $t_{1/2}$ and amplitude of the slow process are not affected by the valinomycin addition. The slow process is of much greater amplitude than the fast process, accounting for the bulk of the Ca^{2+} transported.

Table 3 shows the effect of the K^+ concentration on the rates of the two phases of fluorescence increase associated with two phases of Ca^{2+} influx. Increasing the K^+ concentration from 10 to 100 mM increases slightly the rates of both phases of Ca^{2+} equilibration across the membrane. The table also shows that Mg^{2+} equilibrates across the membrane in two kinetic phases. The slow phase for Mg^{2+} is significantly slower than that measured for Ca^{2+}. The Mg^{2+} influx reaction rates were less affected by the K^+ concentration than were the Ca^{2+} influx rates.

The amounts and rates of Ca^{2+} influx were evaluated semiquantitatively by the graphical procedure described in Materials and Methods. The fifth column of the table shows the calculated internal free Ca^{2+} concentration obtained at $t = t_{1/2,1}$ for the first phase. The ninth column gives the calculated internal free Ca^{2+} concentration obtained after $t = t_{1/2,2}$. Inspection shows that the amount of Ca^{2+} moved in the first half of the fast phase is smaller than that moved in the first half of the slow phase. Division of the changes in internal Ca^{2+} in the first halves of each of these phases by the respective $t_{1/2}$ values gives an indication of the average rates of the two phases, as shown in the 6th and 10th columns. The rates

Table 3. Effect of K^+ concentration on influx of 30 mM Ca^{2+} and Mg^{2+} (100 mM KCl)

$[M^{2+}]$	KCl (mM)	Fast phase				Slow phase			
	(mM)	$A_{i,a}$	$t_{1/2}$ (msec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$\Delta[Ca^{2+}]_i$/sec	$A_{i,b}$	$t_{1/2}$ (sec)	$[Ca^{2+}]$ at $t_{1/2}$	$\Delta[Ca^{2+}]_i$/sec
Ca^{2+}	100	0.20 ± 0.05	40 ± 10	$\sim 1 \times 10^{-3}$	$\sim 2.5 \times 10^{-2}$	1.1 ± 0.1	100 ± 10	$\sim 8 \times 10^{-3}$	$\sim 5 \times 10^{-5}$
Ca^{2+}[a]	100	0.20 ± 0.05	40 ± 10	$\sim 1 \times 10^{-3}$	$\sim 2.5 \times 10^{-2}$	1.1 ± 0.1	100 ± 10	$\sim 8 \times 10^{-3}$	$\sim 5 \times 10^{-5}$
Ca^{2+}	10	0.20 ± 0.05	60 ± 10	$\sim 2 \times 10^{-4}$	$\sim 3.3 \times 10^{-3}$	1.6 ± 0.1	150 ± 20	$\sim 5 \times 10^{-3}$	$\sim 2.7 \times 10^{-5}$
Mg^{2+}	100	0.15 ± 0.05	30 ± 10	$\sim 1 \times 10^{-3}$	$\sim 3.3 \times 10^{-2}$	1.1 ± 0.1	225 ± 25	$\sim 8 \times 10^{-3}$	$\sim 2 \times 10^{-5}$
Mg^{2+}	10	0.10 ± 0.05	30 ± 10	$\sim 2 \times 10^{-4}$	$\sim 6.6 \times 10^{-3}$	1.6 ± 0.1	250 ± 30	$\sim 5 \times 10^{-3}$	$\sim 1.4 \times 10^{-5}$

The indicated divalent cation was jumped to a final concentration of 30 mM. The KCl concentration was constant throughout the experiment. The basic experimental conditions were 0.25 M sucrose, 10 mM Hepes buffer, pH 7.1, 100 mM KCl. 3×10^{-5} M ANS^-, 6 μM valinomycin, and 0.1 mg/ml SR, $T = 23$ °C. The values of $[Ca^{2+}]_i$ were calculated at $t = t_{1/2}$ for the fast phases, respectively, as outlined in Materials and Methods. The average rates for the Ca^{2+} movement, $\Delta[Ca]_{i/sec}$, were calculated from the $t_{1/2}$ values and the values of $\Delta[Ca]_i$ atributed to the first halves of these phase, respectively.

[a] Experiment carried out with valinomycin at $^1/_3$ of the normal concentration.

of Ca^{2+} movement in the first phase (3.3–25 mM/sec) are high compared with the rates in the slow phase (0.014–0.05 mM/sec). Both rates increase with increasing KCl concentration.

Table 4 shows the results of variation of the concentration of Ca^{2+} on the influx reactions at 100 mM K^+. Two phases were observed at all concentrations where measurable rate data could be obtained ($[Ca^{2+}] \geqq 2.0$ mM). The fluorescent amplitudes of both phases increase while the $t_{1/2}$ values remain essentially constant in the concentration range of 2 to 10 mM. The behavior observed with Mg^{2+} influx (not shown) was similar to that observed with Ca^{2+}, except that the values of $t_{1/2}$ for the slow phase were significantly slower, as noted above. As seen in the previous table, the fast phase accounts for only a small portion of the uptake (columns 4 and 8) but the uptake in the fast phase occurs at a much faster rate (columns 5 and 9). Increasing the external Ca^{2+} concentration increases the rate of Ca^{2+} movement in both phases. Kometani and Kasai (1978) have reported permeation times (exponential tau values) for 33 mM Ca^{2+} and Mg^{2+} to be 6,000 sec and 1,800 sec, respectively. Their estimates of the rates are over a magnitude smaller than our estimate of the rate of the slow phase and show Ca^{2+}/Mg^{2+} specificity opposite to what we have observed. We do not believe that their study, carried out on unfractionated SR, can be compared directly with ours carried out on the ATPase-rich SR fraction. We have found that unfractionated SR and the heavy (or calsequestrin-rich) fraction of SR aggregate at Ca^{2+} concentrations above 10 mM, as evidenced by increases in light scattering which do not reverse slowly with time. We have found no evidence for Ca^{2+}-induced aggregation of the ATPase-rich fraction.

The rate of the slow phase can be used to calculate the Ca^{2+} permeability of the SR using $P = {}^1/_3\, rk$ where P is the permeability, r is the SR radius and k is the exponential rate constant for approach to equilibrium. Using the $t_{1/2}$ of the slow phase of Ca^{2+} influx Table 4, we estimate $k = 3\text{–}6 \times 10^{-3}$. Using 250 Å and 750 Å as lower and upper estimates of r, we estimate P to be between 0.75×10^{-8} and 2.2×10^{-8} cm sec^{-1} at 23°. This range encompasses the value of 1.5×10^{-8} cm/sec reported by Inesi (1979) based on isotope flux measurements in a medium containing Mg^{2+} at 37°.

Divalent Cation Efflux Kinetics

The finding of passive influx implies that a passive efflux can also be measured. The latter was studied to test for asymmetry effects of the pump. The SR was incubated with Ca^{2+} at the desired concentration at 23 °C introduced into the stopped-flow apparatus. The experiment was initiated by the addition of EGTA in the following configuration: (SR, ANS^-, K^+, x Ca^{2+}, ${}^1/_2$ x tris EGTA, buffer) *vs.* (ANS^-, K^+, ${}^1/_2$ x Tris EGTA, buffer). The x represents the variable Ca^{2+} concentration. In this particular experimental design Tris EGTA was added to both of the reservoirs to eliminate ionic asymmetries. After the EGTA addition, the external Ca^{2+} concentration was reduced to about 25 μM. Figure 4 shows the results of an experiment of this type. Two kinetic phases of fluorescence decrease are observed with $t_{1/2}$ values of 800 msec and 100 sec, respectively. Table 5 shows the effect of K^+ concentration on the $t_{1/2}$ and amplitude values of the two phases. In the efflux experiments with 2.5 mM Ca^{2+}, the fast phase has the smallest amplitude but represents the largest amount of Ca^{2+} movement. The slow phase represents the

Table 4. Effect of external Ca^{2+} concentration on the Ca^{2+} influx rate (100 mM KCl)

$[Ca^{2+}]$ (mM)	Fast phase				Slow phase			
	$A_{i,a}$	$t_{1/2,1}(Fl)$ (msec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$\Delta[Ca^{2+}]_i$/sec	$A_{i,b}$	$t_{1/2,2}(Fl)$ (sec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$\Delta[Ca^{2+}]_i$/sec
0.0	0.0	–	–	–	0.0	–	–	–
0.5	0.02±0.01	20±10	$<2\times10^{-4}$	$\leqq 1\times10^{-2}$	0.0	–	–	–
2.0	0.02±0.01	20±10	$\leqq 2\times10^{-4}$	$\leqq 1\times10^{-2}$	0.16±0.03	90±10	$\sim 8\times10^{-4}$	$\sim 5\times10^{-6}$
5.0	0.06±0.01	20±10	$\sim 5\times10^{-4}$	$\sim 2.5\times10^{-2}$	0.45±0.05	90±10	$\sim 2\times10^{-3}$	$\sim 1\times10^{-5}$
10.0	0.07±0.01	20±10	$\sim 1\times10^{-3}$	$\sim 5\times10^{-2}$	0.57±0.05	90±10	$\sim 4\times10^{-3}$	$\sim 2\times10^{-5}$
30.0	0.12±0.02	20±10	$\sim 2\times10^{-3}$	$\sim 10\times10^{-2}$	1.00±0.10	150±20	$\sim 12\times10^{-3}$	$\sim 5\times10^{-5}$

The Ca^{2+} concentration was jumped to the final concentration indicated. The basic experimental conditions were 0.25 sucrose, 10 mM Hepes buffer, pH 7.1, 100 mM KCl, 3×10^{-5} M ANS^-, 6 μM valinomycin, and 0.1 mg/ml SR; T=23 °C. The $[Ca^{2+}]_i$ and $\Delta[Ca^{2+}]_{i/sec}$ values were calculated as described on Table 3.

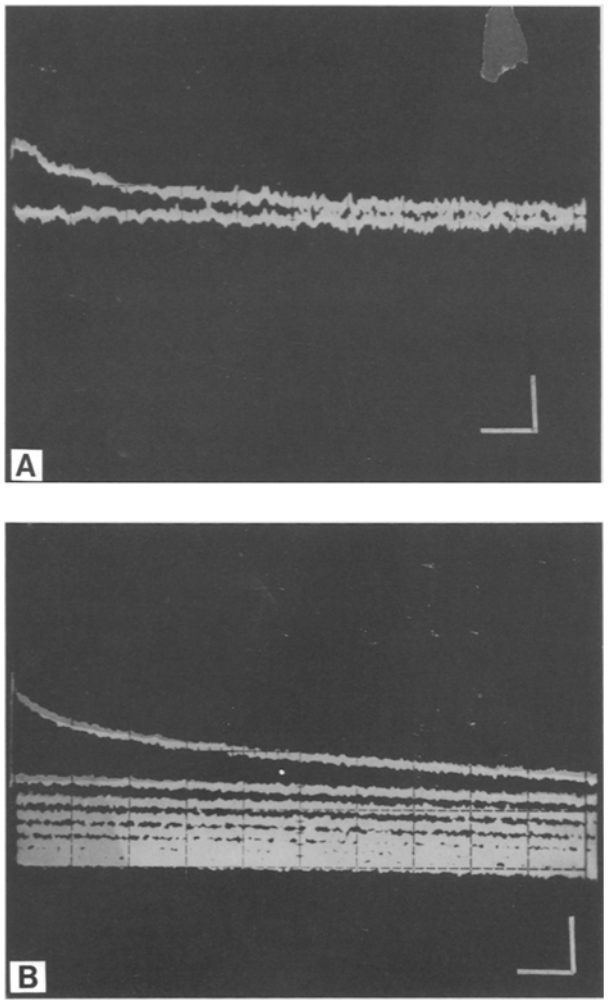

Fig. 4. Example of fluorescence decrease resulting from Ca^{2+} release from SR. The SR vesicles (0.2 mg/ml) suspended in a medium containing 4×10^{-5} M ANS^-, 2.5 mM EGTA, 5 mM Ca^{2+}, 10 mM K^+, 6 μM valinomycin and 0.1 M imidazole, pH 7.1, were mixed rapidly in a stopped-flow apparatus with an equal volume of solution of 3×10^{-5} M ANS^-, 2.5 mM tris-EGTA, 10 mM K^+, 0.1 M imidazole. Temperature was 23 °C. The vertical axis is fluorescence decrease in 0.5 V/division in both pictures. The time scale of fluorescence decrease is 0.5 sec/division on diagram *A* and 5 sec/division on *B*

removal of the small fraction of Ca^{2+} of the remaining (internal concentrations well below 1 mM). The initial phase of efflux has a high average rate (1.9–72 mM Ca^{2+}/sec) comparable to the rates observed for the fast phase of Ca^{2+} influx. The rate is increased by increasing KCl concentration. The rate of the slow phase of efflux, representing the movement of a small amount of Ca^{2+}, seems to be unaffected by the K^+ concentration. The dependence of the half-time for efflux on the internal Ca^{2+} concentration is given in Table 6. The rates of both phases of the Ca^{2+} efflux increase with increasing internal Ca^{2+} concentration. Such behavior could be expected for a carrier system with a K_m on the inside surface greater than 5 mM. The $t_{1/2}$ of the slow phase is not affected by the choice of initial $[Ca^{2+}]_i$. Addition, of 6 μM X537A, a Ca^{2+} ionophore, increased the rates of both phases (not shown).

The rate of the slow phase Ca^{2+} efflux was found to be dependent on the external Ca^{2+} when the latter was varied by varying the [Ca-EGTA]/[EGTA] ratio after mixing. The results, given in Table 7, show that the half-time for efflux of the low level of Ca^{2+} increases from 50 to 625 sec when the external Ca^{2+} concentration is increased from 0.44 to 500 μM. Columns 5–7 show the rates of efflux are expressed as the fraction of the extrapolated maximal (zero Ca^{2+}) values. Half-maximal inhibition was observed at ca. 4 μM external Ca^{2+}, a value close to the dissociation constant reported for high affinity Ca^{2+} binding to the carrier (Ikemoto, 1974; Inesi et al., 1978*a*, 1978*b*). This result suggests carrier-mediated efflux with a trans-inhibition effect which would be predicted from this model. Column 7 shows the fraction of the maximal rate observed at the various Ca^{2+} concentrations. Columns 5 and 6 give calculated values based on plots made under the assumption of one or *n* inhibitory sites per functional translocated unit, respectively. Poor agreement is seen between the observed and calculated data for high degrees of saturation under the assumption of one Ca^{2+} binding site per function-

Table 5. Influence of $[K^+]$ on the kinetics of efflux of 2.5 mM Ca^{2+}

$[K^+]$ (mM)	$-A_a/\Delta S$	$t_{1/2,a}$ (msec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$-\Delta[Ca^{2+}]_i$/sec	$-A_b/\Delta S$	$t_{1/2,b}$ (sec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$-\Delta[Ca^{2+}]_i$/sec
0.6	0.034	4,000			0.034	100		
10	0.023	800	$\sim 1 \times 10^{-3}$	$\sim 1.0 \times 10^{-3}$	0.041	$\geqq 100$	$\leqq 2 \times 10^{-5}$	$\leqq 8 \times 10^{-6}$
25	0.013	200			0.028	$\geqq 100$		
50	0.011	75–100			0.025	$\geqq 100$		
75	0.0048	20–40	$\sim 1 \times 10^{-3}$	$\sim 3.8 \times 10^{-1}$	0.025	100	$\leqq 2 \times 10^{-4}$	$\leqq 4 \times 10^{-6}$

The experimental conditions were described in the legend of Fig. 5. The variable $[K^+]$ is given in the table. The SR vesicles were incubated with 2.5 mM free Ca^{2+} for 1 hr before the initiation of experiments. $-A$ represents the amplitude of fluorescence decrease. ΔS represents the total fluorescence after the release of Ca^{2+}. The subscripts *a*, *b* denote fast phase and slow phase, respectively. The ratio $-A_a/\Delta S$, therefore, represents fraction decrease of the fast phase. The $[Ca]_i$ and $\Delta[Ca^{2+}]_{i/sec}$ values were calculated as described in Table 3.

al unit. Better agreement is observed if a stoichiometry of 0.62 (obtained from a plot of log $((1/t_{1/2})_{max}/(1/t_{1/2})-1)$ *vs.* log $[Ca^{2+}]_o$). is used. This shows that the kinetics of the inhibition of translocation are anticooperative.

Mg^{2+} efflux was studied in the same manner as was Ca^{2+} efflux, except that EDTA was used to chelate the external Mg^{2+}. Figure 5 shows that fluorescence decrease accompanying Mg^{2+} release is also biphasic. However, the decrease in fluorescence is dominated by the fast phase in contrast to the results for Ca^{2+} efflux where the two phases had comparable amplitudes. The figure shows that increasing K^+ concentration serves to increase the rate of both phases.

Measurement of K^+ Permeability of SR Vesicles

It is possible to observe K^+ permeability of the vesicle by the ANS^- method. The following describes the underlying principles involved in the measurement.

As described in the introduction section (Eq. (4)), there are two factors involved in determining the degree of ANS^- binding to the inside membrane surface. The value of exp $(e\psi_{o,i}/kT)$ and the value of $[M^+]_o/[M^+]_i$. In K^+-jump experiments the two factors have influences which are temporally distinct. In the presence of a low catalytic concentration of valinomycin the influence of the increase in the $[M^+]_o/[M^+]_i$ ratio is felt immediately and a large fluorescent increase is observed due to an increase in the $[ANS^-]_{b,i}$. However, as MCl begins to permeate, the ratio decreases and its contribution to the ANS^- binding diminishes. Ideally, this effect would be completely offset by the effect of increasing $[MCl]_i$ on exp $(e\psi_{o,i}/kT)$ since the Gouy-Chapman theory predicts proportionality between these two quantities for high values of surface charge and low values of MCl]. However, the surface potential is low and a less than 1.0 power relationship is observed between these two quantities (Haynes & Simkowitz, 1977). The net effect of the inward MCl movement is thus to decrease the ANS^-

Table 6. Influence of $[Ca^{2+}]_i$ on the kinetics of Ca^{2+} efflux (10 mM KCl)

$[Ca^{2+}]_{free}$ (mM)	$-A_a/\Delta S$	$t_{1/2,a}$ (msec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$\Delta[Ca^{2+}]_i$/sec	$-A_b/\Delta S$	$t_{1/2,b}$ (sec)	$[Ca^{2+}]_i$ at $t_{1/2}$	$\Delta[Ca^{2+}]_i$/sec
0.31	0	–	–	–	0.040	100	–	–
0.62	0.0063	1,000	–	–	0.046	100	–	–
1.25	0.011	800–1,000	$\sim 3\times10^{-4}$	~ 1 –1.2×10^{-2}	0.067	100	$<2\times10^{-5}$	$\leqq 2\times10^{-6}$
2.50	0.013	500–800	$\sim 1\times10^{-3}$	~ 1.8–3.0×10^{-3}	0.058	100	$<2\times10^{-4}$	$\leqq 5\times10^{-6}$
5.00	0.025	500	$\sim 2\times10^{-3}$	$\sim 6\times10^{-3}$	0.093	100	$\sim 5\times10^{-4}$	$\sim 1\times10^{-5}$

The experimental conditions were essentially the same as given in Table 1. The variable $[Ca^{2+}]$ is given in the table. The $[K^+]$ was fixed at 10 mM. The SR vesicles were incubated with the desired $[Ca^{2+}]$ for 1 hr before the initiation of experiments. Release of Ca^{2+} is started by the following mixing configuration: left reservoir – SR, 6 μM valinomycin, 3×10^{-5} M ANS^-, $x[Ca^{2+}]$, $x/2$ Tris-EGTA, and buffer; right reservoir – 3×10^{-5} M ANS^-, $x/2$ Tris-EGTA, and buffer. The buffer contains 0.1 M imidazole, pH 7.1, and 10 mM K^+. Refer to Table 1 for the explanation of the notation. The $[Ca^{2+}]$ and $\Delta[Ca^{2+}]_{i/sec}$ values were calculated as described in Table 3.

Table 7. Effect of external Ca^{2+} concentration on the slow phase of Ca^{2+} efflux kinetics

$[Ca^{2+}]_t$ (mM)	$[EDTA]_t$ (mM)	$[Ca^{2+}]_o$ (μM)	$t_{1/2}$ (sec)	Calculated fraction of max rate assuming 1st power dependence[a]	Calculated fraction of max rate assuming 0.62 power dependence[b]	Fraction of maximal rate[c]
2.5	25	0.44	50	0.911	0.868	0.912
2.5	5.0	3.95	85 (100)[d]	0.533	0.628	0.536
2.5	2.5	99.0	300	0.0437	0.187	0.152
2.5	2.0	500.0	625	0.00897	0.077	0.0912

Ca^{2+} efflux experiments were carried out at 23 °C in the following mixing configuration: (0.2 mg/ml SR, 6 μM valinomycin and 5 mM Ca^{2+} *vs.* (EGTA). The basic medium contained 0.25 M sucrose, 100 mM KCl, 3×10^{-5} M ANS^- and 100 mM HEPES buffer, pH 7.1. The free Ca^{2+} concentrations were calculated using 3.95×10^{-6} M as the K_d of the CaEGTA complex (deMeis & Hasselbach, 1971). The $t_{1/2}$ values refer to the slow phase of efflux. The fast phase was not studied.

[a] A maximal rate ($1/t_{1/2}$ value) was calculated under the assumption of simple competitive inhibition of the efflux rate external Ca^{2+} according to $(1/t_{1/2})/(1/t_{1/2})_{max}=(1+K_i[Ca^{2+}]_o)^{-1}$ where K_i is the association constant for the inhibitory complex. The data of the first two lines were used for this calculation. The values obtained were $(1/t_{1/2})_{max}=2.19\times10^{-2}$ sec^{-1} and $K_i=2.21\times10^{-5}$ M^{-1}.

[b] Values were determined as described above except that a K_i value of 1.323×10^{-3} $(M)^{-0.62}$ and $[Ca^{2+}]_o^{0.62}$ was used.

[c] Actual fractions of the maximal rate were determined using the $(1/t_{1/2})_{max}$ given above.

[d] Value obtained in the presence of 10 mM KCl.

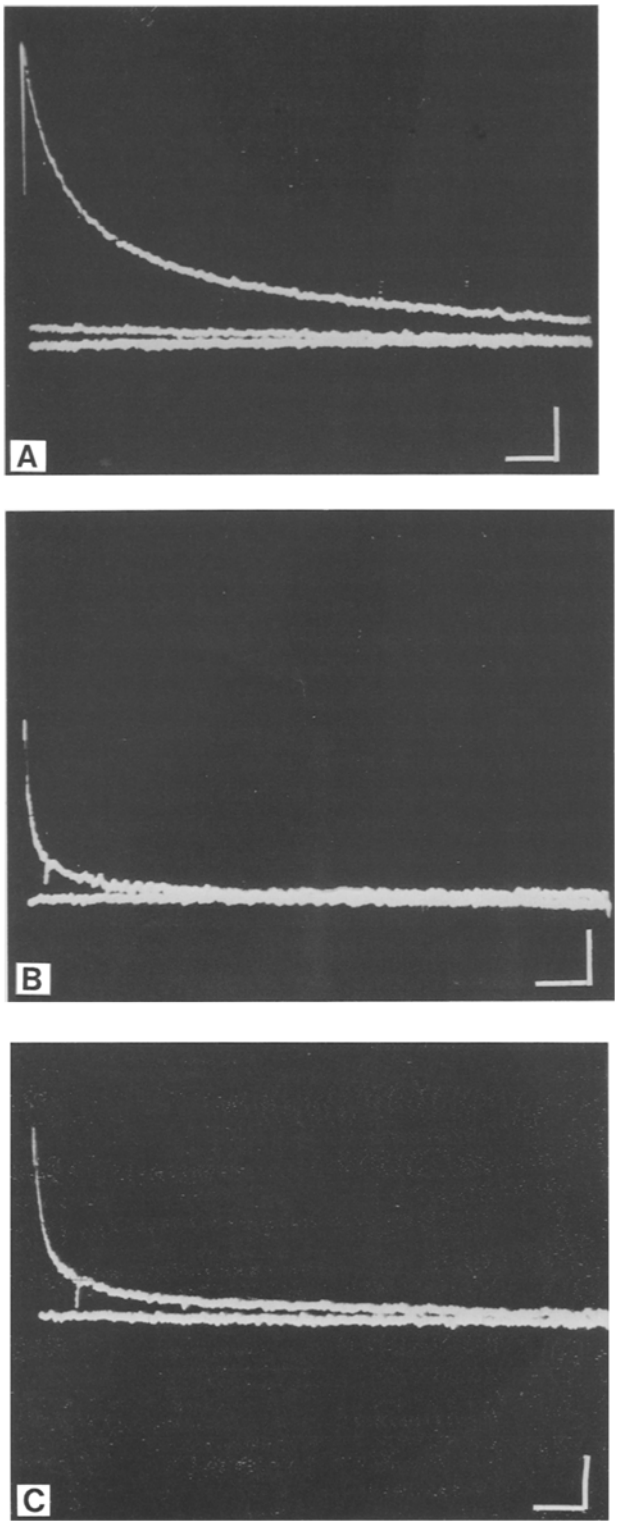

Fig. 5. Examples of fluorescence decrease associated with Mg^{2+} release from SR. Experimental conditions are essentially the same as that given in the legend of Fig. 4 except that Ca^{2+} was replaced with Mg^{2+}, and tris-EDTA instead of tris-EGTA was used. The K^+ concentrations in the medium are: (*A*) 10 mM; (*B*) 25 mM; (*C*) 75 mM. The vertical axis is 0.2 V/division and the horizontal axis is 5 sec/division in all pictures. Temperature was 23 °C

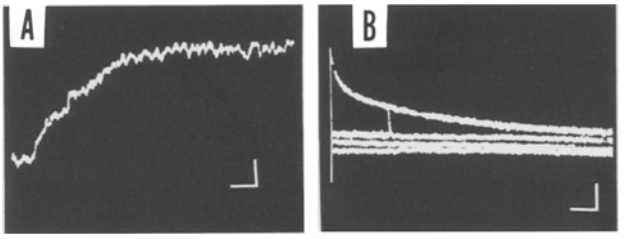

Fig. 6. Triphasic fluorescence behavior resulting from a KCl jump under passive conditions. The vertical axis is fluorescence, 0.2 V/division. The horizontal axis represents time after mixing at (*A*) 10 msec/division and (*B*) 5 sec/division. The upstroke in *B* represents the fast fluorescence increase in *A*. The mixing configuration was 0.2 mg/SR protein, 6×10^{-6} M valinomycin *vs.* 10 mM KCl. Both reservoirs contain 0.6 M sucrose, 2 mM histidine, pH 7.0, 3.17×10^{-5} M ANS^-

fluorescence, and the rate of this phase is used to monitor the rate of *M*Cl permeation. The possibility that the valinomycin contributes to the *M*Cl transport reaction as assessed by experiments in which the ionophore concentration is varied.

Figure 6 shows the result of an experiment in which SR vesicles preincubated with valinomycin and ANS^- are rapidly mixed in the stopped-flow apparatus with 10 mM KCl. Three phases of fluorescence change are seen. The first phase is a rapid increase which results from the shift in the internal ANS^- concentration resulting from the change in the $[K^+]_o/[K^+]_i$ ratio (*cf.* Eq. (4)). This is followed by two phases of fluorescence decrease which result from the effect of KCl influx on the $\exp(e\psi_{o,i}/kT)$ and $[K^+]_o/K^+]_i$ as described above. The first phase of decrease has a $t_{1/2}$ of about 2 sec and accounts for 60–70% of the amplitude. The second phase of decrease has a $t_{1/2}$ of about 100 sec and accounts for 30–40% of the amplitude. The relative amplitudes of the two phases and the $t_{1/2}$ of the slow phase agree well with the findings of McKinley and Meissner (1977) on isotope flux. The results can be accommodated by their explanation that the SR contains two types of vesicle, one containing an efficient permeability mechanism and the other lacking it. The alternative explanation, that there is one type of vesicle with two permeability mechanisms, cannot account for the isotope flux results and does not accommodate our finding that valinomycin affects the rates of both processes.

The effect of the valinomycin on the amplitudes and rates of the three phases was studied for the influx of 400 mM KCl. The results are given in Table 8. Since these results are to be used as a control for the effect of valinomycin on the active transport reaction (Chiu & Haynes, 1980) the experiments were carried out in the presence of 1×10^{-4} M Mg^{2+} and *ca.* 25 μM free Ca^{2+}. The results show that the phases

Table 8. Effect of valinomycin on the rates of equilibration of K^+

Valinomycin (M)	A_1	$t_{1/2\,1}$	A_2	$t_{1/2\,2}$	A_3	$t_{1/2\,3}$
0	1.0 ± 0.1	27 ± 3sec	–	–	–	–
2.2×10^{-7}	1.8 ± 0.2	100 ± 10 msec	-0.4 ± 0.1	2.0 ± 0.5 sec	-0.4 ± 0.1	85 ± 10 sec
6.7×10^{-7}	2.2 ± 0.2	40 ± 5 msec	-0.65 ± 0.05	400 ± 50 msec	-0.45 ± 0.05	80 ± 10 sec
2.0×10^{-6}	2.5 ± 0.2	20 ± 5 msec	-1.1 ± 0.1	200 ± 20 msec	-0.5 ± 0.1	55 ± 5 sec
6.0×10^{-6}	2.5 ± 0.1	10 ± 5 msec	-1.2 ± 0.1	175 ± 25 msec	-0.3 ± 0.1	25 ± 5 sec

The amplitudes of the three phases of fluorescence change observed after a KCl jump. Both syringes contained 0.6 M sucrose, 1×10^{-4} M $MgCl_2$, 4×10^{-4} M CaEGTA, 3.17×10^{-5} M ANS^- and 10 mM histidine buffer, pH 7.0. The *A* syringe contained 0.1 mg/ml SR and the *B* syringe contained 800 mM KCl.

cannot be resolved in the absence of valinomycin. This is due to the slow rate of ANS^- permeation. The lowest valinomycin concentration in this study gave a 100 msec half-time of ANS^- permeation. The second and third phases had $t_{1/2}$ values of 2 and 85 sec, respectively. Reference to Fig. 4 of the previous paper (Chiu et al., 1980) shows that the fluorescence *vs.* K^+ concentration relationship is reasonably linear in this range and that the $t_{1/2}$ values for fluorescent decrease are a reasonable approximation to the $t_{1/2}$ values for KCl permeation. The K^+ permeabilities of the two fractions can be calculated (as for Ca^{2+} above) using *r* values of 250 Å (lower limit) and 750 Å (upper limit). The P_{K^+} value for the KCl permeable vesicles is $(0.86\text{–}2.6) \times 10^{-6}$ cm/sec and the P_{K^+} value for the KCl impermeable vesicles is $(2.0\text{–}6.1) \times 10^{-8}$ cm/sec. Isotope flux and potential-sensing dye experiments of McKinley and Meissner (1978) set this lower limit of 10^{-6} cm/sec for the KCl-permeable vesicles. Increasing the valinomycin increased the rate of both phases, indicating increased rates of K^+ (Cl^-) permeation. The fast phase of KCl equilibration tends to a maximal value (175 msec), while the slow phase gives no indication of reaching a maximal rate.

The above results can be explained by the assumption that the fast phase represents KCl equilibration for an SR fraction containing an active anion channel and a less active cation channel. Valinomycin serves to increase the permeability of the system by contributing an electrical K^+ permeability. This is the model proposed by Kometani and Kasai (1978), based on experiments on unfractionated SR carried out in a manner similar to those reported here. Light scattering charges were used as a measure of electrolyte movement. The results we obtained for the KCl impermeable fraction can be explained by assuming that this fraction contains neither a cation nor an anion channel. The valinomycin effect in this fraction is based on the ability of the ionophore to carry KCl. The postulated reactions are shown in Fig. 7. In the permeable vesicles valinomycin increases the K^+ permeability until the Cl^- permeability becomes limiting. At this point, a maximal rate of KCl entry is observed. In the impermeable vesicles, the electrically active mode of valinomycin is operative, but ineffective, due to the absence of appreciable electrically-active anion permeability. The ionophore makes a small contribution to the slow rates due to its Val-K^+-Cl^- ion pair translocation reaction. The contribution of this reaction to the total rate is roughly proportional to the valinomycin concentration, in keeping with the postulated mechanism.

It is of interest of estimate the turnover numbers of valinomycin for its two modes of action. This can be done using the data of Table 7 for 2×10^{-6} M valinomycin. The data show that this concentration of valinomycin increments the rate ($1/t_{1/2}$) of the K^+ translocation reaction by 4.5 sec^{-1} and increments the rate of the KCl translocation reaction (of the slowly exchanging vesicles) by 6×10^{-3} sec^{-1}. Using 5×10^{-3} mg/ml as the internal water space (Duggan & Martonosi, 1970) and recognizing that roughly 200 mM KCl has been moved into this space during the half-time of the reaction, we estimate the turnover numbers of valinomycin operating in these two modes to be ca. 220 and 0.32 sec^{-1}.

The value for valinomycin operating in the electrically-active mode is much smaller than the values of $1.5\text{–}5 \times 10^4$ sec^{-1} reported for the ionophore in black lipid membranes (Stark et al., 1971; Benz et al., 1973). The turnover number is closer to the value of 2020 turnover number observed in rat liver mitochondria (Haynes et al., 1974). In these experiments the valinomycin was probably operating in an electrically active mode. The low value of 0.32 sec^{-1} observed for the electrically silent mode in the present study can be compared with a turnover number of 328 ± 44 observed for KCNS translocation in phospholipid multilayers (Blok, Gier & Van Deenen, 1974) and 46 sec^{-1} observed for K^+ANS^- translocation in phospholipid bilayer vesicles (Haynes & Simkowitz, 1977). The present results prove what the results cited above suggest – that in the presence of an adequate anion permeability system valinomycin moves

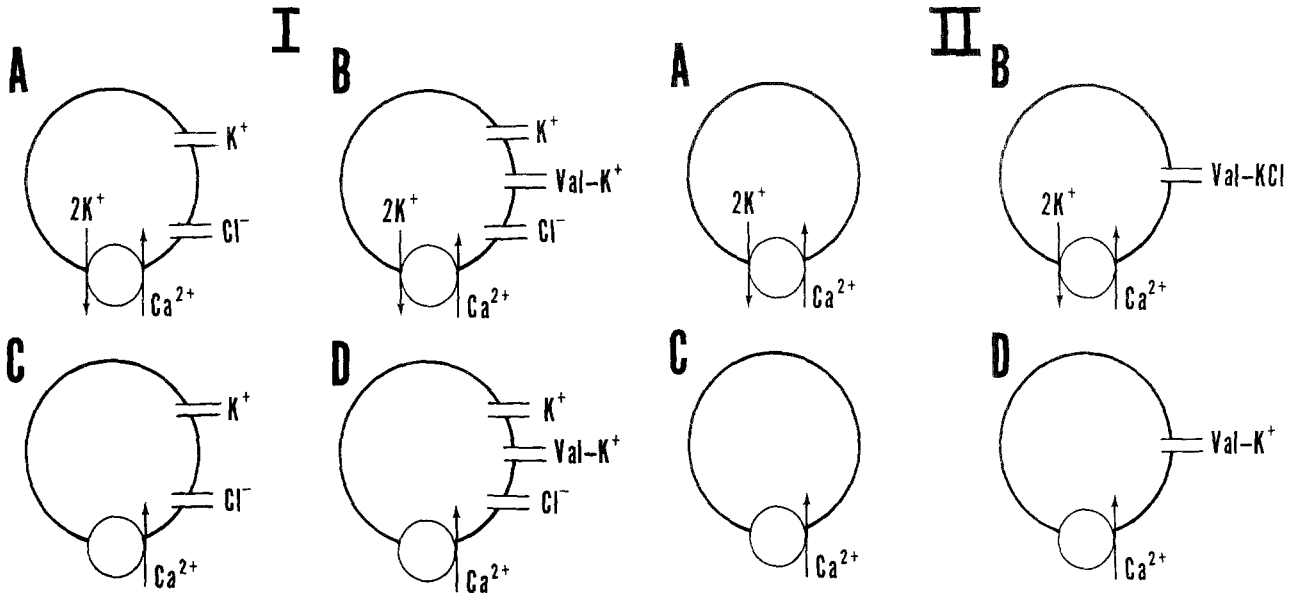

Fig. 7. Mechanism by which valinomycin can facilitate Ca^{2+} transport. Mechanisms for KCl permeable vesicles and impermeable vesicles are given in *I* and *II*, respectively. Mechanisms *A* and *B* are for the Ca-ATPase operating as a $2\,K^+$ for Ca^{2+} exchanger. Mechanisms *C* and *D* are for the enzyme acting in an electrically active fashion

K^+ in the electrically-active mode much more efficiently than it moves KCl in the electrically-silent mode. The ratio of the rates of these two modes is ca. 690.

Discussion

The present study shows that the passive influx and efflux of Ca^{2+} (and Mg^{2+}) can be monitored by the increase or decrease of ANS^- fluorescence accompanying these reactions. The changes in fluorescence result from the changes in the degree of ANS^- binding to the inside surface brought about by changes in the internal concentrations of these divalent cations. The observations that the rate of these processes are increased by the Ca^{2+} ionophore X537A and the results of the tryptophan-ANS^- quantum transfer experiments are evidence that the probe is not reading out slow conformational changes in the membrane protein.

Two phases of fluorescent increase were observed in both the Ca^{2+} influx and in the Ca^{2+} efflux experiments. The $t_{1/2}$ values and amplitudes of the changes in fluorescence were used to estimate the rates of Ca^{2+} movement and the amounts of Ca^{2+} moved. Quantitation of the signal corrects for the nonlinear (quasi-hyperbolic saturation) fluorescence of the signal on the internal Ca^{2+} concentration which makes the fluorescence disproportionately sensitive of changes of Ca^{2+} concentration in the low concentration range. For the influx process the fast phase corresponds to a small amount of Ca^{2+} set free in the SR lumen while the slow phase corresponds to the greater portion of the Ca^{2+} movement. The experimental design for measurement of the kinetics of the efflux process required that lower concentrations of lumenal Ca^{2+} be measured. In these experiments the fast phase was the major contributor to the overall efflux process, while the slow phase corresponded to Ca^{2+} removal at low levels.

Two possible explanations for the passive Ca^{2+} and Mg^{2+} permeability can be considered: (i) The process could be the result of a "leak" or channel mechanism or (ii) the process could be the result of the Ca^{2+}-ATPase operating in its passive mode. This study, with the reports cited in the introduction, gives results which cannot be readily explained by simple forms of the leak or channel mechanisms but which fit well with the carrier model. These results include the finding of two phases of influx and efflux, the finding that the influx and efflux reactions are accelerated by K^+, and the finding that the rate of the efflux is increased with increasing internal and decreasing external Ca^{2+} concentration. The observation that the influx rate increases with increasing external Ca^{2+} concentration is the only observation not explained by the simplest form of the carrier mechanism.

Before proceeding with a detailed evaluation of our results in terms of a carrier mechanism, it is necessary to discuss some quantitative apsects of the ATPase-rich SR preparation. If the ATPase is indeed the mechanism by which the Ca^{2+} crosses the membrane, then only a small number of turnovers is necessary for the complete equilibration of Ca^{2+} in our experiments. Using the pump capacity as 2 moles Ca^{2+}/turnover and the aqueous volume of the internal space as 1.3 μl/mg protein (this study) and the mol wt of the ATPase as 10^5 daltons, we calculate that each pump turnover will increase the internal Ca^{2+} concentration by 15 mM. Since our estimate of the aqueous volume is about one-half of that reported elsewhere (McKinley & Meissner, 1978), a factor of about 7 mM Ca^{2+}/turnover can be taken as the most conservative estimate of the total pump capacity. This calculation shows that most of the influx and efflux experimentation in this paper reports on Ca^{2+} movements of less than one turnover per pump. The major exception to this would be the study of the influx of Ca^{2+} and Mg^{2+} to a final concentration of 30 mM (Tables 3 and 4). The high capacity of the pump also implies that some of the transported Ca^{2+} may remain bound to the translocator site affecting our estimate of the number of Ca^{2+} crossing the membrane per sec. This must be taken into consideration for the evaluation of the rapid phases of the influx and efflux reactions. We will cite evidence that the inwardly-oriented form of the translocator has a K_d for Ca^{2+} which is greater than 5 mM. It would thus seem that contributions of Ca^{2+} bound to the inwardly-directed translocator to the total lumenal Ca^{2+} can be neglected for free Ca^{2+} concentrations of 1 mM or less. A second source of Ca^{2+} binding capacity exposed to the SR lumen is the negatively-charged phospholipids and low-affinity sites on the Ca^{2+}-ATPase. Equilibrium dialysis binding assays of the ATPase-rich SR fraction reported by Meissner (1975) encompass these two categories of sites. That study gave evidence for a low-affinity binding with a K_d of ca. 1.2 mM and a capacity of ca. 75 nmol/mg protein (Fig. 3, Meissner, 1975). About one-half of this activity is referable to the inside surface (G. Meisner, *personal communication*). Using these numbers together with the lumenal volume determined here, we calculated that for 1 mM free Ca^{2+} in the lumen, an amount of Ca^{2+} equivalent to 12 mM lumenal concentration will be bound on the inside surface. Thus the values of $\Delta [Ca^{2+}]_i/dt$ for the fast phases of the influx and efflux reactions could be ca. 13 times as high as reported in Tables 3–6. The rates for the slow phase of influx may also be underestimated, but not seriously.

A second quantitative aspect of the Ca^{2+} permeation is the $K^+(Cl^-)$ permeability or impermeability of the SR on the passive net reactions. The results presented here show that the half-time for KCl movement across $^2/_3$ of the vesicles is ca. 2 sec. This corresponds to rates which are more than 50 times as rapid as the Ca^{2+} translocation. This information shows that the appearance of the slow phase cannot be due to limitations of KCl permeability as proposed by us earlier (Haynes & Chiu, 1977, 1978).

We do believe that the fast phases can be understood as the first partial turnover of the pump in response to the perturbation and that the slow phase represents subsequent turnovers or partial turnovers. We interpret the fast phase to be the changes in internal Ca^{2+} concentration resulting from perturbation of the equilibria of the pump and the slow phase to be representative of steady-state kinetics of the pump. We also believe that the effect of KCl to accelerate the rate of the Ca^{2+} influx and efflux reactions arises from its role as a counter-transported species in the pump mechanisms. Slow Ca^{2+} for 2 K^+ equilibration by the Ca^{2+}-ATPase in the absence of ATP can be explained by a small tendency of the unphosphorylated *E*-Ca form to interconvert between the outwardly- and inwardly-oriented forms, coupled to the K^+-translocating steps postulated by other workers. Most of the information obtained in the present study can be rationalized in terms of the model presented in Fig. 8. The model makes use of an important feature of the model of Kanazawa et al. (1971), that the enzyme (E) can bind 2 K^+ and translocate them in a reaction which does not depend on the formation of a high energy phosphate bond. The model has an added passive translocation reaction for Ca^{2+}, whose rate and characteristics will be discussed below. The model has a 2 Ca^{2+} and 4 K^+ binding stoichiometry for the translocator, in keeping with the model of Kanazawa et al. (1971). However, the results reported here could just as easily be accommodated by the binding of one Ca^{2+} and 2 K^+. The net result of a cycle is the translocation of 2 K^+ out for every Ca^{2+} in. The nomenclature for the rate constants is chosen for ease of comparison with the Kanazawa model presented in the next paper. The subscript "P" is used to denote the reaction under passive conditions in the absence of covalent phosphorylation, while the subscript is omitted for a partial reaction which is considered to be shared with the Kanazawa model.

The enzyme would seem to have a preferential conformation with the translocator exposed to the outside. This can be inferred by the rapid binding of externally-added Ca^{2+} (Chiu & Haynes, 1977; Dupont & Leigh, 1978). Our experimental results can be best accommodated by the assumption that the equilibria of both the *E*Ca and *E*K states favor the

externally oriented translocator. The transformation from the externally- to internally-oriented form would be the rate-limiting step of the passive transport cycle. (In the active transport case this rate would be much accelerated by the phosphorylation of the enzyme). Return of the carrier in the Ca^{2+} or K^+ loaded form would be rapid. The overall equilibrium between the inwardly- and outwardly-oriented forms of the carrier would be modulated by the external and internal Ca^{2+} and K^+ concentrations. The binding of Ca^{2+} to the outwardly-oriented carrier is of extremely high affinity having an apparent K_d of 10^{-6} M (Ikemoto, 1979; Inesi et al., 1978*a*, *b*). Since the low K_d is obtained in the presence of high concentrations of K^+, we can assume a low affinity for K^+ for the outwardly oriented translocator.

With the use of the above assumptions the major observations of the present study can be explained. The slow rate of the major phase of Ca^{2+} influx reaction is due to the slow rate of the outward-to-inward transition ($k_{6\,p}$) of the Ca^{2+}-loaded form of the enzyme. Using the maximal value of $\Delta[Ca]_i$/sec observed (ca. 5×10^{-5} M/sec) and an estimated pump capacity of 15 mM/turnover, a $k_{6\,p}$ value of ca. 3.3×10^{-3} sec^{-1} can be estimated under the assumption that this is the rate-limiting step in the transport reaction.[3] The fast phase of Ca^{2+} influx is not readily explained by the scheme of Fig. 8. The data of Tables 3 and 4 show that at most one crossing of the membrane of the translocator is accomplished in this phase. Ca^{2+} is released to the SR lumen in this phase at a rate 100 times faster than in the slow phase. A possible explanation for this is that the enzyme is initially in a divalent cation-free state and this may represent a conformationally-different state than the one present in the steady-state. The following paper shows that prior occupation of the high affinity Ca^{2+} binding site prior to initiation of active transport by ATP addition gives rise to faster rates of active uptake. This could be considered indirect evidence for the explanation given. The model of Fig. 8 also does not account for the observation that the rates of both phases of Ca^{2+} influx increase with increasing external Ca^{2+} concentration in the 5 to 30 mM range. According to the simple model, the externally oriented binding site should be fully saturated under these situations and the influx kinetics should be zero order with respect to external Ca^{2+}. This would make it necessary to postulate that the conformational equilibria of the carrier are affected by low-affinity Ca^{2+} binding sites exposed to the outside surface. Binding studies of Meissner (1975) have given evidence for low-affinity binding sites possibly associated with the Ca^{2+}-ATPase.

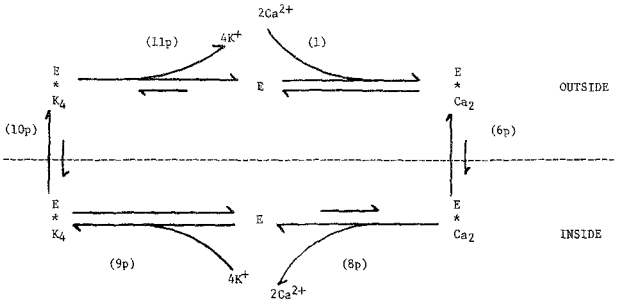

Fig. 8. Cylic mechanism for Ca^{2+}-ATPase-mediated passive equilibration of Ca^{2+} and K^+. The numbering of the reactions is by analogy to the active transport mechanism of Kanazawa et al. (1971) as given in the following paper (Chiu & Haynes, 1980). The subscripts *p* indicate that the reaction is considered to be different from that in the active transport case. Our assumptions about the position of the equilibria are indicated by the length of the arrows (approximate logarithmic scale). The reaction involving Ca^{2+} and K^+ are for reference concentrations of 10^{-6} and 10^{-1} M, respectively

The remaining observations in this paper are readily explained by the mechanism of Fig. 8. The ability of KCl to accelerate the influx and efflux of Ca^{2+} is due to the role of K^+ as a counter-transported cation. The biphasic nature of the Ca^{2+} efflux can be explained as follows: In the beginning of the experiment, the outwardly-oriented translocator is saturated with Ca^{2+} and the inwardly-oriented translocator is partially saturated. Rapid mixing with EGTA removes the external Ca^{2+}, shifting the equilibrium 6_p in favor of outward orientation. This results in a rapid fractional turnover of the pump. This is followed by a slow process of Ca^{2+} efflux which is limited by the slow return of the K^+-loaded form of the carrier (step 10_p). The observation that the rate of the Ca^{2+} efflux reaction increases with increasing internal Ca^{2+} concentration (here and Jilka et al., 1975) is in line with the predicitions of the model if k_{8p}/k_{-8p} is greater than 10 mM. The observation that the rate of the efflux reaction decreases with increasing external Ca^{2+} concentration in the micromolar range is explained directly as a trans-inhibitor effect of Ca^{2+} on the outwardly-oriented form of the carrier. Our observation that the phenomenon shows a ca. 0.6 power dependence on the external Ca^{2+} may be evidence for cooperative/anticooperative interactions between ATPase molecules. Such interactions may provide an explanation for the discrepancies which we have observed between some of our results and the predictions of the simple model of Fig. 8. There is mounting evidence for half-of-site reactivity phenomena in the active transport reaction

[3] In a previous communication (Chiu & Haynes, 1977) we determined a ca. 10^3-fold higher passive Ca^{2+} permeability for the calsequestrin-rich fraction of the SR.

(Froehlich & Taylor, 1975, 1976; *cf.* Chiu & Haynes, 1980), and it is probable that these considerations will require modification of the mechanism of Fig. 8.

The present study gives strong but not conclusive evidence that the passive permeability of Ca^{2+} is due to the action of the pump. The reaction is slow, in keeping with their high thermodynamic efficiency of the pump in the active transport mode. A slow passive Ca^{2+} "leak" mediated by the pump is to be expected since it is inconceivable that conformations brought about by phosphorylation of the enzyme could not occur to a small extent through fluctuations of the unphosphorylated form. This view suggests that the conformational equilibria, and thus the rates of passive equilibration, will be influenced by phosphate, Mg^{2+}, ATP analogues, inhibitors, and factors influencing the active transport process.

This work is supported by NIH grants HL23392 and GM23990. We thank Drs. Giuseppi Inesi and Gerhard Meissner and the Journal referees for their valuable criticism.

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Received 4 March 1980