Kinetics of Sodium Ion Induced Calcium Ion Release in Calcium Ion Loaded Cardiac Sarcolemmal Vesicles: Determination of Initial Velocities by Stopped-Flow Spectrophotometry†

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ABSTRACT: The kinetics of Na^+-dependent Ca^{2+} efflux in preloaded cardiac sarcolemmal (SL) vesicles prepared from bovine ventricular tissue were investigated at 21 °C by using stopped-flow spectrophotometry and the Ca^{2+}-sensitive dye arsenazo III. SL vesicles were loaded with Ca^{2+} in the presence of 160 mM KCl by using one of the following three procedures: (1) passive equilibration, (2) Na^+–Ca^{2+} exchange, and (3) ATP-driven Ca^{2+} uptake. The release of Ca^{2+} from SL vesicles was highly dependent on the concentration of NaCl; in the absence of NaCl, the initial Ca^{2+} efflux velocity in passive- and exchange-loaded vesicles was less than 0.01 nmol (mg of protein)$^{-1}$ s^{-1}, while at 80 mM NaCl, the corresponding value was 200–400 times greater than the unstimulated velocity. Semilogarithmic analysis of the efflux time course revealed that after a brief period of rapid Ca^{2+} efflux the rate declined. Pretreatment of the vesicles with valinomycin increased the duration of the initial phase of efflux while replacement of Cl^- with the impermeant anion gluconate produced the opposite effect in addition to slowing the efflux rate. These results suggest that the initial decline in the efflux rate is due to the accumulation of positive charge resulting from electrogenic Na^+–Ca^{2+} exchange. The initial velocity of Ca^{2+} efflux showed a sigmoidal dependency on the NaCl concentration with a Hill coefficient close to 3, indicating that activation of Ca^{2+} release involves a minimum of three Na^+ sites which interact cooperatively. For vesicles loaded by passive equilibration or by Na^+–Ca^{2+} exchange, the concentration of NaCl producing half-maximal activation of Ca^{2+} release was 31 mM; for vesicles loaded by ATP-driven Ca^{2+} uptake, the $K_{0.5}$ was 22 mM, reflecting an apparent increase in the affinity of these vesicles for Na^+. This difference could be due either to an effect of ATP on the Na^+–Ca^{2+} exchange process itself or to selective loading of a particular subpopulation of vesicles (presumably inside-out vesicles) during ATP-driven Ca^{2+} uptake.

Regulation of the intracellular Ca^{2+} level in cardiac tissue is believed to involve Na^+–Ca^{2+} exchange, a mechanism which couples Na^+ movement in one direction across the sarcolemmal (SL) membrane with Ca^{2+} movement in the opposite direction (Reuter & Seitz, 1968; Langer et al., 1976). We and others (Reeves & Sutko, 1979; Pitts, 1979; Bers et al., 1980) have reported isolation of cardiac sarcolemmal membranes exhibiting Na^+–Ca^{2+} exchange activity. In the presence of an outwardly directed Na^+ gradient, SL vesicles accumulate Ca^{2+} against a concentration gradient. Ca^{2+}-preloaded SL vesicles rapidly release Ca^{2+} when exposed to Na^+-containing media. Moreover, the response triggered by Na^+ is ion specific; K^+, Li, and choline will not substitute for Na^+ in stimulating either the uptake or the release of Ca^{2+}.

Sodium-dependent Ca^{2+} efflux has been shown to be responsive to membrane potential in the squid axon (Mullins & Brinley, 1975), implying that the exchange mechanism is electrogenic (Mullins, 1979). Several different lines of evidence indicate that Na^+–Ca^{2+} exchange activity is electrogenic in the SL vesicle system also. Pitts (1979; Pitts et al., 1980) has found a stoichiometry of three Na^+ per Ca^{2+} for the exchange process by using isotopic flux measurements. As evidence that buildup of charge does indeed occur during Na^+–Ca^{2+} exchange, Reeves & Sutko (1980) showed the SL vesicles transiently accumulate the lipid-soluble cation tetraphenylphosphonium (TPP^+) during Na^+-dependent Ca^{2+} uptake. The latter results were recently confirmed by Caroni et al. (1980). Moreover, increasing the conductivity of the vesicle membrane through the use of ionophores stimulates the rate of Na^+–Ca^{2+} exchange (Reeves & Sutko, 1980; Philipson & Nishimoto, 1980, 1981; Caroni et al., 1980), suggesting that Na^+–Ca^{2+} exchange activity in untreated vesicles becomes self-limiting due to the buildup of charge.

Filtration methods have been used by several groups (Reeves & Sutko, 1979, 1980; Pitts, 1979; Pitts et al., 1980; Philipson & Nishimoto, 1980; Bartschat & Lindenmayer, 1980) to monitor the Na^+-dependent release of $^{45}Ca^{2+}$ from SL vesicles. Because of the time required to mix, pipet, and filter the sample, accurate results can only be obtained in cases where the Ca^{2+} transport activity is linear for several seconds, i.e., under conditions where external Na^+ sites activating Ca^{2+} efflux are only partially saturated. Furthermore, measurements of initial rates of efflux using filtration methods are inherently imprecise because they involve measuring small differences in the amount of intravesicular Ca^{2+} during the initial states of the time course in efflux. In order to avoid the limitations of the filtration technique, we examined the kinetics of Na^+–Ca^{2+} exchange by using stopped-flow mixing and dual-wavelength spectrophotometry. The release of Ca^{2+} was followed by measuring the change in transmittance of arsenazo III, a metallochromic indicator with a sensitivity for Ca^{2+} in the micromolar range (Scarpa et al., 1978; Ogawa et al., 1980). It is shown that after a brief initial period of rapid Ca^{2+} efflux the rate declines as a result of the accumulation of positive charge due to electrogenic Na^+–Ca^{2+} exchange. The relationship between Ca^{2+} efflux velocity and the NaCl concentration is sigmoidal with a Hill coefficient greater than 2, indicating that three or more Na^+ sites are involved in the activation of Ca^{2+} release.

Materials and Methods

Materials. Arsenazo III (Grade I) and the sodium and potassium salts of D-gluconic acid were obtained from Sigma.

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Arsenazo III was used without further purification. Solutions were prepared in once-distilled, once-deionized water which contained about 5 μM Ca^{2+}.

Preparation of Membrane Vesicles. Cardiac sarcolemmal vesicles were prepared from bovine ventricular tissue according to a previously described method (Trumble et al., 1980). Vesicles were rapidly frozen in liquid nitrogen and stored at −80 °C for 1–2 weeks without detectable loss of Na^+–Ca^{2+} exchange activity.

Vesicle Loading Procedures. One of the following three procedures was used to load the vesicles with Ca^{2+}: (1) passive equilibration; (2) Na^+–Ca^{2+} exchange; or (3) ATP-driven Ca^{2+} transport. In the first procedure, SL vesicles (0.25 mg of protein/mL) were incubated for 1 h at 37 °C in the presence of 1 mM $CaCl_2$, 160 mM KCl, and 20 mM 3-(*N*-morpholino)propanesulfonic acid–tris(hydroxymethyl)aminomethane (Mops–Tris), pH 7.4. After the reaction mixture was loaded, it was separated into aliquots and centrifuged at 50000*g* for 30 min. The protein pellet was resuspended in a medium containing 30 μM arsenazo III, 160 mM KCl, and 20 mM Mops–Tris, pH 7.4. A sample of the resuspended material was saved for determination of the protein concentration. Exchange loading was carried out by first preloading the vesicles with 160 mM NaCl and 20 mM Mops–Tris, pH 7.4, and then diluting them 140-fold into a medium containing 30 μM $CaCl_2$, 160 mM KCl, and 20 mM Mops–Tris, pH 7.4. After 2 min at 37 °C, the vesicles were separated from the reaction mixture by centrifugation. ATP-dependent loading of the vesicles was carried out as described by Trumble et al. (1980). In each procedure, the vesicle pellet was rinsed twice before use with the resuspending medium to remove extraneous Ca^{2+}. Vesicles loaded by passive equilibration or Na^+–Ca^{2+} exchange accumulated between 35 and 50 nmol of Ca^{2+} per mg of protein; for vesicles loaded by ATP-driven Ca^{2+} uptake, the maximum amount of accumulation was about 10 times less. When stored on ice, these vesicles retained their Ca^{2+} load for more than 1 h.

Measurement of Ca^{2+} Efflux. The kinetics of Na^+-dependent Ca^{2+} efflux were measured by using an Aminco DW 2A ultraviolet–visible (UV–vis) spectrophotometer equipped with an Aminco-Morrow stopped-flow accessory and the Ca^{2+}-sensitive dye arsenazo III as indicator (Scarpa et al., 1978). In a typical experiment, one syringe contained SL vesicles (0.26–0.32 mg of protein/mL) and 160 mM KCl, and the other syringe contained NaCl (0–160 mM) and sufficient KCl to bring the total monovalent cation concentration to 160 mM. Both syringes contained 30 μM arsenazo III and 20 mM Mops–Tris, pH 7.4. The wavelength pair 575–650 nm was used to monitor the formation of the Ca^{2+}–arsenazo III complex. Unless otherwise indicated, the wavelength chopping frequency was 250 Hz and the time constant 5 ms. The reaction temperature was maintained at 21 °C by a circulating water bath.

Determination of the Rate and Equilibrium Binding Constants for the Ca^{2+}–Arsenazo III Complex. The binding constant of arsenazo III for Ca^{2+} was determined under conditions identical with those of the efflux experiment by using the method described by Ohnishi (1979). In the presence of 30 μM arsenazo III, 160 mM KCl, and 20 mM Mops–Tris, pH 7.4, the apparent binding constant of arsenazo III for Ca^{2+} was 3.3×10^4 M^{-1}. Under these conditions, the relationship between transmittance change and Ca^{2+} concentration was linear up to 10 μM Ca^{2+}. The dissociation rate constant for the Ca^{2+}–arsenazo III complex was determined by mixing a solution containing 30 μM each of $CaCl_2$ and arsenazo III,

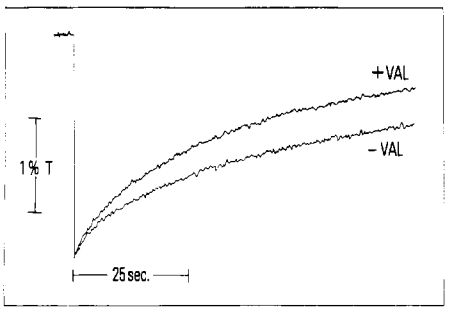

FIGURE 1: Effect of valinomycin on Na^+-dependent Ca^{2+} efflux in Ca^{2+} preloaded SL vesicles. SL vesicles were loaded by exchange of Na^+ for Ca^{2+} and treated with valinomycin as described under Materials and Methods. One syringe contained 30 μM arsenazo III, 160 mM KCl, 20 mM Mops–Tris, pH 7.4, and 0.26 mg/mL Me_2SO-treated (lower trace) or (Me_2SO + valinomycin)-treated vesicles (upper trace). The other syringe contained 30 μM arsenazo III, 100 mM NaCl, 60 mM KCl, and 20 mM Mops–Tris, pH 7.4. Curves shown in the figure are tracings of the original strip chart recordings which have been superimposed in order to facilitate their comparison. In this and all subsequent figures, a change of 1%*T* corresponds to a change in Ca^{2+} concentration of 0.8 μM.

160 mM KCl, and 20 mM Mops–Tris, pH 7.4, with an identical solution containing 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and no added $CaCl_2$. At 21 °C, the decrease in transmittance with time, reflecting dissociation of the dye complex, fit a single exponential with a rate constant of 63 s^{-1}. This value is similar to that obtained by Ogawa et al. (1980). From the dissociation rate constant and the apparent binding constant, the second-order rate constant for the formation of the Ca^{2+}–arsenazo III complex is 2.1×10^6 M^{-1} s^{-1}. Since the concentration of arsenazo III in these experiments (30 μM) greatly exceeded the extravesicular Ca^{2+} concentration, formation of the dye complex obeys pseudo-first-order kinetics with a rate equal to the second-order rate constant times the arsenazo III concentration (=63 s^{-1}). Because this value is 500–1000-fold larger than the observed rate of Ca^{2+} efflux, it was assumed that formation of the Ca^{2+}–dye complex is not rate limiting.

Miscellaneous Assays. SL vesicles were treated with valinomycin by the addition of 10 μL of 9 mM valinomycin in dimethyl sulfoxide (Me_2SO) to 10 mL of a medium containing SL vesicles (0.26 mg of protein/mL). Ca^{2+} efflux activity was measured as described above after the vesicles were spun down and resuspended in 30 μM arsenazo III, 160 mM KCl, and 20 mM Mops–Tris, pH 7.4. In experiments where sodium gluconate replaced NaCl, Ca^{2+} present in the sodium gluconate interfered with the quantitative determination of Ca^{2+}. In order to reduce the level of Ca^{2+} contamination, solutions containing sodium gluconate were treated with Chelex resin. After treatment, the concentration of Ca^{2+} in 160 mM sodium gluconate was 7.5 μM as determined by atomic absorption spectrophotometry. Protein concentrations were determined by the method of Lowry et al. (1951).

Results

The initial time course of Ca^{2+} efflux obtained by mixing Ca^{2+}-loaded SL vesicles in the stopped-flow apparatus at 21 °C with 50 mM NaCl, 110 mM KCl, 30 μM arsenazo III, and 20 mM Mops–Tris, pH 7.4 (final composition), is shown in Figure 1 (lower trace). The velocity of Ca^{2+} efflux was constant for approximately 2 s after mixing and then began to decline. Calcium efflux under these conditions was highly dependent upon the presence of Na^+; for the experiment shown in Figure 1, the initial velocity of Ca^{2+} efflux was 0.35 nmol (mg of protein)$^{-1}$ s^{-1} whereas the corresponding value in the

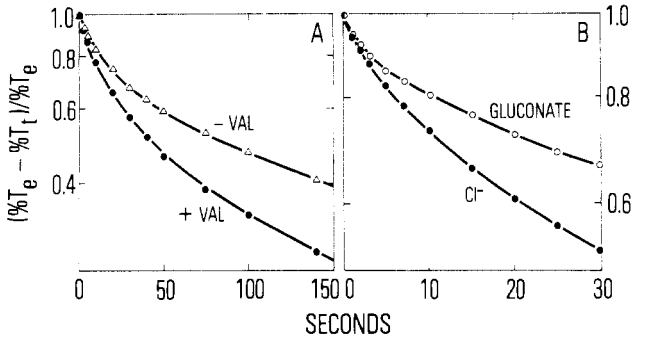

FIGURE 2: Semilogarithmic plot of Ca^{2+} efflux vs. time. (A) Ca^{2+} efflux in the presence (●) or absence (△) of valinomycin. Data were obtained from the experiments in Figure 1. (B) Ca^{2+} efflux in the presence (○) or absence (●) of sodium and potassium gluconate. The solutions were identical with those used in the experiment in Figure 1 except that a different preparation of vesicles was used, the vesicle concentration was 0.42 mg/mL, and sodium gluconate and potassium gluconate replaced NaCl and KCl. $\%T_t$ and $\%T_e$ refer to the percent transmittance at time t and at equilibrium. The data have been normalized and plotted semilogarithmically to compensate for the reduction in sensitivity produced by the binding of Ca^{2+} to gluconate.

absence of Na^+ was less than 0.01 nmol (mg of protein)$^{-1}$ s^{-1} (cf. Figure 3).

As shown by the lower trace in Figure 1, when Ca^{2+} efflux was measured by using valinomycin-treated vesicles under otherwise identical conditions, the initial rate of Ca^{2+} efflux was similar to that obtained in the absence of valinomycin, but there was a prolongation of this initial rapid portion of the time course of efflux. A semilogarithmic plot of these data which is shown in Figure 2A reveals that progressive slowing of the reaction occurs over the entire time course. For ionophore-treated vesicles, the decline in the rate of Ca^{2+} efflux subsequent to the initial rapid phase was more gradual than that observed in untreated vesicles.

The effects produced by valinomycin suggest that the slowing of the rate of Ca^{2+} release during the exchange process results from the buildup of charge across the vesicle membrane. Further support for this interpretation is provided by the effects of replacing Cl^- in the reaction mixture with gluconate, a presumably impermeable anion. Compared to Cl^-, gluconate produced a much more rapid decline in the rate of Ca^{2+} efflux (Figure 2B). Addition of valinomycin to membranes suspended in gluconate-containing media increased the Ca^{2+} efflux activity but not to the level obtained in the presence of Cl^- (data not shown).

Figure 3 (upper trace) shows the time course of Ca^{2+} efflux in SL vesicles loaded by ATP-driven Ca^{2+} transport (see Materials and Methods). The total amount of Ca^{2+} accumulated by these vesicles, and, hence, the total amount released by Na^+, was 5–10 times smaller than for vesicles loaded passively or by Na^+–Ca^{2+} exchange; for the latter vesicles, the total amount of Na^+-releasable Ca^{2+} was 35–45 nmol/mg of protein. This probably represents the loading of a select subpopulation of vesicles (presumably inside-out vesicles) by the ATP-dependent Ca^{2+} transport system. Previously reported measurements of neuraminidase-accessible sialic acid suggest that 10–30% of the vesicles in these preparations have an inside-out orientation (Trumble et al., 1980).

A second difference in the behavior of ATP-loaded and exchange-loaded or passively loaded vesicles is apparent from the time course of Ca^{2+} efflux shown in Figure 3. Compared to the behavior obtained under passive- or exchange-loading conditions, the time course in ATP-loaded vesicles has a more biphasic appearance (compare Figures 1 and 3) due to a slower rate of Ca^{2+} efflux during the late portion of the efflux curve.

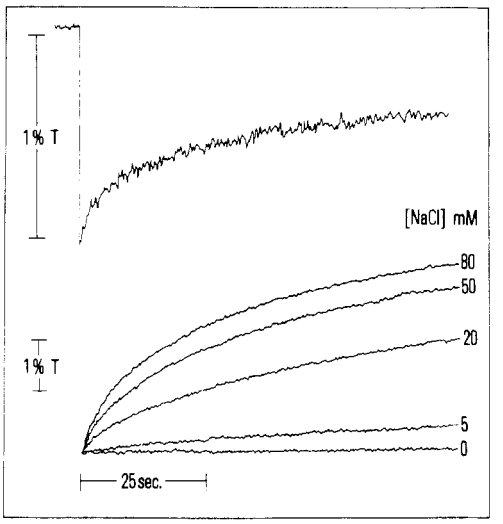

FIGURE 3: Time course of Ca^{2+} efflux in vesicles loaded by using ATP or Na^+–Ca^{2+} exchange: effect of varying the NaCl concentration. The upper trace shows the time course of Ca^{2+} efflux resulting from the addition of 80 mM NaCl to SL vesicles loaded in the presence of ATP (ATP-dependent loading; see Materials and Methods). One syringe contained 30 μM arsenazo III, 160 mM KCl, 20 mM Mops–Tris, pH 7.4, and Ca^{2+}-loaded vesicles (0.16 mg/mL). The other syringe contained an identical solution but without KCl and with 160 mM NaCl added. Lower traces depict Ca^{2+} efflux from exchange-loaded vesicles at different NaCl concentrations. One syringe contained Ca^{2+}-loaded vesicles (0.26 mg/mL) and 160 mM KCl and the other syringe 0–160 mM NaCl and sufficient KCl to bring the final concentration of monovalent cations to 160 mM. Arsenazo III (30 μM) and Mops–Tris (20 mM), pH 7.4, were present in both syringes. Curves shown in the figure are superimposed tracings of the original strip chart recordings.

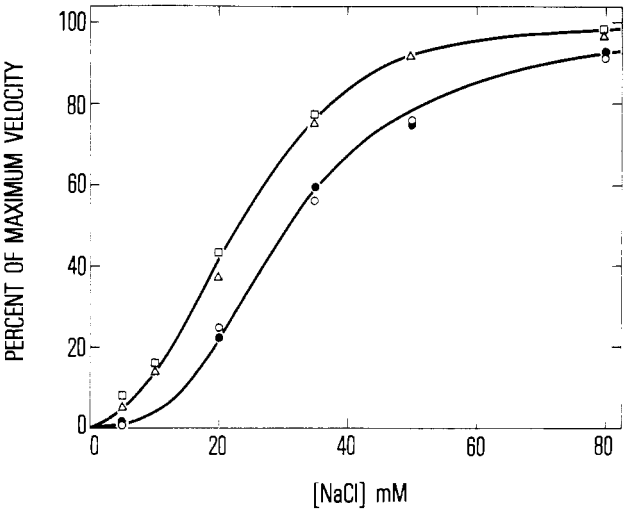

FIGURE 4: Dependence of velocity of Ca^{2+} efflux on [NaCl]: effect of varying the Ca^{2+} loading procedure. Cardiac SL vesicles were loaded with Ca^{2+} by incubating them (○) with 1 mM $CaCl_2$ for 1 h at 37 °C (passive loading), (●) with 30 μM $CaCl_2$ for 2 min at 37 °C after preloading with 160 mM NaCl (exchange loading), or (△, □) with 50 μM $CaCl_2$, 2 mM $MgCl_2$, and 2 mM Tris–ATP for 10 min at 37 °C (ATP-dependent loading). Also included in the incubation media were 160 mM KCl and 20 mM Mops–Tris, pH 7.4. Details of the experimental conditions for measuring Ca^{2+} efflux are given in the legend to Figure 3. The velocities are expressed as a percentage of the maximum Ca^{2+} efflux velocity given in Table I.

As shown in the lower portion of Figure 3, the initial velocity of Ca^{2+} release in exchange-loaded vesicles increased with NaCl concentration. Figure 4 summarizes the results of experiments in which the effect of varying the NaCl concentration on the initial slope of the efflux time course was examined. As seen in the figure, vesicles loaded by different methods gave qualitatively similar results. In each case, the

Table I: Hill Equation Parameters for the Dependence of Ca^{2+} Efflux Velocity on NaCl Concentration under Different Loading Conditions[a]

loading condition	n	$K_{0.5}$ (mM)	V_{max} (nmol mg^{-1} s^{-1})
Na^+ for Ca^{2+} exchange	3.17 ± 0.54	29.50 ± 1.82	2.38 ± 0.29
passive equilibration	2.44 ± 0.24	31.99 ± 1.34	4.08 ± 0.35
ATP dependent	2.60 ± 0.24	23.70 ± 0.95	0.41 ± 0.03
	2.30 ± 0.25	21.20 ± 1.04	0.66 ± 0.05

[a] The data in Figure 5 were fit to the Hill equation by using a nonlinear least-squares curve-fitting procedure (MLAB). $K_{0.5}$, n, and V_{max} refer to the NaCl concentration at half-maximal Ca^{2+} efflux velocity, the Hill coefficient, and the maximum Ca^{2+} efflux velocity, respectively. The Ca^{2+} loading conditions are described under Materials and Methods. Different preparations were used to determine the kinetics of Ca^{2+} efflux in each of the experiments shown in the table. Parameter values are reported as best fit ± the standard error.

efflux velocity varied sigmoidally with [NaCl], rising to a plateau somewhere above 80 mM NaCl. The sigmoid shape of these curves is consistent with a transport mechanism in which the occupation of two or more Na^+ sites is required for the activation of Ca^{2+} efflux. For determination of the number of sites involved in activating Ca^{2+} release, the data in Figure 4 were fit to the Hill equation by computer simulation using a nonlinear least-squares curve-fitting procedure (Knott & Reese, 1972). The results of the curve-fitting operation, summarized in Table I, indicate that the minimum number of Na^+ sites activating Ca^{2+} efflux is three. The NaCl concentration producing half-maximal activation of Ca^{2+} efflux was similar in passive- and exchange-loaded vesicles, about 31 mM. In the case of vesicles loaded by ATP-driven Ca^{2+} transport, however, the apparent half-saturation constant was reduced to 22 mM, reflecting an increase in the affinity of the transport system for Na^+. This shift in affinity for Na^+ is similar to that produced by ATP in guinea pig atria (Jundt & Reuter, 1976) and in squid axon (Blaustein, 1977) and may be related to a direct effect of ATP on the Na^+–Ca^{2+} exchange mechanism.

Discussion

Na^+-stimulated Ca^{2+} efflux from SL vesicles exhibited a complex time course. The velocity of Ca^{2+} efflux was constant for the first 1–2 s after mixing but then declined. Valinomycin treatment and replacement of the Cl^- with gluconate, a less permeable ion, altered the duration of the initial linear phase of efflux and the sharpness of the subsequent decline but did not change the initial velocity of efflux. The results suggest that the duration of the initial phase of Ca^{2+} efflux reflects the time required for the Na^+–Ca^{2+} exchange system to generate a membrane potential of sufficient magnitude to restrain its own activity. One explanation for this behavior is that the initial fast phase of Ca^{2+} efflux is due to a single turnover of the exchange carrier; in this case, charge accumulation would not affect the velocity of Ca^{2+} efflux until after completion of the first cycle of Na^+–Ca^{2+} exchange. If this explanation were correct, the approximate transport site density estimated from the amplitude of the initial phase of Ca^{2+} efflux (Figure 1) would be 1–2 nmol/mg of protein.

In addition to the charge separation generated by the Na^+–Ca^{2+} exchange system, other factors may play a role in the development of a rate-limiting membrane potential during Na^+-dependent Ca^{2+} efflux. Changes in the membrane surface potential as well as the bulk phase transmembrane potential are likely to occur and to influence the rate of Na^+–Ca^{2+} exchange. A large fraction of the Ca^{2+} in preloaded vesicles appears to be bound internally. Thus, for vesicles passively equilibrated with 1 mM $CaCl_2$, the amount of Na^+-releasable Ca^{2+} (35–45 nmol/mg of protein) far exceeds the 5 nmol/mg of protein that would be expected by assuming an internal volume of 5 μL/mg of protein (Reeves & Sutko, 1979). The dissociation of internally bound Ca^{2+} during Ca^{2+} efflux would expose fixed negative charges at the inner membrane surface, and the change in surface potential would counteract to some degree the increase in transmembrane potential generated by the exchange system. Changes in membrane surface potentials have been shown to produce shifts in current–voltage relationships in excitable tissues (McLaughlin, 1977), and it seems likely that such changes might also influence Na^+–Ca^{2+} exchange activity in the vesicle system.

Even under conditions allowing maximal charge compensation (i.e., K^+–valinomycin and Cl^--containing media), the semilogarithmic plot of Ca^{2+} efflux vs. time curved upward, indicating that a progressive slowing of the reaction was taking place (Figure 2A). Several factors could account for this behavior, including (1) nonuniform vesicle size and the consequent variation in the surface-to-volume ratio throughout the vesicle population, (2) collapse of the Na^+ gradient, (3) competition by internal Na^+ for Ca^{2+} binding sites on the exchange carrier, and (4) reuptake of Ca^{2+} through Ca^{2+}–Ca^{2+} exchange as the external Ca^{2+} concentration increases. At present, the relative contributions of each of these factors to the observed deceleration of Ca^{2+} efflux cannot be estimated. The possibility that the rate of Ca^{2+} efflux is limited by a slow dissociation of Ca^{2+} from internal binding sites can be discounted because Ca^{2+} efflux in the presence of the ionophore A23187 is at least 10-fold more rapid than the maximal rate of Na^+-dependent Ca^{2+} efflux.[1]

The data presented in Figures 3 and 4 indicate that the initial rate of Ca^{2+} efflux exhibits a sigmoidal dependence on the Na^+ concentration. The Hill coefficients of 2.3–3.2 (Table I) are consistent with the suggested stoichiometry of three Na^+ ions per Ca^{2+} for the exchange process (Pitts, 1979; Pitts et al., 1980). Philipson & Nishimoto (1981) have also shown a sigmoidal relation between Ca^{2+} efflux and Na^+ concentration; in those studies, however, Ca^{2+} efflux was measured over such prolonged intervals (0.4–4 min) that an accurate measure of initial rates was not possible. The use of noninitial rates, in combination with the complex time course of efflux, may partially explain why the apparent $K_{0.5}$ for Na^+ obtained by Philipson & Nishimoto (12.5 mM) is less than the value of 22–31 mM obtained in the present studies.

The data in Figures 3 and 4 also indicate that the quantitative relationship between the Na^+ concentration and efflux velocity depends on the Ca^{2+} loading procedure. All three methods gave similar values for the Hill coefficient; however, for vesicles loaded by passive equilibration or by Na^+–Ca^{2+} exchange, the threshold for Na^+ activation was higher than for vesicles loaded by ATP-driven Ca^{2+} uptake. A possible explanation for this behavior is that ATP-dependent loading selects from the total population a subpopulation of vesicles whose apparent affinity for Na^+ is greater than that of the remaining vesicles. Because ATP-dependent loading should occur only in vesicles with their ATP binding sites exposed

[1] Vesicles loaded with $^{45}Ca^{2+}$ and diluted 70-fold into 160 mM KCl containing 0.1 mM EGTA and 1 μM A23187 (25 °C) lost 85% of their Ca^{2+} load within 5 s and the remainder within 1 min. In contrast, the half-time of Ca^{2+} efflux was approximately 1.2 min when the vesicles were diluted into 80 mM KCl/80 mM NaCl containing 0.1 mM EGTA but no ionophore.

(inside-out vesicles), this explanation would seem to imply that the carrier is functionally asymmetric, i.e., that its affinity for Na^+ depends on which side of the membrane the Na^+ site is exposed to. An alternative explanation is that ATP, in addition to driving Ca^{2+} uptake, is able to modify the carrier in such a way as to increase its apparent affinity for Na^+. Since this effect persists after the nucleotide has been removed from the vesicles, it may involve the phosphorylation of a regulatory site on the vesicle membrane. Consistent with this proposal, studies of Na^+–Ca^{2+} exchange in the squid axon (Blaustein, 1977) have shown that internal perfusion with ATP increases the affinity for external Na^+ and internal Ca^{2+} and that the nonhydrolyzable ATP analogue, adenosine 5′-(β,γ-methylenetriphosphate), cannot substitute for ATP in producing these effects (DiPolo, 1977). Studies of the kinetics of Na^+–Ca^{2+} exchange in separated subpopulations of inside-out and right-side-out vesicles will be necessary to decide which of these alternative interpretations is correct.

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