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Na^+-dependent alkaline earth metal uptake in cardiac sarcolemmal vesicles

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The ability of alkaline earth metals (M^{2+}) to substitute for Ca^{2+} in Na^+-Ca^{2+} exchange was examined in sarcolemmal vesicles isolated from the canine heart. $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$, in addition to $^{45}Ca^{2+}$, were used to determine the characteristics of Na^+-M^{2+} exchange. The Na_i^+-dependent M^{2+} uptake was measured as a function of time, with t ranging from 0.5 to 360 s, $[Na^+]_i = 140$ mM and $[M^{2+}]_o = 40$ μM. This function was linear for Ca^{2+} and Sr^{2+} uptake for approx. 6 s and for Ba^{2+} for about 60 s. Plateau levels were achieved within 120 s for Ca^{2+} and Sr^{2+} but Ba^{2+} took considerably longer. The K_m values for Na^+-M^{2+} exchange, derived from Eadie-Hofstee plots, were 30, 58, and 73 μM for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively. The Na_i^+-dependent uptake of all three ions was stimulated in the presence of 0.36 μM valinomycin. Na^+-Ca^{2+} exchange was also measured in the presence of either 20 μM Sr^{2+} or 100 μM Ba^{2+}. Both of these ions behaved (at these concentrations) as competitive inhibitors of Na^+-Ca^{2+} exchange with the K_I being 32 μM for Sr^{2+} and 92 μM for Ba^{2+}. Passive efflux was determined by first allowing Na^+-M^{2+} exchange to continue to plateau values and then diluting the loaded vesicles in the presence of EGTA. The rate constants for the passive efflux were 8.4, 6.3 and 4.4 min^{-1} for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively.

Introduction

Myocardial contractility is critically dependent on myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Since the studies of Ringer [1] over one hundred years ago, it has been known that tension generation in the heart has an absolute requirement for Ca^{2+} in the extracellular fluid. Transsarcolemmal Ca^{2+} transport is an essential component in the regulation of $[Ca^{2+}]_i$ and, therefore, contractility [2]. Mechanisms by which Ca^{2+} transport across the sarcolemma is regulated include the calcium channel [3], a Ca^{2+}-ATPase [4], and Na^+/Ca^{2+} exchange [5].

Na^+/Ca^{2+} exchange was unequivocably demonstrated for the first time in cardiac sarcolemma in 1979 by Reeves and Sutko [6]. The exchanger is electrogenic [7–9] and the probably stoichiometry is 3 Na^+/Ca^{2+} [10,11]. In isolated cardiac sarcolemmal vesicles the affinity of the exchanger for Ca^{2+} is reflected in reported K_m values ranging from 2 to 30 μM [12,13]. The activity of the exchanger is markedly dependent on pH [14] and, apparently, anionic moieties within the lipid bilayer [15,16].

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Abbreviations: EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; ANS, 8-anilino-1-naphthalene sulfonate; DMSO, dimethylsulfoxide.

One approach to the study of the role of Ca^{2+} in myocardial contractility has been the substitution of Ca^{2+} by other cations, especially those which also belong to group 2a of the periodic table: the alkaline earth metals. The use of strontium (Sr^{2+}) and barium (Ba^{2+}) has given insight into the mechanisms by which the heart transports and regulates Ca^{2+}. Models of heart function generated by ionic substitution experiments would be greatly enhanced by a detailed knowledge of the characteristics of alkaline earth metal interaction with each organelle, including the sarcolemma, which has a vital role in cardiac excitation-contraction coupling. The present studies were conducted in order to (1) elucidate the details of one aspect of these interactions, the ability of Sr^{2+} and Ba^{2+} to substitute for Ca^{2+} in Na^+/Ca^{2+} exchange, and (2) gain insight into the mechanisms of Na^+-dependent Ca^{2+} transport in myocardial sarcolemma.

Methods

Highly-purified sarcolemmal vesicles were isolated from canine ventricles by a procedure that has been described previously [17]. Vesicles were harvested from a band in 26% (w/w) sucrose from a discontinuous sucrose gradient. This fraction was diluted, spun down ($177\,000 \times g$; 75 min), resuspended in 140 mM NaCl and 10 mM Mops (pH 7.4 at 37°C), and subsequently frozen and stored in liquid N_2.

Na_i^+-dependent alkaline earth metal (M^{2+}) exchange was measured following a procedure described previously for Na^+/Ca^{2+} exchange [16,18], using the rapid uptake device. 4 μl of the Na^+-loaded sarcolemma (1.5–5 mg protein/ml) were suspended on the wall of a plastic tube containing 246 μl of uptake medium maintained at 37°C. The medium contained either 140 mM KCl or NaCl, 10 mM Mops or Hepes (pH 7.4 at 37°C), 5 μCi/ml of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ or $^{133}Ba^{2+}$ and a concentration of M^{2+} ranging from 2–200 μM as indicated below. The uptake was initiated by vortex mixing and quenched at a preset time by the rapid uptake device with the addition of 30 μl of 140 mM KCl and 10 mM $LaCl_3$. An aliquot of 220 μl was then filtered (Millipore, 0.45 μm), and the filters were washed with 2×3 ml of 140 mM KCl, 1 mM $LaCl_3$. La^{3+} was found to be an effective inhibitor of Na^+/M^{2+} exchange for all three alkaline earth metals (data not shown). The filters were dried and counted by liquid scintillation. Uptake by vesicles that were diluted into NaCl represented blanks and was subtracted for all data points. This corrected for M^{2+} uptake that was independent of a Na^+ gradient and M^{2+} that was bound superficially to the sarcolemma.

The kinetics of the Na^+/M^{2+} exchange were determined under the conditions described above with 40 μM M^{2+}, and the uptake quenched at times ranging from 0.5 to 360 s. Because of variations in the absolute magnitude of the uptake between sarcolemmal preparations, these data are expressed as a percent of the vesicular Ca^{2+} content after 120 s of uptake in the same preparation.

The inhibition of Na^+/Ca^{2+} exchange by Sr^{2+} and Ba^{2+} was studied by including either 20 μM Sr^{2+} or 100 μM Ba^{2+} in the uptake medium. The concentration of Ca^{2+} was varied from 2–200 μM, and the reaction was quenched at 2 s. The effect of membrane potential on Na_i^+-dependent alkaline earth metal uptake was studied by including 0.36 μM valinomycin (dissolved in DMSO) in the uptake medium. The final concentration of DMSO was 0.4%, and this amount was also included in tubes without valinomycin without any apparent effect on uptake. The concentration of M^{2+} was 40 μM and the exchange was quenched at 2 s.

Passive M^{2+} efflux was studied by first loading the vesicles by Na^+/M^{2+} exchange. Vesicles were loaded (with $[M^{2+}]_o = 40$ μM) for 2 min for Ca^{2+} and Sr^{2+} and for 6 min for Ba^{2+}. Efflux was initiated by a 5-fold dilution with a solution containing 140 mM KCl and 1 mM EGTA. The final concentrations during the efflux were (in mM): K^+, 140; Na^+, 0.5; EGTA, 0.8. Using the absolute association constants of EGTA for M^{2+} of 10.97, 8.50 and 8.41 for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively (expressed as $\log K$) as determined by Schwarzenbach at $T = 20$°C and ionic strength of 0.1 M [19], and assuming the $[H^+]$ at pH 7.4 to be 50.84 nM, the calculated apparent association constants of EGTA for M^{2+} are $1.72 \cdot 10^7$, $5.83 \cdot 10^4$ and $4.74 \cdot 10^4$ M^{-1} for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively. If one assumes that the actual [EGTA] is 0.77 mM (96% purity) and neglects sarcolemmal M^{2+} buffering, then at time zero extravesicular

pM^{2+} are determined to be pCa 9.2, pSr 6.8 and pBa 6.7. These concentrations are sufficiently low to have no effect on the passive efflux of M^{2+}.

Aliquots of this dilution were taken at 0, 2 and 6 min and then filtered and washed as described above. The M^{2+} content of the vesicles after these periods of passive efflux are expressed as a percentage of the M^{2+} content at time zero.

Protein was determined by the method of Lowry et al. [20]. The radioisotopes $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$ were purchased from New England Nuclear. These isotopes were evaporated to dryness in an oven and then redissolved in distilled water. This procedure was essential for reproducibility. All alkaline earth metals were added as the chloride salt. Valinomycin and EGTA were purchased from Sigma Chemical Company.

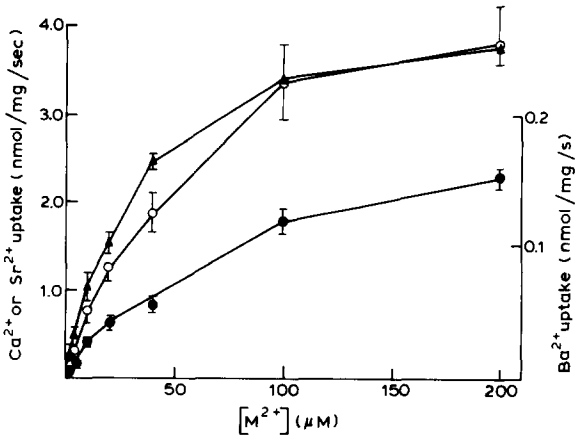

Fig. 2. Na^+/M^{2+} exchange as a function of divalent cation concentration. The uptake was quenched (see Methods for details) at 2 s for Ca^{2+} and Sr^{2+} and at 30 s for Ba^{2+}. Note the difference in scaling for Ba^{2+} on the right-hand ordinate. ▲, Ca^{2+} ($n = 5$); ○, Sr^{2+} ($n = 4$); ●, Ba^{2+} ($n = 3$).

Results

The kinetics of Na^+/M^{2+} exchange are shown in Fig. 1. Both Ca^{2+} and Sr^{2+} uptake increased with time in a linear fashion for approx. the first 6 s. The velocity of Ba^{2+} uptake, however, did not decrease until after 60 s. After 360 s both Ca^{2+} and Sr^{2+} content were reduced compared to 120 s, while Ba^{2+} continued to increase.

By varying $[M^{2+}]$ between 2–200 μM and determining initial rate of Na_i^+-dependent M^{2+} uptake at each concentration (as shown in Fig. 2) the K_m of the exchanger for each M^{2+} could be calculated. Eadie-Hofstee plots of these data indicated that the K_m values of the exchanger for M^{2+} were 30, 58 and 73 μM for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively. The V_{max} values were 4.2, 4.8 and 0.2 nmol/mg per s for the same sequence of cations. There was no significant difference in the V_{max} of the exchanger for Ca^{2+} and Sr^{2+}. Although there was considerable variation in the magnitude of the stimulation of Na^+/M^{2+} exchange by 0.38 μM

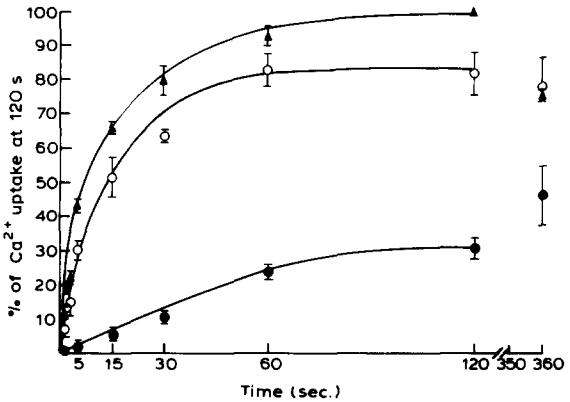

Fig. 1. Time course of Na^+-dependent alkaline earth metal uptake. Data are expressed as a percentage of the Ca^{2+} uptake at 120 s in the same sarcolemmal preparation. The concentration of M^{2+} was 40 μM. ▲, Ca^{2+} ($n = 6$); ○, Sr^{2+} ($n = 5$). ●, Ba^{2+} ($n = 4$). 100% = 23.0 nmol/mg protein.

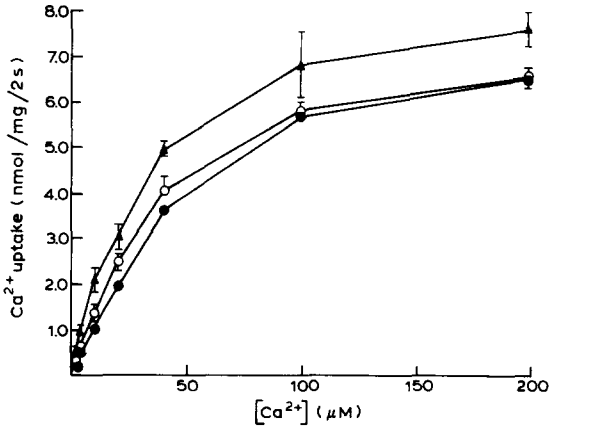

Fig. 3. Inhibition of Na^+/Ca^{2+} exchange by 20 μM Sr^{2+} ($n = 4$) or 100 μM Ba^{2+} ($n = 2$) at various Ca^{2+} concentrations. Uptake was quenched at 2 s. ▲, Ca^{2+} ($n = 4$) as the only divalent cation; ○, + 20 μM Sr^{2+}; ●, +100 μM Ba^{2+}.

TABLE I

RATES OF PASSIVE M^{2+} EFFLUX FROM SARCOLEMMAL VESICLES

M^{2+}	M^{2+} content ($t = 0$) (nmol/mg prot.)	M^{2+} content (% of M^{2+} content at $t = 0$)		k_E (min^{-1})
		120 s	360 s	
Ca^{2+}	22.9	71.9 ± 7.3 ($n = 5$)	32.8 ± 5.3 ($n = 5$)	8.4
Sr^{2+}	19.2	87.8 ± 3.8 [a] ($n = 4$)	55.4 ± 1.7 [b] ($n = 4$)	6.3
Ba^{2+}	11.0	90.0 ± 8.6 ($n = 3$)	70.7 ± 8.9 [b,c] ($n = 3$)	4.4

[a] $p < 0.05$ (Sr^{2+} vs. Ca^{2+}).
[b] $p < 0.001$ (Sr^{2+} or Ba^{2+} vs. Ca^{2+}).
[c] $p < 0.05$ (Ba^{2+} vs. Sr^{2+}).

valinomycin between different sarcolemmal preparations, the results showed a consistent degree of stimulation for all three alkaline earth metals within the same preparation (data not shown).

The inclusion of 20 μM Sr^{2+} or 100 μM Ba^{2+} in the uptake media inhibited the initial rate of Na^+/Ca^{2+} exchange at all $[Ca^{2+}]_o$ studied, as can be seen in Fig. 3. A double-reciprocal plot of these data indicates that the V_{max} of Na^+/Ca^{2+} exchange is unchanged in the presence of Sr^{2+} or Ba^{2+}. The inhibition constant (K_I) was derived from the point at which the graph intercepts the abscissa, as this point represents $-1/[K_m(1 + ([I]/K_I))]$. The K_I for Sr^{2+} and Ba^{2+} on Na^+/Ca^{2+} exchange were determined to be 32 and 92 μM, respectively. Higher concentrations of Ba^{2+} (200 μM) reduced the velocity of Na^+/Ca^{2+} exchange in a manner not strictly consistent with competitive inhibition (not shown).

The time course of passive efflux of the alkaline earth metals from vesicles loaded by Na^+/M^{2+} exchange is shown in Table I. The percent M^{2+} content after 2 min was 71.9 ± 7.3 ($n = 5$), 87.9 ± 3.8 ($n = 4$) and 90.0 ± 8.6 ($n = 3$) for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively. After 6 min these values were reduced to 32.8 ± 5.3 ($n = 5$), 55.4 ± 1.7 ($n = 4$) and 70.7 ± 8.9 ($n = 3$). Within a given sarcolemmal preparation the rate of M^{2+} loss was always $Ca^{2+} > Sr^{2+} > Ba^{2+}$.

Discussion

The experiments clearly indicate that the Na^+/Ca^{2+} exchanger in sarcolemma from the canine heart can transport Sr^{2+} and Ba^{2+} in addition to Ca^{2+}. The selectivity sequence based on the initial velocities of Na^+-dependent uptake at 40 μM M^{2+} is $Ca^{2+} > Sr^{2+} \gg Ba^{2+}$. Because valinomycin stimulated all forms of Na^+/M^{2+} exchange studied, it appears that the transport of all three cations is electrogenic and represents the same stoichiometry. Further experimentation is required, however, to confirm the latter point.

As the V_{max} values of the Na^+-dependent Ca^{2+} and Sr^{2+} uptake are not different, the selectivity appears to be related to differences in the affinity of these metals for the divalent binding site of the exchanger. It is apparent, however, that the low rate of Ba^{2+} uptake (V_{max} being more than an order of magnitude lower than that of Ca^{2+} and Sr^{2+}) is a reflection of more than simply reduced affinity. The possibility of reduced stoichiometry is unlikely since Na^+/Ba^{2+} exchange was stimulated by valinomycin. These data suggest that the efficacy of the translocation of Ba^{2+} across the membrane is also reduced. This selectivity sequence is not unexpected if one considers the crystal ionic radii of these metals (in nm): Ca^{2+}, 0.099; Sr^{2+}, 0.112; Ba^{2+}, 0.134 [21].

The ability of Sr^{2+} and Ba^{2+} to inhibit Na^+/Ca^{2+} exchange was also studied. As expected, Sr^{2+} ($K_I = 32$ μM) was more potent than Ba^{2+} ($K_I = 92$ μM) as an inhibitor of the exchanger. Interestingly, it appears that Sr^{2+} is relatively more effective than Ba^{2+} at competing with Ca^{2+} for the divalent cation binding site as an inhibitor than it is in substituting for Ca^{2+} in

Na^+/Ca^{2+} exchange ($K_M = 58$ and 73 μM for Sr^{2+} and Ba^{2+}, respectively). Since the V_{max} of Na^+/Sr^{2+} exchanger is not different from that of Ca^{2+} and since Sr^{2+} competes favorably for the divalent cation binding site, it appears that the relatively high K_M of Na^+/Sr^{2+} exchange may be related to other factors. It is possible that although Sr^{2+} may substitute for Ca^{2+} with respect to binding and translocation, it may interact differently with other components of the exchanger. This point deserves further investigation as it should give insight into the regulation of Na^+/Ca^{2+} exchange [22].

The rates of passive efflux from the vesicles showed clear differences for the three cations. Because of the extravesicular M^{2+} buffering by EGTA and the attendant high pM_e (> 6.7), the transvesicular movement is unidirectional as the magnitude of the influx will be negligible. The relevant equation describing the rate of vesicular M^{2+} loss is

$$\frac{M_i(t)}{M_i(0)} = e^{-k_E t}$$

where the term k_E is an overall rate constant through one or more efflux pathways. Because the mechanism of the passive efflux of cations in the vesicular preparation is not understood, it is difficult to ascribe the rate constant(s) to any specific membrane components. The overall efflux pathways demonstrates selectivity with a sequence of $Ca^{2+} > Sr^{2+} > Ba^{2+}$. The efflux curves follow approximate first order kinetics, and the rate constants, which should then be independent of small differences in initial load, are 8.4, 6.3 and 4.4 min^{-1} for Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively. Since these k_E values may represent sums of rate constants through various components of the lipid bilayer (possibly including the Ca^{2+} channel), it is conceivable that there are efflux pathways which show no ionic selectivity.

The fact that the exchanger can transport Sr^{2+} and Ba^{2+} was suggested in a previous study by Trosper and Philipson [23]. These experiments demonstrated that these alkaline earth metals were able to stimulate passive Ca^{2+} efflux, suggesting that these ions substitute for Ca^{2+} in Ca^{2+}/Ca^{2+} exchange. Interestingly, Cd^{2+} was found to be more effective than Ca^{2+} (or any other divalent cation) for stimulation of passive Ca^{2+} efflux.

It has been demonstrated that both Sr^{2+} and Ba^{2+} can replaced Ca^{2+} in carrying the transsarcolemmal slow inward current [24] in the mammalian myocardium. Both of these alkaline earth metals can compete with D600 to overcome its inhibition of this current. While increasing $[Ca^{2+}]_o$ tends to abbreviate the action potential, increasing either $[Sr^{2+}]_o$ or $[Ba^{2+}]_o$ prolongs the action potential in the frog heart [25]. This effect appears to result from a differential ability to activate delayed rectification and presumably reflects the ability of these cations to regulate the K^+ channel. In general, the calcium channel of cardiac and skeletal muscle has a selectivity sequence of $Ca^{2+} > Sr^{2+} > Ba^{2+}$ [26–28].

The ability of the alkaline earth metals to interact with cardiac troponin C has a similar sequence as the calcium channel [29]. It is estimated that the affinity of Ba^{2+} for troponin C is approx. 0.03 that of Ca^{2+}. The substitution of Ba^{2+} for Ca^{2+} in the low mM range in the bathing solution of isolated mammalian ventricular muscle produces a contracture [30,31]. The strength of the contracture increases as $[Ba^{2+}]_o$ is increased from 0.1 up to 4.0 mM [31]. Ba^{2+} depolarized the tissue and the magnitude of the depolarization was dependent, in a non-Nernstian fashion, on $[Ba^{2+}]_o$. Interestingly, when 0.5 mM Mn^{2+} was added the contracture, but not the depolarized state, was reversed [31].

It is frequently stated that the sarcoplasmic reticulum sequesters Ca^{2+} and Sr^{2+} but not Ba^{2+} [31–33]. The study that is cited by these articles used ANS fluorescence intensity in rabbit white skeletal muscle microsomes to investigate ATP-dependent alkaline earth metal uptake [34]. However, two points should be considered. First, cardiac sarcoplasmic reticulum Ca^{2+} transport is different from that of skeletal muscle [35], and the ability to sequester Ba^{2+} may differ. Second, ANS fluorescence may not be the most sensitive method for detecting Ba^{2+} transport. More experimentation is required to properly evaluate the capacity of cardiac sarcoplasmic reticulum to transport the alkaline earth metals. These criticisms notwithstanding, it is noted that during the Ba^{2+} contracture in cardiac tissue there is little indication of reversal [31] which may represent supportive evi-

dence that cardiac sarcoplasmic reticulum is unable to transport Ba^{2+}.

In conclusion, the above results clearly show that the Na^+/Ca^{2+} exchanger is also able to transport (albeit to a lesser degree) Sr^{2+} and Ba^{2+}. Passive permeabilities were substantially different for the alkaline earth metals and had the sequence $Ca^{2+} > Sr^{2+} > Ba^{2+}$. The data elucidate the pathways by which alkaline earth metals cross the sarcolemma. The interaction of the cations with the exchanger in different regulatory states [22] may require further examination.

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