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# EFFECTS OF DIVALENT AND TRIVALENT CATIONS ON $Na^+$-$Ca^{2+}$ EXCHANGE IN CARDIAC SARCOLEMMAL VESICLES

TERRY L. TROSPER and KENNETH D. PHILIPSON *

*Departments of Medicine and Physiology, and the American Heart Association, Greater Los Angeles Affiliate, Cardiovascular Research Laboratories, University of California at Los Angeles, Center for the Health Sciences, Los Angeles, CA 90024 (U.S.A.)*

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**Inhibition of $Na^+$-$Ca^{2+}$ exchange processes in canine cardiac sarcolemmal vesicles by several divalent and trivalent cations has been investigated. The order of cation effectiveness in inhibiting initial rates of $Na_i^+$-induced $Ca^{2+}$ uptake in the presence of 140 mM $Na_i^+$ and 20 $\mu$M $Ca_o^{2+}$ is $La^{3+} > Nd^{3+} > Tm^{3+} \sim Y^{3+} > Cd^{2+} \gg Sr^{2+} > Ba^{2+} \sim Mn^{2+} \gg Mg^{2+}$. The effectiveness of the divalent ions is related to their ionic crystal radius as compared with that of $Ca^{2+}$. No such relationship was observed for the trivalent ions, which appeared instead to be more effective the larger their radius. Very low concentrations of trivalent ions ($(1–6)\cdot10^{-7}$ M) caused slight stimulation of $Ca^{2+}$-exchange uptake. The trivalent ions also inhibited passive and $Na_o^+$-induced $Ca^{2+}$ efflux from sarcolemmal vesicles, in the same concentration range as that for inhibiting uptake. The divalent ions, however, stimulated $Ca^{2+}$ efflux, possibly via divalent cation-$Ca^{2+}$ exchange. These various results suggest that the divalent and trivalent cations interact differently with the exchange apparatus in the sarcolemma.**

## Introduction

Since Reeves and Sutko [1] first characterized $Na^+$-$Ca^{2+}$ exchange in isolated cardiac sarcolemmal vesicles, much has been learned about the functional properties of this system. The process is electrogenic [2,3], being affected by membrane potential [4], can occur across the sarcolemma in either direction [5,6], and is sensitive to pH [7]. Exchange is stimulated by phosphorylation of membrane proteins [8] and by mild proteinase treatment of the vesicles [9]. $Sr^{2+}$ and $Ba^{2+}$ can substitute for $Ca^{2+}$ in the exchange process, although the exchange mechanism appears to have lower affinities for these ions [10].

$Na^+$-$Ca^{2+}$ exchange may contribute significantly to $Ca^{2+}$ movements across myocardial sarcolemma during a heart beat cycle, and the exchange mechanism may thus be involved in excitation-contraction coupling [11]. Parallels have been observed between the inhibition, by certain divalent and trivalent cations, of processes observed in intact myocardial tissue, and the displacement of $Ca^{2+}$ from the sarcolemma by these same ions [12,13]. Also, a similar relative effect of some of these ions on plateau levels of $Ca^{2+}$ uptake into sarcolemmal vesicles via $Na^+$-$Ca^{2+}$ exchange has been briefly noted [5].

A more thorough investigation of the effects of several di- and trivalent cations on $Na^+$-$Ca^{2+}$ exchange in cardiac sarcolemmal vesicles is reported here. All of the cations studied ($Ba^{2+}$, $Cd^{2+}$, $La^{3+}$, $Mg^{2+}$, $Mn^{2+}$, $Nd^{3+}$, $Sr^{2+}$, $Tm^{3+}$, $Y^{3+}$) except $Mg^{2+}$ were capable, at appropriate concentrations,

* Correspondence should be addressed to: Dr. K.D. Philipson, Cardiovascular Research Lab, A3-381 CHS, UCLA, School of Medicine, Los Angeles, CA 90024, U.S.A.

of completely inhibiting exchange uptake of $Ca^{2+}$. Their effects on efflux were complex. The relative effectiveness of the various ions in inhibiting $Na^+$-$Ca^{2+}$ exchange phenomena was different in some cases from that previously reported. The divalent and trivalent cations appear to interact with the exchange apparatus in different ways. Not enough information is now available to propose molecular details for the interaction or for the operation of the exchange mechanism.

## Methods

Sarcolemmal vesicles were isolated from trimmed canine ventricles by published procedures [14], and were stored in liquid $N_2$ in 140 mM KCl or 140 mM NaCl plus 5 mM Tris-maleate buffer (pH 7.4 at 37°C) until used. Initial rates of $Na_i^+$-induced $Ca^{2+}$ uptake were measured, using the rapid uptake device and the protocol previously described [7], in the presence or absence of inhibitor ions at the indicated final concentrations. All uptake data were obtained with 20 $\mu$M $CaCl_2$ and 0.36 $\mu$M valinomycin present in the uptake medium, and the filtered vesicles were washed with ice cold 1 mM EGTA in 140 mM KCl. $Ca^{2+}$ uptake (nmol/mg protein per s) was calculated from the counts remaining on the filter, using measured specific activities and protein concentrations. Data on the effects of inhibitors are presented as percent inhibition of the uptake observed in the absence of inhibitor. Each value was corrected for non-specific binding and uptake in the absence of internal sodium.

Efflux experiments were performed using vesicles preloaded with isotopically labeled $Ca^{2+}$ by 2 min of $Na_i^+$-induced $Ca^{2+}$ uptake at 37°C. After $Ca^{2+}$ loading, the vesicle suspension was diluted 15-fold with efflux medium at the same temperature, and $Ca^{2+}$ efflux was measured according to published procedures [6]. All media were buffered at pH 7.4 with 5 mM Tris-maleate and had a total $K^+$ plus $Na^+$ concentration of 140 mM. Efflux was stopped after the times indicated and an aliquot of vesicles filtered and washed as described for uptake experiments. The filters were counted to determine $Ca^{2+}$ remaining in the vesicles. In each experiment the amount of $Ca^{2+}$ loaded (zero efflux time) was also measured by diluting $Ca^{2+}$-loaded vesicles 15-fold with a solution containing 290 $\mu$M $La^{3+}$ in 140 mM KCl. This diluted suspension, in which $Ca^{2+}$ exchange was effectively stopped, was incubated at 37°C for the same length of time as were the efflux samples, and $Ca^{2+}$ content of an aliquot determined as for the efflux samples. Calcium retention by vesicles diluted into the various efflux media is expressed as percent of $Ca^{2+}$ content at zero efflux time. $Ca^{2+}$ lost from vesicles diluted with buffered 140 mM KCl only (passive efflux) was also measured.

Sarcolemmal protein content was determined by the method of Lowry et al. [15]. All inhibitor ions were added as chloride salts. They were obtained from Aldrich or Alfa Thiokol/Ventron Division and were stated to be 99.99% pure, except for $MgCl_2$ and $CdCl_2$, which were reagent grade. Valinomycin was purchased from Sigma Chemical Co. and $^{45}Ca^{2+}$ from New England Nuclear.

## Results

### *Initial rate of $Na_i^+$-induced $Ca^{2+}$ uptake*

The rate of $Na_i^+$-induced $Ca^{2+}$ uptake by the sarcolemmal vesicle preparations used in these experiments was linear for more than one second in the absence of inhibitors. The initial rate of

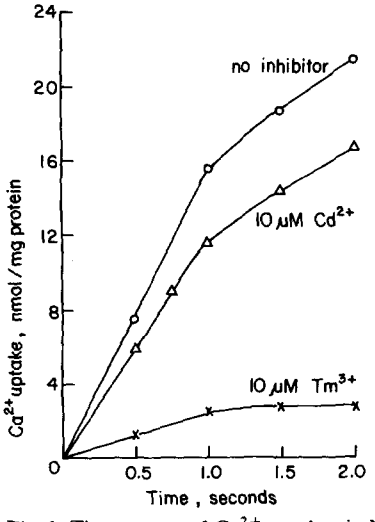


Fig. 1. Time course of $Ca^{2+}$ uptake via $Na^+$-$Ca^{2+}$ exchange in the absence and presence of inhibitors at 37°C. O——O, no inhibitor; Δ——Δ, 10 $\mu$M $Cd^{2+}$; ×——×, 10 $\mu$M $Tm^{3+}$. $Na_i^+$ is 140 mM and $Ca^{2+}$ is 20 $\mu$M in all cases.

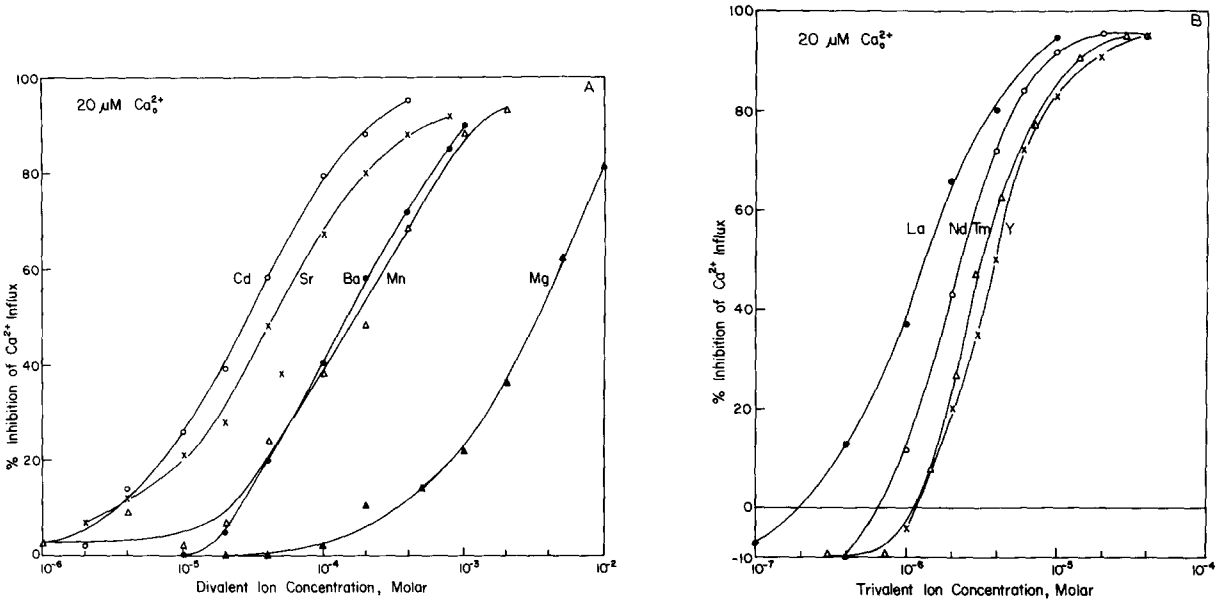


Fig. 2. Inhibition of $Na_i^+$-dependent $Ca^{2+}$ uptake by di- and trivalent cations, as a function of the logarithm of cation concentration. (A) Divalent ion inhibitors. (B) Trivalent ion inhibitors. In all cases, 20 μM $Ca_o^{2+}$ and 0.36 μM valinomycin were present. Inhibition is expressed as percent of control uptake in the absence of inhibitors, and all data are corrected for nonspecific binding and uptake by mechanisms other than $Na_i^+$-induced exchange.

uptake varied with the preparation, but was always within the range of 5 to 14 nmol $Ca^{2+}$/mg membrane protein per s when $Ca^{2+}$ was 20 μM. Both inside-out and right-side-out vesicles were present; the relative contributions of each type were not ascertained. In the presence of the inhibitors, although initial rates were reduced, exchange uptake of $Ca^{2+}$ was linear for ≥ 1.0 s. Data for three particular cases are shown in Fig. 1 for comparative purposes.

*Inhibition of $Na^+$-$Ca^{2+}$ exchange uptake*

Effects of varying inhibitor concentrations on initial rates of $Na_i^+$-induced $Ca^{2+}$ uptake are presented in Fig. 2. Uptake values are quoted as percent of control uptake in the absence of inhibitor cation, and data points are averages from three or more experiments. In all cases external $Ca^{2+}$ concentration, $[Ca]_o$, was 20 μM. The divalent ions investigated become effective over a wide range of concentrations (Fig. 2A). Only $Cd^{2+}$ and $Sr^{2+}$ cause significant inhibition at concentrations equal to or less than $[Ca]_o$, but all except $Mg^{2+}$ are capable of inhibiting exchange uptake by greater than 90% if they are present at high enough concentrations. The order of effectiveness is seen to be: $Cd^{2+} > Sr^{2+} > Ba^{2+} \simeq Mn^{2+} \gg Mg^{2+}$. Even

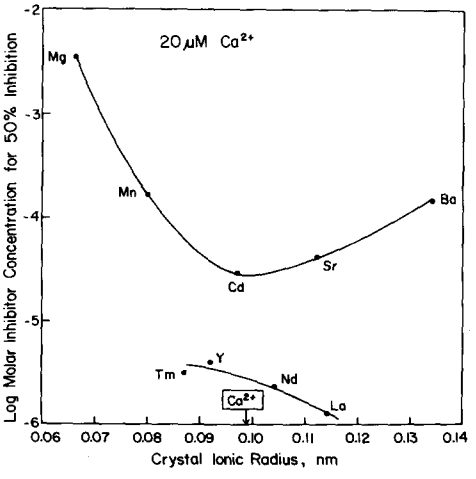


Fig. 3. Relative effectiveness of cations in inhibiting initial rates of $Na_i^+$-dependent $Ca^{2+}$ uptake. Logarithm of cation concentration at which 50% inhibition of exchange uptake was observed is plotted as a function of the nonhydrated crystal ionic radius. Note that $Ca^{2+}$ radius is 0.099 nm.

when present at 10 mM, a 50-fold excess over $Ca^{2+}$, $Mg^{2+}$ is unable to inhibit $Ca^{2+}$-exchange uptake completely.

All of the trivalent cations used were significantly better inhibitors of $Ca^{2+}$-exchange uptake than any of the divalent ions (Fig. 2B). Greater than 90% inhibition was observed at $< 20$ $\mu$M in each case. Again, $[Ca]_o$ was 20 $\mu$M. Percent inhibition showed a much steeper dependence on ion concentration for the trivalent ions than for the divalent ions. The order of effectiveness was $La^{3+} > Nd^{3+} > Tm^{3+} > Y^{3+}$. The most effective, $La^{3+}$, caused 50% inhibition at 1/3 the concentration of the least effective trivalent, $Y^{3+}$. By contrast, the comparable divalent ion concentration range was 30-times as great.

Very low concentrations of trivalent ions ($10^{-7}$ to $10^{-6}$ M) appeared to stimulate $Na_i^+$-induced $Ca^{2+}$ uptake slightly, as shown by the negative inhibition percentages in Fig. 2B. This effect was also observed when 280 mM sucrose, instead of 140 mM KCl, was used to maintain osmolarity in the uptake medium. The phenomenon was observed repeatedly; the largest enhancement obtained for an individual vesicle preparation was approx. 20%. The presence of valinomycin in the uptake medium was not required for enhancement, and 0.4 $\mu$M $Nd^{3+}$ could further enhance exchange uptake already stimulated by chymotrypsin treatment [9]. The other trivalent cations were not tested for this last phenomenon.

The relative effectiveness of all the cations investigated can be compared in Fig. 3, where the logarithm of the inhibitor concentration required for 50% inhibition of $Ca^{2+}$-exchange uptake from media containing 20 $\mu$M $Ca^{2+}$ is plotted as a function of the ionic crystal radius [16]. This graph emphasizes not only the much more potent, but also the qualitatively different, behavior of the trivalent inhibitors. The divalent ion effectiveness is greater, the nearer in size the ion is to $Ca^{2+}$. However, for the trivalent ions, the opposite trend is apparent.

*Inhibition of $Ca^{2+}$ efflux*

Vesicles to be used for efflux studies were loaded with isotopically labeled $Ca^{2+}$ via the $Na^+$-$Ca^{2+}$ exchange mechanism and then diluted with a large volume of efflux medium (see Methods). Dilution after 2 min of exchange uptake ensured that the $Ca^{2+}$ load had reached plateau levels [5]. Determination of an accurate rate of $Ca^{2+}$ efflux immediately following vesicle dilution would be very difficult with the isotope method used in the present investigation. Quantitation of this rate would require detection of a very small change in a large amount of intravesicular $Ca^{2+}$. Consequently, effects of the cation inhibitors on $Ca^{2+}$ efflux occurring over time periods of 30 s or longer were investigated.

After 30 s of efflux, vesicles diluted into Tris-maleate buffer containing 140 mM KCl lost approx. 20 to 35% of their $Ca^{2+}$ load. This loss was essentially completely prevented when 20 $\mu$M of any of the trivalent ions was present in the efflux medium. This concentration of trivalent inhibitor is the same as that which is effective in stopping $Na_i^+$-induced $Ca^{2+}$ uptake. At lower cation concentrations, $Ca^{2+}$ loss was only partially prevented. A clear-cut order of effectiveness for the four ions was not evident; $La^{3+}$ appeared slightly less effective than $Nd^{2+}$, and $Tm^{3+}$ and $Y^{3+}$ somewhat more effective, in some experiments. Larger uncertainties in efflux than in uptake data prevented a more quantitative comparison.

The trivalent cations also reduced $Na_o^+$-independent (passive) $Ca^{2+}$ efflux over longer time periods. After 3 or 5 min, when loss of $Ca^{2+}$ in the absence of inhibitors was substantial, significant reductions in efflux were observed in the presence of inhibitors. At cation concentrations $\leq 10$ $\mu$M, decreases in $Ca^{2+}$ efflux of from 35 to 75% occurred in different experiments, and 20 $\mu$M inhibitor was again completely effective.

$Na_o^+$-induced $Ca^{2+}$ efflux, via the $Na^+$-$Ca^{2+}$ exchange mechanism, was also inhibited by the lanthanides. Extent of inhibition depended not only on concentration of inhibitor and time of efflux, but also on $[Na^+]_o$. For example, 20 $\mu$M $La^{3+}$ completely inhibited $Ca^{2+}$ efflux induced by 25 mM $Na_o^+$ over a 30-s time period. These effects were not investigated in sufficient detail to allow quantitative comparisons.

The divalent ions which inhibited $Na_i^+$-induced $Ca^{2+}$ uptake actually stimulated $Ca^{2+}$ loss in either the absence or the presence of $Na_o^+$. With the exception of $Mg^{2+}$, at concentrations as low as 10 or 20 $\mu$M these cations caused a significant in-

TABLE I

EFFECT OF 20 $\mu$M DIVALENT CATION ON $Ca^{2+}$ EFFLUX IN ABSENCE OF $Na_o^+$

Vesicles were loaded with isotopically labeled $Ca^{2+}$ via 2 min of $Na^+$-$Ca^{2+}$ exchange and then diluted 15-fold with 140 mM KCl, 5 mM Tris-maleate containing 20 $\mu$M of the ion indicated (present as the chloride salt). $Ca^{2+}$ content was determined after 5 min incubation at 37°C.

| Ion | None | $Ba^{2+}$ | $Cd^{2+}$ | $Mg^{2+}$ | $Mn^{2+}$ | $Sr^{2+}$ | $Ca^{2+}$ |
|---|---|---|---|---|---|---|---|
| $Ca^{2+}$ lost (% of load [a]) | 28 | 54 | 85 | 23 | 34 | 74 | 74 |
| $Ca^{2+}$ lost (% of control loss [b]) | | 190 | 300 | 80 | 120 | 260 | 260 |

[a] Load = $Ca^{2+}$ content at zero time in efflux medium.
[b] Control loss = loss in the absence of inhibitors.

crease of $Ca^{2+}$ efflux (Table I) when no external $Na^+$ was added. The order of effectiveness in stimulating $Ca^{2+}$ efflux in the absence of $Na_o^+$ was $Cd^{2+} > Sr^{2+} = Ca^{2+} > Ba^{2+} > Mn^{2+}$, which is approximately the same as their relative effectiveness in preventing $Na_i^+$-induced $Ca^{2+}$ uptake. Relative effectiveness of $Ca^{2+}$ efflux stimulation by these ions in the presence of $Na_o^+$ was not quantitated. However, addition of e.g., 60 $\mu$M $Cd^{2+}$, to an efflux medium containing 10 mM $Na_o^+$ resulted in an increase in the $Ca^{2+}$ efflux above that caused by $Na_o^+$ alone.

## Discussion

The cations used in this study have been previously investigated as inhibitors of various contractile phenomena in intact muscle tissue [12,13] and as agents to displace bound Ca from membranes [13]. In only one of the situations reported here, inhibition of $Na_i^+$-induced $Ca^{2+}$ uptake by divalent cations, are the present results on relative inhibitor effectiveness the same as those in the literature. Reduction in tension maintained by papillary muscle perfused with the ions [13], prevention of creatine kinase release by perfused rabbit septa subjected to 30 min of conditions causing calcium paradox [12], and displacement of bound Ca from tissue cultures of neonatal rat myocardial cells and from sarcolemmal vesicles [13] by the divalent ions, all showed the same qualitative dependence on ionic crystal radius found in the present investigation.

The stimulatory effect of the divalent ions on $Ca^{2+}$ efflux would be consistent with their inhibitory effect on $Ca^{2+}$ exchange uptake, if these ions are capable of substituting for $Ca^{2+}$ in an exchange process. That is, the enhanced loss of $Ca^{2+}$ from sarcolemmal vesicles diluted into media containing another divalent ion may be owing to a $Ca^{2+}$-$Ca^{2+}$ exchange mechanism which will accept these other ions also. Evidence of $Na^+$-divalent cation exchange has been presented by Tibbits and Philipson for $Ba^{2+}$ and $Sr^{2+}$ [10], and the latter was found to be exchanged for $Na^+$ more effectively than the former. The present results suggest that either $Mn^{2+}$ or $Cd^{2+}$ might also exchange for $Na^+$ across the sarcolemma; this possibility was not investigated. In all cases reported for sarcolemmal vesicles and for intact tissue, the site(s) for $Ca^{2+}$ interaction with the membranes appear to discriminate similarly among the divalent cations on the basis of their size.

The results of the trivalent ion inhibition experiments were unexpected in some respects. In a preliminary experiment Bers et al. [5] had found $Cd^{2+}$ to be a better inhibitor than $La^{3+}$ of $Na_i^+$-induced $Ca^{2+}$ uptake by sarcolemmal vesicles, when uptake was measured after it had reached a plateau level (i.e., after 2 min). In the present work, $La^{3+}$ was the best inhibitor of initial rates of exchange uptake, and all four trivalent cations used were significantly more effective than $Cd^{2+}$ ($\geq 90\%$ vs. 40% inhibition at equimolar $Ca^{2+}$ and inhibitor concentrations). However, these different outcomes need not be contradictory. The $Na_i^+$-induced $Ca^{2+}$ uptake level after 2 min will depend on efflux as well as influx behavior. The

lanthanides are very good inhibitors of efflux, whereas $Cd^{2+}$ stimulates efflux markedly, as shown above. A lower plateau level of intravesicular $Ca^{2+}$ might thus be expected at $Cd^{2+}$ concentrations causing only partial inhibition of $Ca^{2+}$ uptake, when loss of $Ca^{2+}$ by exchange with the external divalent could occur.

The order of effectiveness with which the trivalent cations inhibited initial rates of $Na_i^+$-induced $Ca^{2+}$ uptake by the sarcolemmal vesicles is the opposite of that reported for displacement of $Ca^{2+}$ bound to sarcolemmal vesicles, and for inhibition of tension development in rat papillary muscles [13]. The largest ion, $La^{3+}$, is the most effective, and the ions smaller than $Ca^{2+}$ ($Tm^{3+}$, $Y^{3+}$) the least effective in the present studies. This is also at variance with the results of Triggle and Triggle [17], who reported that $La^{3+}$ was one of the least, and $Tm^{3+}$ was the most, effective lanthanide inhibitor of longitudinal smooth muscle mechanical responses. Although the trivalent ions investigated in the present work were also potent inhibitors of $Na_o^+$-independent $Ca^{2+}$ efflux, a clear cut order of effectiveness was not observed. This was probably due to the greater difficulty in obtaining efflux data as accurate as initial influx rates.

The repeated observance of the slight enhancement of initial $Ca^{2+}$-exchange uptake rates by low concentrations of the trivalent cations (Fig. 3) under various conditions tends to rule out the possibility that the phenomenon is an artifact of the procedure. A satisfactory explanation for this unexpected observation has not yet been found.

The results of the present study suggest that the mechanism by which the divalent cations inhibit $Na^+$-$Ca^{2+}$ exchange is different from that by which trivalent cations act. Both classes of cations most probably compete with $Ca^{2+}$ for sites on the exchange apparatus. The fact that their subsequent effects are distinctly different could be owing to discrimination of both ionic size and charge characteristics by the exchanger. The simplest model would be a single type of $Ca^{2+}$-binding site, where the $+3$ charge on a trivalent ion prevents its transport across the sarcolemma, but the bound divalent cations are transported with an ease dependent on their size. Alternatively, the exchange apparatus may have more than one type of site, including a regulatory site not subject to size restraints where trivalent cation binding would result in inhibition of all ion transport. Furthermore, an individual trivalent cation may be able to interact simultaneously with adjacent negative membrane sites to prevent $Na^+$-$Ca^{2+}$ exchange. A detailed description of the interaction of $Ca^{2+}$ with the $Na^+$-$Ca^{2+}$ exchange system must await molecular description of the exchanger itself.

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