Rate-Limiting Mechanisms of Exchange Reactions in the Cardiac Sarcolemma Na^+-Ca^{2+} Exchanger†

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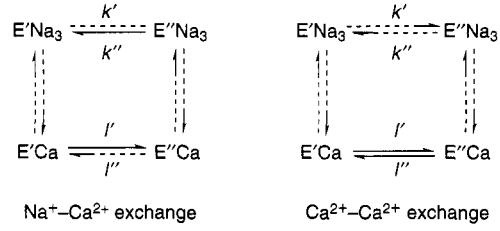
ABSTRACT: The effects of temperature, pH, voltage and K^+ were tested on Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges with a goal to elucidate the rate-limiting mechanisms. The initial rates (t = 1 s) of Na_i- and Ca_i-dependent ^{45}Ca uptakes were measured in the sarcolemma vesicles. At pH 7.4 the $Ca^{2+}-Ca^{2+}$ exchange shows a bell-shaped temperature curve with a maximum at 27−29 °C. This effect is not caused by irreversible inactivation of the exchanger. The increase of pH from pH 6.0 to 7.4 in the K^+-free medium decelerates the $Ca^{2+}-Ca^{2+}$ exchange 1.5−2.0-fold, while the addition of K^+ accelerates the $Ca^{2+}-Ca^{2+}$ exchange 2.0−3.0-fold. Therefore, the accelerating effect of K^+ opposes the decelerating effect of deprotonation. Temperatures increase (6−45 °C) in the K^+-free medium (pH 7.4) elevates the $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange ratio from 0.8 to 5.0. With varying temperatures (6−37 °C) and pH 5.0−9.7, K^+ has no considerable effect on Na^+-Ca^{2+} exchange but accelerates the $Ca^{2+}-Ca^{2+}$ exchange 2−3-fold. At 6−45 °C and fixed pH 7.4, the inside-positive potential ($\Delta\psi \geq +200$ mV) accelerates the Na^+-Ca^{2+} exchange 1.7−2.0-fold, suggesting that the same rate-limiting reaction controls the Na^+-Ca^{2+} exchange at various temperatures. It is concluded that (a) At pH > 6.5 (6−45 °C and 0−100 mM K^+) the voltage-sensitive Na^+ efflux limits the Na^+-Ca^{2+} exchange, while the Ca^{2+} efflux limits the $Ca^{2+}-Ca^{2+}$ exchange. (b) At pH < 6.1 (6−45 °C and 0−100 mM K^+) the voltage-insensitive Ca^{2+} influx limits both Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges (this may represent a reduced voltage sensitivity of Na^+-Ca^{2+} exchange and the similar rates of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges). (c) The bell-shaped temperature curve of $Ca^{2+}-Ca^{2+}$ exchange cannot be described by a simple reversible reaction involving two species (the exchange has to involve at least three species). (d) K^+ interacts with a deprotonated species (pH >6.1) accelerating the rate-limiting Ca^{2+} efflux of $Ca^{2+}-Ca^{2+}$ exchange.

The cardiac sarcolemma (cell membrane) Na^+-Ca^{2+} exchanger can catalyze the electrogenic Na^+-Ca^{2+} exchange ($3Na^+:Ca^{2+}$) as well as the electroneutral $Ca^{2+}-Ca^{2+}$ and Na^+-Na^+ exchanges (Hale & Reeves, 1984; Reeves, 1985; Philipson, 1990; Khananshvili, 1991a,b). These exchange reactions can be described as separate movements of Na^+ and Ca^{2+} ions (the consecutive or ping-pong mechanism) through the exchanger (Khananshvili, 1990a, 1991a,b; Niggli & Lederer, 1991; Hilgemann et al., 1991; Khananshvili & Weil-Maslansky, 1994). It was suggested before that the E·Na_3 species may bear a positive charge, while the E·Ca species carry no charge (e.g., the unloaded cation-binding domain may contain −2 charges) (Khananshvili, 1991a,b; Hilgemann et al., 1991; Matsuoka & Hilgemann, 1992). A similar model has been described before for the Na^+,K^+-ATPase (Goldshleger et al., 1987). Interestingly, both Na^+,K^+-ATPase and Na^+-Ca^{2+} exchangers bind three Na^+ ions and have a homologous sequence in a putative ion-binding domain (Nicoll et al., 1990; Philipson & Nicoll, 1992). Two negatively charged amino acids (Glu-113 and Glu-199) that belong to transmembrane segments 2 and 5 are essential for Na^+-Ca^{2+} exchange (Philipson et al., 1992). The Glu-199 is highly conserved in the Na^+,K^+-ATPase, Ca^{2+}-ATPase, and H^+,K^+-ATPase (Clarke et al., 1989; Philipson & Nicoll, 1992).

Scheme 1



The rate-limiting mechanisms of various exchange modes are still not clear. According to the consecutive mechanism, the Na^+-Ca^{2+}, $Ca^{2+}-Ca^{2+}$, and Na^+-Na^+ exchanges undergo different reaction pathways, but they involve concurring pathways. For example, if we compare the Na_i-dependent ^{45}Ca uptake (Na^+-Ca^{2+} exchange) and Ca_i-dependent ^{45}Ca uptake ($Ca^{2+}-Ca^{2+}$ exchange) in the sarcolemma vesicles, the Ca^{2+} influx (l') can be considered as a concurring partial reaction for both Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges, while the Na^+ efflux (k'') and Ca^{2+} efflux (l'') are explicit for each exchange mode (Scheme 1). The identity of the rate-limiting pathways has a primary importance for determining the properties of exchange modes. For example, a change of the rate-limiting pathway can alter the response of Na^+-Ca^{2+} exchange to voltage and the ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange (Khananshvili & Weil-Maslansky, 1994).

The Na^+-Ca^{2+} exchange exhibits diverse temperature dependence in different species and tissues (Bersohn et al., 1991; Tessari & Rahamimoff, 1991; Tibbits et al., 1992).

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This may reflect a possible diversity in the rate-limiting pathways. Potassium is not cotransported by the cardiac Na^+-Ca^{2+} exchanger (Yasui & Kimura, 1990), but K^+ and other monovalent ions accelerate the $Ca^{2+}-Ca^{2+}$ exchange (Reeves, 1985; Philipson, 1990; DiPolo & Beauge, 1991). Recent findings suggest that the Na^+-Ca^{2+} exchanger can undergo multiple steps of protonation−deprotonation (Khananshvili & Weil-Maslansky, 1994), involving perhaps distinct ion-transport and regulatory sites (Matsuoka & Hilgemann, 1992; Doering & Lederer, 1993; Khananshvili & Weil-Maslansky, 1994).

In this work the combined effects of temperature, pH, diffusion potential (potassium−valinomycin), and K^+ were investigated on Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges with a goal to identify and characterize the rate-limiting partial reactions of exchange modes. The experiments were done under conditions in which the ion binding is not rate-limiting at both sides of the membrane. The preparation of cardiac sarcolemmal vesicles was used, because in this preparation the inside-out vesicles contribute to most, if not all, of the Na^+-Ca^{2+} exchange activity (Li et al., 1991; Ambesi et al., 1991; Khananshvili et al., 1993). All the observed effects of temperature, pH, potassium, and voltage on Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges can be described by the consecutive (ping-pong) mechanism, while the rate-limiting pathways can be modified by various factors. Under most conditions the voltage-sensitive Na^+ efflux is rate limiting for Na^+-Ca^{2+} exchange, while the Ca^{2+} efflux limits the $Ca^{2+}-Ca^{2+}$ exchange. The bell-shaped temperature curve of $Ca^{2+}-Ca^{2+}$ exchange cannot be reconciled with a simple bidirectional reaction involving two elementary rate constants. It is suggested that the Ca^{2+}-transport step involves more than two species.

MATERIALS AND METHODS

The calf cardiac sarcolemmal vesicles (SLV)[1] were isolated as described before (Jones, 1988; Khananshvili et al., 1993) and stored at −70 °C. The vesicles were thawed and loaded with either sodium ($[Na]_i$ = 160 mM) or calcium ($[Ca]_i$ = 250 μM) by incubating them for 14−18 h at 4 °C. With $[^{45}Ca]_i$ = 250 μM and $[Na]_i$ = 160 mM the Na_i-dependent ^{45}Ca uptake of SLV preparations was 1−5 nmol of Ca mg^{-1} s^{-1}. The ^{45}Ca uptake was measured by filtration on glass micro fiber filters (GF/C Whatman) (Reeves, 1988; Cheon & Reeves, 1988; Khananshvili, 1990a, 1991a; Khananshvili & Weil-Maslansky, 1994). The vesicles were preequilibrated for 10−15 s at 6−45 °C and then mixed with the assay medium in thermostated semirapid mixer. The reaction of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges were initiated by a rapid dilution of 5−20 μL of Na- or Ca-loaded vesicles (50−160 μg of protein) in 160−500 μL of assay medium. The reaction mixture contained 20 mM buffer (pH 4.8−9.7), 0.2−0.25 M sucrose with or without choline-Cl or KCl, and 250 μM $^{45}CaCl_2$. The following buffers were used in the assay medium: Mes/Tris, pH 4.8−6.4; Mops/Tris, pH 6.4−8.8; Tris/Ches, pH 8.5−9.7; Caps/Tris, pH 8.0−9.7, bis-tris propane/HCl, pH 6.3−9.5; bis-tris propane/Caps, pH 9.0−9.7. The pH was adjusted at various temperatures, as indicated. Blanks contained 160 mM NaCl in the assay medium. Timing (t = 0.5−15 s) of ^{45}Ca uptake was electronically controlled by injecting 5 mL of quenching buffer (20 mM Mops/Tris, pH 7.4, 160 mM KCl, and 5 mM EGTA) (Khananshvili, 1990a; Khananshvili & Weil-Maslansky, 1994). Quenched samples were filtered on GF/C filters and collected vesicles were washed (5 × 5 mL) with cold Tris/Mops/KCl/EGTA buffer containing 0.5 mM EGTA. For the application of diffusion potential, the Na-loaded vesicles (5−12 mg of protein/mL) were warmed at room temperature (20−25 °C) and then mixed with 0.1−1.0 mM valinomycin (in ethanol) to give a final concentration of 1 μM. Free calcium was detected by arsenazo III (Bauer, 1981). Protein was measured by a modified assay of Lowry (Markwell et al., 1978). Kinetic parameters and their standard errors were estimated by a GraFit program, version 3.0 (written by R. J. Leatherbarrow, Erithacus Software Ltd.).

RESULTS

Effect of Temperature on the Time Course of Na_i- and Ca_i-Dependent ^{45}Ca Uptake. The effect of temperature was tested on the time course of Na_i-dependent ^{45}Ca uptake (Na^+-Ca^{2+} exchange) and Ca_i-dependent ^{45}Ca uptake ($Ca^{2+}-Ca^{2+}$ exchange) (Figure 1). The cardiac sarcolemma vesicles were preloaded either with 250 μM $CaCl_2$ or 160 mM NaCl, and reaction of ^{45}Ca uptake was initiated by a rapid dilution (50-fold) of Ca-loaded or Na-loaded vesicles in the assay medium (20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, and 250 μM $^{45}CaCl_2$). The ^{45}Ca internalization was quenched by addition of EGTA in the semirapid mixer, and intravesicular ^{45}Ca was measured by filtration (see Materials and Methods). The time course (t = 1−15 s) of Na_i-dependent ^{45}Ca uptake (Figure 1A) and Ca_i-dependent ^{45}Ca uptake (Figure 1B) was measured at two fixed temperatures, 28 or 37 °C. As can be seen from Figure 1A, the temperature increase from 28 to 37 °C accelerates the initial raters of Na^+-Ca^{2+} exchange ~2-fold. In contrary, the temperature increase from 28 to 37 °C decelerates the $Ca^{2+}-Ca^{2+}$ exchange ~2−3-fold (Figure 1B) (n = 7).

The time course (t = 0.5−2.5 s) of Ca_i-dependent ^{45}Ca uptake was measured in the presence or absence of potassium at two fixed temperatures, 28 °C (Figure 2A) or 37 °C (Figure 2B). The Ca-loaded (250 μM $CaCl_2$) vesicles were mixed with the assay medium (Mops/Tris/sucrose buffer plus 250 μM $^{45}CaCl_2$) containing either 100 mM KCl or choline-Cl. As can be seen from Figure 2, at both temperatures potassium accelerates the initial rates of $Ca^{2+}-Ca^{2+}$ exchange 2−3-fold (n = 5).

Effect of Temperature and Potassium on $Ca^{2+}-Ca^{2+}$ Exchange. The effect of varying temperature (6−45 °C) was investigated on $Ca^{2+}-Ca^{2+}$ exchange in the absence (100 mM choline-Cl) or presence of potassium (100 mM KCl) in the assay medium. The initial rates (t = 1 s) of $Ca^{2+}-Ca^{2+}$ exchange were measured under equilibrium exchange conditions $[^{45}Ca]_o = [Ca]_i$ = 250 μM. In the absence of potassium the $Ca^{2+}-Ca^{2+}$ exchange exhibits a bell-shaped curve with a broad maximum at 26−33 °C (Figure 3A). At each temperature K^+ accelerates the $Ca^{2+}-Ca^{2+}$ exchange 1.3−3.0-fold, exhibiting a prominent maximum at 27−29 °C (Figure 3A). A similar bell-shapped pattern was observed

[1] Abbreviations: bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; arsenazo III, 2,7-bis-(arsenophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SLV, sarcolemmal membrane vesicles.

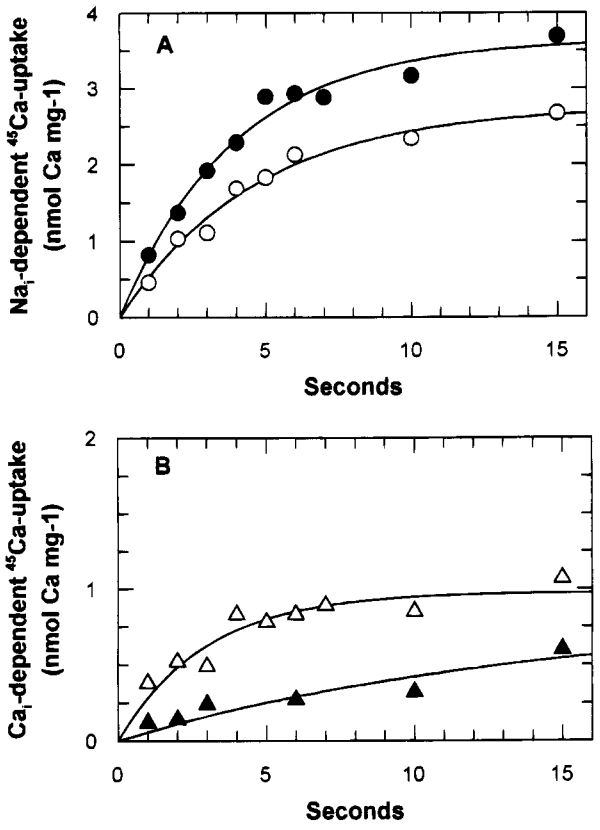

FIGURE 1: Time course of Na_i- or Ca_i-dependent ^{45}Ca uptake at 28 and 37 °C. The time course of Na_i-dependent (A) or Ca_i-dependent (B) ^{45}Ca uptake was measured in the absence of monovalent cations in the medium and two fixed temperatures, 28 °C (△, ○) or 37 °C (●, ▲). The cardiac sarcolemma vesicles were loaded with either 250 μM $CaCl_2$ (△, ▲) or 160 mM NaCl (○, ●) at 4 °C overnight. Before the experiment the Na (○, ●)-loaded (A) or Ca (△, ▲)-loaded (B) vesicles were preequilibrated for 10−15 s at 28 or 37 °C (see Materials and Methods) and then mixed with the assay medium 20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, and 250 μM $^{45}CaCl_2$ (66 900 cpm/nmol) at the same temperature. The reaction of ^{45}Ca uptake was quenched after t = 1−15 s, and intravesicular ^{45}Ca was measured as described under Materials and Methods.

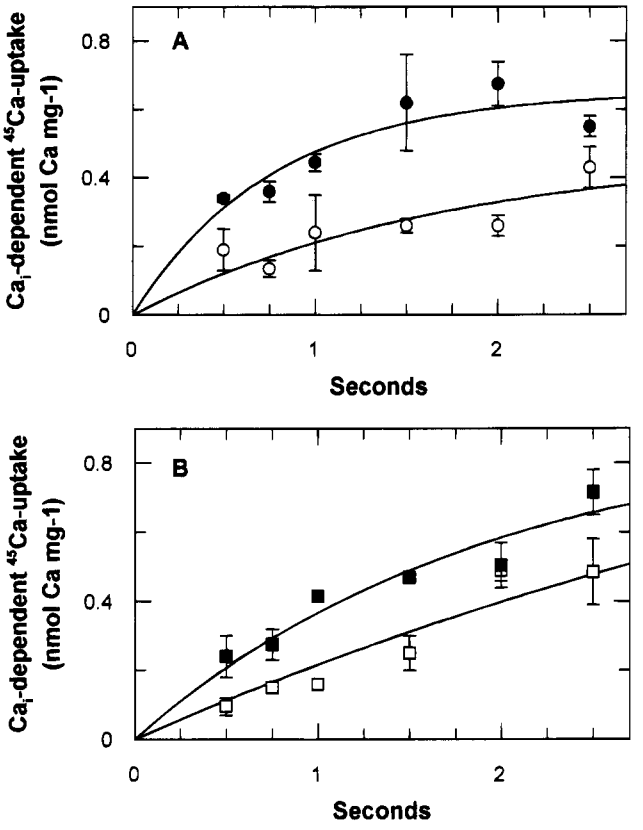

FIGURE 2: Effect of potassium on the initial rates of $Ca^{2+}-Ca^{2+}$ exchange. The effect of K^+ on the time-course of $Ca^{2+}-Ca^{2+}$ exchange was tested at 28 °C (A) or 37 °C (B). The sarcolemma vesicles were loaded with calcium as described in Figure 1, and the Ca_i-dependent ^{45}Ca-uptake was measured at 28 °C (○, ●) or 37 °C (□, ■). The Ca-loaded vesicles were mixed with the assay medium containing 20 mM Mops/Tris, pH 7.4, 0.2 M sucrose, and 250 μM $^{45}CaCl_2$ (88 970 cpm/nmol) plus either 100 mM KCl (●, ■) or 100 mM choline-Cl (○, □). ^{45}Ca uptake was quenched (t = 0.5−2.5 s) by addition of EGTA containing buffer, and intravesicular ^{45}Ca was measured as described under Materials and Methods. Each point is a mean of duplicate measurements (bars indicate ± SD mean).

with $[^{45}Ca]_o = [Ca]_i = 500$ μM (not shown) suggesting that the ion binding is not rate-limiting under given experimental conditions. The bell-shaped temperature curve of the $Ca^{2+}-Ca^{2+}$ exchange was observed in 12 independent experiments with five different preparations of sarcolemma vesicles. Control experiments show that a short-time exposure (10−15 s) of vesicles to 29−45 °C does not cause an irreversible inactivation of $Ca^{2+}-Ca^{2+}$ exchange (not shown). Therefore, the descendic shoulder of $Ca^{2+}-Ca^{2+}$ exchange (Figure 3A) cannot be caused by "thermal inactivation" of the exchanger.

Temperature Dependence of $Ca^{2+}-Ca^{2+}$ Exchange in the Passively and Actively Ca-Loaded Vesicles. In order to avoid any ambiguities with a vesicular orientation of sarcolemma vesicles, the temperature dependence of $Ca^{2+}-Ca^{2+}$ exchange was tested in a total preparation of vesicles and in the inside-out vesicles. Two different protocols were used for Ca-loading. The total preparation of vesicles was passively loaded overnight at 4 °C (standard conditions for loading). The inside-out vesicles were actively loaded by Ca^{2+}-ATPase (the vesicles were incubated with 10 μM ATP and 20 μM $CaCl_2$ for 2 min at 37 °C). The passively or actively Ca-loaded vesicles were rapidly diluted at various temperatures (9−45 °C) in the assay medium containing 250 μM $^{45}CaCl_2$, and the initial rates (t = 1 s) of $Ca^{2+}-Ca^{2+}$ exchange were measured. Two distinct preparations of Ca-loaded vesicles show similar rates of $Ca^{2+}-Ca^{2+}$ exchange at each fixed temperature displaying a typical bell-shaped temperature curve (Figure 4). By using the Ca^{2+}-ATPase loading protocol (2 min and 37 °C) the intracellular concentrations of calcium can easily reach ≥1 mM in the inside-out vesicles. Under these conditions the temperature dependence of $Ca^{2+}-Ca^{2+}$ exchange is still bell-shaped, exhibiting a temperature curve very similar to that of the vesicles that were passively loaded with $[Ca^{2+}]_i$ = 250 μM (Figure 4). Therefore, the bell-shaped temperature curve is obtained under conditions in which the Ca^{2+} binding is not rate-limiting at both sides of the membrane.

Effect of Temperature and Potassium on Na^+-Ca^{2+} Exchange and the Ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ Exchange. The effect of varying temperature (6−45 °C) was tested on the initial rates (t = 1 s) of Na^+-Ca^{2+} exchange in the assay medium with fixed pH 7.4 containing 100 mM of either choline-Cl or KCl. Temperatures increase (6−45 °C) in the potassium-free medium accelerates the Na^+-Ca^{2+} exchange 15−20 fold (Figure 5A). The Na^+-Ca^{2+} exchange is apparently insensitive to potassium in the range of 6−35 °C, while a modest activation (10−15%) by potassium is

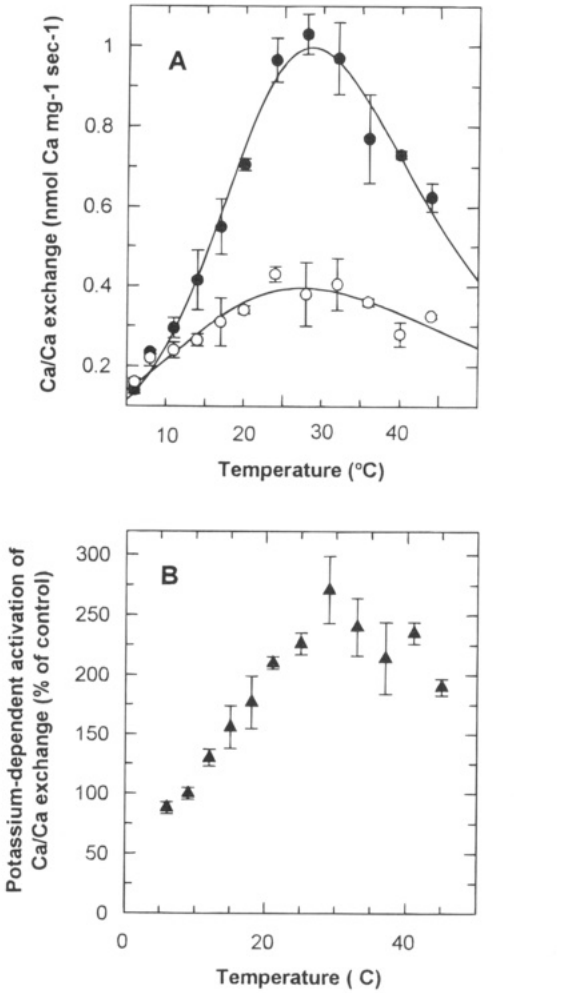

FIGURE 3: Effect of temperature and potassium on $Ca^{2+}-Ca^{2+}$ Exchange. (A) The vesicles were loaded with calcium as described in Figure 1. The Ca-loaded vesicles were mixed ($t = 1$ s) at 6−45 °C with the assay medium, 20 mM Mops/Tris, pH 7.4, 0.2 M sucrose, and 250 μM $^{45}CaCl_2$ (121 760 cpm/nmol) plus 100 mM of either KCl (●) or choline-Cl (○). Each point represents a mean of four independent measurements (bars indicate ± SD mean). The lines were calculated according to eq 4. It is assumed that each rate constant is temperature-dependent $k^i = k^i_o\, Q_i(\Delta T/10)$, while k^i represents a specific rate constant (l', l'', f', and f'') at a given temperature, k^i_o is the rate constant (l_o', l_o'', f_o', and f_o'') at a reference temperature (6 °C), and Q_i is an appropriate Q_{10} for each rate constant ($Q_{l'}$, $Q_{l''}$, $Q_{f'}$, and $Q_{f''}$). ΔT is a difference between a given and reference temperatures (6 °C). In the presence of potassium the values of Q_{10} were calculated as follows: $Q_{l'} = 1.45 \pm 0.19$; $Q_{l''} = 2.77 \pm 0.40$; $Q_{f'} = 1.30 \pm 0.15$; and $Q_{f''} = 3.71 \pm 0.48$. In the presence of choline-Cl the Q_{10} values were estimated as follows: $Q_{l'} = 1.48 \pm 0.23$; $Q_{l''} = 2.80 \pm 0.40$; $Q_{f'} = 1.30 \pm 0.20$; and $Q_{f''} = 2.82 \pm 0.43$. (B) The experimental data described in (A) were plotted as K^+-dependent activation of $Ca^{2+}-Ca^{2+}$ exchange. Control (100%) represents the $Ca^{2+}-Ca^{2+}$exchange activities in the absence of potassium, measured at the indicated temperatures.

observed ($n = 5$) at >37 °C (Figure 5A). Presumably at high temperatures Ca^{2+} transport becomes partially rate limiting.

Under standard assay conditions (K^+-free medium, pH 7.4, and 37 °C), the ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange (the R value) is $R \geq 2.5-3.0$. To investigate the effect of temperature and potassium on the ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange, the initial rates ($t = 1$ s) of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges were measured at various temperatures (6−45 °C) and fixed pH 7.4 in the presence or absence

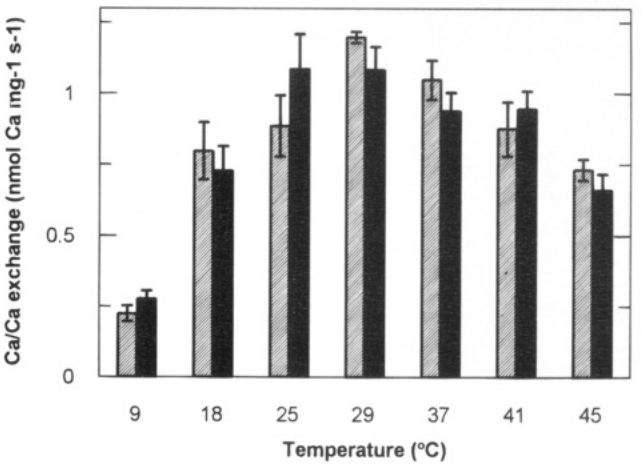

FIGURE 4: Temperature-dependence of $Ca^{2+}-Ca^{2+}$ exchange in the passively and actively loaded vesicles. The vesicles were actively loaded with calcium by using the Ca^{2+}-ATPase protocol (dark histograms) or the vesicles were passively loaded (light histograms) by incubation with calcium in the absence of ATP. Passive loading was carried out at 4 °C for 14−18 h. For active loading the sarcolemma vesicles (8.5 mg/mL) were incubated with 1 mM $MgCl_2$, 20 μM $CaCl_2$, and 10 μM ATP for 2 min at 37 °C. The Ca-loaded vesicles were preequilibrated at 9−45 °C for 10−15 s and then immediately diluted 27-fold in the assay medium containing 20 mM Mops/Tris, pH 7.4 (temperature adjusted), 0.2 M sucrose, 250 μM $^{45}CaCl_2$ (91 590 cpm/nmol), and 100 mM KCl. The initial rates ($t = 1$ s) were measured as described in Materials and Methods. Each point represents a mean of four independent measurements (bars indicate ± SD mean).

(choline-Cl) of potassium. Increasing the temperature (6−45 °C) in the potassium-free medium the ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange increases from 0.8 to 5.0 (Figure 6). In contrast, the addition of extravesicular potassium reduces the R values by 30−200%, achieving a maximal effect at 27−33 °C (Figure 6). These data suggest that the increasing temperatures and K^+ have opposite effects on the ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange.

Effect of Inside-Positive Potential on Na^+-Ca^{2+} Exchange at Various Temperatures. In a wide range of temperature potassium accelerates the $Ca^{2+}-Ca^{2+}$ exchange (Figure 3) but has a little (if any) affect on Na^+-Ca^{2+} exchange (Figure 5). Therefore, the voltage-sensitive Na^+ efflux may still limit the Na^+-Ca^{2+} exchange at various temperatures. If so, the Na^+-Ca^{2+} exchange rate has to response to voltage in a wide range of temperature. In order to test this possibility the effect of diffusion potential (potassium valinomycin) was tested on the initial rates of Na^+-Ca^{2+} exchange at 6−45 °C. The Na-loaded (160 mM) vesicles were treated with or without valinomycin and then mixed ($t = 1$ s) with Mos/Tris/sucrose buffer containing 250 μM $^{45}CaCl_2$ and 100 mM KCl. Different voltages were clamped by exposing the valinomycin untreated ($\Delta\psi = 0$ mV) or valinomycin treated vesicles ($\Delta\psi \geq +200$ mV) to K^+ containing medium (Figure 7A). The inside-positive potential accelerates the Na^+-Ca^{2+} exchange at each temperature by 150−230% (Figure 7B), suggesting that the Na^+ efflux is voltage-sensitive and rate-limiting at various temperatures.

Effect of Potassium and pH on $Ca^{2+}-Ca^{2+}$ Exchange. Under the standard experimental conditions (potassium-free medium and 37 °C), the cardiac sarcolemma Na^+-Ca^{2+} exchanger undergoes multiple steps of protonation−deprotonation, affecting the Na^+ and Ca^{2+} ion movements (Khananshvili & Weil-Maslansky, 1994). The effect of potassium

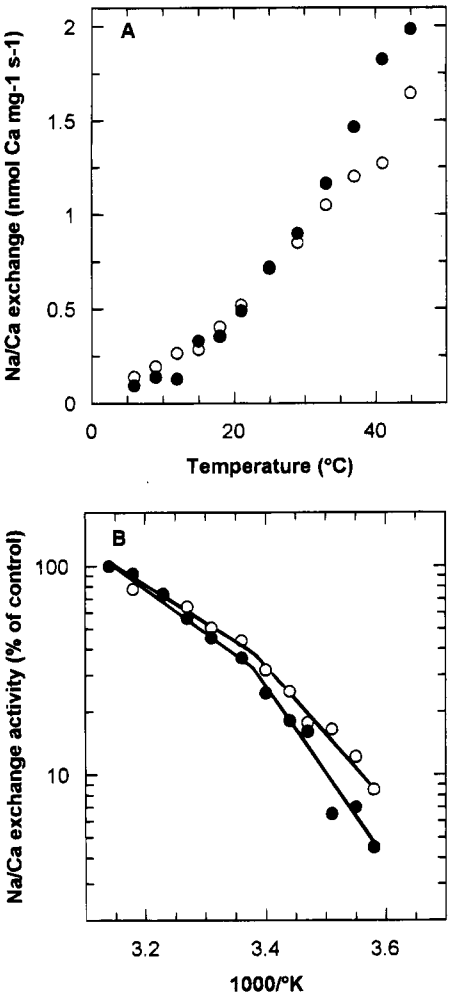

FIGURE 5: Effect of temperature and potassium on Na^+-Ca^{2+} exchange. (A) The vesicles were loaded with 160 mM NaCl at 4 °C for 14−18 h. The Na-loaded vesicles were mixed (t = 1 s) at 6−45 °C in the assay medium with 20 mM Mops/Tris, pH 7.4, 0.2 M sucrose, and 250 μM $^{45}CaCl_2$ (121 800 cpm/nmol) containing 100 mM of either KCl (●) or choline-Cl (○). ^{45}Ca uptake was quenched and intravesicular ^{45}Ca measured as described under the Materials and Methods. Each point represents a mean of four measurements (bars indicate ± SD mean). (B) The experimental data described in A were plotted as Arrhenius plot. The Na^+-Ca^{2+} exchange activities at 45 °C were taken as 100%.

and pH 4.8−9.7 was examined on $Ca^{2+}-Ca^{2+}$ exchange at two fixed temperatures, 27 °C (Figure 8A) and 37 °C (Figure 8B). The initial rates (t = 1 s) of $Ca^{2+}-Ca^{2+}$ exchange were measured with $[^{45}Ca]_o = [Ca]_i = 250$ μM in the presence of 100 mM choline-Cl or KCl in the assay medium. In the absence of K^+ the pH-titration curve of $Ca^{2+}-Ca^{2+}$ exchange shows a bell-shaped pattern in the acidic range (pK_{a1} = 5.2−5.4 and pK_{a2} = 6.0−6.4) followed by the exchange acceleration in the alkaline range (pK_{a3} = 8.7−9.5) (Figure 8). These data suggest that a deprotonation of the exchanger in the range of pH 6.0−7.5 decelerates the $Ca^{2+}-Ca^{2+}$ exchange. Addition of extravesicular K^+ has a little (if any) effect at low pH 5.0−6.0, while it affects the pK_{a2} and pK_{a3} values (Figure 8). Therefore, the accelerating effect of K^+ opposes the inhibitory effect of deprotonation. Likewise, in the range of pH 6.0−7.5 the effect of K^+ causes a characteristic shift and overlap of pK_{a2} and pK_{a3} components (Figure 8) and, thereby, reduces a difference between the pK_{a2} and pK_{a3} values ($\Delta pK_a = pK_{a3} - pK_{a2}$ is declined by K^+ from 2.7−3.2 to 0.9−1.0) (Figure 8). Therefore, K^+ may interact with

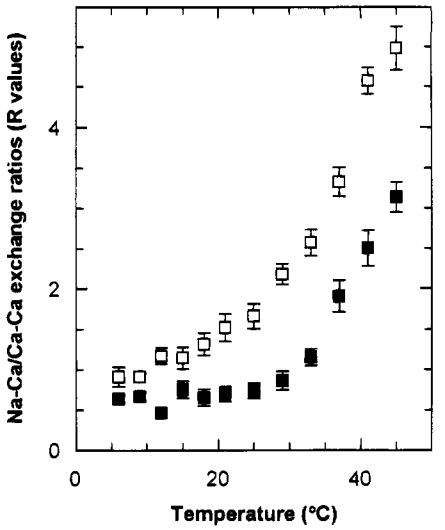

FIGURE 6: Effect of temperature and potassium on the ratios of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange. The initial rates (t = 1 s) of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges were measured at various temperatures (6−45 °C) in the presence (■) or absence (□) of potassium as described in Figure 3. The ratios (R) of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange were plotted vs temperature. Each point represents a mean of four measurements of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges and bars indicate ± SD mean.

the deprotonated species (pH 6.0−8.0), which in turn accelerates the rate-limiting Ca^{2+} efflux of Ca^+-Ca^{2+} exchange. At pH 5.0−9.7 and fixed 37 °C, K^+ has no effect on Na^+-Ca^{2+} exchange (not shown), suggesting that even so K^+ binds to the exchanger it cannot alter the rate-limiting Na^+ efflux of Na^+-Ca^{2+} exchange.

DISCUSSION

Rate-Limiting Reactions of Na^+-Ca^{2+} and Ca^+-Ca^{2+} Exchange Modes. At various temperatures (6−45 °C) and pH (6.0−9.7), potassium affects the Ca^+-Ca^{2+} exchange (Figures 2 and 3) but has a very little (if any) affect on Na^+-Ca^{2+} exchange (Figure 5). In the frame of consecutive (ping-pong) mechanism the Ca^{2+} influx (l') can contribute to either Ca^+-Ca^{2+} or Na^+-Ca^{2+} exchange (Scheme 1). If we assume that the Ca^{2+} influx is a rate-limiting partial reaction for both exchange modes, (a) the rates of Ca^+-Ca^{2+} and Na^+-Ca^{2+} exchanges [$V_{max}(Na/Ca) = E_t l'k''/(l' + k'')$ and $V_{max}(Ca/Ca) = E_t l'l''/(l' + l'')$] might be comparable, and (b) K^+ has to accelerate (or decelerate) both Ca^+-Ca^{2+} and Na^+-Ca^{2+} exchanges in a similar manner. The present data (Figures 1−3 and 5) do not support these predictions, suggesting that the Ca^{2+} influx (l') cannot be a rate-limiting step for both exchange modes. The data can be successfully described by a model in which the different partial reactions, the Na^+ efflux (k'') and Ca^{2+} efflux (l''), are rate-limiting for Na^+-Ca^{2+} and Ca^+-Ca^{2+} exchanges, respectively (Figure 9).

The effect of inside-positive potential was examined on Na^+-Ca^{2+} exchange in the range of 6−45 °C with a goal to test a possible alteration of voltage sensitivity of Na^+-Ca^{2+} exchange. It was found that the increasing temperatures accelerate the Na^+-Ca^{2+} exchange 15−20-fold (Figure 7A), although the fraction of voltage-induced effect (1.5−2-fold acceleration) is similar at various temperatures (Figure 7B). These findings suggest that, in a wide range of conditions (6−45 °C, pH 7.0−9.7, and 0−100 mM K^+) the voltage-

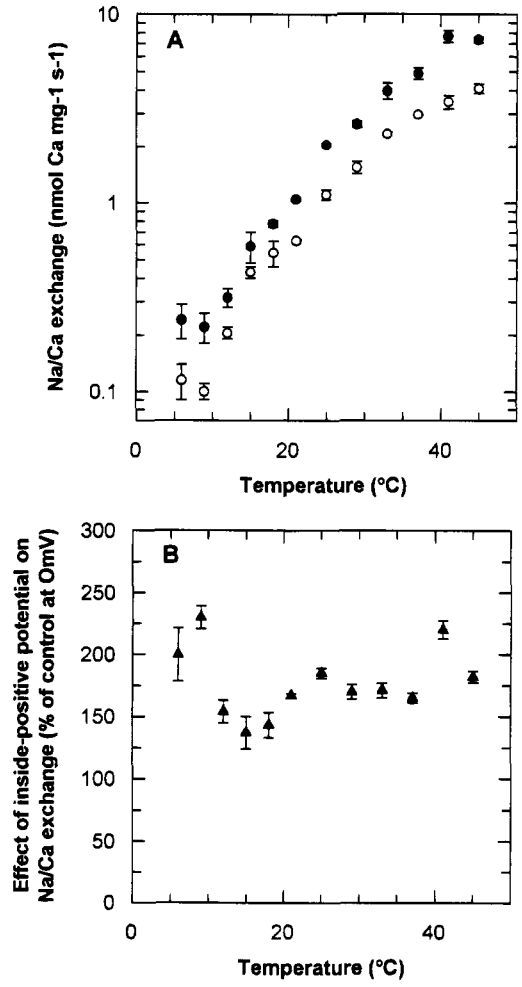

FIGURE 7: Effect of inside-positive potential and temperature on Na^+-Ca^{2+} exchange. (A) The Na-loaded vesicles were obtained as described in Figure 5. Before the experiment, the Na-loaded vesicles were treated with (●) or without (○) valinomycin (see Materials and Methods). The valinomycin treated or untreated vesicles were mixed (t = 1 s) at 6−45 °C with the assay medium containing 20 mM Mops/Tris pH 7.4, 0.2 M sucrose, 100 mM KCl, and 250 μM ^{45}Ca (147 230 cpm/nmol). Each point represents a mean of duplicate measurements (bars indicate ± SD mean). (B) The data described in A were plotted as an effect of inside-positive potential on the Na^+-Ca^{2+} exchange. 100% represents the control activity of Na^+-Ca^{2+}exchange at 0 mV, measured at various temperatures.

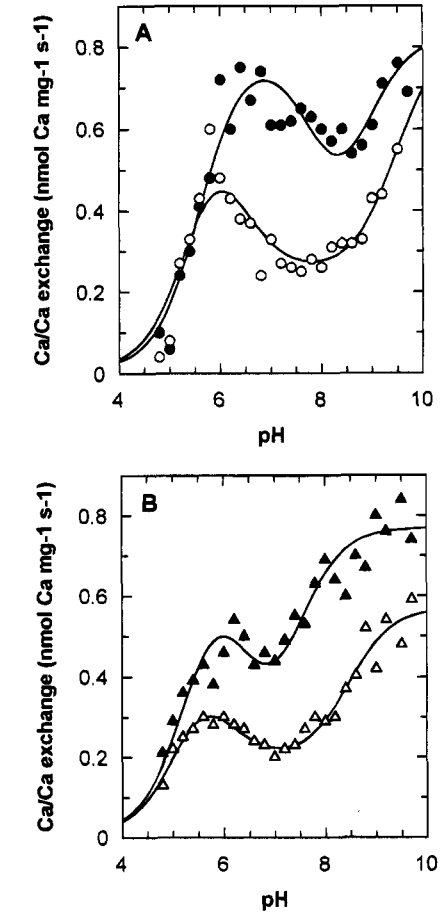

FIGURE 8: Effect of potassium on $Ca^{2+}-Ca^{2+}$exchange at various pH. The effect of potassium on the $Ca^{2+}-Ca^{2+}$ exchange was tested by varying pH 4.8−9.7 at two fixed temperatures, 27 °C (A) or 37 °C (B). The vesicles were loaded with calcium as described in the legend to Figure 1. The Ca-loaded vesicles were mixed (t = 1 s) with the assay medium containing 20 mM buffer pH 4.8−9.7 (adjusted at the indicated temperatures), 0.2 M sucrose, and 250 μM $^{45}CaCl_2$ (127 620 cpm/nmol) plus 100 mM of either KCl (●, ▲) or choline-Cl (○, △). Each point represents a mean value of duplicate measurements. The lines were computed to give an optimal fit to the experimental points: $V_{max}(Ca/Ca) = [(Lim_0 + Lim_1 \times 10^{pH-pK_{a1}})/(1 + 10^{pH-pK_{a1}})] - [(Lim_0' + Lim_2 \times 10^{pH-pK_{a2}})/(1 + 10^{pH-pK_{a2}})] + [(Lim_0' + Lim_3 \times 10^{pH-pK_{a3}})/(1 + 10^{pH-pK_{a3}})]$. The pK_a values were calculated as (A) $pK_{a1} = 5.4 \pm 0.1$, $pK_{a2} = 6.3 \pm 0.1$, and $pK_{a3} = 9.5 \pm 0.1$ in the absence of K^+ (○) and $pK_{a1} = 5.6 \pm 0.1$, $pK_{a2} = 7.9 \pm 0.1$, and $pK_{a3} = 8.8 \pm 0.2$ in the presence of K^+ (●), and (B) $pK_{a1} = 5.2 \pm 0.1$, $pK_{a2} = 6.0 \pm 0.1$, and $pK_{a3} = 8.7 \pm 0.2$ in the absence of K^+ (△) and $pK_{a1} = 5.3 \pm 0.1$, $pK_{a2} = 6.4 \pm 0.1$, and $pK_{a3} = 7.4 \pm 0.1$ in the presence of K^+ (▲). The Lim_0, Lim_0', Lim_1, Lim_2, and Lim_3 terms represent the fitted limits of specific pK_a values.

sensitive Na^+ transport is still rate-limiting of Na^+-Ca^{2+} exchange. The situation may be different at pH < 6.1, when the Ca^{2+} influx (I') can become rate-limiting for both exchange modes (the Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchange rates are similar, and the Na^+-Ca^{2+} exchange is voltage-insensitive) (Khananshvili & Weil-Maslansky, 1994).

The Bell-Shaped Temperature Curve of $Ca^{2+}-Ca^{2+}$ Exchange. When temperatures increases from 29 to 45 °C, there is a decline in the rate of Ca^+-Ca^{2+} exchange (Figures 1B and 3A) while the rate of Na^+-Ca^{2+} exchange increases (Figures 1A and 5A). In the K^+-free medium (pH 7.4) the $Ca^{2+}-Ca^{2+}$ exchange shows a bell-shaped temperature curve with a broad maximum at 26−33 °C (Figure 3A). At various temperatures K^+ accelerates the $Ca^{2+}-Ca^{2+}$ exchange by 130−300% with a pick at 27−29 °C (Figure 3A). The bell-shaped temperature curve of $Ca^{2+}-Ca^{2+}$ exchange cannot be explained by irreversible inactivation (e.g., thermal denaturation) of the exchanger or by heterogeneity of vesicular orientation (Figure 4).

Previous studies suggest that the temperature dependence of Na^+-Ca^{2+} exchange represents the intrinsic properties of the exchanger protein rather than the property of lipid environment (Bersohn et al., 1991; Tessari & Rahamimoff, 1991; Tibbits et al., 1992). Possible changes in lipid fluidity cannot attribute the bell-shaped temperature curve of $Ca^{2+}-Ca^{2+}$ exchange, because a simple bidirectional reaction (E'Ca ⇆ E''Ca) cannot exhibit a bell-shaped temperature curve (the Q_{10} values of elementary reactions cannot be less then the unity) (Londesborough, 1980). Therefore, the present data cannot be described by a simple bidirectional reaction involving two elementary rate constants.

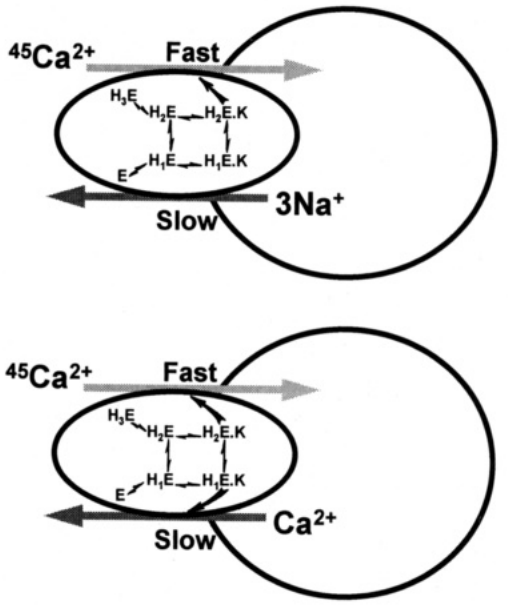

FIGURE 9: Rate-limiting pathways of Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges. The Na^+-Ca^{2+} exchanger is described as a system which can undergo three steps of protonation−deprotonation ($H_3E \rightleftharpoons H_2E \rightleftharpoons H_1E \rightleftharpoons E$). Potassium can interact with a deprotonated species of the exchanger (e.g., H_2E and H_1E species), yielding the $H_2E{\cdot}K$ and $H_1E{\cdot}K$ species. Potassium-bound species cannot affect the rate-limiting and voltage-sensitive Na^+ efflux (k'') of Na^+-Ca^{2+} exchange, but they can speed up the rate-limiting Ca^{2+} efflux (l'') of $Ca^{2+}-Ca^{2+}$ exchange.

Possible Mechanisms Involving the Ca^{2+} Transport. For description of the bell-shaped temperature curve of $Ca^{2+}-Ca^{2+}$ exchange, it is essential to assume that at least two reactions are involved in Ca^{2+} transport. Three possible mechanisms can be considered: (a) A ground-state intermediate (E°Ca) may be involved in the Ca^{2+} transport (eq 1), which may reflect a specific conformation (e.g., an

$$\begin{array}{ccccc} E'Ca & \underset{l''}{\overset{l'}{\rightleftharpoons}} & (E°Ca) & \underset{f''}{\overset{f'}{\rightleftharpoons}} & E''Ca \\ \downarrow\uparrow & & & & \downarrow\uparrow \\ E' & & & & E'' \end{array} \qquad (1)$$

"occluded" state) of the transporter. According to this model the E°Ca intermediate can be accumulated at transient temperatures, resulting the bell-shaped temperature curve.

(b) Inactive conformation (E*Ca) of the exchanger ("inactive" species) may be in a rapid equilibrium with other active species (eq 2). According to this model the E*Ca species

$$\begin{array}{ccccc} E'Ca & \underset{l''}{\overset{l'}{\rightleftharpoons}} & E''Ca & \underset{f''}{\overset{f'}{\rightleftharpoons}} & E^*Ca \\ \downarrow\uparrow & & \downarrow\uparrow & & \\ E' & & E'' & & \end{array} \qquad (2)$$

can be accumulated by increasing temperatures resulting the bell-shaped temperature curve.

(c) Two (or more) putative conformational states (e.g., E_f and E_s) may be in rapid equilibrium and operate in parallel (eq 3). According to this mechanism, the temperature-induced changes can control not only the rate constants of ion-transport reactions (l', l'', f', and f'') but also the equilibrium between the "fast" (E_f) and "slow" (E_s) conformers. Therefore, a fractional contribution of different conformational states may determine the bell-shaped temperature curve of $Ca^{2+}-Ca^{2+}$ exchange.

$$\begin{array}{ccc} E'_fCa & \underset{l''}{\overset{l'}{\rightleftharpoons}} & E''_fCa \\ \downarrow\uparrow & & \downarrow\uparrow \\ E'_sCa & \underset{f''}{\overset{f'}{\rightleftharpoons}} & E''_sCa \end{array} \qquad (3)$$

Since the calcium concentrations are nearly saturating under the conditions tested, the fraction of ligand free species (E′ and E″) can be assumed as negligible. In this case the mechanisms *a* and *b* become indistinguishable, which can be described by eq 4. A reasonable fit to the experimental

$$E'Ca \underset{l''}{\overset{l'}{\rightleftharpoons}} E{\cdot}Ca \underset{f''}{\overset{f'}{\rightleftharpoons}} E''Ca \qquad (4)$$

data can be obtained by using this formalism (Figure 3), and the Q_{10} values of partial reactions can be calculated ($Q_{l'} = 1.5$, $Q_{l''} = 2.8$, $Q_{f'} = 1.3$, and $Q_{f''} = 3.7$) (Figure 3). In this simple model the Q_{10} values are the same in a wide range of temperature. Although the validity of this assumption is limited, the main future of the model is still valid: The bell-shaped temperature curve is obtained because the Q_{10} values of forward reactions ($Q_{l'} = 1.5$ and $Q_{f'} = 1.3$) are much lower than the Q_{10} values of reverse reactions ($Q_{l''} = 2.8$ and $Q_{f''} = 3.7$).

Potassium Interacts with a Deprotonated Form(s) of the Exchanger. The effect of potassium and varying pH was examined on $Ca^{2+}-Ca^{2+}$ exchange at 27 °C (Figure 8A) or 37 °C (Figure 8B). In the absence of potassium (choline-Cl medium) the pH-titration curve of $Ca^{2+}-Ca^{2+}$ exchange shows a bell-shaped curve in the acidic range with $pK_{a1} = 5.1-5.4$ and $pK_{a2} = 6.2-6.5$, followed by a rate increase in the alkaline range ($pK_{a3} = 8.5-9.0$) (Figure 8). These data suggest that a deprotonation of the exchanger in the range of pH 6.0−8.5 suppresses the rate of $Ca^{2+}-Ca^{2+}$ exchange. Addition of extravesicular potassium has a little effect (if any) on $Ca^{2+}-Ca^{2+}$ exchange in the range of pH 5.0−6.0, indicating that potassium may not bind to the protonated species of the exchanger. Potassium accelerates the $Ca^{2+}-Ca^{2+}$ exchange 2−3-fold in the range of pH 6.0−8.5 (Figure 8). Therefore, the accelerating effect of K^+ opposes the inhibitory effect of deprotonation at pH >6.0. Likewise, potassium results in a characteristic shift and overlap of the pK_{a2} and pK_{a3} components and, thereby, reduces the difference between the two pK_a values from the 2.7−3.2 to the 0.9−1.0 pH unit (Figure 8). It is conceivable to assume that a putative binding site becomes accessible for K^+ when pH >6.1. In contrast to $Ca^{2+}-Ca^{2+}$ exchange, potassium has no detectable effect on Na^+-Ca^{2+} exchange in the range of pH 5.0−9.7 (not shown). Even so, K^+ interacts with the deprotonated species during Na^+-Ca^{2+} exchange; this cannot affect the rate-limiting and voltage-sensitive Na^+ efflux (k'') of Na^+-Ca^{2+} exchange (Figure 9).

Effect of Temperature on the Ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ Exchange. A theoretical and experimental analysis shows that the ratio of $Na^+-Ca^{2+}/Ca^{2+}-Ca^{2+}$ exchange (signed as the R value) may reflect a degree of asymmetry of bidirectional Ca^{2+} movements during the $Ca^{2+}-Ca^{2+}$ exchange (Khananshvili, 1991; Khananshvili & Weil-Maslansky, 1994). A bottom line of this analysis is that when the R values exceed the unity ($R \gg 1$), it can be

concluded that (a) the Ca^{2+} influx is faster than the Ca^{2+} efflux ($l' > l''$), and (b) the Ca^{2+} efflux is slower than the Na^+ efflux ($l'' < k''$). However, when the Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchange rates are similar ($R \sim 1$), the bidirectional Ca^{2+} movements may or may not be asymmetric. Temperature decreases from 45 to 6 °C (K^+-free medium and pH 7.4) reduces the R values from 4.8−5.0 to 0.8−1.0 (Figure 6). Similarly, the decrease of pH from 9.0 to 6.0 reduces the R values from 4.0 to 0.9−1.0 (Khananshvili & Weil-Maslansky, 1994). Despite these similarities, the underlying mechanisms of pH and temperature-induced effects must be different. The crucial difference is that the Na^+-Ca^{2+} exchange becomes voltage-insensitive in the acidic range (Khananshvili & Weil-Maslansky, 1994), while at low temperatures the Na^+-Ca^{2+} exchange is still voltage-sensitive (Figure 6). Therefore, at low pH the voltage-sensitive Ca^{2+} influx may limit both Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges. In contrast, the Na^+ efflux (k'') and Ca^{2+} efflux (l''), which are rate-limiting for Na^+-Ca^{2+} and $Ca^{2+}-Ca^{2+}$ exchanges respectively, may become equated ($k'' \simeq l''$) at low temperatures resulting similar rates of exchange modes ($R \sim 1$).

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