The Cardiac Na^+–Ca^{2+} Exchanger: Relative Rates of Calcium and Sodium Movements and Their Modulation by Protonation–Deprotonation of the Carrier†

Daniel Khananshvili* and Evelyne Weil-Maslansky

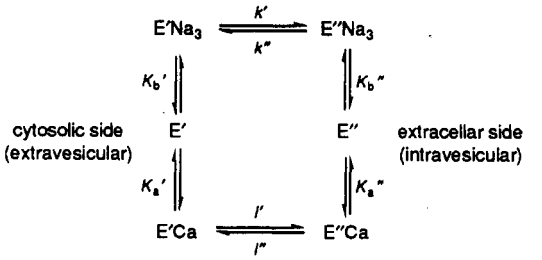
The Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel

Received June 24, 1993; Revised Manuscript Received October 25, 1993®

ABSTRACT: The exchange cycle of the cardiac Na^+–Ca^{2+} exchanger can be described as separate steps of Ca^{2+} and Na^+ transport [Khananshvili, D. (1990) *Biochemistry 29,* 2437–2442]. In order to determine the relative rates of Na^+ and Ca^{2+} movement during the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange modes, the ratios (R) of Na^+–Ca^{2+}/Ca^{2+}–Ca^{2+} exchanges were estimated with saturating concentrations of ions at both sides of the membrane. The effect of extravesicular pH and voltage (potassium valinomycin) on the initial rates (t = 1 s) of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange were investigated by assuming that, under the conditions tested, the intravesicular pH (pH 7.4) is not affected. Na^+- or Ca^{2+}-preloaded sarcolemma vesicles were diluted rapidly in assay medium containing ^{45}Ca and buffer (pH 5.0–10.9), and the reaction of ^{45}Ca uptake was quenched by using a semi-rapid-mixing device. Under conditions in which $[^{45}Ca]_o$ = $[Ca]_i$ = 250 μM, the pH-dependent curve of Ca^{2+}–Ca^{2+} exchange shows a bell shape in the acidic range (pK_{a1} = 5.1 ± 0.1 and pK_{a2} = 6.5 ± 0.2) followed by activation of the exchange in the alkaline range (pK_{a3} = 10.0 ± 0.2). With $[^{45}Ca]_o$ = 250 μM and $[Na]_i$ = 160 mM, the Na^+–Ca^{2+} exchange increases monotonically from pH 5.0 to 9.5 (pK_{a1} = 5.1 ± 0.1, pK_{a2} = 7.2 ± 0.2, and pK_{a3} = 9.1 ± 0.2). At pH <6.1, the ratio of Na^+–Ca^{2+}/Ca^{2+}–Ca^{2+} exchange is close to unity ($R \approx 1$), while it increases to R = 3–4 in the range of pH 7.1–9.3. These data suggest that, in the absence of monovalent ions and at pH >6.5, Ca^{2+} moves severalfold faster from the extravesicular to the intravesicular side than in the opposite direction, meaning that the bidirectional movement of Ca^{2+} is an intrinsically asymmetric process. The pH–voltage curve analysis shows that at pH >6.5 the inside positive potential accelerates Na^+–Ca^{2+} exchange by 2–3-fold. However, at pH <6.1 the voltage response of Na^+–Ca^{2+} exchange is lost, suggesting that the deprotonation of the carrier determines a voltage response of Na^+–Ca^{2+} exchange. In the range of pH 5.0–10.8 and $\Delta\psi$ = 0–200 mV, the Ca^{2+}–Ca^{2+} exchange is voltage-insensitive, indicating that the Ca^{2+} transport is not affected by membrane potential. The data can be interpreted as follows: (a) When the carrier is protonated (pH <6.1), the "electroneutral" movement of Ca^{2+} from the extravesicular (intracellular) side to the intravesicular (extracellular) side limits the rates of both Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchanges. (b) In the deprotonated carrier (pH >6.5), the ion movements in the opposite direction (from the intravesicular side to the extravesicular side) become rate-limiting: the voltage-sensitive Na^+ movement controls the rate of Na^+–Ca^{2+} exchange, and a Ca^{2+} transport limits the Ca^{2+}–Ca^{2+}exchange rate. (c) Deprotonation of the carrier (pH >6.5) has opposite effects on Na^+ and Ca^{2+} movements: it accelerates the voltage-sensitive and rate-limiting transport of Na^+ during the Na^+–Ca^{2+} exchange, while it decelerates the rate-limiting Ca^{2+} transport during the Ca^{2+}–Ca^{2+} exchange.

The cardiac sarcolemma (cell membrane) Na^+–Ca^{2+} exchange protein provides a predominant mechanism for electrogenic ($3Na^+$:Ca^{2+}) transport of calcium during the action potential, thereby regulating transient levels of intracellular calcium and muscle contraction–relaxation (Reuter & Seitz, 1968; Mullins, 1977; Reeves & Hale, 1984; Hilgemann & Nobel, 1987; Nobel et al., 1991; Blaustein et al., 1991). The ion-exchange cycle exhibits a turnover number of 1000–5000 s^{-1} (Cheon & Reeves, 1988; Niggli & Lederer, 1991; Hilgemann et al., 1991), representing a typical carrier-type mechanism that differs from channel-type machinery (Stein, 1986; Lauger, 1987; Khananshvili, 1990b, 1991b). During the action potential, the Na^+–Ca^{2+} exchanger is able to reverse the calcium entry/extrusion processes in a voltage- and $[Ca]_i$-dependent manner (Eisner & Lederer, 1985; Nobel et al., 1991; Bridge et al., 1991). The effects of voltage and other regulatory modes on the partial reactions of Na^+–Ca^{2+} exchange have not yet been resolved (Khananshvili, 1991a,b; Hilgemann et al., 1992; Matsuoka & Hilgemann, 1992).

Scheme 1



Recent kinetic analyses of ion fluxes in the reconstituted proteoliposomes (Khananshvili, 1990a, 1991a,b) and patch-clamp studies on intact cell membranes (Niggli & Lederer, 1991; Hilgemann et al., 1991; Li & Kimura, 1991; Matsuoka & Hilgemann, 1992) provide an increasing body of evidence that the Na^+–Ca^{2+} exchange cycle can be described as separate steps of calcium and sodium transport (the ping-pong or consecutive mechanism, Scheme 1). Although the binding of

† This work is supported by an Igal Alon Career Development Fellowship and by an Israeli Science Foundation grant to D.K. The financial support from the Tiber Foundation is gratefully acknowledged.

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/94/0433-312$04.50/0

either Na^+ or Ca^{2+} to the exchanger is a weakly voltage-sensitive process, the electrogenic feature of the Na^+–Ca^{2+} exchange cycle arises primarily within the rate-limiting movement of the ion (presumably sodium) through the exchanger (Khananshvili, 1991a,b; Hilgemann et al., 1991; Niggli & Lederer, 1991; Matsuoka & Hilgemann, 1992). A gene-sequence analysis of the cardiac Na^+–Ca^{2+} exchanger reveals a single polypeptide (108 kDa) with 11 putative transmembrane segments and a large regulatory intracellular loop (Nicoll et al., 1990; Nicoll & Philipson, 1991). The membrane-spanning segments 4 and 5 contain a putative ion-binding domain (23 amino acids), which is homologous to the Na^+,K^+-ATPase and other ion pumps (Nicoll et al., 1990; Nicoll & Philipson, 1991). Complete deletion of the intracellular loop (520 amino acids) does not alter the voltage response properties of the Na^+–Ca^{2+} exchange currents, suggesting that the voltage-dependent modulation of the exchange cycle is determined by ion movement pathways through the transmembrane portion of the protein (Matsuoka et al., 1993). The structure–function similarities between Na^+,K^+-ATPase and the Na^+–Ca^{2+} exchanger seem to be encouraging, because the specific charge-carrying intermediates may determine ion-transport specificities along the transport cycle (Goldshlegger et al., 1987; Gadsby & Nakao, 1989; Khananshvili, 1991a,b; Hilgemann et al., 1991, 1992).

In cardiac cells, the extracellular $K_m(Ca^{2+})$ of Na^+–Ca^{2+} exchange is 10^2–10^3-fold higher than the intracellular K_m-(Ca^{2+}) (Hilgemann et al., 1991; Li & Kimura, 1991; Matsuoka & Hilgemann, 1992). Although a major physiological demand of this phenomenon is to extrude intracellular calcium, this asymmetry could be based on different mechanisms (e.g., the intrinsic binding affinity of Ca^{2+} and/or Na^+ at the opposite sides of the membrane, the difference in the rates of ion movements and their modulation by secondary regulatory modes, the effect of voltage on specific ion-transport steps, etc.) (Khananshvili, 1991b; Matsuoka & Hilgemann, 1992). It is well established that the Na^+–Ca^{2+} exchanger can be modulated by pH [for a review, see Reeves and Philipson (1989)], but the underlying mechanisms are not known (Doering & Lederer, 1992; Hilgemann et al., 1992). Since the intracellular pH in cardiac and other cells can be affected under pathological conditions (e.g., within 1 min after the onset of myocardial ischemia the intracellular pH decreases by 0.5–0.8 unit), the interplay and structure–function relationships between specific Ca^{2+}-transport systems may have considerable significance from mechanistic and physiological points of view (Dixon & Haynes, 1990).

It has been suggested previously that, under the conditions in which the ion binding is not rate-limiting, the Na^+–Ca^{2+}/Ca^{2+}–Ca^{2+} exchange ratios may reflect the relative rates of Na^+ and Ca^{2+} movement through the Na^+–Ca^{2+} exchanger (Khananshvili, 1991b). This approach could be useful for identification and characterization of the rate-limiting and voltage-sensitive steps of the exchange cycle, because no experimental setup is available at this moment for measuring the absolute rate constants of partial reactions (Khananshvili, 1991b). In this work, we have examined the combined effects of pH and voltage (potassium valinomycin) on the initial rates of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange modes. The main goal was to find a relationship between the protonation–deprotonation states of the carrier and the rate-limiting and voltage-sensitive partial reactions of the transport cycle. To achieve this goal, isolated cardiac sarcolemmal vesicles were used, in which 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). The semi-rapid-mixing technique allowed us to study the exchange reactions during a short-time (1 s) exposure of the extravesicular (cytosolic) side of the membrane to various pH's. The experimental data show that the protonation–deprotonation states of the carrier modulate the relative rates of Na^+ and Ca^{2+} movements and the voltage sensitivity of Na^+–Ca^{2+} exchange.

MATERIALS AND METHODS

Materials. $^{45}CaCl_2$ (0.8–1.5 Ci/mmol) was obtained from New England Nuclear/DuPont (Boston, MA). The scintillation cocktail Opti-Fluor for radioactivity counting was from Packard (Groningen, Netherlands). Calcium atomic absorption standard, poly(ethyleneimine) and EGTA[1] were purchased from Sigma (St. Louis, MO). Chelex-100 was from Bio-Rad Laboratories (Hercules, CA). Arsenazo III was obtained from ICN Pharmaceuticals (Plainview, NY). Protease inhibitors (PMSF, pepstatin, leupeptin, and aprotenin) and buffers (Mes, Mops, Tris, Ches, and Caps) were purchased from Sigma. The glass microfiber filters (GF/C Whatman) were obtained from Tamar (Jerusalem, Israel). All other reagents used in this work were of analytical or reagent (>99.9%) grade purity. The solutions were prepared with deionized water (18 MΩ/cm) which contained no more than 1–3 μM ambient calcium.

Methods. Sarcolemmal membrane vesicles (SLV) were isolated from calf heart according to the modified procedure of Jones (1988) in the presence of protease inhibitors PMSF (0.2 mM), pepstatin, leupeptin, and aprotenin (1 μg/mL of each) (Khananshvili, 1990, 1991a; Khananshvili et al., 1993). SLV (3–7 mg of protein/mL preparations, equilibrated with 20 mM Mops/Tris, pH 7.4, and 0.25 M sucrose) were stored at −70 °C (no change in exchange activity was detected for at least 3 months). Before the experiment, vesicles were thawed at room temperature and loaded with either sodium ($[Na]_i$ = 160 mM) or calcium ($[Ca]_i$ = 250 μM) by incubating them overnight (14–18 h) at 4 °C. Under the standard assay conditions (20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, 250 μM extravesicular ^{45}Ca, and 160 mM intravesicular NaCl), the Na_i-dependent ^{45}Ca uptake of different SLV preparations was 3–6 nmol mg^{-1} s^{-1}.

Initial rates of the Na_i- or Ca_i-dependent ^{45}Ca uptake were measured at 37 °C for 1 s (Reeves, 1988; Khananshvili, 1990, 1991a; Khananshvili et al., 1993). GF/C filters were presoaked in 0.3% poly(ethyleneimine) (Cheon & Reeves, 1988) and prewashed with 5 mL of 20 mM Mops/Tris, pH 7.4, 160 mM KCl, and 0.5 mM EGTA. Blanks contained 160 mM NaCl in the assay medium. The reaction mixture (0.5 mL) contained 20 mM buffer with different pH's (Mes/Tris, pH 5.0–6.4; Mops/Tris, pH 6.4–8.8; Tris/Ches, pH 8.5–9.3; Caps/Tris, pH 9.0–10.0; Caps/LiOH, pH 9.0–11.0), 0.25 M sucrose (or KCl + LiCl = 160 mM), and 5–250 μM $^{45}CaCl_2$. For the application of diffusion potential, SLV (3–7 mg of protein/mL) were preincubated first with 0.1 mM KCl plus 160 mM NaCl at 4 °C overnight. These vesicles were mixed thoroughly with valinomycin (in ethanol) to give a final concentration of 1 μM (final concentration of ethanol was <0.2%), and different values for the diffusion potential were

[1] Abbreviations: 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(arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SLV, sarcolemmal membrane vesicles.

clamped by 25–50-fold dilution of the vesicles in assay medium containing 0.1–160 mM KCl and LiCl ($[K]_o + [Li]_o = 160$ mM). The reaction of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchanges was initiated by the dilution of 5–20 μL of Na- or Ca-loaded vesicles in 0.25–0.5 mL of assay medium. The timing of ^{45}Ca uptake was controlled electronically by the injection of 5 mL of quenching buffer (5 mM EGTA, 20 mM Mops/Tris, pH 7.4, and 160 mM KCl) (Khananshvili, 1990, 1991a). Quenched solutions were filtered on GF/C filters (the filtration rate was controlled by a Gilford-3021 pressure regulator), and collected vesicles were washed within 9–12 s with cold 3 × 8 mL Tris/Mops/KCl/EGTA buffer with 0.5 mM EGTA.

In experiments in which varying concentrations of ^{45}Ca were added to the assay medium, the calcium concentrations were plotted as $[Ca]_o = [^{45}Ca]_a + [Ca]_e + [Ca]_v$, and the specific radioactivity was corrected. In this equation, $[^{45}Ca]_a$ is the added ^{45}Ca, $[Ca]_e$ is the endogenous (ambient) calcium in assay medium (1–3 μM), and $[Ca]_v$ is the final concentration of calcium obtained after 20–70-fold dilution of Ca-loaded vesicles (in the case of Na-loaded vesicles, $[Ca]_v = 0$). For each $[^{45}Ca]_a$ in the assay medium, the specific radioactivity was corrected as $[^{45}Ca]_a/[Ca]_o$. In assay solutions, $[Ca]_e$ was measured with ±10% accuracy by using the optical probe, arsenazo III (Bauer, 1981). Protein was measured according to a modified assay of Lowry (Markwell et al., 1978). The kinetic parameters and their standard errors were estimated by the Enzfitter program (Hippo II, IBM-compatible 486) as outlined before (Khananshvili, 1990, 1991a).

RESULTS

Ratio of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} Exchanges. In order to compare the initial rates of the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchanges, a time course of Na_i- or Ca_i-dependent ^{45}Ca uptake was measured in the isolated cardiac sarcolemma vesicles (Figure 1A). The concentrations of intravesicular (either Na^+ or Ca^{2+}) and extravesicular ions (^{45}Ca) were saturating. The reaction of ^{45}Ca uptake was initiated by 20-fold dilution of Ca-loaded (250 μM $CaCl_2$) or Na-loaded (160 mM NaCl) vesicles in assay medium containing 20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, and 250 μM $^{45}CaCl_2$, and the reaction was quenched after t = 1–40 s by using a semirapid mixer. As can be seen from Figure 1A, the initial rates of Na^+–Ca^{2+} exchange are at least 2–3-fold faster as compared to the initial rates of Ca^{2+}–Ca^{2+} exchange.

In order to compare the V_{max} and K_m values of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange, the initial rates (t = 1 s) of Na_i- or Ca_i-dependent ^{45}Ca uptake were measured with extravesicular pH 7.4 or 9.1 and varying $[^{45}Ca]_o$. Before the experiment, the vesicles were preequilibrated with saturating concentrations of unlabeled calcium (250 μM $CaCl_2$) or sodium (160 mM NaCl) as described in Materials and Methods. The Na^+- or Ca^{2+}-loaded vesicles were diluted (70-fold) in assay medium containing varying concentrations of ^{45}Ca (5–120 μM), 0.25 M sucrose, and 20 mM buffer with pH 7.4 (Figure 1B) or 9.1 (Figure 1C). Since the vesicles were prepared and equilibrated with 20 mM Mops/Tris, pH 7.4, it was assumed that during the assay (t = 1 s) the intravesicular pH is not affected. The observed K_m (μM) values were as follows: $K_m(Na/Ca) = 43.6 \pm 6.3$ and $K_m(Ca/Ca) = 16.7 \pm 1.6$ at pH 7.4 (Figure 1B); $K_m(Na/Ca) = 55.2 \pm 6.2$ and $K_m(Ca/Ca) = 17.3 \pm 2.5$ at pH 9.1 (Figure 1C). The V_{max} (nmol mg^{-1} s^{-1}) values were estimated as follows: $V_{max}(Na/Ca) = 5.1 \pm 0.3$ and $V_{max}(Ca/Ca) = 2.0 \pm 0.1$ at pH 7.4 (Figure 1B); $V_{max}(Na/Ca) = 8.9 \pm 0.4$ and $V_{max}(Ca/Ca) = 2.5 \pm 0.1$ at pH 9.1 (Figure 1C). Therefore, at pH 7.4 the ratios of the kinetic parameters were $K_m(Na/Ca)/K_m(Ca/Ca) = 2.6 \pm 0.2$ and $V_{max}(Na/Ca)/V_{max}(Ca/Ca) = 2.5 \pm 0.2$. At pH 9.1, the ratios were $K_m(Na/Ca)/K_m(Ca/Ca) = 3.2 \pm 0.2$ and $V_{max}(Na/Ca)/V_{max}(Ca/Ca) = 3.6 \pm 0.3$. These data show that, within experimental error, the ratio (R) between the observed K_m values for the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchanges is equal to the ratio of the V_{max} values. This relationship (see eq 1) is expected in the frame of a ping-pong (consecutive) mechanism [for details on the origin and derivation of eq 1, see Khananshvili (1991b)].

$$R = \frac{K_m(Na/Ca)}{K_m(Ca/Ca)} = \frac{V_{max}(Na/Ca)}{V_{max}(Ca/Ca)} = \frac{1 + (l'/l'')}{1 + (l'/k'')} \quad (1)$$

Although the absolute values of the kinetic parameters varied from experiment to experiment (12 independent experiments, obtained from five different preparations of vesicles) under fixed experimental conditions, the R value was constant for a given preparation of vesicles. The R values were different at pH 7.4 ($R \approx 2.5$) and pH 9.1 ($R \approx 3.4$), suggesting that the ratios of the specific rate constants are controlled by pH. These data provide independent evidence in favor of a consecutive (ping-pong) mechanism (Khananshvili, 1990a, 1991a,b; Niggli & Lederer, 1991; Hilgemann et al., 1991; Li & Kimura, 1991; Matsuoka & Hilgemann, 1992), yielding new information about the relative movement of Ca^{2+} and Na^+ (see the Discussion).

pH-Titration Curves of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} Exchanges. In order to examine the effect of a wide range of pH's on R values, the initial rates (t = 1 s) of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange [$V_{max}(Na/Ca)$ and $V_{max}(Ca/Ca)$] were measured with saturating concentrations of intravesicular ions (either 250 μM Ca^{2+} or 160 mM Na^+ for Ca^{2+}–Ca^{2+} and Na^+–Ca^{2+} exchange, respectively) and saturating concentrations of extravesicular ^{45}Ca (250 μM). Under these conditions, the ion-binding events are not rate-limiting (Khananshvili, 1990a, 1991a,b). In these experiments the Na-loaded or Ca-loaded (Figure 2A) vesicles were diluted in assay medium containing 20 mM buffer with varying pH (5.0–10.8). The pH–titration data of Ca^{2+}–Ca^{2+} exchange can be fit to a bell-shaped pattern in the acidic range with apparent $pK_{a1} = 5.1 \pm 0.1$ and $pK_{a2} = 6.5 \pm 0.2$, followed by an alkaline-dependent increase in the exchange with an apparent $pK_{a3} = 10.0 \pm 0.2$ (Figure 2A). A similar pattern was observed in nine independent experiments (four different preparations of vesicles). It is worth noting that, in the range of pH 5.0–10.8, potassium has no effect on Na^+–Ca^{2+} exchange, while the bell-shaped pH–titration curve of Ca^{2+}–Ca^{2+} exchange can be observed only in the absence of monovalent ions (D. Khananshvili & E. Weil-Maslansky, unpublished data).

The bell-shaped pattern of Ca^{2+}–Ca^{2+} exchange in the acidic range has been reported previously under conditions in which the calcium concentration was not saturating (Slaughter et al., 1983). The pH–titration curve of Na^+–Ca^{2+} exchange (Figure 2A) shows a monotonic increase from pH 5.0 to 9.5. The experimental data can be fit to either two pK_a values ($pK_{a1} = 5.8 \pm 0.3$ and $pK_{a2} = 8.6 \pm 0.4$) or three pK_a values ($pK_{a1} = 5.1 \pm 0.1$, $pK_{a2} = 7.2 \pm 0.2$, and $pK_{a3} = 9.1 \pm 0.2$). In most cases, the experimental data were better fit with the three- rather than the two-pK_a option. No reasonable fit could be obtained for a one-pK_a model (n = 9).

The ratio of Na^+–Ca^{2+}/Ca^{2+}–Ca^{2+} exchange (R) shows a pH-dependent bell-shaped curve (Figure 2B). In the range of pH 5.0–6.0, the R values are close to the unity ($R \approx 1$).

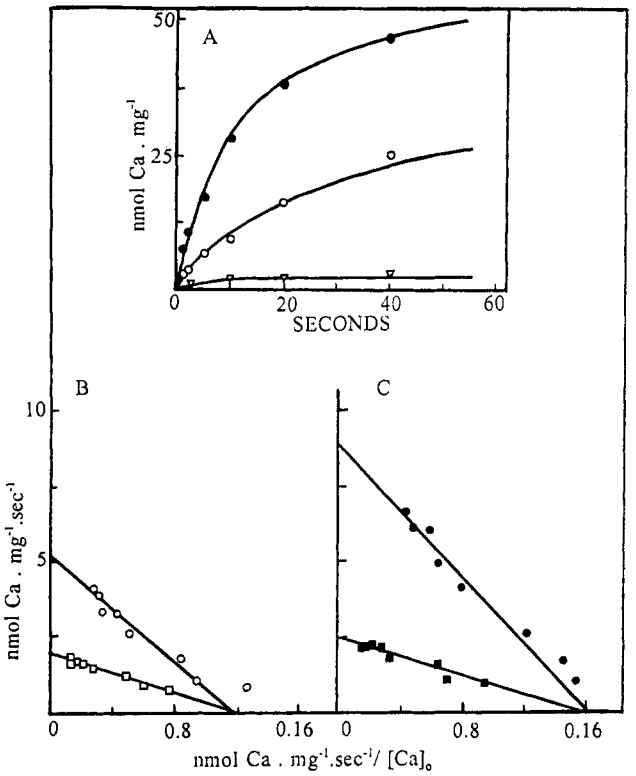


FIGURE 1: Kinetic parameters of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange at pH 7.4 and 9.1. (A) The cardiac sarcolemma vesicles were prepared in 20 mM Mops/Tris, pH 7.4, and 0.25 M sucrose and loaded with either 250 μM $CaCl_2$ or 160 mM NaCl as described in Materials and Methods. The Na-loaded (●) or Ca-loaded (○) vesicles were diluted in assay medium with 20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, and 250 μM $^{45}CaCl_2$ (21 000 cpm/nmol). Blanks (▽) contained 160 mM NaCl in assay medium. The reaction of ^{45}Ca uptake was quenched after 1–40 s, and intravesicular ^{45}Ca was measured on GF/C filters as described in Materials and Methods. (B and C) The Na-loaded (160 mM) and Ca-loaded (250 μM) vesicles were obtained as described in A. Initial rates (t = 1 s) of Na_i- or Ca_i-dependent ^{45}Ca uptake were measured by dilution of Ca-loaded (■, □) or Na-loaded (●, ○) vesicles in the assay medium, buffered at pH 7.4 (○, □) or 9.1 (●, ■). The other components of the assay medium were 0.25 M sucrose, $[^{45}Ca]_a$ = 5–120 μM (98 000 cpm/nmol), and $[Ca]_e$ = 1.5 μM. The pH in assay medium was controlled with 20 mM Mops/Tris, pH 7.4 (B) or 20 mM Tris/Mops, pH 9.1 (C). The reaction of ^{45}Ca uptake was terminated by injection of EGTA buffer in the semirapid mixer, and intravesicular ^{45}Ca was measured by filtration (see Materials and Methods). The specific radioactivity for each $[Ca]_o$ value was corrected for unlabeled calcium in the assay medium, and the lines were calculated to give an optimal fit to the experimental points (see Materials and Methods). The K_m (μM) values were 43.6 ± 6.3 (○), 16.7 ± 1.6 (□), 55.2 ± 6.2 (●), and 17.3 ± 2.5 (■). The V_{max} (nmol mg^{-1} s^{-1}) values were 5.1 ± 0.3 (○), 2.0 ± 0.1 (□), 8.9 ± 0.4 (●), 2.5 ± 0.1 (■).

By increasing the pH from 8.1 to 9.1, the R values reach a maximum (R = 4), followed by a decrease to R < 2 at more alkaline pH values (Figure 2B). These data indicate that the rate-limiting step, as well as the ratios between the rate constants of Na^+ and Ca^{2+} movement, is modulated by pH (see eq 1).

Combined Effects of pH and Voltage on Na^+–Ca^{2+} Exchange. In order to examine the capacity of response to voltage of various protonated forms of the carrier, the effect of diffusion potential (potassium valinomycin) on Na^+–Ca^{2+} exchange was studied at various pH's in the assay medium. Saturating concentrations of extravesicular ^{45}Ca (250 μM) and intravesicular Na (160 mM) were used in these experiments. Before the experiment, the Na-loaded vesicles were treated with or without valinomycin. "0 mV" or inside-positive potential (195 mV) was clamped by exposure of the vesicles

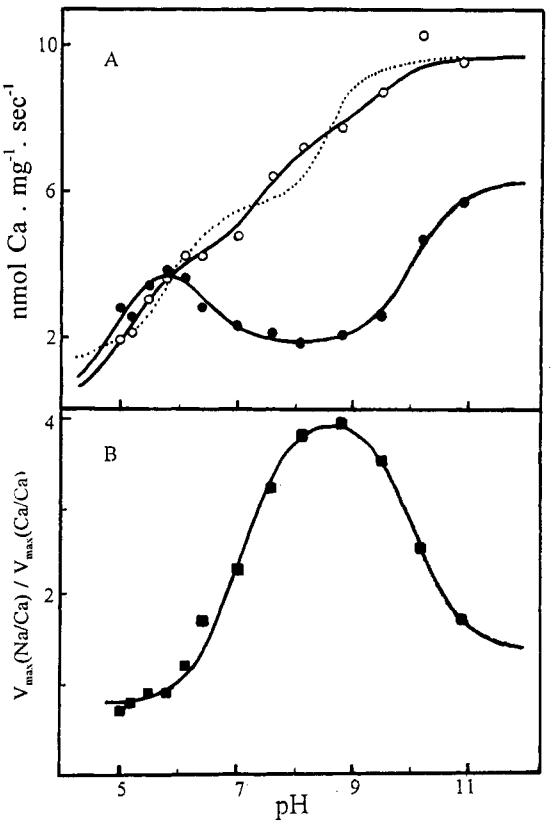


FIGURE 2: Effect of pH on Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange at fixed, saturating $[^{45}Ca]_o$ and $[Na]_o$. (A) The Na-loaded (160 mM) or Ca-loaded (250 μM) vesicles were rapidly diluted in the assay medium containing 20 mM buffer with different pH's (5.0–10.8), 0.25 M sucrose, and 250 μM ^{45}Ca (27 500 cpm/nmol). The Na^+–Ca^{2+} (○) and Ca^{2+}–Ca^{2+} (●) exchange reactions were quenched after 1 s by the automatic addition of EGTA containing buffer (pH 7.4) in the semirapid mixer (see Materials and Methods and Figure 1). Each point represents the average value of duplicate measurements and reflects the V_{max}(Na/Ca) (○) or V_{max}(Ca/Ca) (●) value at each given pH. The lines were computed to give an optimal fit to the experimental points. The fitting equations were written as follows: $V_{max}(Ca/Ca) = [(Lim_0 + Lim_1 10^{pH-pK_{a1}})/(1 + 10^{pH-pK_{a1}})] - [(Lim_0' + Lim_2 10^{pH-pK_{a2}})/(1 + 10^{pH-pK_{a2}})] + [(Lim_0' + Lim_3 10^{pH-pK_{a3}})/(1 + 10^{pH-pK_{a3}})]$ and $V_{max}(Na/Ca) = [(Lim_0 + Lim_1 10^{pH-pK_{a1}})/(1 + 10^{pH-pK_{a1}})] + [(Lim_2 10^{pH-pK_{a2}})/(1 + 10^{pH-pK_{a2}})] + [(Lim_3 10^{pH-pK_{a3}})/(1 + 10^{pH-pK_{a3}})]$. The apparent pK_a values were estimated as pK_{a1} = 5.2 ± 0.1 (Lim_0 = 2.0 ± 0.1 and Lim_1 = 4.2 ± 0.3), pK_{a2} = 7.4 ± 0.2 (Lim_2 = 3.3 ± 0.5), and pK_{a3} = 9.4 ± 0.4 (Lim_3 = 2.1 ± 0.5) for the Na^+–Ca^{2+} exchange and pK_{a1} = 5.0 ± 0.2 (Lim_0 = 1.0 ± 0.1 and Lim_1 = 5.2 ± 0.2), pK_{a2} = 6.5 ± 0.4 (Lim_0' = 1.8 ± 0.1 and Lim_2 = 5.2 ± 0.2), and pK_{a3} = 10.0 ± 0.2 (Lim_0' = 1.8 ± 0.1 and Lim_3 = 6.3 ± 0.4) for the Ca^{2+}–Ca^{2+} exchange. (B) The experimental data described in A were plotted as the ratio of V_{max}(Na/Ca)/V_{max}(Ca/Ca) values. The line was calculated as the best fit to the experimental points according to the following equation: $[V_{max}(Na/Ca)/V_{max}(Ca/Ca)] = [(Lim_0 + Lim_1 10^{pH-pK_{a1}})/(1+10^{pH-pK_{a1}})] - [(Lim_0 + Lim_1 10^{pH-pK_{a1}})/(1 + 10^{pH-pK_{a2}})]$. The estimated values were pK_{a1} = 7.1 ± 0.1 (Lim_0 = 0.8 ± 0.1 and Lim_1 = 4.1 ± 0.1) and pK_{a2} = 10.0 ± 0.1 (Lim_0 = 0.8 ± 0.1 and Lim_2 = 2.7 ± 0.1).

(–Val- or +Val-treated) in assay medium containing 20 mM buffer (pH 5.5–10.0), 250 μM ^{45}Ca, and 160 mM LiCl or KCl, respectively, and the initial rates of ^{45}Ca uptake (t = 1 s) were measured. Control experiments showed that the potassium itself had no effect on the pH–titration curves of Na^+–Ca^{2+} exchange (not shown). The pH–voltage titration curves of Na^+–Ca^{2+} exchange can be fit to three pK_a values for both valinomycin-untreated (pK_{a1} = 5.3 ± 0.1, pK_{a2} = 6.9 ± 0.1, and pK_{a3} = 9.2 ± 0.1) and valinomycin-treated (pK_{a1} = 5.3 ± 0.3, pK_{a2} = 7.1 ± 0.1, and pK_{a3} = 9.1 ± 0.1) vesicles (Figure 3, main panel). However, positive-inside potential has no effect on Na^+–Ca^{2+} exchange at pH <6.1, while with

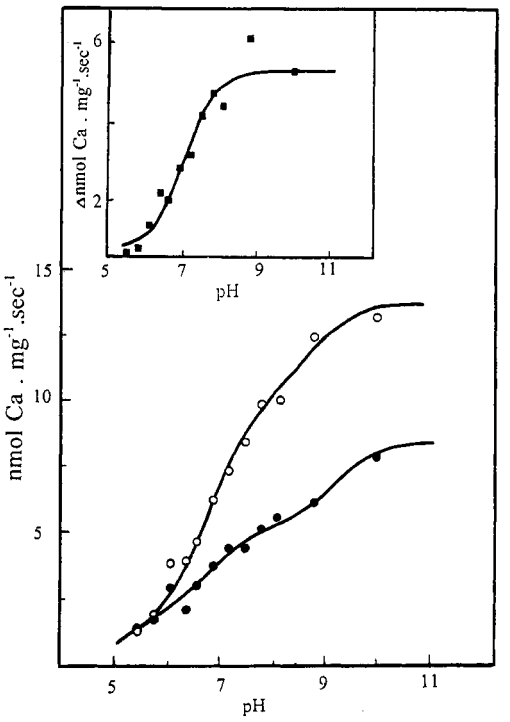


FIGURE 3: Effect of pH and inside-positive potential on Na^+–Ca^{2+} exchange. Before the experiment, the Na-loaded vesicles were treated with (○) or without (●) 1 μM valinomycin (see Materials and Methods). By using the semi-rapid-mixing device, the vesicles were diluted in the assay medium containing 20 mM buffer (pH 5.5–10.0), 160 mM KCl, and 250 μM ^{45}Ca (23 300 cpm/nmol), and the reaction of ^{45}Ca uptake was quenched after 1 s, as described in Materials and Methods (also see Figures 1 and 2). The lines were calculated to give an optimal fit to the experimental points, as described for the Na^+–Ca^{2+} exchange in Figure 2. For the valinomycin-untreated vesicles, the estimated values were $pK_{a1} = 5.3 \pm 0.1$, $pK_{a2} = 6.9 \pm 0.1$, and $pK_{a3} = 9.2 \pm 0.1$ ($Lim_0 = 0.1$, $Lim_1 = 2.1$, $Lim_2 = 3.1$, and $Lim_3 = 3.2$). For the valinomycin-treated vesicles, the kinetic parameters were computed as $pK_{a1} = 5.3 \pm 0.3$, $pK_{a2} = 7.1 \pm 0.1$, and $pK_{a3} = 9.1 \pm 0.1$ ($Lim_0 = 0.1$, $Lim_1 = 2.1$, $Lim_2 = 9.2$, and $Lim_3 = 2.2$). Inset: The difference in Na^+–Ca^{2+} exchange activities [ΔV_{max}(Na/Ca)] between the valinomycin-treated and -untreated vesicles was plotted as a function of pH. The fitted line was calculated according to the following equation: $\Delta V_{max}(Na/Ca) = [(Lim_0 + Lim_1 10^{pH-pK_a})/(1 + 10^{pH-pK_a})$. The estimated value was $pK_a = 7.0 \pm 0.1$ ($Lim_0 = 0.2 \pm 0.1$ and $Lim_1 = 5.3 \pm 0.3$).

increasing pH values the membrane potential activates the rate of the exchange reaction. The difference between the Na^+–Ca^{2+} exchange activities of valinomycin-treated and -untreated vesicles (Figure 3, inset) can be fit to a single $pK_a = 7.0 \pm 0.1$. Under similar conditions, the Ca^{2+}–Ca^{2+} exchange is voltage-insensitive in the pH range 5.0–10.8 (not shown).

The above data suggest that, at low pH values, Na^+–Ca^{2+} exchange becomes nearly voltage-insensitive, while the capacity of the exchange reaction for voltage response appears with increasing pH, exhibiting an apparent $pK_a = 7.0$. In order to confirm this conclusion, an experiment was designed to test Na^+–Ca^{2+} exchange at two fixed pH's (5.5 and 7.6) and at membrane potentials ($\Delta\psi$) varying from 0 to 195 mV. In these experiments, the vesicles (preequilibrated with 20 mM Mops/Tris, pH 7.4, 160 mM NaCl, and 0.1 mM KCl) were treated with valinomycin before the experiment. Different values of the diffusion potential were clamped by a 25-fold dilution of valinomycin-treated vesicles in assay medium containing either 20 mM Mops/Tris (pH 7.6) or Mes/Tris (pH 5.5) and varying concentrations of K^+ (the total concentration of monovalent ions was balanced by LiCl,

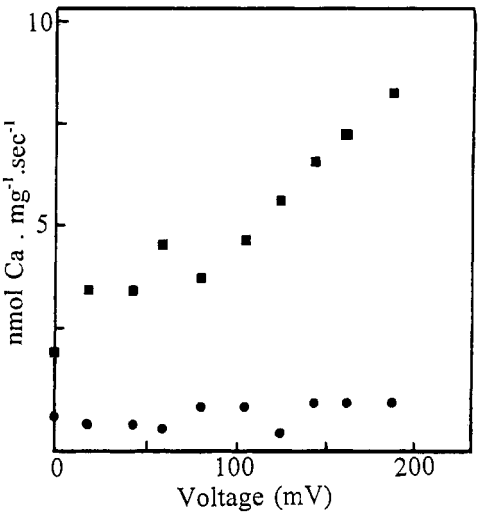


FIGURE 4: Effect of membrane potential on the initial rate of the Na^+–Ca^{2+} exchange with saturating [^{45}Ca]$_o$ and [Na]$_i$, at pH 5.5 and 7.6. Cardiac sarcolemma vesicles were preequilibrated with 20 mM Mops/Tris, pH 7.4, 160 mM NaCl, and 0.1 mM KCl at 4 °C overnight and pretreated with 1 μM valinomycin before the experiment, as outlined in Materials and Methods. Different values of the diffusion potential were clamped by 25-fold dilution of vesicles in assay medium containing various concentrations of KCl and LiCl ([K]$_o$ + [Li]$_o$ = 160 mM), 250 μM $^{45}CaCl_2$ (28 000 cpm/nmol), and either 20 mM Mops/Tris, pH 7.6 (■), or Mes/Tris, pH 5.5 (●). Each point represents duplicate measurements of the initial rate (t = 1 s) of Na_i-dependent ^{45}Ca uptake. Blanks were taken at each clamped voltage and subtracted as described (see Materials and Methods). The membrane potential was calculated according to $\Delta\psi = 61.6$ mV log ([K]$_o$/[K]$_i$).

[K]$_o$ + [Li]$_o$ = 160 mM). A typical voltage–flux relationship was obtained at pH 7.6, while at pH 5.5 the voltage response of Na^+–Ca^{2+} exchange was nearly lost over the range 0–195 mV (Figure 4). Likewise, at pH 5.5 the Na^+–Ca^{2+} exchange became voltage-insensitive as negative-inside potential ($\Delta\psi$ was varied from −80 to 0 mV) was clamped in the vesicles (not shown). The typical profiles of pH–voltage titration curves of Na^+–Ca^{2+} exchange were observed in seven independent experiments obtained from three different preparations of cardiac sarcolemma vesicles.

DISCUSSION

Measurement of absolute rate constants is the most direct way to characterize partial reactions; however, no such experimental setup is available yet for the Na^+–Ca^{2+} exchanger and similar proteins. In an attempt to overcome this problem, we offer here an analytical approach to estimate the ratios of rate constants involving the specific steps of the Na^+–Ca^{2+} exchange cycle. The advantage of this approach is that the kinetic parameters of various exchange modes (Na^+–Ca^{2+}, Ca^{2+}–Ca^{2+}, and Na^+–Na^+ exchange) can be analyzed by measuring the relative rather than the absolute rate constants. In this work, the pH- and voltage-induced (potassium valinomycin) effects were investigated on the initial rates (t = 1 s) of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange in cardiac sarcolemma vesicles by using a semi-rapid-mixing technique. It is assumed that, under the explored experimental conditions, there is a unidirectional movement of ions during the Na^+–Ca^{2+} exchange cycle [for details, see Stein (1986) and Khananshvili (1991a,b)]. This is a reasonable assumption (at least for the steady-state conditions), because the experimental data (Figure 1) satisfy a formalism (eq 1) predicted

by the consecutive mechanism of unidirectional ion fluxes (Scheme 1).

Although the preparation of isolated sarcolemma vesicles consists of a mixture of inside-out and outside-out vesicles, a number of recent observations suggest that under specific experimental conditions only inside-out vesicles may contribute to most, if not all, of the Na^+–Ca^{2+} exchange activity: (a) The XIP peptide, which was originally designed to interact with the cytoplasmic loop of the exchanger molecule (Li et al., 1991), and a number of synthetic tetrapeptides inhibit the exchange activities (Khananshvili et al., 1993). (b) In the range of 5–250 μM extravesicular calcium, no heterogeneity in K_m(Ca) values has been observed for both Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange (see Figure 1). (c) The exchange activity of outside-out vesicles cannot be seen, because of a probable leak in this population of vesicles (Ambesi et al., 1991). Although it is clear that in the range of 5–250 μM there is a saturable and obligatory site of Ca^{2+} for exchange reactions, the present work cannot exclude the possible existence of a distinct low-affinity Ca^{2+} site. This putative Ca^{2+}-binding site could be located on the extravesicular or intravesicular surface of either inside-out or outside-out vesicles. Since no significant differences were observed in the initial rates of Ca^{2+}–Ca^{2+} exchange with intravesicular calcium of 0.25–0.75 mM (not shown), a putative low-affinity Ca^{2+} site may operate under distinct experimental conditions, described in this work. If this is the case, further systematic investigation is necessary for the resolution of a possible role for this putative site in the regulation of relative rates of Na^+ and Ca^{2+} ion movement through the exchanger.

The present experimental approaches seem promising for the study of intrinsic properties (rate limiting, kinetic asymmetry, etc.) and modulatory factors (e.g., voltage sensitivity, pH, monovalent ions, proteolytic enzymes, phospholipase C, regulatory peptides, etc.) that are valid in vesicular preparations. However, the Na^+–Ca^{2+} exchanger of cardiac sarcolemma vesicles is insensitive to some important cellular regulators (Reeves & Philipson, 1989). For example, the patch–clamp studies suggest that intracellular sodium, calcium, and ATP modulate the exchange cycle via reactions that do not involve the ion-transport pathway (Blaustein et al., 1991; Matsuoka & Hilgemann, 1992). The relevant regulatory domains are located on the intracellular loop of the exchanger molecule (Matsuoka et al., 1993), and they should be exposed to the extravesicular medium in inside-out vesicles. Nevertheless, there is no evidence that these regulatory domains are active in our experimental system. Therefore, vesicular and electrophysiological approaches should be complementary for understanding the fine regulatory mechanisms of the exchange cycle.

Na^+–Ca^{2+}/Ca^{2+}–Ca^{2+} Exchange Ratios As an Expression of Relative Rates of Ion Movement. Figure 1 shows that, at fixed extravesicular pH (7.4 or 9.1), varying concentrations of extravesicular ^{45}Ca, and saturating concentrations of intravesicular Na^+ or Ca^{2+}, the ratio (R) between the observed K_m values of the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange reactions is equal to the ratio of the V_{max} values (see eq 1). These data provide kinetic evidence that the Ca^{2+}–Ca^{2+} and Na^+–Ca^{2+} exchange reactions have common ion-transport pathways (e.g., l'), as predicted by the consecutive (ping-pong) mechanism (Khananshvili, 1991b). Examination of eq 1 allows us to conclude that when $R > 2$–3 (Figures 1 and 2), a relationship between the rate constants can be written as $[1 + (l'/l'')] > [1 + (l'/k'')]$. This means that the observed $R > 2$–3 values (Figures 1 and 2) can be obtained only if a specific relationship

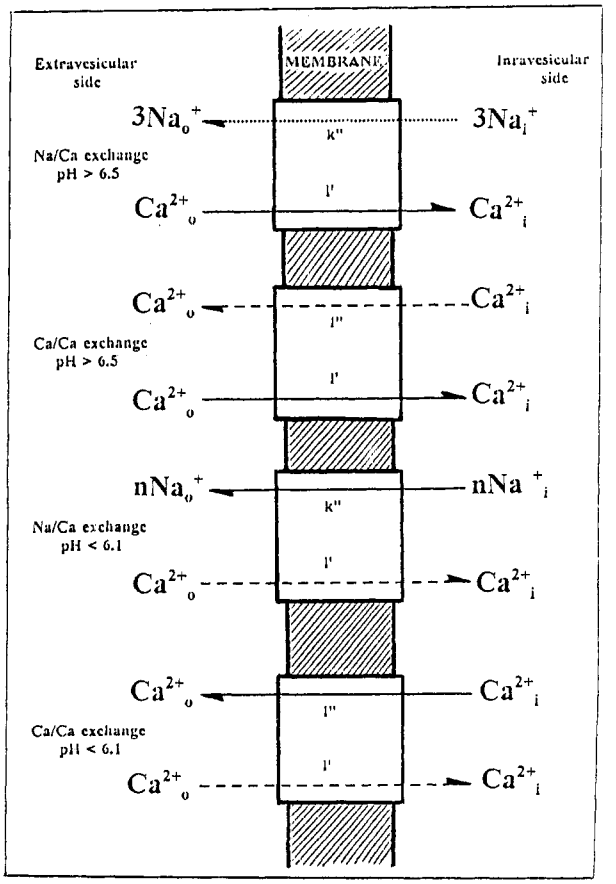


FIGURE 5: pH-dependent modulation of rate-limiting and voltage-sensitive properties of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange reactions. The rate constants (l', l'', k', and k'') represent unidirectional movement of sodium or calcium through the Na^+–Ca^{2+} exchanger during the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchanges. It is assumed that the concentrations of ions are saturating on both sides of the vesicular membrane, and the intravesicular pH is 7.4 under the experimental conditions tested. - - - depicts the rate-limiting and voltage-insensitive reaction, and ··· corresponds to the rate-limiting and voltage-sensitive reaction.

between the rate constants is realized: $l' > l''$ and $k'' > l''$ (also see Scheme 1). Likewise, as can be seen from eq 1, the maximal values of R = 3–4 are observed, only if Ca^{2+} moves severalfold faster from the extravesicular (cytosolic) to the intravesicular (extracellular) side than in the opposite direction ($l' > l''$). This means that at pH >6.5 the bidirectional movement of Ca^{2+} through the Na^+–Ca^{2+} exchanger is an intrinsically asymmetric process, while the transport of Ca^{2+} from the intravesicular to the extravesicular side (l'') is a rate-limiting step of Ca^{2+}–Ca^{2+} exchange (Figure 5).

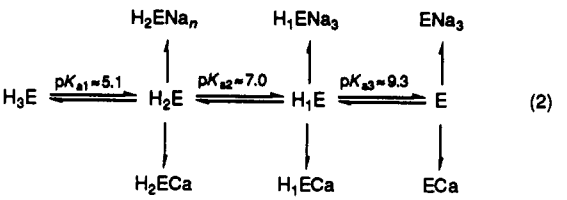
The $V_{max}/K_m = K_a'[E]_T/l'$ values of both the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange (see interceptions on the abscissas of Figure 1B,C) are not significantly affected when the pH is increased from 7.4 to 9.1, supporting the idea that the dissociation constant for extravesicular Ca binding (K_a') and the rate constant of unidirectional Ca^{2+} movement (l') are not significantly modified by deprotonation of the exchanger. Likewise, the pH change from 7.4 to 9.1 has little effect on $V_{max}(Ca/Ca) = l'l''[E]_T/(l' + l'')$, suggesting that under the conditions tested, the bidirectional movements of Ca^{2+} (l' and l'') are weakly modulated by deprotonation of the carrier (Figure 1B,C). Therefore, at pH >6.5 when $V_{max}(Na/Ca) = l'k''[E]_T/(l' + k'')$ increases by severalfold (Figures 1 and 2), the rate of Na^+–Ca^{2+} exchange is limited by pH-sensitive movement of Na^+ (k'') rather than by pH-insensitive movement of Ca^{2+} (l') (Figure 5). Although in the pH range 7.0–

9.0 the unidirectional movement of Ca^{2+} (l') is "pH-insensitive", acidic pH (<6.1) may slow down l' in such a way that l' determines the rates of both Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange [the $V_{max}(Na/Ca) = l'k''[E]_T/(l' + k'')$ and $V_{max}(Ca/Ca) = l'l''[E]_T/(l' + l'')$ values may become nearly equal if l' is rate-limiting] (Figure 5).

pH–Voltage Titration Curves and Voltage Sensitivity of Exchange Reactions. Under conditions in which saturating concentrations of calcium exist at both sides of the membrane (the ion binding is not rate-limiting), the Ca^{2+}–Ca^{2+} exchange shows a bell-shaped pattern (pK_{a1} = 5.1 and pK_{a2} = 6.5) followed by activation of the exchange in the alkaline range (pK_{a3} = 10.0) (Figure 2A). Under similar conditions, the Na^+–Ca^{2+} exchange increases from pH 5.0 to 9.5, exhibiting apparent pK_a values of pK_{a1} = 5.1, pK_{a2} = 7.2, and pK_{a3} = 9.1 (Figure 2A). The pattern of these pH–titration curves indicates that a number of protonated forms are involved in these ion-transport mechanisms [for reviews, see Tipton and Dixon (1979) and Cleland (1979)]. The most striking finding is that at pH <6.1, the pH–voltage titration of Na^+–Ca^{2+}-exchange is not affected by inside-positive potential, while at higher values of pH the same voltage clamp results in 2–3-fold activation of the exchange reaction (Figures 3 and 4). Interestingly enough, this voltage-dependent activation of Na^+–Ca^{2+} exchange appears with a single pK_a of 7.0 (Figure 3, inset), which is comparable to the $pK_{a2}(Na/Ca)$ = 6.9–7.4 and $pK_{a2}(Ca/Ca)$ = 6.5–7.2 values (n = 9) derived from pH–titration curves (see Figures 2 and 3).

Another "coincidence" is that the increase in R values also shows an apparent pK_a = 7.1 (Figure 2B). Proton-dependent inactivation of voltage response has also been demonstrated when the pH was fixed and the membrane potential varied over a wide range. At fixed pH 5.5, no measurable voltage response of Na^+–Ca^{2+} exchange was obtained when $\Delta\psi$ was gradually increased from 0 to +195 mV (Figure 4). Similar results were obtained by varying $\Delta\psi$ from –80 to 0 mV (not shown). A typical voltage-dependent activation of the exchange reaction could be demonstrated at pH 7.6 by inside-positive potential (Figure 4). Two principal mechanisms may account for proton-dependent modulation of Na^+–Ca^{2+} exchange voltage response: (a) The protonation of the ion-transport and/or regulatory sites of the carrier slows down the electroneutral movement of Ca^{2+} (l') in such a way that it becomes a rate-limiting step ($l' < k''$), thereby masking the electrogenic transport of Na^+ (k'') (the kinetic mechanism). (b) There is no net charge transfer per exchange cycle, because the electrogenic stoichiometry ($3Na^+$:Ca^{2+}) becomes electroneutral (e.g., $2Na^+$:Ca^{2+} or $3Na^+$:Ca^{2+},H^+) at low pH (the equilibrium mechanism). In conclusion, at pH <6.1 the electroneutral movement of Ca^{2+} from the extravesicular to the intravesicular side (l') may limit the Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+}exchange rates, while at pH >6.5 the ion movements in the opposite direction may become rate-limiting [voltage-sensitive Na^+ movement (k'') controls Na^+–Ca^{2+} exchange, and electroneutral Ca^{2+} transport (l'') limits Ca^{2+}–Ca^{2+}-exchange] (Figure 5).

A Simple Model for the Functional Protonation–Deprotonation State of the Carrier. A simple model can be presented describing the relationship between the protonated–deprotonated states of the membrane-bound Na^+–Ca^{2+} exchanger and its functional properties (eq 2). Although the observed pK_a values do not necessarily represent the pK_a values of specific functional groups involving ion-transport and/or modulatory activities, three functional states (H_2E, H_1E, and E) of the exchanger can be proposed: (a) Deprotonation of



(2)

the protonated carrier (H_3E) in the acidic range ($pK_{a1} \approx 5.1$) accelerates both voltage-insensitive Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange in a similar way, exhibiting the $R \approx 1$ values (see Figures 2 and 3), suggesting that the electroneutral H_2ECa forms may involve the rate-limiting step of both exchange modes (no matter whether the H_2ENa_n forms are charged or not). An alternative possibility is, of course, that the H_2ENa_n species are involved in the rate-limiting step, but they are voltage-insensitive. Whatever the exact mechanism of proton-dependent modulation of voltage response is (see above the proposed kinetic and equilibrium mechanisms), the H_2E species cannot provide a voltage-sensitive intermediate at the rate-limiting step of the Na^+–Ca^{2+} exchange cycle (Figure 5). (b) Further deprotonation of the carrier ($pK_{a2} \approx 7.0$) is necessary for expressing the voltage response of Na^+–Ca^{2+} exchange, which is supported by voltage-sensitive and rate-limiting transport of Na^+ (the H_1ENa_3 species are voltage-sensitive). The H_1E forms are able to accelerate the rate-limiting Na^+ transport (k'') of Na^+–Ca^{2+} exchange, but they decelerate the rate-limiting Ca^{2+} transport (l'') of Ca^{2+}–Ca^{2+}exchange (R > 2–3; see Figure 2B); therefore, the H_1E species have opposing effects on Na^+ and Ca^{2+} transport when the ions move from the intravesicular to the extravesicular side (Figure 5). (c) deprotonation of the carrier in the alkaline range ($pK_{a3} \approx 9.3$) has a higher accelerating effect on the rate-limiting step of Ca^{2+}–Ca^{2+} exchange (l'') than on the rate-limiting step (k'') of Na^+–Ca^{2+} exchange (the R values decrease from 4 to 2 in the pH range 9.0–10.8; see Figure 2B). There is no evidence that the E forms have any special property for controlling the voltage response of Na^+–Ca^{2+} exchange.

Physiological Relevance. In light of the present findings, it is suggested that the asymmetry of bidirectional Ca^{2+} movement across the sarcolemma membrane may account for (at least partially), but not limit, a 200–300-fold asymmetry in the intracellular vs extracellular $K_m(Ca^{2+})$ exhibited by the Na^+–Ca^{2+} exchanger in intact cardiac cells (Hilgemann et al., 1991; Li & Kimura, 1991; Matsuoka & Hilgemann, 1992). It is possible that in intact cells, the R values are even larger than the values observed here in sarcolemmal membranes (Figure 2B). Likewise, the specific regulatory modes may control the relative movements of Na^+ and Ca^{2+} according to the physiological needs of the cell. The present study shows that the decrease in extravesicular (intracellular) pH from 7.4 to <6.1 reduces the overall activity of Na^+–Ca^{2+} exchange by only 30–50% (Figure 2), but the same drop in pH results in a nearly complete loss of measurable voltage response of Na^+–Ca^{2+} exchange (Figures 3 and 4). If this phenomenon also operates in intact cardiac cells, the acidic conditions might diminish the voltage response of the Na^+–Ca^{2+} exchanger during the action potential, which might be reflected in an altered pattern of action potential and an elevation of intracellular calcium levels. This situation might be relevant to some pathological conditions (e.g., acidosis and ischemia) when the intracellular pH drops considerably (0.5–0.8 unit) within 1 min. In contrast to the Na^+–Ca^{2+} exchanger, it has been reported that the V_{max} values of the cardiac sarcolemmal and sarcoplasmic reticulum Ca^{2+}-ATPase can be dramatically activated at pH 6.0–7.0 (Dixon & Haynes, 1990). This means

that the interrelationship between the two major Ca^{2+}-extruding systems, the Na^+–Ca^{2+} exchanger and Ca^{2+}-ATPase (both sarcolemmal and sarcoplasmic), could markedly differ under normal and pathological conditions. Further investigation is necessary for the elucidation of relevant structure–function interrelationships between the different Ca^{2+}- and H^+-transport systems.

REFERENCES

Ambesi, A., Van Alstyne, E. L., Bagwell, E. E., & Lindenmayer, G. E. (1991) *Ann. N.Y. Acad. Sci. 639*, 245–247.

Bauer, P. J. (1981) *Anal. Biochem. 87*, 206–210.

Blaustein, M. P., Di Polo, R., & Reeves, J. P. (1991) *Ann. N.Y. Acad. Sci. 639*, 99–177.

Bridge, J. H., Smolley, J., Spitzer, K. W., & Chin, T. K. (1991) *Ann. N.Y. Acad. Sci. 639*, 34–47.

Cheon, J., & Reeves, J. P. (1988) *J. Biol. Chem. 263*, 2309–2315.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Doering, A. E., & Lederer, J. W. (1992) *J. Mol. Cell Cardiol. 24* (Suppl. IV), p-55, S.54.

Dixon, D. A., & Haynes, D. H. (1990) *Biochim. Biophys. Acta 1029*, 274–284.

Eisner, D. A., & Lederer, J. W. (1985) *Am. J. Physiol. 248*, C189–202.

Gadsby, D. C., & Nakao, M. (1989) *J. Gen. Physiol. 94*, 511–537.

Goldshlegger, R., Karlish, S. J. D., Rephaeli, A., & Stein, W. D. (1987) *J. Physiol. (London) 387*, 331–355.

Hilgemann, D. W., & Nobel, D. (1987) *Proc. R. Soc. London B230*, 163–205.

Hilgemann, D. W., Nicoll, D. A., & Philipson, K. D. (1991) *Nature 352*, 715–718.

Hilgemann, D. W., Matsuoka, S., Nagel, G. A., & Collins, A. (1992) *J. Gen. Physiol. 100*, 905–931.

Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.

Jones, L. R. (1988) *Methods Enzymol. 157*, 85–91.

Khananshvili, D. (1990a) *Biochemistry 29*, 2437–2442.

Khananshvili, D. (1990b) *Curr. Opin. Cell Biol. 2*, 731–734.

Khananshvili, D. (1991a) *J. Biol. Chem. 266*, 13764–13769.

Khananshvili, D. (1991b) *Ann. N.Y. Acad. Sci. 639*, 85–98.

Khananshvili, D., Price, D. C., Greenberg, M. J., & Sarne, Y. (1993) *J. Biol. Chem. 268*, 200–205.

Lauger, P. (1987) *J. Membr. Biol. 99*, 1–11.

Li, J., & Kimura, J. (1991) *Ann. N.Y. Acad. Sci. 639*, 48–60.

Li, Z., Nicoll, D. A., Collins A., Hilgemann, D. W., Filoteo, A. G., Penniston, J. T., Tomich, J. M., & Philipson, K. D. (1991) *J. Biol. Chem. 266*, 1014–1020.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Matsuoka, S., & Hilgemann, D. W. (1992) *J. Gen. Physiol. 100*, 963–1001.

Matsuoka, S., Nicoll, D. A., Reilly, R. F., Hilgemann, D. W., & Philipson, K. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3870–3874.

Mullins, L. J. (1977) *J. Gen. Physiol. 70*, 681–695.

Nicoll, D. A., & Philipson, K. D. (1991) *Ann. N.Y. Acad. Sci. 639*, 181–188.

Nicoll, D. A., Longoni, S., & Philipson, K. D. (1990) *Science 250*, 562–564.

Niggli, E., & Lederer, W. J. (1991) *Nature 349*, 621–624.

Nobel, D., Nobel, S. J., Bett, G. C. L., Earm, Y. E., Ho, W. K., & So, I. K. (1991) *Ann. NY Acad. Sci. 639*, 334–354.

Reeves, J. P. (1988) *Methods Enzymol. 157*, 505–510.

Reeves, J. P., & Hale, C. C. (1984) *J. Biol. Chem. 259*, 7733–7739.

Reeves, J. P., & Philipson, K. D. (1989) in *Sodium-Calcium exchange* (Allen et al., Eds.) pp7–53, Oxford University Press, Oxford, U.K.

Reuter, H., & Seitz, N. (1968) *J. Physiol. 195*, 451–470.

Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183–3190.

Stein, W. D. (1986) in *Transport and Diffusion across Cell Membranes*, Academic Press, New York.

Tipton, K. F., & Dixon, H. B. F. (1979) *Methods Enzymol. 63*, 183–234.