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Distinction between the Two Basic Mechanisms of Cation Transport in the Cardiac Na^+–Ca^{2+} Exchange System†

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ABSTRACT: In order to distinguish between the Ping-Pong and sequential mechanisms of cation transport in the cardiac Na^+–Ca^{2+} exchange system, the initial rates of the Na_i-dependent ^{45}Ca uptake (t = 1 s) were measured in reconstituted proteoliposomes, loaded with a Ca chelator. Under "zero-trans" conditions ($[Na]_o = [Ca]_i = 0$) at a fixed $[Na]_i$ = 10–160 mM with varying $[^{45}Ca]_o$ = 2.5–122 μM for each $[Na]_i$, the K_m and V_{max} values increased from 7.7 to 33.5 μM and from 2.3 to 9.0 nmol·mg^{-1}·s^{-1}, respectively. The V_{max}/K_m values show a ±2–10% deviation from the average value of 0.274 nmol·mg^{-1}·s^{-1}·μM^{-1} over the whole range of $[Na]_i$. These deviations are within the standard error of V_{max} (±3–7%), K_m(±11–17%), and V_{max}/K_m (±11–19%). This suggests that, under conditions in which V_{max} and K_m are $[Na]_i$ dependent and vary 4–5-fold, the V_{max}/K_m values are constant within the experimental error. In the presence of K^+–valinomycin the V_{max}/K_m values are 0.85 ± 0.17 and 1.08 ± 0.18 nmol·mg^{-1}·s^{-1}·μM^{-1} at $[Na]_i$ = 20 and 160 mM, respectively, suggesting that under conditions of "short circuit" of the membrane potential the V_{max}/K_m values still exhibit the $[Na]_i$ independence. At a very low fixed $[^{45}Ca]_o$ = 1.1 μM with varying $[Na]_i$ = 10–160 mM, the initial rates were found to be $[Na]_i$ independent. At a high fixed $[^{45}Ca]_o$ = 92 μM the initial rates show a sigmoidal dependence on the $[Na]_i$ with V_{max} = 13.8 nmol·mg^{-1}·s^{-1}, K_m^{Na} = 21 mM, and Hill coefficient n_H = 1.5. The presented data support a Ping-Pong (consecutive) mechanism of cation transport in the Na^+–Ca^{2+} exchanger.

Coupled vectorial systems differ from ordinary enzymes in that they undergo large changes in chemical and vectorial specificity toward their ligands (Jencks, 1980; Hill, 1981; Tanford, 1981). Exchange-only systems, like the Na^+–Ca^{2+} exchanger, are especially interesting because they catalyzed stoichiometric translocation of ions without formation and breakdown of covalent bonds. This is a relatively simple system for understanding basic principles of coupling and catalysis (Stein, 1986; Krupka, 1989a,b).

A membrane-bound Na^+–Ca^{2+} exchanger of the heart cell is involved in controlling Ca^{2+} homeostasis and regulation of the contractile force in this organ (Luttgau et al., 1958; Reeves, 1985; Philipson, 1985). Ion transport by the Na^+–Ca^{2+} exchange system exhibits a stoichiometry of three Na^+ per Ca^{2+} and is controlled by a membrane potential (Pitts, 1979; Reeves, & Hale, 1984). Several groups have solubilized and reconstituted an active Na^+–Ca^{2+} exchange protein into liposomes (Miaymoto & Racker, 1980; Soldati et al., 1985; Barzilai & Rahamimoff, 1987; Vemuri & Philipson, 1987; Cheon & Reeves, 1988).

The Na^+–Ca^{2+} exchange is a bireactant reaction, which can be classified basically as either sequential or Ping-Pong (Cleland, 1963). In the Ping-Pong mechanism the first substrate adds and the first product is released before binding of the second substrate. In the sequential mechanism both substrates add before products are released. Both basic mechanisms have subtypes (for example, the sequential mechanism could be ordered, random, etc.). Distinction between the two basic mechanisms is an initial task for the resolution of any unknown mechanism. In the past, the sequential mechanism has been favored over the Ping-Pong mechanism (Blaustein, 1977; Ledvora & Hegyvary, 1983) This conclusion has been disputed by theoretical analysis, suggesting that initial rate measurements did not meet the requirements of the zero-trans reaction (Johnson & Kootsey, 1985; Lauger, 1987). The Ping-Pong mechanism is established for Na^+,K^+-ATPase (Sachs, 1980) and for some other systems (Stein, 1986), and it is thought that the same cation binding

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sites are involved in the transport of Na^+ and K^+ (Shani-Sekler et al., 1988).

Application of classical steady-state kinetic approaches (Cleland, 1963; Stein, 1986) to the Na^+–Ca^{2+} exchange system has been seriously hampered due to the fact that in sarcolemmal and nerve membrane vesicles Ca_i is accumulated, accelerating Na^+–Ca^{2+} exchange (Reeves & Poronnik, 1987; Condrescu et al., 1988). $[Ca]_i$ may also induce Ca^{2+}–Ca^{2+} exchange at low $[Na]_i$. Subtraction of Ca^{2+}–Ca^{2+} exchange is not helpful as this is sensitive to Na^+ (Philipson, 1985; Reeves 1985). In this work an attempt has been made to overcome these problems by measuring the initial rates of the Na_i-dependent Ca uptake in the reconstituted proteoliposomes by use of incorporated Ca chelator. The data are consistent with the Ping-Pong mechanism.

Materials and Methods

Asolectin (L-α-phosphatidylcholine, type II-S, soybean), DNase (DN-25), Triton X-100, and calcium atomic absorption standard were purchased from Sigma. $^{45}CaCl_2$ and [^{14}C]-EDTA-Na_2[1] were obtained from Amersham. [^{14}C]Sucrose was from New England Nuclear. Bio-Beads SM-2 were purchased from Bio-Rad. Arsenazo III was obtained from ICN Pharmaceuticals. Asolectin was precipitated from acetone and stored in chloroform at −20 °C (Hale et al., 1984). The concentration of endogenous calcium in the assay buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose) was decreased with a Tris/Dowex 50W-X8 column. K^+-containing solutions were treated by K/Chelex-100. Bio-Beads SM-2 were treated and stored as described by Holloway (1973).

Sarcolemmal membrane vesicles (SLV) were isolated from calf heart according to Jones (1988) with modifications: (A) Medium I contained 20 mM Tris-HCl, pH 7.4, and 0.7 M NaCl, and medium II was 20 mM Tris-HCl, pH 7.4. (B) After homogenation (3 × 30 s) in a Polytron PT-20, DNase was added (10 μg/mL) and the homogenate stirred at 25 °C for 1 h. (C) After a 17000*g* centrifugation step, the membranes were collected at 180000*g* for 30 min in two Ti-45 rotors. (D) A four-step gradient was carried out in the Ti-45 tubes with 1 M (25 mL), 0.8 M (15 mL), 0.6 M (15 mL), and 0.25 M (15 mL) sucrose at 180000*g* for 2 h. The SLV preparations were stored in liquid nitrogen with 20 mM Tris-HCl, pH 7.4, and 160 mM NaCl. Different preparations of SLV's show Na_i-dependent ^{45}Ca uptake of 1–2 nmol·mg^{-1}·s^{-1} with $[Na]_i$ = 160 mM and $[^{45}Ca]_o$ = 20 μM.

A SLV–Triton X-100 extract was obtained by mixing equal volumes of SLV and extraction buffer to give 1.0–1.1 mg/mL protein, 300 mM NaCl, 15 mg/mL asolectin, and 1.5% Triton X-100. After a 20–30-min incubation at 4 °C the suspension was centrifuged at 250000*g* for 45 min, and 100 mM of EGTA/KOH, pH 7.4 (or EDTA/KOH), was added to the supernatant to give 10 mM EGTA. Triton X-100 was removed by SM-2 Bio-Beads (0.4 g/mL) with gentle agitation for 2 h at 25 °C. This procedure was repeated with fresh SM-2 Bio-Beads. Proteoliposomes were centrifuged at 250000*g* for 1 h and resuspended in 20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 10 mM EGTA (or EDTA); 1–2 mL of this suspension was sonicated for 30 s and stored at 4 °C overnight. Proteoliposomes were diluted in 20 mM Tris-HCl, pH 7.4, and 0.25 M sucrose, centrifuged twice, resuspended in Tris/sucrose, and loaded with NaCl for 4 h at 4 °C. The internal volume of proteoliposomes, estimated by [^{14}C]sucrose (Vemuri & Philipson, 1987), was 3–7 μL/mg of protein.

Table I: Incorporation of [^{14}C]EDTA in the Reconstituted Proteoliposomes[a]

time after dilution (h)	entrapped [^{14}C]EDTA (nmol of [^{14}C]EDTA/mg of protein)	[^{14}C]EDTA remaining (% of control)
0	77.5	100
2	74.2	96
4	72.0	93
18	52.2	67
18 (+ 2% Triton)	4.4	5

[a] [^{14}C]EDTA-entrapped proteoliposomes were obtained as described under Materials and Methods and diluted 50-fold in Tris/KCl buffer (20 mM Tris-HCl, 160 mM KCl) at 4 °C. Aliquots were removed at the indicated times and filtered on GF/C filters. After incubation at 4 °C for 18 h, 10% Triton X-100 was added to yield 2%, incubated at 4 °C for 10 min, and subsequently filtered. 100% control reflects zero time point, obtained immediately after the dilution of [^{14}C]EDTA-entrapped proteoliposomes in Tris/KCl.

Initial rates of the Na_i-dependent ^{45}Ca uptake were measured at 37 °C for 1 s (Reeves, 1988). GF/C filters were presoaked in 0.3% poly(ethylenimine) (Cheon & Reeves, 1988) and prewashed with 5 mL of 20 mM Tris-HCl, pH 7.4, 160 mM KCl, and 0.5 mM EGTA. Unloaded proteoliposomes (no NaCl addition during the loading procedure) plus different $[^{45}Ca]_o$ in the assay medium were used as blanks. Reaction mixture (0.5 mL) contained 20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.1–120 μM added $[^{45}Ca]_a$, and 1–2 μM endogenous $[Ca]_e$. Proteoliposomes (5–10 μL) were applied on the plastic tube (15 × 100 mm), and the reaction was initiated by vortexing. Timing (0.5–30 s) was electronically controlled by a RTB-MP-2N timer (IDEC) automatically injecting 3 mL of cold Tris/KCl/EGTA (with 5 mM EGTA) from the liquid transferer (GME) into the plastic tube. Quenched solutions were filtered and collected vesicles washed with cold Tris/KCl/EGTA (3 × 5 mL) (containing 0.5 mM EGTA). $[Ca]_o$ in the assay medium was plotted as $[Ca]_o = [^{45}Ca]_a + [Ca]_e$. Specific radioactivity for each $[Ca]_o$ in the assay medium was corrected as $[^{45}Ca]_a/([^{45}Ca]_a + [Ca]_e)$. In assay buffers $[Ca]_e$ was measured with ±10% accuracy by using an atomic absorption technique (Tris/sucrose buffer) and an arsenazo III procedure (Tris/sucrose or Tris/KCl buffers) (Bauer, 1981). Protein was measured according to a modified assay of Lowry (Markwell et al., 1978).

Best fit of V_{max} and K_m values and their standard errors, [K_m ($A \pm a$) and V_{max} ($B \pm b$)], were estimated by the Enzfitter (1987) PC-IBM program. The standard error for V_{max}/K_m [$(B/A) \pm c$] was calculated as $c = ([(Ba/A)^2 \pm b^2]/A)^{1/2}$.

Results

[^{14}C]EDTA Entrapment into the Reconstituted Proteoliposomes. The goal was to entrap EGTA (or EDTA) into the reconstituted proteoliposomes in order to measure the "true" initial rates of the Na_i-dependent ^{45}Ca uptake. To achieve this, the Triton–SLV extract was incubated with 10 mM Ca chelator under conditions in which all lipids are solubilized (1.5% Triton and 15 mg/mL lipid). After detergent removal, excess external EGTA was washed out, and EGTA-entrapped proteoliposomes were collected (see Materials and Methods). To test the trapping efficiency of the Ca chelator and its concentration during Na loading, trapping of [^{14}C]EDTA was measured. [^{14}C]EDTA-loaded proteoliposomes were diluted in Tris/KCl buffer at 4 °C, and aliquots were filtered after 0–18 h. Table I shows that initially 77.5 nmol of [^{14}C]-EDTA/mg of protein was incorporated inside the proteoli-

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; arsenazo III, 2,7-bis[(2-arsenophenyl)azo]-1,8-dihydroxynaphthalene-3,6-disulfonic acid; SLV, sarcolemmal membrane vesicles.

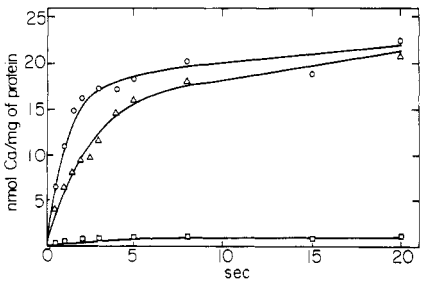
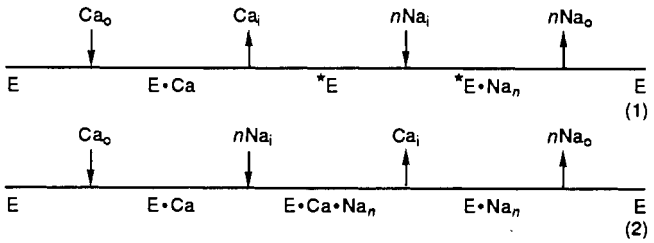


FIGURE 1: Time dependence of Na_i-dependent ^{45}Ca uptake in reconstituted proteoliposomes with or without entrapped EGTA. Proteoliposomes were prepared with (△, □) or without (○) 10 mM EGTA and loaded with (△, ○) or without (□) 40 mM NaCl (see Materials and Methods). The reaction medium contained Tris/sucrose buffer with $[^{45}Ca]_e = 25\ \mu M$ and $[Ca]_e = 2\ \mu M$. The Na_i-dependent ^{45}Ca uptake was quenched at the indicated times, and internalized ^{45}Ca was analyzed (see Materials and Methods). $+EGTA_i + Na_i$ (△); $-EGTA_i + Na_i$ (○); $+EGTA_i - Na_i$ (□).

posomes. If it is assumed that the concentration of entrapped Ca chelator is 10 mM, the internal volume of proteoliposomes can be estimated as 7–8 μL/mg of protein. This value is reasonably close to the internal volume estimated by means of [^{14}C]sucrose (see Materials and Methods), suggesting that there is no significant dilution of entrapped Ca chelator. Table I shows that, after dilution of [^{14}C]EDTA-loaded proteoliposomes in Tris/sucrose buffer at 4 °C for 4 h, ~95% of the initial radioactivity remains on the filter. This suggests that entrapped [^{14}C]EDTA is highly impermeable to the membranes. Triton X-100 at 2% removes ~95% of initial radioactivity (Table I), demonstrating that most of the [^{14}C]EDTA is indeed incorporated inside of the vesicles.

Time Course of Na_i-Dependent Ca Uptake in EGTA-Entrapped Proteoliposomes. In order to test the effect of entrapped Ca chelator on the initial rates, the Na_i-dependent Ca uptake was measured with $[Ca]_o = 27\ \mu M$ and $[Na]_i = 40$ mM in the presence (Figure 1, triangles) or absence (Figure 1, circles) of entrapped EGTA. The reaction was quenched after $t = 0.5$–20 s. As can be seen from Figure 1, EGTA effectively decreases initial rates of ^{45}Ca uptake. EGTA-entrapped proteoliposomes without Na_i (Figure 1, squares) show no significant internalization of Ca^{2+} in the range of 0.5–5 s, suggesting that there is no Ca^{2+}–Ca^{2+} exchange during this period. A slow increase in Ca uptake at higher time points (presumably reflecting slow diffusion of Ca^{2+}) cannot interfere with the signal/background ratio for the initial rate measurements. With $[Ca]_o = 27\ \mu M$ and varying $[Na]_i$ from 20 to 160 mM the entrapped EGTA decreases initial rates from 50% to 20% (not shown). In order to avoid any possible uncertainties, caused by $[Ca]_i$ under varying conditions, EGTA- (or EDTA-) loaded proteoliposomes were used for further measurements of the initial rates ($t = 1$ s).

Estimation of V_{max}, K_m, and V_{max}/K_m for the Na-Dependent Ca Uptake in the EGTA-Entrapped Proteoliposomes. In order to distinguish between the Ping-Pong (eq 1) and sequential (eq 2) mechanisms of cation exchange, it is necessary to analyze the dependence of V_{max}, K_m, and V_{max}/K_m on $[Na]_i$ and $[Ca]_o$.



If we assume that under given experimental conditions the concentration of products of the Na_i-dependent Ca uptake reaction are very low ($[Na]_o \rightarrow 0$ and $[Ca]_i \rightarrow 0$), the general equation for the bireactant exchange can be expressed as (Cleland, 1963; Rudolph & Fromm, 1979; Fromm, 1979; Stein 1986)

$$v = \frac{V_o[Ca]_o[Na]_i^n}{K_{ia}K_b + K_a[Na]_i^n + K_b[Ca]_o + [Na]_i^n[Ca]_o} \quad (3)$$

in which V_o is a maximal rate at saturating concentrations of $[Ca]_o$ and $[Na]_i$. K_a and K_b are Michaelis constants for Ca_o and Na_i (at saturating $[Na]_i$ and $[Ca]_o$, respectively), and K_{ia} is a dissociation constant for the E·Ca complex. In the case of the Ping-Pong mechanism, $K_{ia}K_b = 0$, as no ternary enzyme–substrate complex (E·Na·Ca) is formed. By dividing the denominator and numerator of eq 3 by $K_b + [Na]_i^n$, the rate equation can be rewritten as

$$v = \frac{V_o[Ca]_o/(1 + K_b/[Na]_i^n)}{K_a/(1 + K_b/[Na]_i^n) + [Ca]_o} \quad (4)$$

in which $V_{max} = V_o/(1 + K_b/[Na]_i^n)$ and $K_m = K_a/(1 + K_b/[Na]_i^n)$. For the sequential mechanism ($K_{ia}K_b \neq 0$), eq 3 gives

$$v = \frac{V_o[Ca]_o/(1 + K_b/[Na]_i^n)}{K_a(1 + K_{ia}K_b/K_a[Na]_i^n)/(1 + K_b/[Na]_i^n) + [Ca]_o} \quad (5)$$

in which the observed values are $V_{max} = V_o/(1 + K_b/[Na]_i^n)$ and $K_m = K_a(1 + K_{ia}K_b/K_a[Na]_i^n)/(1 + K_b/[Na]_i^n)$. This analysis shows that with increasing $[Na]_i$ the V_{max} increases by the same extent for both mechanisms (eqs 4 and 5). The $[Na]_i$ dependence of K_m and V_{max}/K_m, however, is different. For the Ping-Pong mechanism K_m can only increase, while for the sequential mechanism it may increase ($K_{ia} < K_a$), decrease ($K_{ia} > K_a$), or remain unchanged ($K_{ia} = K_a$). The most important point for distinguishing between the two mechanisms is the fact that for the Ping-Pong mechanism the ratio of $V_{max}/K_m = V_o/K_a$ is $[Na]_i$ independent, while in the case of the sequential mechanism $V_{max}/K_m = V_o/K_a(1 + K_{ia}K_b/K_a \cdot [Na]_i^n)$ is $[Na]_i$ dependent. Thus for the Ping-Pong mechanism, V_{max} and K_m increase proportionally with increasing $[Na]_i$, while for the sequential mechanism all other possibilities can be realized.

The initial rates of the Na_i-dependent ^{45}Ca uptake were measured in the EGTA-entrapped proteoliposomes at four fixed concentrations of $[Na]_i$ (Figure 2), 10 (A), 20 (B), 40 (C), and 160 mM (D). At each fixed $[Na]_i$ the $[^{45}Ca]_o$ was varied from 2.5 to 122 μM (Figure 2). The observed values for K_m and V_{max} increased from 7.7 ± 0.8 to 33.5 ± 5.8 μM and from 2.3 ± 0.06 to 9.0 ± 0.6 nmol·mg^{-1}·s^{-1}, respectively. At the four fixed $[Na]_i$, the V_{max}/K_m ratios show very similar values of 0.299 ± 0.032, 0.270 ± 0.039, 0.257 ± 0.036, and 0.269 ± 0.05 nmol·mg^{-1}·s^{-1}·μM^{-1}. Likewise, the observed V_{max}/K_m values are not significantly different from the average value of 0.274 nmol·mg^{-1}·s^{-1}·μM^{-1}, deviating by only ±2–10%. These deviations of the V_{max}/K_m values are within the standard error for the experimental measure of V_{max} (±3–7%), K_m (±11–17%), and V_{max}/K_m (±11–19%). Thus under conditions in which the V_{max} and K_m vary 4–5-fold, the V_{max}/K_m is constant within the experimental error. Similar relationship between the $[Na]_i$ and V_{max}/K_m is observed, when, instead of EGTA, EDTA is incorporated in the reconstituted proteoliposomes (not shown). V_{max}/K_m was $[Na]_i$ independent in nine different preparations of EGTA- or EDTA–proteoliposomes,

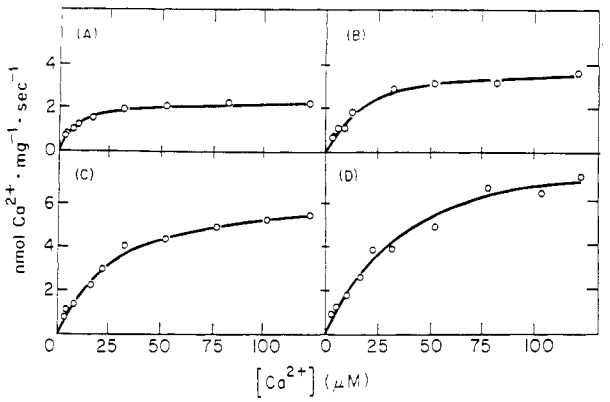


FIGURE 2: Assay of V_{max}, K_m, and V_{max}/K_m values for Na_i-dependent ^{45}Ca uptake in EGTA-entrapped proteoliposomes at fixed $[Na]_i$ and varying $[Ca]_o$. EGTA-entrapped proteoliposomes were loaded with different $[Na]_i$, 10 (A), 20 (B), 40 (C), and 160 mM (D) (see Materials and Methods). Initial rates (t = 1 s) of the Na_i-dependent ^{45}Ca uptake were measured in Tris/sucrose medium with $[^{45}Ca]_a$ = 0.5–120 μM and $[Ca]_e$ = 2 μM. $[Ca]_o$ in the assay medium was plotted as $[Ca]_o = [^{45}Ca]_a + [Ca]_e$, and the specific radioactivity for each $[Ca]_o$ was corrected as $[^{45}Ca]_a/([^{45}Ca]_a + [Ca]_e)$. The lines were calculated according to $v = V_{max}[Ca]_o/(K_m + [Ca]_o)$ to give a maximal fit of K_m and V_{max} to the experimental points (Enzfitter PC-IBM program). The K_m (μM) values were 7.7 ± 0.8 (A), 14.8 ± 2.1 (B), 26.1 ± 3.2 (C), and 33.5 ± 5.8 (D). The V_{max} (nmol·mg^{-1}·s^{-1}) values were 2.3 ± 0.1 (A), 4.0 ± 0.2 (B), 6.7 ± 0.3 (C), and 9.0 ± 0.6 (D).

Table II: V_{max}/K_m of Na_i-Dependent ^{45}Ca Uptake in the Valinomycin/KCl Medium, at Two Fixed $[Na]_i$[a]

$[Na]_i$ (mM)	V_{max} (nmol·mg^{-1}·s^{-1})	K_m (μM)	V_{max}/K_m (nmol·mg^{-1}·s^{-1}·μM^{-1})
20	10.7 ± 0.7	12.6 ± 2.3	0.85 ± 0.17
160	45.2 ± 3.4	41.9 ± 6.5	1.08 ± 0.18

[a] The EGTA-entrapped proteoliposomes with $[Na]_i$ = 20 or 160 mM were prepared as described in Figure 1. Before the experiment the proteoliposomes were treated with 2 μM valinomycin and diluted in the assay medium with 20 mM Tris-HCl, pH 7.4, 160 mM KCl, $[Ca]_e$ = 1 μM, and $[^{45}Ca]_e$ = 2–120 μM. The Na_i-dependent ^{45}Ca uptake was measured as described under Materials and Methods.

obtained from the three preparations of SLV.

Because of the electrogenic nature of the Na^+–Ca^{2+} exchange and the low ion conductivity, a large membrane potential will build up, tending to reduce the velocity of the exchange reaction. In order to test the $[Na]_i$ dependence of V_{max}/K_m under the conditions of "short circuit" of the membrane potential, the experiment was done in the presence of K^+/valinomycin. Under these conditions the V_{max}/K_m values were 0.85 ± 0.17 and 1.08 ± 0.18 nmol·mg^{-1}·s^{-1}·μM^{-1} at $[Na]_i$ = 20 and 160 mM, respectively (Table II). These values are somewhat higher when compared to the values obtained in the Tris/sucrose medium; however, V_{max}/K_m is $[Na]_i$ independent within the experimental error under these conditions too.

Initial Rates of Na_i-Dependent Ca Uptake at Fixed $[Ca]_o$ and Varying $[Na]_i$. By inspection of eq 3 it can be seen that at a very low $[Ca]_o$ and $K_{ia}K_b = 0$ (the Ping-Pong mechanism) the rate equation can be presented as

$$v = V_o[Ca]_o/K_a \quad (6)$$

That means that when the $[Ca]_o \ll K_a$ and the Na_i-dependent Ca uptake is still detectable, the observed initial rates must be independent of the $[Na]_i$. For the sequential mechanism at a very low $[Ca]_o$ and $K_{ia}K_b \neq 0$, eq 3 can be rewritten as

$$v = \frac{V_o[Ca]_o[Na]_i^n}{K_{ia}K_b + K_a[Na]_i^n} \quad (7)$$

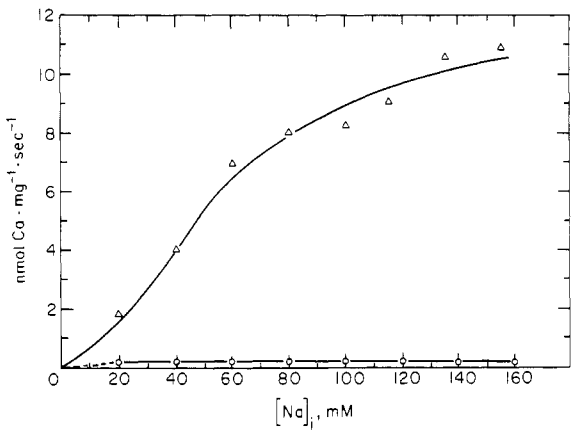


FIGURE 3: Initial rates of Na_i-dependent ^{45}Ca uptake in EGTA-entrapped proteoliposomes at fixed $[Ca]_o$ and varying $[Na]_i$. EGTA-entrapped proteoliposomes were loaded with 10–160 mM $[Na]_i$ (see Materials and Methods). The reaction medium contained Tris/sucrose buffer with $[^{45}Ca]_a$ = 0.1 (○) or 91 μM (△) plus $[Ca]_e$ = 1 μM. The line for $[Ca]_o$ = 92 (△) was calculated according to $v = V_{max} \cdot [Na]_i^n/(K_m + [Na]_i^n)$, showing a best fit with V_{max} = 13.8 nmol·mg^{-1}·s^{-1}, K_m^{Na} = 21 mM, and n_H = 1.5.

In order to test these possibilities, Ca uptake was measured at a fixed $[^{45}Ca]_o$ = 1.1 μM and varying $[Na]_i$ = 10–160 mM. Figure 3 (circles) shows that the initial rates remain at the same level as predicted by the Ping-Pong mechanism. In this experiment the signal/background ratio was 8–9 (5000–6000 cpm/600–700 cpm) with the standard error of ±10–15%, suggesting that any changes in the rates (expected according to the sequential mechanism) should have been detectable.

At a fixed concentration with a high $[^{45}Ca]_o$ = 92 μM and varying $[Na]_i$ from 10 to 160 mM, the initial rates of the Na_i-dependent Ca uptake show a sigmoidal dependence on the $[Na]_i$ (Figure 3, triangles). The V_{max} is 13.8 ± 3.15 nmol·mg^{-1}·s^{-1}, K_m^{Na} is 21.2 ± 3.36 mM, and n_H = 1.5 ± 0.42. Similar kinetic parameters have been observed in SLV (Kadome et al., 1982; Philipson, 1985; Reeves, 1985) indicating that the Na^+–Ca^{2+} exchange system, reconstituted under our experimental conditions, still has common properties with the native transporter. At the fixed concentration with a high $[Ca]_o$ and varying $[Na]_i$ the rate equation for the Ping-Pong mechanism can be described as

$$v = \frac{V_o[Na]_i^n/(1 + K_a/[Ca]_o)}{K_b/(1 + K_a/[Ca]_o) + [Na]_i^n} \quad (8)$$

in which $V_{max} = V_o/(1 + K_a/[Ca]_o)$ and $K_m^{Na} = K_b/(1 + K_a/[Ca]_o)$. Under the same conditions with a high $[Ca]_o$ and varying $[Na]_i$ a similar rate equation with apparent $V_{max} = V_o/(1 + K_a/[Ca]_o)$ and $K_m^{Na} = K_b(1 + K_{ia}K_a/K_b[Ca]_o)/(1 + K_a/[Ca]_o)$ can be written for the sequential mechanism. In this case it is impossible to distinguish between the two basic mechanisms, because initial rates are $[Na]_i$ dependent in both cases.

Discussion

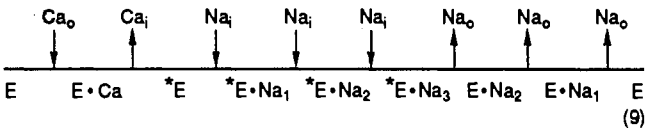
In this work Triton X-100 extraction–reconstitution techniques (Soldati et al., 1985; Vemuri & Philipson, 1987; Cheon & Reeves, 1988) were adapted to entrap a Ca chelator inside of proteoliposomes (Table I). In these preparations the initial rates of the Na_i-dependent Ca uptake were effectively decreased by entrapped EGTA (Figure 1), suggesting that the entrapped Ca chelator (10 mM) may decrease the free concentration of Ca_i.

Previously, proposals have been made in favor of the sequential mechanism rather than the Ping-Pong mechanism (Blaustein, 1977; Ledvora & Hegyvary, 1983), suggesting that K_m for Ca^{2+} is constant over the whole range of sodium concentrations. The present analysis shows that K_m is $[Na]_i$ dependent for both basic mechanisms [eqs 3–5; see also Lauger (1987) and Johnson and Kootsey (1985)]. $[Na]_i$-independent K_m is an extreme case, in which $K_{ia} = K_a$ (eq 5). Straightforward experimental distinction between the simultaneous (sequential) and Ping-Pong (consecutive) mechanisms is possible by studying the concentration dependence of fluxes under zero-trans conditions ($[Ca]_i = [Na]_o = 0$) [Lauger, 1987; for review, see Stein (1986)]. The experimental conditions used in the present work meet this demand ($[Ca]_i \rightarrow 0$ and $[Na]_o \rightarrow 0$). There are also some experimental conditions that were ignored in previous works. For example, in this study $[Ca]_e$ in assay buffers were reduced to 1–2 μM (without preliminary purification of Tris/sucrose or Tris/KCl buffers, $[Ca]_e$ = 12–17 μM), and the diluted specific radioactivity of added ^{45}Ca was carefully estimated (see Materials and Methods). Any disregard of these corrections underestimates the ^{45}Ca uptake readings (especially at low $[^{45}Ca]_a/([^{45}Ca]_a + [Ca]_e)$ ratios, when $[^{45}Ca]_a < [Ca]_e$) and overestimate the K_m values. Such a mistake is not proportional over the whole range of $[Na]_i$ (it is bigger for the lower K_m values rather than for the higher K_m values).

The most striking observation in the presented work is that, over the range of fixed $[Na]_i$ = 10–160 mM, V_{max} and K_m values for the Na_i-dependent ^{45}Ca uptake vary 4–5-fold, while the V_{max}/K_m values are constant within the experimental error (Figure 2). Likewise, under conditions in which an adequate charge compensating mechanism is provided (K^+/valinomycin assay medium) the V_{max}/K_m values are independent of the $[Na]_i$ (Table II). All these data support the Ping-Pong mechanism. Another piece of evidence is that at a very low concentration of fixed $[Ca]_o \ll K_m$ and varying $[Na]_i$ the initial rates of the Na_i-dependent ^{45}Ca uptake are independent of the $[Na]_i$ (eq 6 and Figure 3, circles). By contrast, sequential mechanisms predict that V_{max}/K_m at varing $[Ca]_o$, and initial rates at very low fixed $[Ca]_o$, are $[Na]_i$ dependent (eqs 5 and 7).

The steady-state approach has limitations in distinguishing between the two basic mechanisms. For example, if $K_{ia}K_b$ is very low, as compared to the $K_a[Na]_i^n + K_b[Ca]_o + [Na]_i^n \cdot [Ca]_o$ term (eqs 3 and 4), the sequential mechanism reduces to a form of the "pseudo-Ping-Pong" mechanism. Since the value of $K_b = K_m^{Na}$ = 25–40 mM [eq 8, and triangles in Figure 3; see also Kadoma et al. (1982), Reeves (1985), and Philipson (1985)], one can calculate that an upper limit for K_{ia} would have to be $<10^{-7}$–10^{-8} M in order to fit a hypothetical pseudo-Ping-Pong mechanism. Under fixed conditions the experimental resolution between the two basic mechanisms depends mainly on the K_{ia} value. If $K_{ia} < 3 \times 10^{-7}$ M for the sequential mechanism, it would be impossible to detect $[Na]_i$-dependent changes in V_{max}/K_m within the experimental error of ±10–20%. However, for higher values of K_{ia} the $[Na]_i$-dependent changes in V_{max}/K_m should have been detectable under the given experimental conditions. For example, a 2-fold increase in V_{max}/K_m is expected to be observed by varying $[Na]_i$ from 10 to 160 mM, if $K_{ia} \sim 4 \times 10^{-6}$ M. The $[Na]_i$-dependent V_{max}/K_m can be increased 10-fold (or even more) if $K_{ia} \sim 4 \times 10^{-5}$ M.

The simplest Ping-Pong mechanism (Uni-Uni Ter-Ter Ping-Pong) can be represented as



(9)

The Uni-Uni Ter-Ter Ping-Pong mechanism is not the only mechanism that can be proposed for the Na^+–Ca^{2+} exchange system. This stems from the fact that different Ping-Pong mechanisms can be written for a four-substrate ($Ca_o + 3Na_i$) and four-product ($Ca_i + 3Na_o$) reaction (Cleland, 1963; Rudolph & Fromm, 1979; Fromm, 1979) with similar (or identical) rate equations. These mechanisms cannot be easily distinguished by the steady-state approach.

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Articles

Dynamic Nature of the Quaternary Structure of the Vesicular Stomatitis Virus Envelope Glycoprotein†

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ABSTRACT: The envelope glycoprotein (G protein) of vesicular stomatitis virus probably exists in the viral envelope as a trimer of identical subunits. Depending on the conditions of solubilization, G protein may dissociate into monomers. G protein solubilized with the detergent octyl glucoside was shown to exist as oligomeric forms by sedimentation velocity analysis and chemical cross-linking. G protein was modified with either fluorescein isothiocyanate or rhodamine isothiocyanate. Resonance energy transfer between fluorescein and rhodamine labels was observed upon mixing the two labeled G proteins in octyl glucoside. This result provided further evidence that G protein in octyl glucoside is oligomeric and indicated that the subunits are capable of exchange to form mixed oligomers. Resonance energy transfer was independent of G protein concentration in the range examined (10–80 nM) and was not observed when labeled G proteins were mixed with fluorescein or rhodamine that was not conjugated to protein. Resonance energy transfer decreased upon incorporation of G protein into Triton X-100, consistent with sedimentation velocity data that G protein in Triton X-100 is primarily monomeric. Kinetic analysis showed that the subunit exchange reaction had a half-time of about 3 min at 27 °C that was independent of G protein concentration. These data indicate that the exchange occurs through dissociation of G protein trimers into monomers and dimers followed by reassociation into trimers. Thus, in octyl glucoside, G protein must exist as an equilibrium between monomers and oligomers. This implies that monomers are capable of self-assembly into trimers.

Many viral and cellular plasma membrane proteins consist of assemblies of polypeptide subunits; that is, they display some type of quaternary structure (Carlin & Merlie, 1986). Some surface proteins are composed of two or more different polypeptide chains, i.e., are heterooligomers. Examples include B cell surface immunoglobulins and T cell antigen receptors, class I and class II major histocompatibility antigens (MHC antigens),[1] and the acetylcholine receptor. Other membrane proteins consist of multiple copies of a single polypeptide. For example, the influenza virus hemagglutinin is synthesized as a single polypeptide precursor, which self-associates into a trimer of identical subunits (Wilson et al., 1981). Similarly, the influenza virus neuraminidase consists of a tetramer of identical subunits (Varghese et al., 1983). In some cases, such as surface immunoglobulins, the subunits are held together by disulfide bonds, while in other cases, such as MHC antigens, the subunit interactions are noncovalent.

Determination of the subunit structure of a membrane protein usually requires solubilization of the protein from the membrane either by detergent treatment or by proteolytic cleavage of the membrane-anchor domain. Such treatments may alter the subunit interactions, especially if they are noncovalent in nature. The vesicular stomatitis virus surface glycoprotein (G protein) was originally reported to be a monomer following solubilization with detergent or proteolytic cleavage of the membrane-anchor sequence (Crimmins et al., 1983). However, other evidence has suggested that G protein is oligomeric, most likely a trimer of identical subunits (Dubovi & Wagner, 1977; Kreis & Lodish, 1986; Doms et al., 1987).

Assembly of membrane protein subunits is thought to occur at the site of synthesis in the endoplasmic reticulum, and in several cases, it has been shown that acquisition of the correct quaternary structure is a regulatory step required for transport to Golgi membranes [reviewed by Rose and Doms (1988)]. Subunit assembly is usually a posttranslational event, and most polypeptides appear to exist as monomers in the endoplasmic reticulum membrane for a period of time following synthesis prior to interaction with other subunits. In the case of the

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[1] Abbreviations: DSS, disuccinimidyl suberate; G protein, envelope glycoprotein of VSV; MHC antigens, major histocompatibility antigens; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; VSV, vesicular stomatitis virus.

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