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# COMPARISON OF KINETIC CHARACTERISTICS OF $Na^+$-$Ca^{2+}$ EXCHANGE IN SARCOLEMMA VESICLES AND CULTURED CELLS FROM CHICK HEART

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**The kinetic characteristics of $Na^+$-$Ca^{2+}$ exchange in isolated sarcolemma vesicles from new-borne chick heart, which contain about 70% of right-side-out vesicles, were compared with those of cultured embryonic chick heart cells. $Na^+$-$Ca^{2+}$ exchange was monitored as $Na_i$-dependent $Ca^{2+}$ uptake. Increase in the internal concentration of $Na^+$ ($[Na^+]_i$) in these two preparations caused increase in both the initial rate and the saturation-level of $Ca^{2+}$ uptake. Plots of the rate of $Ca^{2+}$ uptake against $[Na^+]_i$ showed similar saturation-kinetics in these two preparations. The apparent Michaelis constant ($K_m$) (0.35 mM) for $Ca^{2+}$ uptake by the intact cells was much higher than that (0.031 mM) for $Ca^{2+}$ uptake by the vesicles. The degree of inhibition by $Mg^{2+}$ was also higher in the cells than in the vesicles. Some possible reasons (age of the chicks used, membrane potential, etc.), for these differences were examined and are discussed.**

## Introduction

Calcium is an important regulator of contraction of cardiac muscle. During excitation, calcium enters the cell by a slow inward current. It is obvious that the same amount of calcium as that gained by the cells must be again extruded before the next excitation. Reuter et al. [1,2] first demonstrated a $Na^+$-$Ca^{2+}$ exchange mechanism in sarcolemma membranes and suggested that this system is important in determining the intracellular concentration of free $Ca^{2+}$ [3].

We have developed a technique for measuring $Na^+$-$Ca^{2+}$ exchange in cultured myocardial cells [4–6]. In the previous paper, we reported several kinetic properties of $Na^+$-$Ca^{2+}$ exchange in cultured mouse myocardial cells [5] including the stoichiometry (three $Na^+$ for one $Ca^{2+}$), the $K_m$ value (0.1 mM) and the affinity sequence of divalent cations ($Co^{2+} > Sr^{2+} > Mg^{2+}$). Single isolated myocardial cells obtained from mammalian or chicken heart are known to beat spontaneously in culture [7–9]. Thus, cultured myocardial cells contain functionally intact sarcolemma membranes. Moreover, cultured myocardial cells are separated anatomically and functionally from nerves, connective tissue and blood vessels, and are in direct contact with the extracellular medium. Therefore, their use could greatly simplify studies on $Na^+$-$Ca^{2+}$ exchange.

Reeves and Sutko [10] recently developed another simple experimental system, a technique for measuring $Na^+$-$Ca^{2+}$ exchange in cardiac sarcolemma vesicles. Using this simple system, several groups have recently reported some important characteristics of $Na^+$-$Ca^{2+}$ exchange: the $Na^+$-$Ca^{2+}$ exchange system mediates the exchange of three $Na^+$ for one $Ca^{2+}$ [11], generates a electric current [12] and is affected by the membrane potential [13]. These findings were obtained using extensively homogenized membrane preparations, which may not be comparable to intact cells. Therefore, it is very important to compare the characteristics of $Na^+$-$Ca^{2+}$ exchange in sarcolemma vesicles with those in intact cells.

In the present study, we compare several basic properties of $Na^+$-$Ca^{2+}$ exchange in sarcolemma

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

vesicles from chick heart with those in cultured chick heart cells. We found a large difference between the apparent Michaelis constants ($K_m$) for $Ca^{2+}$ of the two types of preparation.

## Methods

*Materials.* Materials were obtained from the following sources: fetal bovine serum from Flow Laboratories; 4-morpholine-propanesulfonic acid (Mops) (one of the Good's buffers) and glycol ether-diaminetetraacetic acid (EGTA) from Dojindo Co.; ouabain and valinomycin from Sigma Chemical Co.; nigericin from Nippon Roche Co.; $^{45}CaCl_2$ and [$^3$H]-ouabain from New England Nuclear; $^{24}$NaCl from Japan Radioisotope Association.

*Preparation of sarcolemma vesicles.* Sarcolemma vesicles were isolated from 1-day-old chick heart by a method similar to that described previously [10,11]. Hearts were removed from the chicks and homogenized in a solution of 0.6 M sucrose, 10 mM imidazole/HCl (pH 7.0) for 20 s in an Ultra Turrax Tissuemizer (IKA-Werk). The homogenate was centrifuged at 12 000 $\times g$ for 30 min and the supernatant was diluted with 1.5 Vol. of a solution of 140 mM KCl, 20 mM Mops/Tris, 1 mM EGTA (pH 7.4) and centrifuged at 70 000 $\times g$ for 30 min. The pellet was suspended in a solution of 140 mM KCl, 20 mM Mops/Tris (pH 7.4), layered over 30% (w/v) sucrose solution buffered with 10 mM imidazole/HCl (pH 8.3) and centrifuged at 220 000 $\times g$ (max) for 150 min. The band at the sample-sucrose interface was collected and diluted with 3 vol. of a solution of 140 mM KCl, 20 mM Mops/Tris(pH 7.4) and then centrifuged at 80 000 $\times g$ for 30 min. The pellet was resuspended in a solution of 0 to 140 mM NaCl (substituted for 0 to 140 mM KCl), 20 mM Mops/Tris (pH 7.4) at a final protein concentration of at least 2 mg/ml. The purified vesicles exhibited high activities of sarcolemma markers ($K^+$-stimulated *p*-nitrophenylphosphatase, 7.5 $\mu$mol/mg per h; ($Na^+ + K^+$)-ATPase, 27.5 $\mu$mol/mg per h; sialic acid, 120 nmol/mg). These values for the purified sarcolemmal vesicles were about 6-times those of the initial homogenate. Contaminations with mitochondrial markers (succinate dehydrogenase and azide-sensitive ATPase) and a sarcoplasmic reticulum marker (($Ca^{2+} + Mg^{2+}$)-ATPase) were slight. After purification, sarcolemma vesicles maintained high $Na^+$-$Ca^{2+}$ exchange activity for at least 5 h during experiments on $Ca^{2+}$ uptake.

*Culture of chick heart cells.* Hearts were removed from 6-day-old chick embryos and dissociated into single isolated cells by trypsinization as described previously [14]. Cells were seeded into 35- or 60-mm diameter Petri dishes (Falcon) at a density of $2 \cdot 10^5$ to $4 \cdot 10^6$ cells per dish at 37°C in modified Eagle's minimum essential medium (containing 1 mM KCl instead of the 5.5 mM KCl present in normal Eagle's medium) supplemented with 8% fetal bovine serum under a water-saturated atmosphere of 5% $CO_2$ in air. On incubation for 10 h, the single heart cells became attached to the surface of the Petri dishes, and about 60% of these single cells beat spontaneously.

*Pre-loading of vesicles or cells with $Na^+$.* (1) vesicles. For the measurement of $^{24}$Na uptake shown in Fig. 1(a), 190 $\mu$l of reaction medium containing 37 to 147 mM NaCl (substituted for 0 to 103 mM KCl), $^{24}$Na (20 $\mu$Ci/ml) and 20 mM Mops/Tris (pH 7.4) was added to 10 $\mu$l of vesicle suspension in 140 mM KCl, 20 mM Mops/Tris (pH 7.4). After incubation for 15 s to 50 min, the reaction was stopped by adding 5 ml of a cold (0°C) solution of 280 mM sucrose, 5 mM $CoCl_2$ and 5 mM Mops/Tris (pH 7.4) (stopping solution). The mixture was filtered through a Millipore filter (pore size: 0.45 $\mu$m) and the filter was washed four times with 5 ml of the stopping solution at 25°C by filtration. Then the $^{24}$Na-radioactivity on the filter was measured in a well-type gamma spectrometer. As shown in Fig. 1(a), during incubation the intravesicular $^{24}$Na activity reached a steady level within 50 min, and the value at 50 min was exactly proportional to the extravesicular concentration of $Na^+$ ($[Na^+]_o$). Moreover, the $^{24}$Na activity at 50 min was not changed by further incubation for 5 min in the presence of 5 $\mu$M nigericin. These results show that the concentration of $Na^+$ in the vesicles became the same as that in the extravesicular medium on incubation for 50 min. Thus, vesicles loaded with various concentrations of $Na^+$ were easily prepared by incubating them for 50 min at 37°C in medium containing various concentrations of $Na^+$.

(2) Cells. For pre-loading of cultured cells with $Na^+$, the culture medium was discarded and the cells were washed three times with 2.5 ml of medium containing 0 to 140 mM NaCl (substituted for 0 to

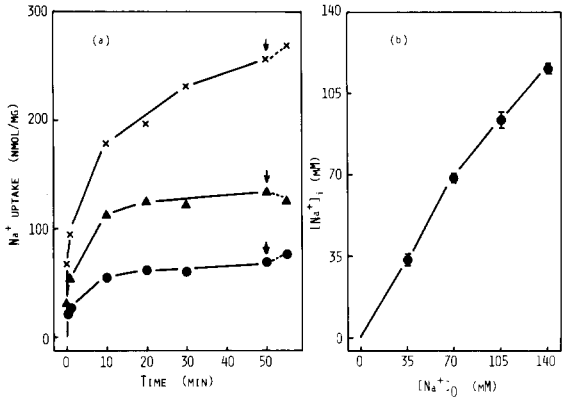


Fig. 1. (a). Pre-loading of sarcolemma vesicles with $Na^+$. $^{24}Na$ uptake was performed in medium containing 35 mM (●), 70 mM (▲) or 140 mM NaCl (X) at 37°C as described in Methods. Nigericin (5 μM) was added at the time shown by the arrows. (b). Pre-loading of cultured myocardial cells with $Na^+$. After incubation in $Ca^{2+}$-free medium containing 0 to 140 mM $Na^+$ (in stead of 0 to 140 mM choline$^+$) at 25°C for 10 min, the intracellular concentration of $Na^+$ ($[Na^+]_i$) was measured as described in Methods. Points and bars are means ±S.D. for three independent experiments.

140 mM choline chloride), 20 mM Mops/Tris, 0.1 mM EGTA (pH 7.4) and incubated in the same medium at 25°C for 10 min. For the results shown in Fig. 1(b), after incubation at 25°C for 10 min, the intracellular concentration of $Na^+$ ($[Na^+]_i$) was measured with a flame photometer as described in detail previously [5]. As shown in Fig. 1(b), $[Na^+]_i$ increased in parallel with increase in $[Na^+]_o$. When cultured cells were incubated in $Ca^{2+}$-free medium containing 140 mM KCl or 140 mM choline chloride, $[Na^+]_i$ became almost zero. Thus, cells loaded with various concentrations of $Na^+$ were easily prepared by incubating them for 10 min at 25°C in $Ca^{2+}$-free medium containing various concentrations of $Na^+$.

*Measurement of $Na^+$-$Ca^{2+}$ exchange.*

(1) Vesicles: $Na^+$-$Ca^{2+}$ exchange in sarcolemmal vesicles was measured at 25°C as described previously [10,11]. The reaction medium (190 μl), containing 140 mM KCl, 20 mM Mops/Tris, $^{45}Ca$ (0.5 μCi/ml) and 25 μM to 3200 μM $Ca^{2+}$, was added to 10 μl of vesicles loaded with 0 to 140 mM NaCl (usually 0 and 140 mM). At appropriate times, $^{45}Ca$ uptake by the vesicles was stopped by adding 5 ml of the cold (0°C) stopping solution described above. Then the total mixture was filtered through a Millipore filter (pore size: 0.45 μm) and the filter was washed four times with 5 ml of the stopping solution at 25°C by filtration. The filters were then dried and the radioactivity of $^{45}Ca$ was measured in a liquid scintillation spectrometer (Aloka). Vesicles loaded with 140 mM KCl exhibited low $^{45}Ca$ uptake. Intravesicular $Na^+$ ($Na_i$)-dependent $^{45}Ca$ uptake was estimated as the difference between the values of $^{45}Ca$ uptake by NaCl-loaded vesicles and KCl-loaded vesicles.

(2) Cells. $Na^+$-$Ca^{2+}$ exchange in monolayer cultured cells was measured at 25°C as described previously [4–6]: After pre-loading the cells with $Na^+$, the pre-incubation medium was rapidly replaced by 1 to 2 ml of reaction medium containing $^{45}Ca$ (2 μCi/ml) and 50 μM to 5000 μM $Ca^{2+}$. At appropriate times, the reaction medium was discarded and the cells were washed four times with 2.5 ml of the stopping solution described above. Then the cells were suspended in 0.7 ml of 0.1 M NaOH solution and the $^{45}Ca$ in an aliquot of the suspension was measured. Almost all cells adhered to the bottom of the Petri dish throughout the experiments. Intracellular $Na^+$($Na_i$)-dependent $^{45}Ca$ uptake was estimated as the difference between the values for $^{45}Ca$ uptake by cells with and without $Na^+$-loading.

*Experiments on sidedness of vesicles.*

(1) ATP-dependent $^{24}Na$ uptake. To determine whether our vesicle preparation contained inside-out vesicles, we measured $^{24}Na$ uptake in the presence and absence of ATP. Reaction medium (190 μl) containing 10 mM $^{24}NaCl$, 5 mM $MgCl_2$, 130 mM choline chloride, 0.1 mM EGTA and 20 mM Mops/Tris, pH 7.4, ± 2 mM ATP was added to 10 μl of 140 mM KCl-loaded vesicles at 37°C. The reaction was stopped, the mixture was filtered and $^{24}Na$-radioactivity on the filters was measured as described above.

(2) Accessibility of acetylcholine esterase. Before enzyme assay, KCl-loaded vesicles were treated with 0.1% Triton X-100 at room temperature for 5 min. The activity of acetylcholine esterase in untreated vesicles and those treated with Triton X-100 was measured by the method of Ellman et al. [15].

(3) Accessibility of ouabain-binding sites. Before binding assay, NaCl-loaded vesicles were treated with 0.1% Triton X-100 at room temperature for 5 min. Untreated vesicles and those treated with Triton X-100 (40 μl) were added to 160 μl of a medium

containing 140 mM NaCl, 5 mM $MgCl_2$, 20 mM Mops/Tris, 200 nM–0.1 mM ouabain and 10 $\mu$Ci/ml [$^3$H]ouabain, pH 7.4, ±3 mM ATP, and incubated at 37°C for 10 min. Then 5 ml of a solution of 140 mM NaCl, 20 mM Mops/Tris (pH 7.4) was added, the mixture was filtered, and the filter was washed twice with 5 ml of the same medium, and its $^3$H-radioactivity was counted. Specific ouabain binding was monitored as increase in binding in the presence of ATP.

*Other measurements.* $K^+$-Stimulated *p*-nitrophenylphosphatase assay was performed as described previously [16]. The amount of total sialic acid released by acid-hydrolysis (0.05 M $H_2SO_4$, 80°C, 1 h) was measured directly as described previously [17]. ($Na^+ + K^+$)-ATPase assay was performed by spectrophotometric measurement coupled with pyruvate kinase. Protein was measured by the method of Lowry et al. [18].

## Results and Discussion

For the results shown in Fig. 2(a), vesicles loaded with various concentrations of $Na^+$ were incubated in medium containing $^{45}$Ca and 50 $\mu$M $Ca^{2+}$. $Ca^{2+}$ uptake increased linearly during the first 30 s and reached a saturation-level in 1 min or more. $Ca^{2+}$ uptake depended on the intravesicular concentration of $Na^+$ ($[Na^+]_i$): increase in $[Na^+]_i$ caused marked increase in both the initial rate and the saturation-level of $Ca^{2+}$ uptake by the vesicles. A similar time course of $Ca^{2+}$ uptake was observed in cultured chick myocardial cells (Fig. 2(b)): increase in $[Na^+]_i$ caused marked increase in both the initial rate and the saturation level of $Ca^{2+}$ uptake by the cells. These observations show that these two types of preparation exhibit $Na_i$-dependent $Ca^{2+}$ uptake. Plots of the rates of $Ca^{2+}$ uptake against $[Na^+]_i$ for these two preparations showed similar saturation-kinetics (no figure). The $[Na^+]_i$ for the half maximal initial rate of $Ca^{2+}$ uptake was 25 to 50 mM.

Fig. 3 shows Lineweaver-Burk plots of the initial rate of $Na_i$-dependent $Ca^{2+}$ uptake by the vesicles and cells against the external concentration of $Ca^{2+}$ ($[Ca^{2+}]_o$). The data fitted linear regression lines ($r > 0.95$) and showed saturation kinetics. These

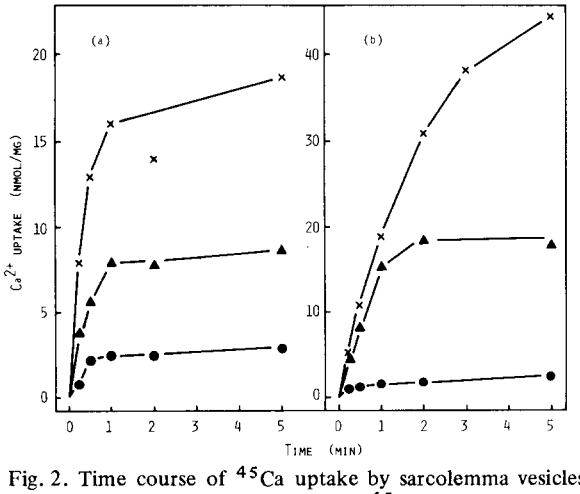


Fig. 2. Time course of $^{45}$Ca uptake by sarcolemma vesicles (a) and cultured myocardial cells (b). $^{45}$Ca uptake in (a) and (b) was studied in 140 mM KCl, 20 mM Mops/Tris, 50 $\mu$M $CaCl_2$ ($^{45}$Ca), pH 7.4, at 25°C using vesicles loaded with 0 (•), 35 (▲) or 140 mM $Na^+$ (X) and cells loaded with 0 (•), 34 (▲), or 113 mM $Na^+$ (X), respectively.

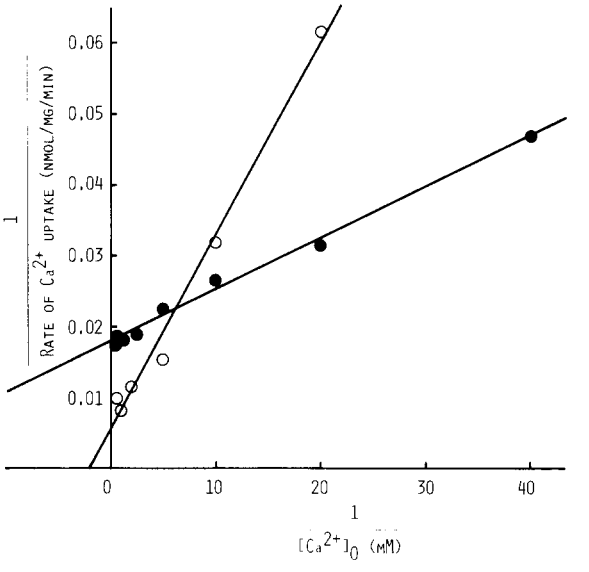


Fig. 3. Lineweaver-Burk plots of the initial rate of $Na_i$-dependent $Ca^{2+}$ uptake (in the first 15 s, 25°C) by sarcolemma vesicles (•) loaded with 140 mM $Na^+$ and cultured myocardial cells (○) loaded with 113 mM $Na^+$ against the external concentration of $Ca^{2+}$ ($[Ca^{2+}]_o$). The straight lines for vesicles and cells were obtained by linear regression ($r$ 0.997 and 0.994, respectively). The $K_m$ and $V$ values for the vesicles were estimated to be 40 $\mu$M and 56 nmol/mg per min, respectively. The $K_m$ and $V$ values for the cells were estimated to be 0.48 mM and 175 nmol/mg per min, respectively.

results suggest that the $Na_i$-dependent $Ca^{2+}$ uptakes by the vesicles and cells are both due to a carrier-mediated $Na^+$-$Ca^{2+}$ exchange system. The apparent Michaelis constant ($K_m$) and the maximal initial rate ($V$) were obtained from the substrate-axis intercept and velocity-axis intercept, respectively, on the Lineweaver-Burk plot.

As shown in Table I, the apparent $K_m$ value for $Ca^{2+}$ uptake by the vesicles was estimated to be 31 $\mu$M. This value is similar to those reported by others: 18 $\mu$M by Reeves and Sutko [10] and 30 $\mu$M by Bers et al. [19]. However, the apparent $K_m$ value (0.35 mM) for $Ca^{2+}$ uptake by cultured chick myocardial cells (Table I) was about 10-fold more than that by the vesicles. $Ca^{2+}$ uptake was inhibited by $Mg^{2+}$. We estimated the value of the inhibition constant ($K_i$) for $Mg^{2+}$ from the competitive mode of inhibition by $Mg^{2+}$. Table I shows that the $K_i$ values for $Mg^{2+}$ in two types of preparation are similar. Nevertheless, the degree of inhibition by $Mg^{2+}$ at 50 $\mu$M $[Ca^{2+}]_0$ was much higher in the cells than in the vesicles (Table I). Thus the large difference in the $K_m$ values in the two preparations must have caused the difference in the degree of inhibition by $Mg^{2+}$ at the same external concentration of $Ca^{2+}$ (50 $\mu$M); that is, the strong inhibition by $Mg^{2+}$ in the cells depends on the high $K_m$ value for $Ca^{2+}$, while the weak inhibition by $Mg^{2+}$ in the vesicles depends on the low $K_m$ value for $Ca^{2+}$.

There are several possible explanations for the large difference in the $K_m$ values of the two types of preparation.

*1. Age.* The large difference between the $K_m$ values of the vesicles and cells could depend on the difference in the age of the chicks from which the preparations were obtained. So we compared the $K_m$ value of $Ca^{2+}$ uptake in vesicles from the hearts of 6-day-old chick embryos and of 1-day-old chicks. The $K_m$ value of the embryonic heart vesicles was similar to that of vesicles from 1-day-old chicks (Table II). Moreover the percentage inhibition by $Mg^{2+}$ at 50 $\mu$M $[Ca^{2+}]_0$ (5% at 1 mM $Mg^{2+}$, 45% at 10 mM $Mg^{2+}$) in embryonic heart vesicles was also close to that in vesicles from newly hatched chicks. These results show that these characteristics of $Na^+$-$Ca^{2+}$ exchange are not dependent on the age of the chicks.

*2.* $[Na^+]_i$. A previous report [5] showed that the intracellular concentration of $Na^+$ had no effect on the $K_m$ value for $Ca^{2+}$ uptake by cultured myocardial cells. Consistent with this, a change in the intravesicular concentration of $Na^+$ from 140 to 70 or 35 mM had no significant effect on the $K_m$ for $Ca^{2+}$ uptake by the vesicles (Table II), though it reduced the $V$ (67% of the control at 70 mM $[Na^+]_i$, 21% of the control at 35 mM $[Na^+]_i$).

*3. Membrane potential.* The resting membrane potential of cultured chick myocardial cells was $-59 \pm 7$ mV ($n = 6$) in normal culture medium. After pre-incubation in $Ca^{2+}$-free medium for 10 min, it was only $-4 \pm 1$ mV ($n = 11$). We also examined the effect of the membrane potential on the $K_m$ value of vesicles. An inside-positive or inside-negative membrane potential was induced by addition of 1 $\mu$M valinomycin to vesicles loaded with 70 mM $Na^+$ by

TABLE I

COMPARISON OF VARIOUS KINETIC PROPERTIES OF $Na_i$-DEPENDENT $Ca^{2+}$ UPTAKE BY VESICLES AND CELLS

Inhibition by $Mg^{2+}$ was studied in medium containing 50 $\mu$M $Ca^{2+}$. Vesicles loaded with 140 mM $Na^+$ and cells loaded with 113 mM $Na^+$ were used for measurements. The values are presented as mean ± S.D. for three independent experiments. Percentage of inhibition by $Mg^{2+}$ is given in parentheses.

| | | Vesicles | Cells |
|---|---|---|---|
| Rate of $Ca^{2+}$ uptake (nmol/mg/min) | $Mg^{2+}$-free | 30.9 ± 1.9 (0) | 14.4 ± 1.8 (0) |
| | 1 mM $Mg^{2+}$ | 27.4 ± 1.4 (11) | 10.3 ± 0.9 (28) |
| | 10 mM $Mg^{2+}$ | 18.1 ± 0.4 (41) | 1.9 ± 0.5 (87) |
| $K_m$ for $Ca^{2+}$ (mM) | | 0.031 ± 0.009 | 0.35 ± 0.13 |
| $K_i$ for $Mg^{2+}$ (mM) | | 1–5 | 1–5 |

the methods of Philipson and Nishimoto [13] and Bers et al. [19]. An inside-positive potential markedly stimulated the initial rate of $Ca^{2+}$ uptake, whereas an inside-negative potential reduced it (data not shown). These stimulatory and inhibitory effects seemed to be due to increase (159% of the control) and decrease (68% of the control), respectively, in the $V$ value, not to change in the $K_m$ value, which varied only from about 40 to 60 $\mu$M (Table II). These results show that change in the membrane potential is not responsible for the difference in the $K_m$ values of the two types of preparation.

*4. Conformational change of $Na^+$-$Ca^{2+}$ transporter or deletion of factors that modify $Na^+$-$Ca^{2+}$ exchange during purification of sacrolemma vesicles.* These possibilities are of interest, but their effects are difficult to demonstrate. The mucopolysaccharide coated layer on the outer surface of the membrane [20] may participate in these effects.

*5. Sidedness.* We measured $^{24}Na$ uptake by vesicles in the presence and absence of 2 mM ATP. The results showed the presence of ATP-stimulated $^{24}Na$ uptake (data not shown). The $^{24}Na$ accumulated in the vesicles was rapidly released on addition of 5 $\mu$M nigericin or 1 mM $Ca^{2+}$. Addition of 1 mM ouabain had no effect on ATP-dependent $^{24}Na$ uptake (data not shown). These results show that our preparation contained inside-out vesicles with an inverted configuration of $Na^+$-pump, which transported $Na^+$ inward and that this transport was not inhibited by ouabain in the medium. Table III shows the percentage accessibilities of acetylcholine esterase and specific ouabain-binding sites. These results suggest that about 70% of the total sarcolemma vesicles are right-side-out. This percent is similar to the value (80%) obtained by Bers et al. [19]. If we suppose that the $K_m$ values, but not the $V$ values, are different in inside-out and right-side-out vesicles, the initial rate of $Ca^{2+}$ uptake by whole vesicles can be written in the following equation:

TABLE III

ACCESSIBILITIES OF ACETYLCHOLINE ESTERASE AND SPECIFIC OUABAIN-BINDING SITES

Values are means ± S.D. for three independent experiments. Activities of acetylcholine esterase and ouabain binding are indicated as $\mu$mol/mg per h and pmol/mg, respectively.

| | Treatment | Activity | Accessibility |
|---|---|---|---|
| Acetylcholine esterase | None | 10.5 ± 0.4 | |
| | 0.1% Triton | 14.5 ± 1.4 | 72% |
| Ouabain binding | None | 16.2 ± 0.6 | |
| | 0.1% Triton | 22.2 ± 2.5 | 73% |

Rate of $Ca^{2+}$ uptake ($v$)

$$= \left( \frac{P_i [Ca^{2+}]_o}{K_{mi} + [Ca^{2+}]_o} + \frac{P_r [Ca^{2+}]_o}{K_{mr} + [Ca^{2+}]_o} \right) V$$

where $P_i$ and $P_r$ are the ratios of inside-out and right-side-out vesicles to total sarcolemmal vesicles, respec-

TABLE II

EFFECTS OF VARIOUS EXPERIMENTAL CONDITIONS ON THE $K_m$ VALUE FOR $Ca^{2+}$ UPTAKE BY THE VESICLES

| Age of chick | $[Na^+]_i$ (mM) | Treatment | Expected membrane potential | $K_m$ (mM) |
|---|---|---|---|---|
| 6-day-old embryos | 140 | None | 0 | 0.051 |
| 1-day-old | 140 | None | 0 | 0.031 |
| 1-day-old | 70 | None | 0 | 0.054 |
| 1-day-old | 35 | None | 0 | 0.035 |
| 1-day-old | 70 | VM [a] | Inside-positive | 0.057 |
| 1-day-old | 70 | VM [b] | Inside-negative | 0.043 |

[a] For measurement of $Ca^{2+}$ uptake, reaction medium containing 140 mM KCl, 20 mM Mops/Tris (pH 7.4), $^{45}Ca$ (25 to 800 $\mu$M $Ca^{2+}$) and 1 $\mu$M valinomycin (VM) was added to vesicles loaded with 70 mM $Na^+$ + 70 mM $choline^+$.

[b] For measurement of $Ca^{2+}$ uptake, reaction medium containing 140 mM choline chloride, 20 mM Mops/Tris (pH 7.4), $^{45}Ca$ (25 to 800 $\mu$M $Ca^{2+}$) and 1 $\mu$M valinomycin (VM) was added to vesicles loaded with 70 mM $Na^+$ + 70 mM $K^+$.

tively, and $K_{mi}$ and $K_{mr}$ are the apparent Michaelis constants for $Ca^{2+}$ uptake by inside-out and right-side-out vesicles, respectively. Even if $K_{mr}$ is ten times $K_{mi}$, the theoretical Lineweaver-Burk plots for the equation would show only slight curvature. It would be impossible to detect this small curvature by kinetic analysis of a mixture of inside-out and right-side-out vesicles. So the true $K_m$ value at the inner surface of the cell membrane may be lower than the value obtained from Fig. 3. The possibility that the affinity for $Ca^{2+}$ of the inner surface of the membrane is much higher than that of the outer surface is very fascinating. Any $Ca^{2+}$ gained during the depolarizing cycle must be lost during the repolarization phase, which can last for two-thirds of the cardiac cycle as discussed recently by Mullins [21]. Therefore, the cells require rapid extrusion of $Ca^{2+}$ from the cytoplasm during the repolarization phase. However, it is known that the intracellular concentration of free $Mg^{2+}$ is in the order of millimoles [22,23], which may be high enough to inhibit $Ca^{2+}$ efflux through $Na^{+}$-$Ca^{2+}$ exchange. A low $K_m$ value at the inner surface of the membrane may allow rapid $Ca^{2+}$ extrusion from the cytoplasm, in a way which is insensitive to intracellular $Mg^{2+}$.

In this work we found a large difference between the $K_m$ values for $Ca^{2+}$ of sarcolemma vesicles and intact cells. We examined the reasons for this difference, but could not test some interesting possibilities (points 4 and 5 described above); these must be examined using purified sarcolemma vesicles in a form similar to that of intact cell membranes. We are now attempting to separate inside-out vesicles completely from right-side-out vesicles.

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