Sodium-Calcium Ion Exchange in Skeletal Muscle Sarcolemmal Vesicles

John R. Gilbert and Gerhard Meissner

Departments of Biochemistry and Nutrition, and Physiology, and the Neurobiology Program, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Summary. The Ca^{2+} permeability of rabbit skeletal muscle sarcolemmal vesicles was investigated by means of radioisotope flux measurements. A membrane vesicle fraction highly enriched in sarcolemma, as revealed by enzymatic markers, was obtained from the 22–27% region of sucrose gradients after isopycnic centrifugation. The ability of sarcolemmal vesicles to exchange Na^+ for Ca^{2+} was investigated by measuring Ca^{2+} influx into and efflux from sarcolemmal vesicles in the presence and absence of a Na^+ gradient. It was found that Ca^{2+} movements were enhanced in the direction of the higher Na^+ concentration. When intra- and extravesicular Na^+ concentrations were high, $Na^+ - Na^+$ exchange predominated and $Na^+ - Ca^{2+}$ exchange was low or absent. The presence of the Ca^{2+} ionophore A23187 in the dilution medium resulted in the rapid release of Ca^{2+} and the elimination of the Na^+-enhanced efflux of Ca^{2+}, suggesting that internal rather than bound external Ca^{2+} was exchanged with Na^+. La^{3+} abolished $Na^+ - Ca^{2+}$ exchange and decreased overall membrane permeability. $Na^+ - Ca^{2+}$ exchange was not due to sarcoplasmic reticulum or mitochondrial contaminants. This investigation suggests that skeletal muscle, like cardiac muscle and neurons, is capable of a transmembranous $Na^+ - Ca^{2+}$ exchange.

Key words sodium-calcium ion exchange · sarcolemmal vesicles · skeletal muscle · ion permeability

Introduction

Information concerning Ca^{2+} transport mechanisms across the sarcolemma[1] of vertebrate skeletal muscle is limited at present. It has, however, been known for more than two decades that resting skeletal muscle possesses a Ca^{2+} "leak" influx and that under normal conditions this Ca^{2+} entry is increased several fold during muscle activity [3, 4, 9]. Thus to maintain a steady-state, Ca^{2+} must be extruded from muscle fibers against an electrochemical gradient [28]. Estimates have been made that resting skeletal muscle cells use approximately 1% of their energy to maintain the Ca^{2+} gradient across their sarcolemma [3, 12].

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[1] The sarcolemma is defined as consisting of two components: the surface membrane proper and the invaginations of the surface membrane into the muscle cell known as the transverse-tubular system (T-system).

Two mechanisms of Ca^{2+} transport across mammalian sarcolemma are an ATP-driven pump and a $Na^+ - Ca^{2+}$ exchange system. Both Ca^{2+} transport systems have been recently identified in membranous vesicle fractions isolated from cardiac muscle [2, 7, 22, 24, 25]. In rabbit skeletal muscle, an ATP-energized Ca^{2+} pump has been demonstrated in vesicle fractions enriched in transverse-tubule [5]. Physiological studies with intact muscle cells have demonstrated $Na^+ - Ca^{2+}$ exchange, where transmembrane movement of Ca^{2+} is directly linked to reciprocal movements of Na^+, in frog skeletal muscle [6, 8] and guinea pig diaphragm [31]. In this report we provide evidence that sarcolemmal vesicles isolated from rabbit skeletal muscle are also capable of catalyzing the exchange of Na^+ for Ca^{2+}.

Materials and Methods

Materials

Preparation of Vesicles

The sarcolemmal vesicles used in this study were prepared by zonal gradient centrifugation. Rabbits (~ 3 kg) were killed by the injection of 180 mg nembutal. The white muscle of the back and legs were removed in the cold, cut into small pieces, and homogenized in aliquots of 40 g in 320 ml of 0.3 M sucrose with two 30-sec bursts in a Waring Blendor set at high speed. The homogenate was centrifuged in a Sorvall centrifuge for 12 min at $8,000 \times g$. The supernatants ($\sim 1,500$ ml) were decanted through eight layers of cheesecloth, placed in a Beckman Ti 15 zonal rotor and accelerated to 3,000 rpm. A discontinuous 20/50% sucrose interface was created by pumping in 100 ml of 20% sucrose followed by 150 ml of 50% sucrose. After centrifugation for 3 hr at 29,000 rpm, a crude rabbit skeletal muscle "microsomal fraction" present at the 20/50% interface was collected, diluted 1:1.5 with H_2O, and centrifuged for 75 min at 33,000 rpm ($90,000 \times g$) in a Beckman 35 rotor.

Table 1. Yield and enzymatic marker activities of sucrose gradient fractions derived from rabbit skeletal muscle

Fraction	% sucrose	Yield (mg protein/ 1,000 g muscle)	"Basic" ATPase (μmol/mg protein/min)	Leucyl β-naph-thylamidase (μmol/mg protein/min)	^{32}P-labeled phosphoenzyme (nmol/mg protein)	Succinate-cyto-chrome c reductase (μmol/mg protein/min)
CF	–	2886	0.25	0.01	2.20	–
1	19–22	10	3.27	0.08	0.20	0.002
2	22–27	29	4.50	0.12	0.25	0.008
3	27–29	17	3.48	0.10	0.52	0.012
4	29–31	275	0.84	0.04	1.73	0.014
5	31–36	568	0.26	0.01	4.50	0.066
6	36–45	525	0.16	0.01	3.20	0.240

Crude microsomal fractions (CF) and sucrose gradient fractions (1–6) were obtained and enzymatic assays were carried out as described in Materials and Methods. The data are the average of three preparations. Standard errors were less than $\pm 10\%$. % sucrose – region of gradient from which vesicular fraction was obtained

Pellets were collected in approximately 25 ml of 0.5 M sucrose, 2 mM K Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.2, frozen and stored at -70 °C.

Microsomal fractions from four rabbits were combined after rapid thawing at 32 °C, and diluted with KCl to a final concentration of 0.25 M sucrose, 0.45 M KCl, 5 mM K Hepes, pH 7.1. The vesicle suspension was kept at 2 °C for 4 hr, then subjected to sucrose isopycnic zonal centrifugation at 29,000 rpm in a Beckman Ti 15 for 18 hr on a 1,000-ml continuous 20–50% sucrose gradient containing 50 mM KCl and 10 mM K Hepes, pH 7.1. After deceleration to 2,000 rpm the gradient was pumped out with 55% sucrose, fractions were collected, diluted with H_2O and centrifuged at 33,000 rpm (90,000 $\times g$) for 75 min in a Beckman 35 rotor. The pellets were taken up in 0.5 M sucrose, 4 mM K Hepes, pH 7.1, and stored at -70 °C until use. Control experiments with unfrozen fractions indicated that initial freezing and thawing during vesicle preparation did not appreciably affect $Na^+ - Ca^{2+}$ exchange activity. Final gradient fractions were, however, frozen in small aliquots since a decline in the rate of $Na^+ - Ca^{2+}$ exchange was seen with repeated thawing and freezing of the purified vesicles.

Isotope Flux Measurements

Unless otherwise indicated, isotope flux measurements were carried out at 22 °C. Vesicle incubation was as outlined in the figure legends. Briefly, vesicles were incubated in the experimental media for 4 hr at 22 °C. After incubation vesicles were centrifuged for 30 min at 20,000 rpm (33,000 $\times g$) in a Beckman 42.1 rotor. The pellet was taken up in a minimum amount (~ 0.2 ml) of the original incubation mixture at a concentration of approximately 5 mg/ml protein. In those experiments involving $^{45}Ca^{2+}$ efflux the vesicles were first osmotically shocked in a $^{45}Ca^{2+}$ solution of low osmolality to ensure rapid $^{45}Ca^{2+}$ entry into the vesicles, then incubated in the media for an additional 4 hr. Efflux or influx of radioactive compounds from or into the vesicles was initiated by diluting vesicles 200-fold into a dilution medium under rapid mixing. The reaction was followed by placing aliquots at various time intervals on a 0.45 μm HAWP Millipore filter followed by rapid rinsing (3 ×) with unlabeled medium. The radioactivity retained on the filters was counted in 4.5 ml of a scintillation liquid which completely dissolved the filters. The fluid contained 60 g naphthalene, 4.2 g 2,5-diphenyloxazole, 180 mg 1,4-bis-[2-(5-phenyloxazolyl)] benzene and 20 ml of water in 900 ml dioxane. Counting of singly and doubly labeled samples was carried out in a LKB Liquid Scintillation System, using mini vials.

Biochemical Assays

Protein was determined by the procedure of Lowry et al. [17] using bovine serum albumin as a standard. Free Ca^{2+} concentrations were determined with a HNU systems (Newton Upper Falls, MA) Ca^{2+} electrode. Mg^{2+}- or Ca^{2+}-activated ("basic") ATPase [18] was determined at 32 °C in 2 ml of a medium containing 10 mM K Hepes, 0.1 M KCl, 2.5 mM ATP, 6 mM Mg^{2+} plus 1 mM EGTA (ethyleneglycol bis-[β-aminoethyl ether]-N, N'-tetraacetic acid), pH 7.3. The reaction was started by the addition of ATP and stopped after 5 and 10 min with 0.7 ml of 1.5 M $HClO_4$. Inorganic phosphate was determined on 1 ml of the protein-free supernatant [10] using Elon as a reducing agent. The enzyme concentration used resulted in less than 10% hydrolysis of ATP. The concentration of the Ca^{2+}-dependent, ^{32}P-labeled phosphoenzyme intermediate of sarcoplasmic reticulum was measured as previously described [20]. Leucyl-β-naphthylamidase activity was determined at 32 °C by the method of Goldbarg and Rutenberg [14]. Succinate-cytochrome c reductase activity was estimated at 32 °C according to Fleischer and Fleischer [11].

Results

Properties of Sarcolemmal Fractions

The sarcolemmal fractions used for this study were derived from the 22–27% region of the sucrose gradient. This region was found to be enriched in two sarcolemma-associated activities, "basic" ATPase [18] and leucyl β-naphthylamidase [29] (Table 1). The level of ^{32}P-phosphoenzyme intermediate and succinate-cytochrome c reductase activities were used to indicate the presence of sarcoplasmic reticulum [30] and inner mitochondrial membranes [11], respectively. The two latter activities were low in membranes recovered from the 22–27% sucrose region. All results were consistent with the view that the 22–27% sucrose region was greatly enriched in and chiefly composed of membranes derived from the sarcolemma.

Sarcolemmal fractions derived from the 22–27% region of the sucrose gradient were capable of en-

hanced D-glucose (3.1 nmol/mg protein/min) versus L-glucose (2.1 nmol/mg protein/min) uptake. A maximal D-glucose isotope space of 1.5–3.0 µl/mg protein suggested that an appreciable fraction of these membranes were present in the form of enclosed vesicular compartments.

Calcium Flux Experiments

Ca^{2+} movements across sarcolemmal vesicles were studied in the presence and absence of Na^+ gradients by incubating the vesicles in $^{45}Ca^{2+}$ medium and observing $^{45}Ca^{2+}$ efflux upon dilution into media of varying composition, or by observing $^{45}Ca^{2+}$ influx into vesicles incubated in either K^+ or Na^+ media.

The initial experiments were designed to determine the effect of extravesicular Na^+ on the rate of $^{45}Ca^{2+}$ efflux from sarcolemmal vesicles. Vesicles were equilibrated in media containing 100 mM KCl, 2 mM Mg^{2+}, and 30 µM Ca^{2+} with trace amounts of $^{45}Ca^{2+}$. Vesicles were then diluted 200-fold into KCl media containing increasing amounts of Na^+ (Fig. 1). Dilution media contained either 1 mM EGTA, to maintain external Ca^{2+} at a concentration of less than 10^{-7} M, or 1 mM La^{3+}. As will be shown below (Fig. 4), La^{3+} "tightens" sarcolemmal vesicles, rendering them less permeable to non-mediated ion fluxes and eliminating $Na^+ - Ca^{2+}$ exchange. Figure 1 demonstrates that as the extravesicular Na^+ concentration was increased from 0 to 100 mM, in media containing 1 mM EGTA, an enhancement in $^{45}Ca^{2+}$ efflux was observed. $^{45}Ca^{2+}$ efflux was approximately half-maximal at 10 mM external Na^+ and nearly maximal at 50 mM external Na^+.

Our explanation for Na^+-mediated $^{45}Ca^{2+}$ efflux is that skeletal muscle sarcolemma is capable of $Na^+:Ca^{2+}$ exchange, as has been demonstrated for vesicles derived from heart sarcolemmal membranes [2, 22, 24, 25]. In agreement with such an interpretation were the following observations. First, in the presence of the ionophore A23187, greater than 95% of $^{45}Ca^{2+}$ was rapidly released from the vesicles in K^+ or Na^+ media (Fig. 2), an indication that the retained $^{45}Ca^{2+}$ was trapped inside the vesicles. The elimination of Na^+-mediated Ca^{2+} efflux in the presence of A23187 also suggested that the increased loss in $^{45}Ca^{2+}$ in the presence of extravesicular Na^+ was due to greater efflux of internal Ca^{2+} rather than Na^+ replacement of externally bound Ca^{2+}. Second, filling the vesicles with 100 mM Na^+ instead of 100 mM K^+ gave identical $^{45}Ca^{2+}$ efflux on dilution into 100 mM K^+ or 50 mM K^+ plus 50 mM Na^+ media (data not shown). Thus as shown for the cardiac
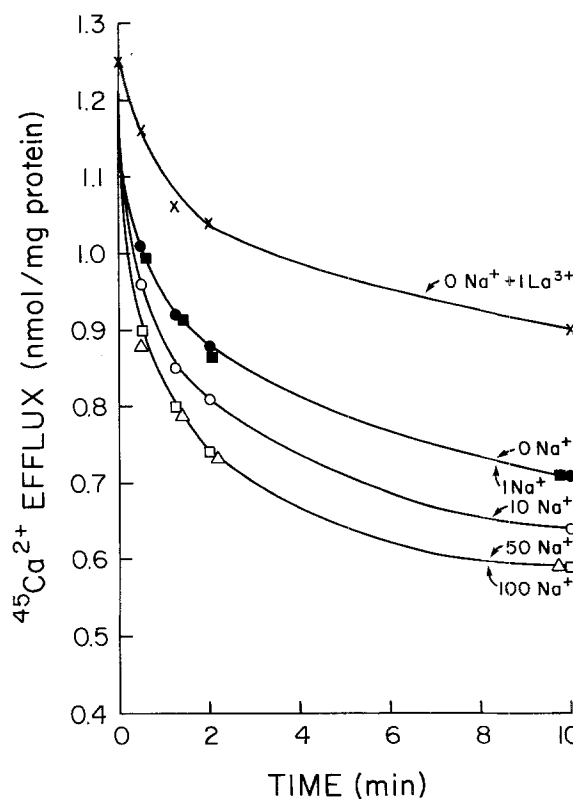

Fig. 1. Measurement of $^{45}Ca^{2+}$ efflux from sarcolemmal vesicles in the presence of various amounts of extravesicular Na^+. Sarcolemmal vesicles (Fraction 2 of Table 1) present in 0.5 M sucrose, 4 mM Hepes, pH 7.1, were osmotically shocked by 10-fold dilution into 30 µM $^{45}CaCl_2$, incubated at 20 °C for 4 hr in 100 mM KCl, 2 mM $MgCl_2$, 30 µM $^{45}CaCl_2$, 20 mM Hepes, pH 7.3, and centrifuged for 30 min at 20,000 rpm ($33,000 \times g$) in a 42.1 rotor. The pellets were taken up at a concentration of ~5 mg/ml in the incubation medium. $^{45}Ca^{2+}$ efflux was initiated by diluting the vesicles 200-fold into a medium containing 20 mM Hepes, pH 7.3, 2 mM $MgCl_2$, 100 mM KCl and 1 mM La^{3+} (×), or 1 mM EGTA and either 0 mM (●), 1 mM (■), 10 mM (○), 50 mM (□), or 100 mM (△) NaCl. Where NaCl was less than 100 mM, KCl was added to maintain osmolality. Aliquots of 0.4 ml were caught on Millipore filters and rinsed. Efflux of $^{45}Ca^{2+}$ was determined by measuring the amount of radioactivity (cf. Materials and Methods) remaining with the filters. Amounts initially present in the vesicles were determined by extrapolating back to zero time the $^{45}Ca^{2+}$ efflux curves in the presence of 1 mM La^{3+}. Data are the average of four experiments. SE were less than ±10% within a given preparation

$Ca^{2+}:Na^+$ exchange system [25], internal Na^+ could block Na^+-mediated Ca^{2+} efflux. Third, sarcoplasmic reticulum (Fraction 5 of Table 1) and mitochondrial (Fraction 6 of Table 1) enriched fractions did not demonstrate significant Na^+-mediated $^{45}Ca^{2+}$ efflux (data not shown). Fourth, Na^+-mediated Ca^{2+} efflux from sarcolemmal vesicles was abolished by 1 mM La^{3+} (see below), a blocker of transmembrane Ca^{2+} movements [16, 21, 24, 27].

Figure 2 compares the effect of 1 mM La^{3+} on Ca^{2+} efflux when added prior to vesicle dilution and
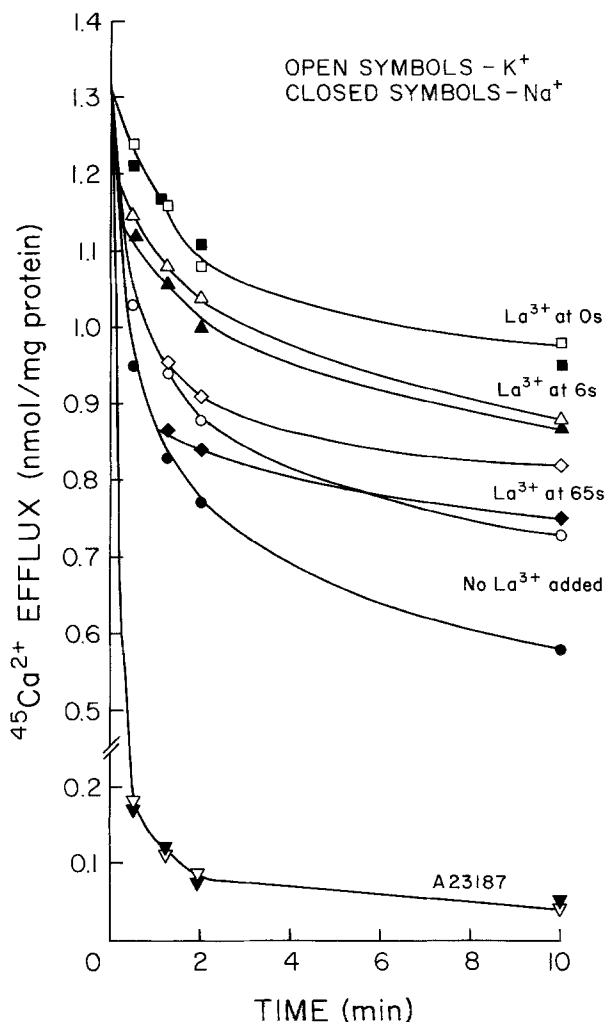
Fig. 2. Effect of La^{3+} on Ca^{2+} efflux from sarcolemmal vesicles. Sarcolemmal vesicles were filled with 100 mM KCl, 2 mM Mg^{2+}, 30 μM $^{45}Ca^{2+}$ and 20 mM Hepes, pH 7.3, as described in Fig. 1. $^{45}Ca^{2+}$ efflux was initiated by diluting the vesicles 200-fold into media containing 100 mM K^+ (open symbols) or 50 mM Na^+ plus 50 mM K^+ (filled symbols), 2 mM Mg^{2+}, 20 mM Hepes, pH 7.3, 0.1 mM EGTA (o, ●), or 0.1 mM EGTA plus 1 mM La^{3+} (□, ■) or 0.1 mM EGTA plus the ionophore A23187 (2 μg/ml) (▽, ▼). In a second type of experiment 1/100 volume of 100 mM La^{3+} in release medium was added to vesicles, at 6 (△, ▲) or 65 (◇, ◆) sec time points, already present in release medium. Aliquots of 0.4 ml were caught on Millipore filters, rinsed, and counted (cf. Materials and Methods)
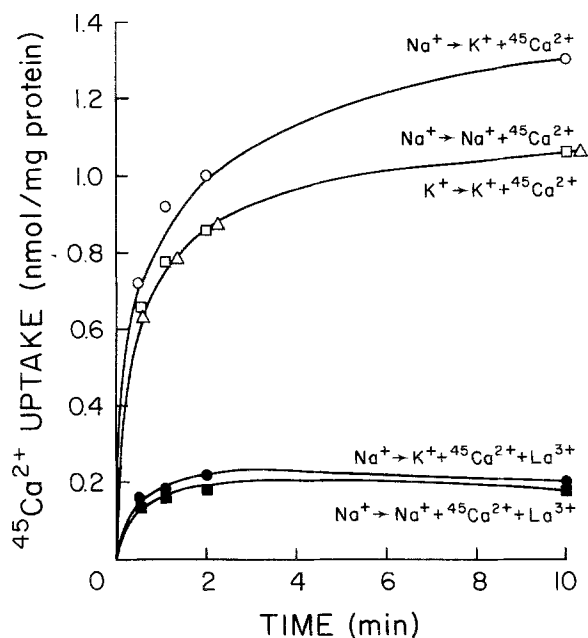

Fig. 3. Measurement of Na^+-mediated $^{45}Ca^{2+}$ influx into sarcolemmal vesicles. Sarcolemmal vesicles were incubated at 22 °C for 4 hr in 100 mM KCl (△) or 50 mM KCl plus 50 mM NaCl (o, □, ●, ■), 2 mM $MgCl_2$, 90 μM EGTA, and 20 mM Hepes, pH 7.3. Vesicles were then centrifuged for 30 min at 20,000 rpm (33,000 × g) in a 42.1 Beckman rotor and taken up in the original incubation solution at a concentration of ~5 mg/ml. Vesicles were diluted 200-fold into the indicated incubation medium except that 30 μM $^{45}CaCl_2$ was substituted for 100 μM EGTA. In experiments involving La^{3+}, La^{3+} was present in the dilution medium at a concentration of 1 mM. At the indicated time points, 0.4 ml aliquots were placed on a 0.45 μm Millipore filter and rapidly rinsed with dilution medium. Radioactivity remaining with the vesicles was determined. Data are the average of a minimum of six experiments and standard errors were less than ±10%

at 6 and 65 sec after the initial vesicle dilution into Na^+ or K^+ media. There was a rapid initial release of Ca^{2+} that could be blocked by the prior addition of 1 mM La^{3+}. At 6 sec, a consistent difference in the amount of Ca^{2+} efflux in K^+ and Na^+ media was observed. The amount of Na^+-mediated $^{45}Ca^{2+}$ efflux increased at the 65 sec time point. The addition of La^{3+} at each time point slowed subsequent Ca^{2+} efflux from the vesicles in both K^+ and Na^+ media so that no Na^+-mediated Ca^{2+} efflux was seen in

the presence of 1 mM La^{3+}. Differences in Na^+-mediated and -nonmediated $^{45}Ca^{2+}$ efflux prior to the addition of La^{3+} were, however, preserved in the presence of La^{3+}.

When vesicles were preincubated at 30 μM Ca^{2+} in the presence of 100 mM KCl and 2 mM Mg^{2+} and diluted into KCl medium containing 1 mM La^{3+}, about 1.25–1.35 nmol $^{45}Ca^{2+}$/mg protein were retained by the vesicles on the filters (Figs. 1 and 2). This value corresponded to an apparent $^{45}Ca^{2+}$ space of about 40 μl/mg protein. At 1 mM Ca^{2+} the $^{45}Ca^{2+}$ space decreased to about 10 μl/mg protein (not shown). For comparison, ^3H-sucrose spaces of about 2 μl/mg protein were measured. Differences in isotope spaces suggested that a large portion of $^{45}Ca^{2+}$ retained by the vesicles on the filters was present in an internally bound form.

$^{45}Ca^{2+}$ influx into sarcolemmal vesicles was investigated in the presence and absence of intravesicular Na^+. Sarcolemmal vesicles equilibrated in either 100 mM KCl or 50 mM KCl plus 50 mM NaCl were

Table 2. Initial rates of $^{45}Ca^{2+}$ efflux and influx

	Composition		$^{45}Ca^{2+}$ flux rates at 3 sec	Na^+-mediated increment
	Incubation medium Temperature (°C)	Dilution medium	(nmol/mg prot/min)	
Ca^{2+} efflux	100 KCl ($-Mg^{2+}$) (20°)	100 KCl 100 NaCl	3.32 ± 1.24 6.43 ± 1.78	3.11 ± 1.73 ($P < 0.001$)
	100 KCl+2 Mg^{2+} (4°)	100 KCl+2 Mg^{2+} 100 NaCl+2 Mg^{2+}	4.00 4.02	–
	100 KCl+2 Mg^{2+} (20°)	100 KCl+2 Mg^{2+} 100 NaCl+2 Mg^{2+}	4.06 ± 0.68 5.17 ± 1.13	1.11 ± 0.91 ($P < 0.001$)
	100 KCl+2 Mg^{2+} (37°)	100 KCl+2 Mg^{2+} 100 NaCl+2 Mg^{2+}	4.58 ± 0.70 6.02 ± 0.79	1.44 ± 0.23 ($P < 0.001$)
Ca^{2+} influx	100 KCl+2 Mg^{2+} 100 NaCl+2 Mg^{2+} (20°)	100 KCl+2 Mg^{2+} 100 KCl+2 Mg^{2+}	18.70 ± 0.40 21.11 ± 1.26	2.41 ± 0.76 ($P < 0.025$)

Initial $^{45}Ca^{2+}$ efflux and influx rates from and into sarcolemmal vesicles were approximated as described in Figs. 2 and 3 by adding 1 mM La^{3+} to the vesicles 3 sec after dilution. "Free" $^{45}Ca^{2+}$, as measured by a HNU systems Ca^{2+} electrode, was present at a concentration of 45 μM and incubation and dilution media contained in addition to the indicated cations (in mM) 20 mM K Hepes at pH 7.2. Experimental temperatures were as indicated. In influx experiments, the intravesicular EGTA concentration was 1 mM. In efflux experiments, dilution media contained 100 μM EGTA. In both efflux and influx experiments 2 μl of sarcolemmal vesicles (5 mg protein/ml) were placed in 0.4 ml of isoosmolal dilution medium. Exchange was carried out under rapid mixing and was stopped at 3 sec by the addition of 0.4 ml of reaction medium containing 2 mM La^{3+}. An aliquot (0.7 ml) of the vesicle suspension was then immediately filtered. $^{45}Ca^{2+}$ flux rates were calculated as the difference between the 3-sec time point and a "zero" time point derived by directly diluting vesicles into media containing 1 mM La^{3+}. Standard deviations in the absence of Mg^{2+} ($-Mg^{2+}$) and at 37 °C are based on a minimum of six experiments. Other standard deviations are based on a minimum of 12 experiments. The relatively large standard deviations reflect experimental differences between preparations. Significance (P) was determined by Students t Test of significance for paired experiments.

diluted into media containing 30 μM $^{45}Ca^{2+}$ and either 100 mM KCl or 50 mM KCl plus 50 mM NaCl. Figure 3 demonstrates that Ca^{2+} uptake into the vesicles was enhanced in the presence of an inside-outside Na^+ gradient. That a Na^+ gradient was necessary for the enhancement of Ca^{2+} uptake is indicated by the observation that diluting vesicles incubated in Na^+ medium into Na^+ medium and vesicles incubated in K^+ medium into K^+ medium gave similarly reduced Ca^{2+} uptake rates. Addition of 1 mM La^{3+} to the dilution media greatly reduced $^{45}Ca^{2+}$ influx and essentially eliminated Na^+-mediated influx (Fig. 3).

Effects of temperature and Mg^{2+} on the initial Ca^{2+} efflux rates for sarcolemmal vesicles at 45 μM Ca^{2+} are compared in Table 2. Omission of 2 mM Mg^{2+} from the incubation and dilution media enhanced Na^+-mediated Ca^{2+} efflux. At 45 μM internal Ca^{2+} the efflux rate increased from 1.11 nmol/mg protein/min in the presence of Mg^{2+} to 3.11 nmol/mg protein/min in the absence of Mg^{2+}. Increases in Na^{2+} mediated and nonmediated $^{45}Ca^{2+}$ efflux rates were observed when $^{45}Ca^{2+}$ efflux was measured at

37 °C instead of 20 °C. Na^{2+}-mediated efflux was, on the other hand, abolished when $^{45}Ca^{2+}$ efflux was measured at 4 °C. This temperature dependence provides additional evidence for an enzyme-mediated $Na^+ - Ca^{2+}$ exchange reaction.

Table 2 further shows that Ca^{2+} influx into the vesicles at 3 sec postdilution, both in the presence and absence of a transmembrane Na^{2+} gradient, was faster than the rates of Ca^{2+} efflux from the vesicles: 21.11 vs. 5.17 and 18.7 vs. 4.06 nmol Ca^{2+}/mg protein/min, in the presence and absence of a Na^{2+} gradient, respectively. One possible explanation for these different rates is that in the Ca^{2+} influx experiments vesicles were preincubated in 1 mM EGTA for prolonged periods. The EGTA may affect the vesicular membranes so as to make them more permeable to Ca^{2+}. On dilution Ca^{2+} moves into the vesicles more rapidly than during efflux, where the vesicles are only exposed to EGTA for a short period. This interpretation is supported by findings in preliminary influx experiments that at 200 μM external Ca^{2+}, utilizing 5-sec time points, incubation in EGTA increased nonmediated Ca^{2+} influx over 200% (not shown). EGTA
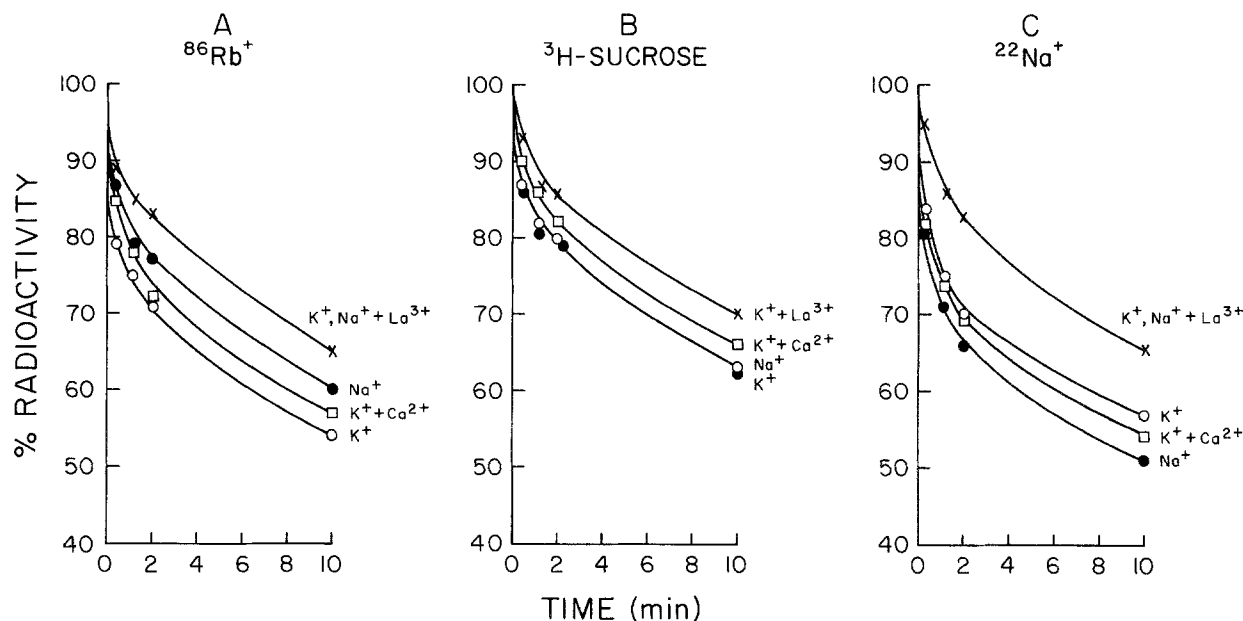
Fig. 4. $^{86}Rb^+$, ^3H-sucrose, and ^{22}Na efflux from sarcolemmal vesicles. Sarcolemmal vesicles (5 mg protein/ml) were incubated for 4 hr at 22 °C in media containing either (*A* and *B*) 10 mM $^{86}RbCl$, 30 mM ^3H-sucrose, 90 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM Hepes, pH 7.3 or (*C*) 10 mM $^{22}NaCl$, 30 mM sucrose, 90 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM Hepes, pH 7.3. Aliquots of vesicles were diluted 200-fold into 100 mM Na^+ ($\bullet$, $\times$) or (*A* and *B*) 10 mM Rb^+, 90 mM K^+ or (*C*) 100 mM K^+ (o, $\square$, $\times$) medium containing 30 mM sucrose, 2 mM $MgCl_2$, 20 mM Hepes, pH 7.3 and either 1 mM EGTA (o, $\bullet$), 1 mM Ca^{2+} ($\square$) or 1 mM La^{3+} ($\times$). Vesicles were caught on Millipore filters, rinsed, and radioactivity remaining with the vesicles on the filters was determined. All data are expressed as percent radioactivity remaining with vesicles on the filter. 100% was determined by extrapolating back to zero time the efflux curves in the presence of 1 mM La^{3+}

was used in influx experiments to maintain low free internal Ca^{2+} concentrations and increase the Ca^{2+} capacity of the vesicles.

The effect of a membrane potential (inside negative) was investigated by diluting vesicles filled with KCl and $^{45}Ca^{2+}$ into NaCl medium containing the K^+-ionophore valinomycin. Similar Na^+-mediated Ca^{2+} efflux rates were observed in the presence and absence of the ionophore.

Kinetic parameters of Ca^{2+} influx were determined for one preparation under conditions similar to those used in Table 2 except that the concentration of free extravesicular Ca^{2+} was varied from 10 μM to 1 mM. The double reciprocal Lineweaver-Burk plot of the data yielded a 0.91 correlation to a straight line with an apparent K_m value of 28 μM Ca^{2+} and a V_{max} value of 6.25 nmol Ca^{2+}/mg protein/min.

$^{22}Na^+$, $^{86}Rb^+$ and ^3H-sucrose Flux Experiments

The above data suggests that skeletal muscle sarcolemma is capable of Na^+-Ca^{2+} exchange. One might expect then that external Ca^{2+} will increase Na^+ efflux from sarcolemmal vesicles as well. To test this hypothesis, vesicles filled with 10 mM $^{22}NaCl$ were diluted into KCl media containing less than 10^{-7} M Ca^{2+}, or 1 mM free Ca^{2+}, or 1 mM La^{3+}.

We also tested for Na^+-Na^+ exchange and in control experiments for $^{86}Rb^+$ and ^3H-sucrose efflux in the presence and absence of La^{3+} and Ca^{2+}.

The effect of external Ca^{2+} and La^{3+} on the efflux of $^{86}Rb^+$, a substitute for K^+, and ^3H-sucrose from the sarcolemmal vesicles is shown in Fig. 4*A* and *B*. On dilution into an unlabeled medium with a composition identical to the incubation medium, intravesicular $^{86}Rb^+$ and ^3H-sucrose levels declined within 10 min post-dilution by 47 and 36%, respectively. Dilution of the vesicles into Na^+ medium retarded $^{86}Rb^+$ efflux, raising the possibility that the vesicles were capable of Rb^+-Rb^+ exchange. Addition of 1 mM La^{3+} to the dilution media retarded both $^{86}Rb^+$ and ^3H-sucrose efflux. In the presence of 1 mM Ca^{2+}, $^{86}Rb^+$ and ^3H-sucrose efflux were also slowed down, although to a lesser extent. Our interpretation of the data of Fig. 4*A* and *B* is that extravesicular La^{3+} and Ca^{2+} act to "tighten" the vesicles, thereby rendering them less permeable to nonmediated ion fluxes. In addition, it appeared that La^{3+} inhibited Rb^+-Rb^+ exchange.

The effect of extravesicular ion composition on $^{22}Na^+$ efflux from sarcolemmal vesicles is illustrated in Fig. 4*C*. On dilution into the control KCl medium the percent of $^{22}Na^+$ radioactivity retained by the vesicles decreased to 57% of the initial value within

the first 10 min. A decrease to 51% within the first 10 min on dilution into Na^+ medium was in accord with the ability of the vesicles to exchange ^{22}Na for Na. Placement of the vesicles into K^+ or Na^+ media containing 1 mM La^{3+} retarded Na^+ efflux from the vesicles and eliminated $Na^+ - Na^+$ exchange. The effect of 1 mM extravesicular Ca^{2+} on $^{22}Na^+$ efflux was somewhat variable. In some experiments, addition of 1 mM Ca^{2+} to K^+ media showed no effect; in other experiments $^{22}Na^+$ efflux was slightly increased (Fig. 4C). Lack of a clear dependence of $^{22}Na^+$ efflux on external Ca^{2+} may have been due to the "tightening" effect of Ca^{2+} on overall membrane permeability (Fig. 4A and B).

Discussion

The results presented in this study demonstrate that $^{22}Na^+$ and $^{45}Ca^{2+}$ movements are increased in the direction of opposing Na^+ gradients across sarcolemmal vesicles derived from rabbit skeletal muscle. $Na^+ - Ca^{2+}$ exchange has not been previously demonstrated in skeletal muscle vesicle preparations but has been the object of extensive recent investigation in cardiac muscle. We find that in skeletal muscle, as in heart muscle, La^{3+} inhibits $Na^+ - Ca^{2+}$ exchange. We also find that when intra- and extravesicular Na^+ concentrations are high no Na^+-mediated Ca^{2+} flux is observed. Lowering the temperature to 4 °C eliminates Na^+-mediated Ca^{2+} flux, while raising it to 37 °C increases it. This Na^+-mediated increase is not observed in vesicle fractions enriched in sarcoplasmic reticulum or mitochondria. Further, essentially all of the $^{45}Ca^{2+}$ retained by the vesicles on the filters seems to be trapped inside the vesicles since it is rapidly released on addition of A23187. The most straight-forward interpretation of these results is that sarcolemmal vesicles derived from rabbit skeletal muscle, like those from cardiac muscle, are capable of $Na^+ - Na^+$ and $Na^+ - Ca^{2+}$ exchange.

In heart, K_m values for Ca^{2+} and Na^+ have been calculated to be 18 μM and 12.5 mM, respectively [23, 25]. We estimate that Na^+-mediated Ca^{2+} efflux from skeletal muscle sarcolemma has a K_m of about 28 μM and is half-maximally stimulated at an external Na^+ concentration of approximately 10 mM.

In studies of $Na^+ - Ca^{2+}$ exchange in cardiac vesicles derived from rabbit, Ca^{2+} rapidly accumulated with an initial rate of approximately 20–30 nmol/mg protein/min at an external Ca^{2+} concentration of 40 μM [25]. For comparison, the rate of exchange at 3 sec observed in the present study for skeletal sarcolemmal vesicles at 45 μM $^{45}Ca^{2+}$ was about 2.4 nmol/mg protein/min (Table 2). In principle, this lower rate

of exchange could be due to an exchange system with a low exchange rate, or it may, as discussed below, reflect a high capacity, rapid turnover system present in only a small percentage of the vesicles.

Enzymatic data as well as isotope flux and membrane polarization studies using the fluorescent dye 3,3′dipentyl-2,2′-oxacarbocyanine [13, 19] indicate that our vesicle fraction (Fraction 2 of Table 1) is of heterogeneous origin, being composed primarily of membranes derived from the sarcolemma, but containing also 10–30% contaminating sarcoplasmic reticulum depending upon the individual preparation. As a general rule, we found that sarcolemmal vesicles in comparison with sarcoplasmic reticulum vesicles are quite impermeable to small ions, including Ca^{2+}. About two-thirds of the sarcoplasmic reticulum (SR) vesicle fraction is highly permeable to K^+, Rb^+ and Na^+ due to the presence of a K^+, Na^+ channel [19]. Accordingly, we interpret the Ca^{2+} flux data of the present study to indicate the presence of four populations of vesicles in Fraction 2: two populations of SR vesicles relatively permeable to Ca^{2+}; a population of impermeable sarcolemmal vesicles that are capable of $Na^+ - Ca^{2+}$ exchange; and, finally, highly impermeable sarcolemmal vesicles that are not capable of $Na^+ - Ca^{2+}$ exchange. The initial rapid efflux and influx components would be mainly due to SR and under exchange conditions to the sarcolemmal vesicles containing the $Na^+ - Ca^{2+}$ exchange system. The remaining slow efflux component would represent highly impermeable sarcolemmal vesicles lacking the $Na^+ - Ca^{2+}$ exchange system.

The existence of a $Na^+ - Ca^{2+}$ exchange system in only a fraction of the vesicles could explain some of the apparent discrepancies observed in studies with skeletal and cardiac membrane vesicles. In cardiac membrane vesicles, a membrane potential-stimulated $Na^+ - Ca^{2+}$ exchange has led to the suggestion of an electrogenic $3:1$ $Na^+ - Ca^{2+}$ exchange system [21, 22, 26]. The absence of a large stimulatory effect by membrane potentials in our vesicle fraction may have been due to a lowering of nonmediated or passive Ca^{2+} fluxes. For example, a negative inside potential was formed on dilution of vesicles containing K^+ and $^{45}Ca^{2+}$ into a Na^+ medium in the presence of valinomycin. This inside negative potential would stimulate Ca^{2+} efflux from vesicles containing a $3:1$ $Na^+ - Ca^{2+}$ exchange system [22]. On the other hand, it may act to slow nonmediated efflux of positively charged Ca^{2+} from the remaining large fraction of vesicles lacking the $Na^+ - Ca^{2+}$ system. A similar explanation may be given for our inability to demonstrate clearly enhanced $^{22}Na^+$ flux rates in the presence of a Ca^{2+} gradient. A reduction of overall membrane permeability in all vesicles by Ca^{2+} (Fig. 4)

would be expected to mask the increase in Ca^{2+}-mediated $^{22}Na^+$ efflux in the small vesicle population possessing $Na^+ - Ca^{2+}$ exchange.

Finally, it may be noted that a possibility we cannot totally rule out at present is that our preparation contained vesicles derived from the plasmalemma of cells other than skeletal muscle and that these contaminating vesicles were responsible for the observed $Na^+ - Ca^{2+}$ exchange activity. In this case, it would have to be postulated that the contaminating vesicles accounted for at least 10% of the total vesicle population. It should be noted, however, that studies with intact frog skeletal and guinea pig diaphragm muscle cells have demonstrated $Na^+ - Ca^{2+}$ exchange [6, 8, 31].

Unlike cardiac muscle, excitation-contraction coupling in skeletal muscle is not believed to be dependent on external Ca^{2+} [1]. It is therefore unlikely that the $Na^+ - Ca^{2+}$ exchange system has a specific role in the process of excitation-contraction coupling in skeletal muscle. On the other hand, the $Na^+ - Ca^{2+}$ exchange system might, in conjunction with a Ca^{2+}-ATPase [5], act to remove Ca^{2+} that enters the sarcoplasm in direct response to an action potential or as a leak current [3, 4, 9, 15].

References

1. Armstrong, C.M., Bezanilla, F.M., Horowicz, P. 1972. Twitches in the presence of ethylene glycol bis (β-aminoethyl ether)-N,N′-tetracetic acid. *Biochim Biophys. Acta* **267**:605–608
2. Bartschat, D.K., Lindenmayer, G.E. 1980. Calcium movements promoted by vesicles in a highly enriched sarcolemma preparation from canine ventricle. *J. Biol. Chem.* **255**:9626–9634
3. Bianchi, C.P. 1968. Cell Calcium. Buttersworth, London
4. Bianchi, C.P., Shanes, A.M. 1959. Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *J. Gen. Physiol.* **42**:805–815
5. Brandt, N.R., Caswell, A.H., Brunschwig, J.P. 1980. ATP-energized Ca^{2+} pump in isolated transverse tubules of skeletal muscle. *J. Biol. Chem.* **255**:6290–6298
6. Caputo, C., Bolãnos, P. 1978. Effect of external sodium and calcium on calcium efflux in frog striated muscle. *J. Membrane Biol.* **41**:1–14
7. Caroni, P., Carafoli, E. 1981. The Ca^{2+}-pumping ATPase of heart sarcolemma. *J. Biol. Chem.* **256**:3263–3270
8. Cosmos, E.E., Harris, E.J. 1961. *In vitro* studies of the gain and exchange of calcium in frog skeletal muscle. *J. Gen. Physiol.* **44**:1121–1130
9. Curtis, B.A. 1966. Ca^{2+} fluxes in single twitch muscle fibers. *J. Gen. Physiol.* **50**:255–267
10. Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375–400
11. Fleischer, S., Fleischer, B. 1967. Removal and binding of polar lipids in mitochondria and other membrane systems. *Methods Enzymol.* **10**:406–433
12. Gilbert, D.L., Fenn, W.O. 1957. Calcium equilibrium in muscle. *J. Gen. Physiol.* **40**:393–408
13. Gilbert, J.R. 1980. K^+, Na^+ and Cl^- permeability of sarcolemmal vesicles from rabbit skeletal muscle. *Fed. Proc* **39(6)**:2176
14. Goldbarg, J.A., Rutenberg, A.M. 1958. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. *Cancer* **11**:283–291
15. Hille, B. 1972. The permeability of the sodium channel to metal cations in myelinated nerve. *J. Gen. Physiol.* **59**:637–658
16. Horackova, M., Vassort, G. 1979. Sodium-calcium exchange in regulation of cardiac contractility. *J. Gen. Physiol.* **73**:403–424
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275
18. Malouf, N.N., Meissner, G. 1979. Localization of a Mg^{2+}- or Ca^{2+}-activated ("Basic") ATPase in skeletal muscle. *Exp. Cell Res.* **122**:233–250
19. McKinley, D., Meissner, G. 1978. Evidence for a K^+, Na^+ permeable channel in sarcoplasmic reticulum. *J. Membrane Biol.* **44**:159–186
20. Moore, B., Lentz, B.R., Meissner, G. 1978. Effects of sarcoplasmic reticulum Ca^{2+}-ATPase on phospholipid bilayer fluidity: Boundary lipid. *Biochemistry* **17**:5248–5254
21. Mullins, L.J. 1979. The generation of electric currents in cardiac fibers by Na/Ca exchange. *Am. J. Physiol.* **236**:C103–C110
22. Philipson, K.D., Nishimoto, A.Y. 1981. $Na^+ - Ca^{2+}$ exchange is affected by membrane potential in cardiac sarcolemmal vesicles. *J. Biol. Chem.* **255**:6880–6882
23. Philipson, K.D., Nishimoto, A.Y. 1981. Efflux of Ca^{2+} from cardiac sarcolemmal vesicles. *J. Biol. Chem.* **256**:3698–3702
24. Pitts, B.J.R. 1979. Stoichiometry of sodium-calcium exchange in cardiac sarcolemma vesicles. *J. Biol. Chem.* **254**:6232–6235
25. Reeves, J.P., Sutko, J.L. 1979. Sodium-calcium ion exchange in cardiac membrane vesicles. *Proc. Natl. Acad. Sci. USA* **76**:590–594
26. Reeves, J.P., Sutko, J.L. 1980. Sodium-calcium exchange activity generates a current in cardiac membrane vesicles. *Science* **208**:1461–1463
27. Reuter, H., Seitz, N. 1968. The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol. (London)* **195**:451–470
28. Rosenberger, L., Triggle, D.J. 1977. Calcium, calcium translocation, and specific calcium antagonists. *In:* Calcium in Drug Action. G.B. Weiss, editor. pp. 3–31. Plenum Press, New York
29. Schimmel, S.D., Kent, C., Bischoff, R., Vagelos, P.R. 1973. Plasma membranes from cultured muscle cells: Isolation procedure and separation of putative plasma-membrane marker enzymes. *Proc. Natl. Acad. Sci. USA* **70**:3195–3199
30. Tada, M., Yamamoto, T., Tonomura, Y. 1978. Molecular mechanism of active calcium transport by sarcoplasmic reticulum. *Physiol. Rev.* **58**:1–79
31. Yamamoto, S., Greeff, K. 1981. Effect of intracellular sodium on calcium uptake in isolated guinea-pig diaphragm and atria. *Biochim. Biophys. Acta* **646**:348–352