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Sodium–calcium exchange in transverse tubules isolated from frog skeletal muscle

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Transverse tubule vesicles isolated from frog skeletal muscle display sodium–calcium exchange activity, which was characterized measuring ^{45}Ca influx in vesicles incubated with sodium. The initial rates of exchange varied as a function of the membrane diffusion potentials imposed across the membrane vesicles, increasing with positive intravesicular potentials according to an electrogenic exchange with a stoichiometry greater than 2 sodium ions per calcium ion transported. The exchange activity was a saturable function of extravesicular free calcium, with an apparent $K_{0.5}$ value of 3 μM and maximal rates of exchange ranging from 3 to 5 nmol/mg protein per 5 s. The exchange rate increased when intravesicular sodium concentration was increased; saturation was approached when vesicles were incubated with concentrations of 160 mM sodium. The isolated transverse tubule vesicles, which are sealed with the cytoplasmic side out, had a luminal content of 112 ± 39 nmol calcium per mg protein. In the absence of sodium, the exchanger carried out electroneutral calcium–calcium exchange, which was stimulated by increasing potassium concentrations in the intravesicular side. Calcium–calcium exchange showed an extravesicular calcium dependence similar to the calcium dependence of the sodium–calcium exchange, with an apparent $K_{0.5}$ of 6 μM. Sodium–calcium and calcium–calcium exchange were both inhibited by amiloride. The sodium–calcium exchange system operated both in the forward and in the reverse mode; sodium, as well as calcium, induced calcium efflux from ^{45}Ca-loaded vesicles. This system may play an important role in decreasing the intracellular calcium concentration in skeletal muscle following electrical stimulation.

Introduction

The physiological action of calcium in triggering contraction in skeletal muscle requires maintaining an intracellular calcium concentration of about 10^{-7} M at rest. Following muscle stimulation, calcium is rapidly liberated from the terminal cisternae of the SR; the increase in its intracellular concentration to 10^{-6} M or higher, allows muscle contraction to proceed [1,2]. Relaxation takes place when the intracellular calcium concentration returns to its resting level by the action of the calcium pump of the SR, which reaccumulates calcium in the SR lumen [2].

In muscle cells at rest, there is a background influx of calcium into the cells down a steep electrochemical gradient [3]. Following stimulation, depolarization of the T-tubule membrane causes opening of voltage-sensitive calcium channels, which results in an influx of calcium that contributes to the intracellular calcium concentration during activity [4]. The SR has a considerable calcium storage capacity but, being a strict intracellular compartment, it is saturable. So, long-term regulation of intracellular calcium concentration depends on the extrusion of calcium across the cell membrane against its electrochemical gradient.

Two different calcium transport systems exist in the plasma membranes of most excitable cells: a calcium-pumping ATPase and a sodium–calcium exchange system [5]. Both have been found in membrane fractions isolated from rabbit [6] and pig skeletal muscle [7]. Furthermore, T-tubule membranes isolated from rabbit skeletal muscle possess a high-affinity ATP-dependent calcium transport system [8] but they lack a measurable sodium–calcium exchange activity, suggesting that in

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; NTA, nitrilotriacetic acid; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

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the rabbit the latter is probably located in the surface membrane.

In frog skeletal muscle, where most of the research on muscle physiology has been done, studies in intact skeletal fibers suggested that the sodium–calcium exchange system is an important calcium-transporting system [9]. However, there are no studies on calcium transport using isolated membrane preparations.

This work describes the presence of a sodium–calcium exchange system in highly purified T-tubule membranes isolated from frog skeletal muscle. This system shares several properties with the cardiac sarcolemmal sodium–calcium exchange system, and it may play an important role in the decrease of intracellular calcium concentration in frog skeletal muscle following stimulation.

Materials and Methods

Isolation of T-tubule membranes

T-tubules were isolated from frog skeletal muscle as described in detail elsewhere [10], except that the buffer used was 20 mM Tris-Hepes (pH 7.5). Membranes were frozen rapidly by immersion in solid CO_2/acetone and were stored frozen at −20 °C. T-tubule membranes obtained by this procedure have a high cholesterol and phospholipid content and a characteristic protein composition as evidenced by their electrophoretic pattern in SDS-containing polyacrylamide gels. They also exhibit a high density of nitrendipine and ouabain-binding sites and they are devoid of SR contamination, as indicated by the protein composition and the lack of measurable Ca^{2+}-ATPase activity [10,11].

Sodium-dependent calcium uptake

Preincubation of vesicles. T-tubule vesicles were incubated at 25 °C for 1.5 h in 100 mM NaCl/0.28 M sucrose/20 mM Tris-HCl (pH 7.5), with or without 5–10 mM KCl. As controls, we used vesicles in which NaCl was replaced by 100 mM choline chloride or, in some experiments, with 100 mM KCl, or 100 mM LiCl or only with 0.28 M sucrose/20 mM Tris-HCl (pH 7.5). We chose 1.5 h as incubation time, because experiments with ^{22}NaCl and ^{14}C-choline chloride showed that in 1.5 h equilibrium was reached. Longer incubation times did not show differences in the amount of calcium taken up after dilution in a calcium-containing solution.

Calcium uptake. At the end of the preincubation period vesicles were diluted (60–100-fold) in a solution containing 0.1 mM $^{45}CaCl_2$ (10–15 μCi/μmol)/0.28 M sucrose/20 mM Tris-HCl (pH 7.5)/1 μM valinomycin and KCl at variable concentrations. Osmolarity was kept constant with choline chloride. Incubation temperature was 25 °C. To stop the exchange reaction, 0.3 ml fractions were added at different times to 0.3 ml of an ice-cold solution containing 5 mM $MgCl_2$/10 mM EGTA/20 mM Tris-HCl (pH 7.5) (quench solution). The change in osmolarity following dilution from the reaction solution to the quench solution did not affect the amount of calcium transported by the vesicles. After addition of the quench solution, 0.5 ml fractions were filtered under suction through Millipore filters (HA, 0.45 μm) and the filters were washed three times with 3 ml of ice-cold quench solution. The filters had been previously soaked with 0.1 mM $CaCl_2$/5 mM $MgCl_2$/20 mM Tris-HCl (pH 7.5).

This procedure lowers nonspecific calcium binding to the filter, yielding very low blanks. The time from quenching to filtration and washing was about 20 s. The filters were dried and the radioactivity was determined in a liquid scintillation counter.

In most experiments, the initial rate of exchange was measured with reaction times of 5, 10 and 15 s, using the procedure described by Reeves and Sutko [12]: 3–6 μl of sodium-incubated vesicles were placed as a drop on the side of a polystyrene tube containing 300 μl of uptake solution, and the reaction was begun by touching the tube on a vortex allowing both solutions to mix. The uptake reaction was stopped by adding ice-cold quench solution as described above. In most experiments, rates were determined in quadruplicate.

Calcium efflux

Equilibrium of T-tubule vesicles with ^{45}Ca. T-tubule vesicles were incubated at 25 °C with 2 mM $CaCl_2$ containing $^{45}CaCl_2$ (20–30 μCi/μmol) and 30 mM KCl/70 mM choline chloride in 0.23 M sucrose and 20 mM Tris-HCl (pH 7.5). The time-course of calcium entry was determined by diluting aliquots of the reaction solution with quench solution and filtering through Millipore filters as above.

Calcium efflux. Vesicles loaded with ^{45}Ca were diluted (30–50-fold) in 10 mM EGTA/30 mM KCl/0.23 M sucrose/20 mM Tris-HCl (pH 7.5) and either 70 mM NaCl or 70 mM choline chloride. Alternatively, vesicles were diluted in a solution containing 30 mM KCl/70 mM choline chloride/0.95 mM $CaCl_2$/1 mM HEDTA/0.23 M sucrose/20 mM Tris-HCl (pH 7.5)/valinomycin 1 μM. This solution had a free calcium concentration of 20 μM, as measured directly with a calcium electrode [13]. The amount of ^{45}Ca remaining in the vesicles was determined after filtration through Millipore filters and washing with a solution similar to quench solution, except that 5 mM $MgCl_2$ was replaced by 5 mM $LaCl_3$.

Determination of free calcium concentration

Free-calcium concentrations were controlled with Calcium-HEDTA (for free calcium concentrations up to 20 μM) and with calcium-NTA buffers (for higher concentrations). Ligand concentration was 1 mM and free calcium concentration was calculated using the

computer program described by Goldstein [14] and the constants described by Martell [15]. Free calcium was measured with calcium electrodes [13]. The free calcium concentrations indicated in Fig. 6 correspond to this measured free calcium concentration.

Determination of the endogenous calcium content in T-tubule vesicles

Total calcium content in the vesicles was determined by atomic absorption spectrophotometry in a Perkin-Elmer 303 spectrophotometer. Determination was done in the presence of 0.4% $SrCl_2$ and 0.1 M $HClO_4$. Vesicles were ashed prior to calcium determination.

Protein determination

Protein concentration was determined according to Hartree [16], using bovine serum albumin as standard.

Reagents

Amiloride was added from a stock solution in dimethylsulfoxide. Final concentration of dimethylsulfoxide was 2% by volume, which did not affect the exchange reaction. Ionophores A23187 and monensin were added in ethanol. Final ethanol concentration was 1%, and did not affect the exchange reaction. All the reagents used were of analytical grade. Ionophores A23187, valinomycin and monensin were obtained from Calbiochem. Amiloride was obtained from Sigma and $^{45}CaCl_2$ from Amersham.

Results

Calcium uptake in T-tubule vesicles

T-tubule vesicles incubated with sodium took up calcium when diluted in a sodium-free solution containing 0.1 mM $^{45}CaCl_2$ (Fig. 1A). Calcium was rapidly transported in the presence of a sodium gradient and apparent equilibrium was obtained after 4 min reaching final calcium levels between 8 and 14 nmol/mg protein. This calcium was rapidly liberated by the ionophore A23187, indicating that it had been transported to the intravesicular space. Linear rates of calcium uptake were observed until 15 s (Fig. 1B). Gradual replacement of intravesicular sodium with potassium led to a progressive decrease in calcium uptake (Fig. 2). However, there was still appreciable calcium uptake when sodium was completely replaced by potassium (Fig. 2, Table I) or by other monovalent ions, such as lithium or choline. Likewise, similar levels of calcium uptake were observed when the sodium gradient was dissipated with monensin or when no monovalent ions, except for Tris-HCl, were added during the preincubation of the vesicles (Table I).

Calcium uptake in sodium-incubated vesicles was 90% inhibited by amiloride (Table I). A similar percent of inhibition was observed in choline- or lithium-incubated vesicles (Table I).

It is likely that in the presence of a sodium gradient, calcium uptake proceeded by sodium–calcium exchange, whereas in the presence of other monovalent ions, calcium uptake proceeded by calcium–calcium exchange. In this case, calcium inside the vesicles (en-

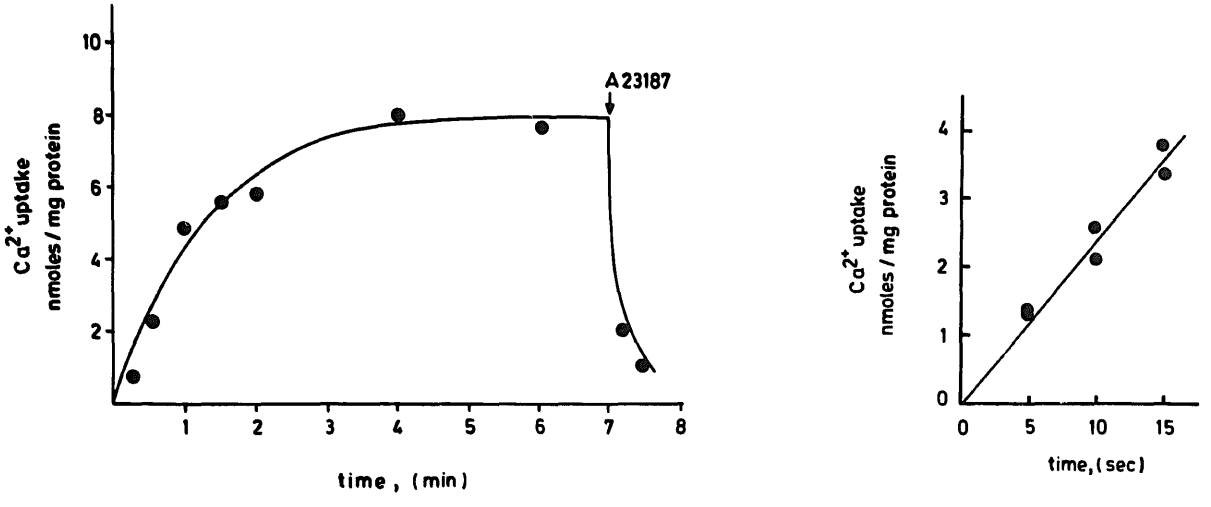

Fig. 1. (A) Calcium uptake in sodium incubated T-tubule vesicles from frog muscle. Vesicles were incubated in 100 mM NaCl/0.28 M sucrose/20 mM Tris-maleate (pH 7.0) for 1.5 h at 25 °C, and were diluted in 0.1 mM $^{45}CaCl_2$/100 mM KCl/0.28 M sucrose/20 mM Tris-maleate (pH 7.0). The reaction was stopped with quench solution (see text) at the indicated times and the vesicles were harvested by filtration as described under Materials and Methods. The final concentration of A23187 used was 6 μM. (B) Initial rates of calcium transport in sodium-incubated T-tubule vesicles. Vesicles were incubated in 100 mM NaCl/10 mM KCl/0.28 M sucrose/20 mM Tris-HCl (pH 7.5). Calcium uptake was measured by diluting 5 μl of sodium-incubated vesicles in 300 μl of a solution containing 0.1 mM $^{45}CaCl_2$/100 mM KCl/1 μM valinomycin/0.28 M sucrose/20 mM Tris-HCl (pH 7.5). The reaction was stopped by adding 300 μl of quench solution (see text) and vesicles were harvested by filtration as described under Materials and Methods. A T-tubule preparation different to that shown in Fig. 1A was used.

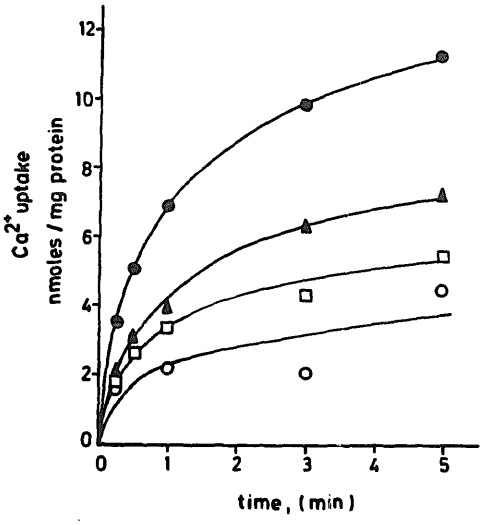

Fig. 2. Effect of intravesicular sodium. Vesicles were incubated in 0.28 M sucrose/20 mM Tris-HCl (pH 7.5) plus: 100 mM NaCl/20 mM KCl (●); 50 mM NaCl/70 mM KCl (▲); 20 mM NaCl/100 mM KCl (□); 120 mM KCl (○). Vesicles were diluted in 0.1 mM $^{45}CaCl_2$/120 mM KCl/0.28 M sucrose/20 mM Tris-HCl (pH 7.5). The reaction was stopped at different times and the intravesicular calcium was determined as described in the text.

dogenous calcium) would be required to exchange with extravesicular calcium. To investigate this possibility we measured the endogenous calcium content of the vesicles by atomic absorption spectrophotometry. The calcium content in eight different preparations of T-tubule vesicle was 112 ± 39 nmol/mg protein and it did not change after the sodium loading procedure. We do not know how much of this intravesicular calcium is free, but, given its high content, only a fraction of free calcium would be sufficient to carry out calcium–calcium exchange (see Discussion).

Table I

Effect of monovalent ions and amiloride on calcium uptake in T-tubule vesicles

Vesicles were incubated in 5 mM KCl/0.28 M sucrose/20 mM Tris-HCl (pH 7.5), plus the additions indicated above. Calcium uptake was measured by dilution in 0.1 mM $^{45}CaCl_2$/105 mM KCl/1 μM valinomycin/0.28 M sucrose/20 mM Tris-HCl (pH 7.5). The rates of ^{45}Ca uptake were measured as described in the text. Rates of 100%, obtained by diluting vesicles incubated with 100 mM NaCl, ranged between 1.2 and 3.5 nmol/mg protein per 5 s. Values represent the mean ± S.D.

Additions to the incubation solution		Rate of calcium uptake (%)	Number of experiments
NaCl	100 mM	100	
NaCl	100 mM + 10 μM monensin	45.0 ± 8.6	4
NaCl	100 mM + 10 mM amiloride	9.1 ± 2.3	7
KCl	100 mM	41.7 ± 9.0	6
LiCl	100 mM	49.0 ± 8.9	4
LiCl	100 mM + 10 mM amiloride	25.9 ± 5.7	3
Choline Cl	100 mM	33.1 ± 4.6	3
Choline Cl	100 mM + 10 mM amiloride	13.9 ± 5.7	3
None		34.4 ± 5.6	3

Electrogenicity and potential-dependence of calcium uptake

Most evidence indicates that sodium–calcium exchange is electrogenic [17,18,19] with a stoichiometry of 3 sodiums per each calcium exchanged [20,21]. In consequence, sodium-dependent calcium uptake involves a net outward movement of positive charge that tends to inhibit further exchange. This effect can be overcome by imposing a constant membrane potential, inside positive, during the exchange. In many vesicular systems, a diffusion potential can be established for several seconds with potassium gradients in the presence of valinomycin. To investigate if an inside-negative potential is generated in T-tubule vesicles, as a consequence of calcium uptake in sodium-incubated vesicles, initial rates of calcium uptake were measured in the presence of a potassium gradient ($K_i = 10$ mM; $K_e = 100$ mM) with or without 1 μM valinomycin. Sodium-incubated vesicles exhibited an initial rate of uptake in the presence of valinomycin that was 50% higher than in its absence. In contrast, the presence of valinomycin made no difference on the initial rate of calcium uptake in the vesicles incubated with choline. This result suggests an

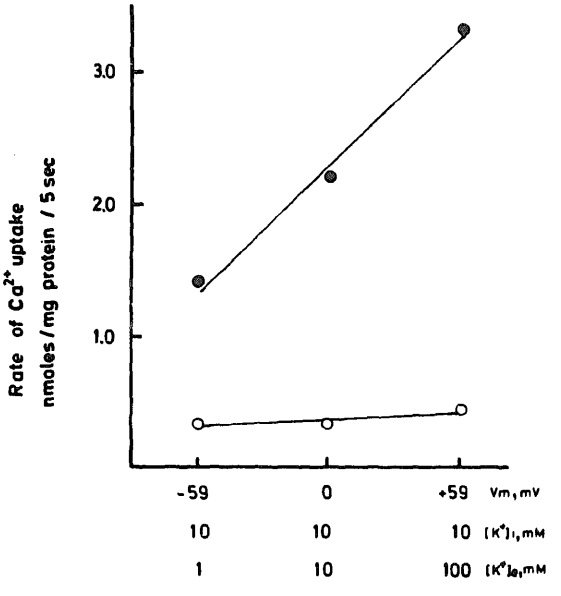

Fig. 3. Effect of membrane potential on calcium uptake. Vesicles were incubated in 0.28 M sucrose/20 mM Tris-HCl (pH 7.5) with 100 mM NaCl/10 mM KCl (●) or 100 mM choline chloride/10 mM KCl (○) and were diluted in 0.1 mM $^{45}CaCl_2$/0.28 M sucrose/20 mM Tris-HCl (pH 7.5)/1 μM valinomycin, containing different concentrations of KCl to obtain the indicated membrane potentials. Ionic strength was kept constant with choline chloride. Potentials are given as inside minus outside.

electrogenic process in the sodium-incubated vesicles and an electroneutral exchange in the vesicles incubated with choline.

In order to investigate the potential-dependence of the calcium uptake in T-tubule vesicles, initial rates of exchange in the presence of different potassium gradients and valinomycin were measured. In the experiment shown in Fig. 3, the potassium diffusion potentials used were −59 mV ($K_i = 10$ mM, $K_e = 1$ mM), 0 mV ($K_i = K_e = 10$ mM) and 59 mV ($K_i = 10$ mM, $K_e = 100$ mM). Ionic strength was kept constant with choline chloride. As shown in Fig. 3, initial rates of uptake increased by 50% when the membrane potential increased from 0 to +59 mV and decreased by about the same magnitude if the change in membrane potential had the opposite sign. The same change in membrane potential had no effect on calcium uptake in choline-incubated vesicles (Fig. 3).

Effects of potassium on calcium uptake

Some studies have suggested that potassium per se activates the exchanger and that the activation seen by an inside-positive potential is caused by the increase in potassium used to establish the membrane potential [22]. To discover whether this is the case in our T-tubule vesicles we investigated the effect of different potassium concentrations on calcium uptake in sodium-incubated vesicles, keeping constant the K_i/K_e ratio in order to maintain the membrane diffusion potential constant. Fig. 4 (solid circles) shows no effect of increasing potassium concentration when $K_i/K_e = 1$, i.e., when the membrane potential was 0 mV. No effect was seen either when $K_i/K_e = 0.1$, i.e., the membrane potential was +59 mV (not shown).

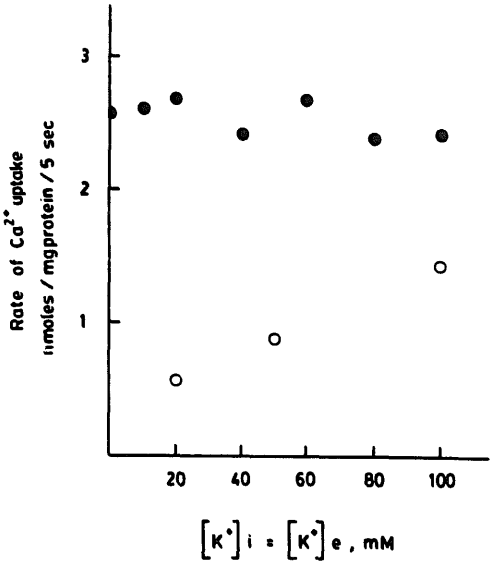

Fig. 4. Effect of potassium on calcium uptake. Vesicles were incubated with 100 mM NaCl (●) or 100 mM choline chloride (○) and different concentrations of KCl in 0.28 M sucrose/20 mM Tris-HCl (pH 7.5) and were diluted in 0.1 mM $^{45}CaCl_2$/0.28 M sucrose/20 mM Tris-HCl (pH 7.5) and the same KCl concentration as in the previous incubation to keep $E_k = E_m = 0$ mV in the presence of 1 μM valinomycin. Ionic strength was kept constant with choline chloride.

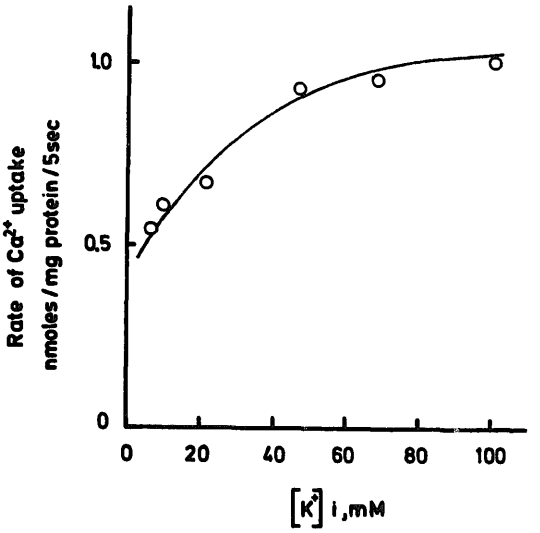

Fig. 5. Effect of intravesicular potassium on calcium uptake in the absence of sodium. Vesicles were incubated with different concentrations of KCl in 0.28 M sucrose/20 mM Tris-HCl (pH 7.5), and were diluted in 0.1 mM $^{45}CaCl_2$/100 mM KCl/1 μM valinomycin/0.28 M sucrose/20 mM Tris-HCl (pH 7.5). Choline chloride was used to keep the ionic strength constant.

In cardiac sarcolemma [23,24] and in squid axon [25], it has been shown that potassium, lithium and other monovalent ions activate calcium–calcium exchange. In T-tubule vesicles, intravesicular potassium increased the initial rate of calcium uptake in choline-incubated vesicles. The experiment shown in Fig. 4 (open circles) was performed at 0 mV ($K_i = K_e$), keeping the ionic strength constant with choline chloride. When the intravesicular potassium concentration was varied, keeping the extravesicular potassium constant, we obtained the same effect on the initial rate of calcium uptake as when potassium was changed on both sides (Fig. 5). In this experiment the calculated membrane potential was different in each case, with values in the range 0–70 mV. Since calcium uptake in choline-incubated vesicles was not potential-sensitive, these results indicate that the activating effect is due to intravesicular potassium.

Extravesicular calcium dependence of calcium uptake

Fig. 6 shows the dependence on free calcium concentration of the initial rate of calcium uptake in sodium-incubated T-tubule vesicles. In this experiment, the free calcium concentration was controlled with HEDTA (for free calcium up to 10 μM) and NTA (for upper free calcium concentration). Membrane potential was +59 mV. As shown in Fig. 6, the initial rate of exchange was a hyperbolic function of extravesicular free calcium concentration, with an apparent $K_{0.5}$ of 3.0 μM and a V_{max} of 2.7 nmol/mg protein per 5 s. A

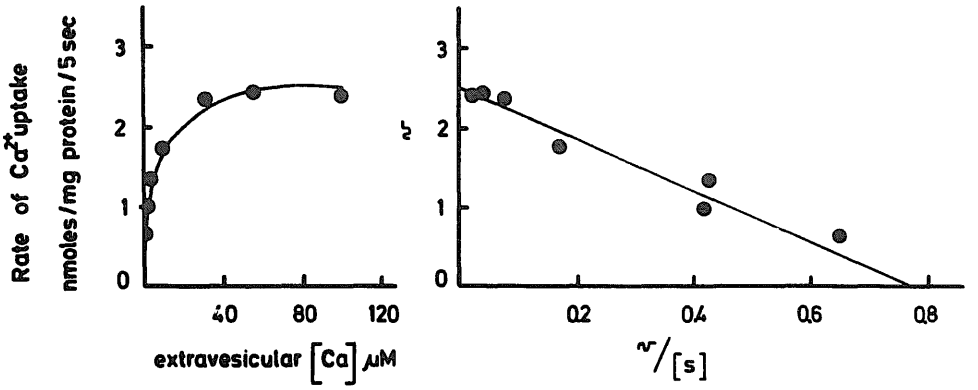

Fig. 6. Calcium dependence of calcium uptake in sodium-loaded vesicles. Vesicles were incubated with 100 mM NaCl/10 mM KCl in 0.28 M sucrose/20 mM Tris-HCl (pH 7.5). Calcium uptake was determined by dilution in 100 mM KCl/10 mM choline chloride/1 μM valinomycin/0.28 M sucrose/20 mM Tris-HCl (pH 7.5), plus variable concentrations of $^{45}CaCl_2$. $^{45}CaCl_2$-HEDTA and $^{45}CaCl_2$-NTA buffers were used to obtain the free calcium concentrations indicated.

similar kinetic behavior was observed for the initial rate of calcium uptake in choline-incubated vesicles. The apparent $K_{0.5}$ determined was 5.9 μM (data not shown).

Intravesicular sodium-concentration dependence of calcium uptake

The dependence of Na/Ca exchanger on intravesicular sodium concentration was determined by incubating the vesicles at different sodium concentrations, keeping constant the total ion concentration with choline chloride. The rate of calcium uptake increased when the sodium concentration used in the preincubation stage was increased (Fig. 7). In the absence of sodium, the initial rate of calcium uptake was approx. 1/3 of the rate found in 160 mM sodium, the highest concentration used. If we assume that (a) the rate of calcium uptake in the absence of sodium represents the rate of calcium–calcium exchange and (b) this rate is independent of the internal sodium concentration, it is possible to subtract the basal rate of calcium–calcium exchange. The curve obtained after this correction showed an apparent $K_{0.5}$ for sodium of 72 mM (not shown).

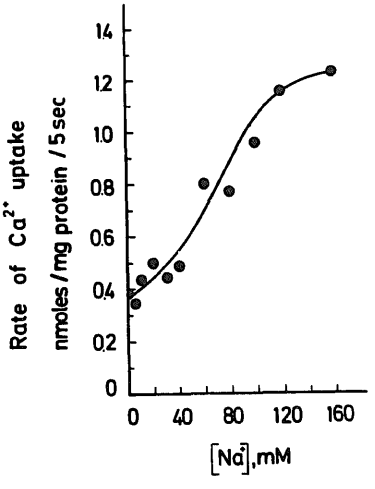

Fig. 7. Intravesicular sodium dependence of calcium uptake. Vesicles were incubated with 5 mM KCl and the indicated sodium concentrations, in 0.28 M sucrose/20 mM Tris-HCl (pH 7.5). Calcium uptake was measured by dilution in 0.1 mM $^{45}CaCl_2$/100 mM KCl/60 mM choline chloride/1 μM valinomycin/0.28 M sucrose/20 mM Tris-HCl (pH 7.5).

Calcium efflux from T-tubule vesicles incubated with $^{45}CaCl_2$

In order to study the reversibility of the sodium–calcium exchange system, we equilibrated the vesicles with ^{45}Ca prior to dilution in sodium-contain-

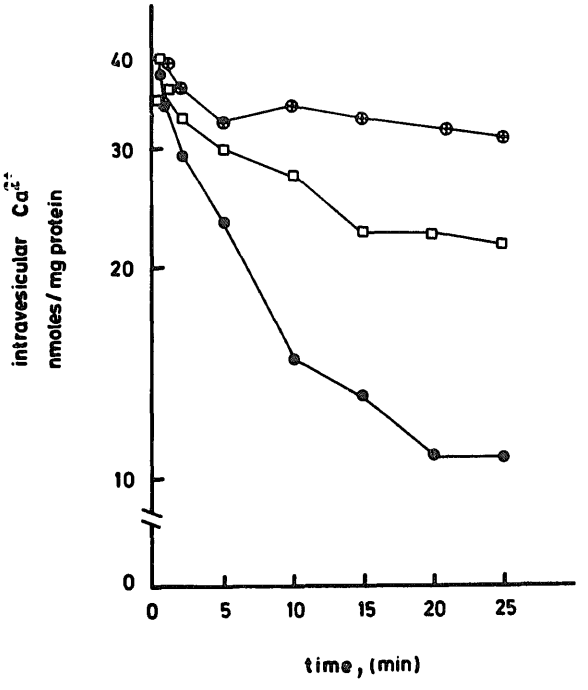

Fig. 8. Calcium efflux from vesicles incubated with $^{45}CaCl_2$. Effect of extravesicular sodium and calcium. Vesicles were equilibrated with 2 mM $^{45}CaCl_2$ 30 mM KCl/70 mM choline chloride/0.24 M sucrose/20 mM Tris-HCl (pH 7.5). Efflux was measured by dilution in 70 mM NaCl/10 mM EGTA (□); 70 mM choline chloride 10 mM EGTA (⊕), or in $CaCl_2$-HEDTA buffer yielding 20 μM free calcium concentration (●). The efflux solution in addition containing 30 mM KCl 1 μM valinomycin/0.24 M sucrose/20 mM Tris-HCl (pH 7.5).

ing or sodium-free solutions. The amount of calcium taken up by the vesicles after a 6 h incubation period was a hyperbolic function of the external calcium concentration; saturation was calculated at 83 nmol/mg protein (data not shown). This value is within the range of intravesicular calcium content determined experimentally (112 ± 39 nmol/mg protein). In the experiment shown in Fig. 8, vesicles equilibrated for 3 h with 2 mM $^{45}CaCl_2$ were diluted in calcium-free solution containing sodium or choline, or in a solution containing choline and 20 μM free calcium. Sodium as well as calcium promoted the rapid efflux of the ^{45}Ca trapped inside the vesicles, whereas choline failed to promote calcium efflux (Fig. 8). The results suggest that the exchanger acts reversibly and it catalyzes sodium-inside calcium uptake, sodium-outside calcium efflux, as well as calcium–calcium exchange.

Discussion

Sodium–calcium exchange

The results obtained in this work are consistent with the presence of sodium–calcium exchange in T-tubule membranes from amphibian skeletal muscle. The T-tubule vesicles used in this study have the inside-out orientation [10]. Thus, calcium uptake by sodium-loaded vesicles reflects the calcium-extruding capacity of these membranes from the cytoplasm to the extracellular medium.

That the observed calcium accumulation is due to sodium–calcium exchange is supported by the fact that replacement of intravesicular sodium by potassium gradually decreases calcium uptake, and also by the effect of monensin, a sodium ionophore, which lowers calcium uptake to the level observed in the absence of sodium. The lower-calcium uptake observed following sodium replacement by potassium is not due to an inhibitory effect of intravesicular potassium, since increasing intravesicular potassium increases calcium uptake in the absence of sodium (see below). Furthermore, similar values of uptake were obtained when LiCl, choline chloride or KCl replaced NaCl (Table I).

In contrast to cardiac sarcolemmal vesicles where very little calcium accumulation is observed in the absence of a sodium gradient [12], in T-tubule vesicles it accounts for 30–50% of the level obtained with a sodium gradient (Fig. 2, Table I). However, the exchange system in T-tubule vesicles shows a low rate of exchange compared to the rates reported in the literature for cardiac sarcolemmal vesicles, where the exchange rates are on average 30-times greater than in T-tubule vesicles. It is conceivable that the sodium–calcium exchange system is a minor membrane component in frog skeletal muscle, although in vivo conditions may account for rates higher than those found in vitro. The use of vesicles in transport studies has the disadvantage that due to their small size, intravesicular concentrations change very rapidly and initial velocities are difficult to measure. As far as our detection methods allow, our measurements were done on the initial rate (Fig. 1B), most of them within 5 or 10 s of uptake. Furthermore, rates of exchange similar to these of isolated T-tubule membranes have been reported in chicken cardiac sarcolemma [26], pituitary plasmatic membrane [27] and bovine adrenal cells [28].

The response of the sodium-dependent calcium uptake in T-tubule vesicles to valinomycin-induced membrane potential is consistent with an electrogenic process, with at least 3 sodium ions exchanging for each calcium. The relatively low stimulation obtained after valinomycin addition might reflect a high intrinsic potassium permeability in T-tubule membranes, so that a positive intravesicular potential may develop even in the absence of valinomycin.

The electrogenic nature of the exchange is further supported by the 240% increase of activity obtained by changing the intravesicular membrane diffusion potential from -59 to $+59$ mV. Although we did not measure the membrane potential obtained, we assume that, in the short time used to measure the uptake (15 s), no appreciable dissipation of the potassium gradient had taken place. If this had been the case, non-linear rates of exchange, instead of the linear rates we measured in 15 s, would have been obtained. Changes in potassium concentration had no effect on sodium–calcium exchange; in consequence, the effect of the potassium gradient can be ascribed entirely to membrane potential.

Sodium–calcium exchange in this preparation shows a relatively high affinity for calcium (apparent $K_{0.5} = 3$ μM). Although affinity constants of this magnitude have been determined in other cells, values reported for cardiac sarcolemmal sodium–calcium exchange systems are, on the average, one order of magnitude higher. Moreover, there is a broad range of affinity constants reported, with differences up to two orders of magnitude among them. This variability has been attributed to the many factors that modify the exchanger activity increasing its calcium affinity. One of these factors is the intravesicular calcium activation, which modifies the kinetic behavior from a linear relationship between initial rate and external calcium concentration, which shows no saturation in EGTA-treated vesicles, to Michaelis-Menten kinetics in vesicles treated with calcium [29]. If the same regulation operates in skeletal muscle, the high endogenous calcium content in our T-tubule vesicles can account for the low $K_{0.5}$ reported by maintaining a fully activated exchange system. Moreover, in many cases, constants were determined without calcium buffers. The impossibility of keeping precise micromolar calcium concentrations under these conditions can account for the differences reported. The

sodium–calcium exchange reaction seems to have a sigmoidal dependence on the internal sodium concentration, suggesting a high degree of cooperativity in the binding of sodium. This would be expected if the stoichiometry of the exchanger is 3 sodium ions per each calcium transported.

Calcium–calcium exchange

It is likely that calcium uptake in the absence of sodium proceeds by calcium–calcium exchange with endogenous intravesicular calcium; the similar degree of amiloride inhibition of calcium uptake obtained in vesicles loaded with sodium, or with other monovalent ions, suggests that the same transporter may be operating in both cases. The lack of valinomycin effect and the insensitivity to changes in membrane potential from −59 to +59 mV in choline-loaded vesicles are in agreement with the operation of an electroneutral mechanism of uptake. Furthermore we obtained similar calcium affinities for calcium–calcium exchange (calcium uptake in choline-loaded vesicles) and sodium–calcium exchange, suggesting that the external calcium affinity of the exchanger is independent of internal sodium.

In order to carry out calcium–calcium exchange, the isolated T-tubule vesicles must have intravesicular free calcium at a concentration high enough to carry out the exchange reaction. We found that the T-tubule vesicles had a very high calcium content (112 nmol/mg protein); assuming an intravesicular lumen of 4 μl per mg protein, similar to that of SR vesicles [30], this calcium content would correspond to a free intravesicular calcium concentration of 28 mM. Most likely, a large fraction of this calcium is bound to luminal T-tubular components, a finding which may have important physiological implications. But even if 99% of this calcium is bound, the remaining free calcium would have a concentration of 0.28 mM, and in other vesicular systems only micromolar calcium concentrations are required to carry out efficient calcium–calcium exchange [23].

Calcium uptake in choline-loaded vesicles was activated by intravesicular potassium. In many systems it has been shown that calcium–calcium exchange is activated by monovalent cations, including potassium, lithium or even sodium in low concentrations [23,24]. It is likely that this potassium-activated calcium uptake in T-tubule vesicles proceeds via calcium–calcium exchange with endogenous calcium.

External calcium promoted calcium efflux from ^{45}Ca-loaded vesicles, which further supports the idea that calcium uptake in the absence of sodium takes place via calcium–calcium exchange. Although sodium was also able to induce calcium efflux, indicating that the exchanger also operates in the reverse mode, it was less effective than calcium. The most likely explanation for this finding is that the T-tubules have intravesicular calcium-binding sites, which exchange efficiently with external calcium but not with sodium. Alternatively, the sodium concentration inside the vesicles might rapidly reach a value high enough to inhibit further exchange [12]. In any case, it has been shown in the squid axon [31] that the exchanger exhibits an asymmetrical behavior with precludes straightforward comparisons between the forward and the reverse modes of the exchange reaction.

From the results presented here, we can conclude that T-tubule membranes from skeletal muscle display sodium–calcium (and calcium–calcium) exchange activity. It is probable that this system plays a role in the regulation of intracellular calcium concentration after increased muscular activity or after tetanic stimulation, conditions which cause a significant increase in intracellular calcium concentration.

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