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Sodium/calcium exchange in amphibian skeletal muscle fibers and isolated transverse tubules

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Cifuentes, Fredy, Julio Vergara, and Cecilia Hidalgo. Sodium/calcium exchange in amphibian skeletal muscle fibers and isolated transverse tubules. Am J Physiol Cell Physiol 279: C89–C97, 2000.—The Na+/Ca2+ exchanger participates in Ca2+ homeostasis in a variety of cells and has a key role in cardiac muscle physiology. We studied in this work the exchanger of amphibian skeletal muscle, using both isolated inside-out transverse tubule vesicles and single muscle fibers. In vesicles, increasing extravascular (intracellular) Na+ concentration cooperatively stimulated Ca2+ efflux (reverse mode), with the Hill number equal to 2.8. In contrast to the stimulation of the cardiac exchanger, increasing extravascular (cytoplasmic) Ca2+ concentration ([Ca2+]i) inhibited this reverse activity with an IC50 of 91 nM. Exchanger-mediated currents were measured at 15°C in single fibers voltage clamped at −90 mV. Photolysis of a cytoplasmic caged Ca2+ compound activated an inward current (forward mode) of 28 ± 10 nA (n = 3), with an average current density of 0.6 μA/μF. External Na+ withdrawal generated an outward current (reverse mode) with an average current density of 0.36 ± 0.17 μA/μF (n = 6) but produced a minimal increase in cytosolic [Ca2+]i. These results suggest that, in skeletal muscle, the main function of the exchanger is to remove Ca2+ from the cells after stimulation.

intracellular calcium regulation; electrogenic ion transport; calcium fluxes; calcium permeability; plasma membrane transporters

The sodium/calcium exchanger (NCX) is an electrogenic and reversible countertransport system with a well-established role in Ca2+ homeostasis in a variety of cells (6). In its forward mode, the exchanger transports Ca2+ against its transmembrane electrochemical gradient, making use of the Na+ electrochemical gradient. Given the right balance between the respective electrochemical gradients, the NCX also operates in the reverse mode, transporting Ca2+ into cells and Na+ out (6).

Three mammalian isoforms of the NCX protein, products of three different genes, have been cloned (28, 31, 32) and appear to have very similar properties (29). Mammalian cardiac muscle expresses high levels of the NCX1 isoform (31), whereas amphibian cardiac muscle has an NCX that presents a novel molecular determinant that causes different regulation by cAMP from that observed in mammalian cardiac muscle (37). The mammalian NCX1 isoform has been found in varying amounts in most other tissues, including kidney, brain, pancreas, liver, placenta, and skeletal muscle, where it is present both in transverse tubule (T tubule) and surface plasma membranes (35). In addition, the mammalian isoforms NCX2 (28) and NCX3 (32) are present in skeletal muscle and brain.

Most of the current knowledge of NCX properties has been obtained from the many studies performed on mammalian cardiac muscle, where the NCX plays a central role in transporting Ca2+ out of the cells during cardiac muscle relaxation (6, 34). By allowing Ca2+ entry during membrane depolarization, the exchanger might also participate in cardiac excitation-contraction coupling, but this alleged role remains controversial (6).

The NCX of skeletal muscle has been less studied than its cardiac counterpart, and a clear-cut physiological role has not been defined yet. Small bundles of muscle fibers from amphibian skeletal muscle (7), as well as single muscle fibers (22), exhibit Na+-dependent Ca2+ fluxes. This trait is shared by sarcolemmal fractions isolated from mammalian muscle (14, 30) and by T tubules isolated from amphibian muscle (10, 18). The NCX present in T tubules from amphibian muscle displays in the forward mode a Michaelis constant of 2.7 μM for intracellular Ca2+ (10). In agreement with this rather low Ca2+ affinity, to detect Na+-dependent Ca2+ efflux in amphibian single fibers, it is necessary to increase the intracellular Ca2+ concentration ([Ca2+]i) well above its resting level (22). The reverse mode of the exchanger may cause the enhancement of contraction that takes place after external Na+ withdrawal in phasic (8, 13, 17, 26) or tonic (24) amphibian skeletal muscle fibers.

In mammalian skeletal muscle, exchanger reverse currents, associated with net Na+ efflux, have been measured in giant inside-out excised sarcolemmal patches (17). However, these currents are of a lower magnitude than those observed in heart (10). This difference might be due to the fact that amphibian skeletal muscle fibers are relatively smaller than cardiac fibers. The amphibian exchanger might also present properties different from those of its mammalian homologues.

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magnitude than their cardiac counterparts measured under similar conditions (20). Recent experiments suggest that intact single skeletal muscle fibers from the mouse have an NCX that becomes activated in its forward mode when cytoplasmic [Ca\textsuperscript{2+}] is increased to levels similar to those produced by tetanic stimulation (3). Other observations, albeit of a more indirect nature, also support the presence of a functional NCX in mammalian skeletal muscle (4, 39).

The purpose of this work was to further characterize the NCX transporter of amphibian muscle, using both isolated sealed inside-out T tubule vesicles and single fibers. Vescicular experiments indicate that increasing extravesicular (intracellular) Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]) from 0 to 120 mM resulted in a marked and cooperative stimulation of Ca\textsuperscript{2+} efflux from the vesicles (reverse mode). Increasing extravesicular (intracellular) [Ca\textsuperscript{2+}] inhibited the reverse skeletal NCX activity in contrast to the stimulation reported in cardiac muscle. In single fibers, photolysis of a cytoplasmic caged Ca\textsuperscript{2+} compound activated an inward current, as expected from the exchanger operating in its forward mode. Na\textsuperscript{+} withdrawal from the external solution activated an outward current, indicative of the reverse mode, but produced only a very modest increase in cytosolic [Ca\textsuperscript{2+}].

**MATERIALS AND METHODS**

**Isolation and Characterization of Amphibian Membrane Fractions**

T tubule vesicles were isolated from the skeletal muscle of the Chilean frog *Caudiverbera caudiverbera* using a procedure described in detail elsewhere (19). After isolation, vesicles were resuspended in 0.3 M sucrose and 20 mM Tris-maleate, pH 7.0, frozen in liquid nitrogen, and stored at −80°C for up to 1 mo. The density of dihidropyridine and ouabain binding sites and vesicle sidedness were determined as described in previous work (19, 25).

**Vesicular Ca\textsuperscript{2+} Fluxes**

To determine Ca\textsuperscript{2+} influx, T tubule vesicles in 0.3 M sucrose and 20 mM Tris-maleate, pH 7.0, were diluted 5- to 10-fold to 0.1 mg protein/ml in loading solution. The loading solution contained variable 45CaCl\textsubscript{2} at a specific activity of 15–20 mCi/mmol, 10 mM potassium gluconate, 150 mM HEPES-Tris, pH 7.4, plus 1 μM valinomycin. After dilution, vesicles were incubated for 3 h at the temperatures specified in text. To determine exchanger-mediated Ca\textsuperscript{2+} influx, the vesicles were incubated for 3 h as above, except that the loading solution contained 2 mM nonradioactive CaCl\textsubscript{2}; after this time, an aliquot of 45CaCl\textsubscript{2} was added, and the incubation was continued for up to 15 min. To stop Ca\textsuperscript{2+} influx, a 0.01-ml fraction of the incubation solution was mixed with 1 ml of an ice-cold solution (quench solution) containing (in mM) 5 MgCl\textsubscript{2}, 10 EGTA-Tris, 10 LaCl\textsubscript{3}, and 20 HEPES-Tris, pH 7.4. A 0.9-ml fraction was immediately filtered through Millipore filters (HA 0.45 μm) previously soaked with (in mM) 0.1 CaCl\textsubscript{2}, 5 MgCl\textsubscript{2}, and 20 HEPES-Tris, pH 7.4. The filters were washed three times with 3 ml of quench solution, and the radioactivity retained in the dried filters was determined by liquid scintillation counting. For Ca\textsuperscript{2+} efflux determinations, T tubule vesicles (1 mg/ml) were first equilibrated for 3 h at the temperatures specified in the text in a loading solution containing (in mM) 10 potassium gluconate, 2 CaCl\textsubscript{2} plus 45CaCl\textsubscript{2} to a specific activity of 15–20 mCi/mmol, and 150 HEPES-Tris, pH 7.4. All efflux experiments were carried out in the presence of 1 μM valinomycin and equal K\textsuperscript{+} concentrations inside and outside the vesicles to maintain the membrane potential clamped at 0 mV. To determine passive Ca\textsuperscript{2+} efflux, vesicles were diluted 100-fold in a solution containing (in mM) 10 potassium gluconate, 10 EGTA, and 160 HEPES-Tris, pH 7.4. To measure Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux, vesicles were diluted 100-fold in a solution that contained (in mM) 140 sodium gluconate, 10 potassium gluconate, 10 EGTA, and 20 HEPES-Tris, pH 7.4. To study the effect of extravesicular [Na\textsuperscript{+}] or [Ca\textsuperscript{2+}] on Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux, vesicles were diluted in solutions with varying [Na\textsuperscript{+}], replacing Na\textsuperscript{+} with choline, or at different free [Ca\textsuperscript{2+}], calculated using published values of binding constants (16). Ca\textsuperscript{2+} efflux was stopped by rapid filtration of 1 ml of each dilution through Millipore filters (HA 0.45 μm) previously soaked in the solution described above. The filters were rinsed three times with 3 ml of ice-cold quench solution and dried, and their radioactivity was measured in a liquid scintillation counter.

**Electrophysiological Measurements**

The experiments were carried out using short segments of single muscle fibers dissected from the semitendinosus muscle of *Rana catesbeiana*. Individual fibers were mounted on a triple Vaseline gap chamber as described (2, 21).

Fibers, first dissected in normal Ringer solution (in mM: 115 NaCl, 2.5 KCl, 1.8 CaCl\textsubscript{2}, and 10 Na-MOPS, pH 7.0), were bathed with a relaxing solution containing (in mM) 94 K\textsubscript{2}SO\textsubscript{4} and 10 K-MOPS, pH 7.0. Fibers were then transferred to the Vaseline gap chamber and laid across the three grease seals that divided the chamber into four pools. The end pool solutions were exchanged for an internal solution and allowed to equilibrate for 45–90 min before the experiment was initiated. The composition of the internal solution was (in mM) 110 potassium aspartate or 110 cesium aspartate, 20 MOPS, pH 7.0, adjusted with KOH or CsOH, 2 MgCl\textsubscript{2}, 5 K\textsubscript{2}-ATP, 0.1 mg/ml creatine phosphokinase, 5 Na\textsubscript{2}-phosphate, and 0.2 EGTA. One of the Ca\textsuperscript{2+} indicators (calcium orange-5N, fluo 3, or rhod 2), alone or in combination with the cage-Ca\textsuperscript{2+} compound DM-nitrophen (Molecular Probes), was added to the end pool solution. In some experiments, rhodamine B (Sigma) was also added to the end pool solution to monitor non-Ca\textsuperscript{2+}-specific changes in fluorescence. The [Ca\textsuperscript{2+}] of the internal solution was measured using a Ca\textsuperscript{2+}-sensitive microelectrode (calibrated with a commercial kit from WPI, Sarasota, FL) and was adjusted to pCa 7.0. A segment of the fiber lying in one of the two central pools (pool A) was voltage clamped, and the external solution bathing this segment was changed by a steady flow of Ringer solution into the pool while removing the excess. Membrane currents were normalized by fiber capacitance rather than by current density and are expressed as microammere per microfarad. This normalization eliminates possible errors in estimating the area of outer membrane in the central pool A, which might occur due to irregularities in the fiber cross-sectional area and in the grease seal boundaries. Fiber capacity (μF) was calculated from the integral of the transient currents elicited in response to hyperpolarizing pulses. The voltage clamp chamber was mounted on the stage of a modified compound fluorescence microscope used as a vertical optical bench. This setting was designed to allow epi-illumination of the voltage-clamped segment of muscle fiber by focusing the
luminous field diaphragm on the preparation with a ×20 objective (Fluo-20, numerical aperture 0.75; Nikon). Fluorescent light from the preparation was focused on a low-noise photodiode, converted to voltage, and amplified (27, 38).

**Flash Photolysis**

Ultraviolet (UV) flashes from an argon laser were delivered to the muscle fiber segment in pool A to elicit photolysis of DM-nitrophen, following a similar procedure to that described by Sanchez and Vergara (36) but adapted for Ca\(^{2+}\) detection, as illustrated by Escobar et al. (11, 12).

**RESULTS**

**Vesicular Experiments**

The amphibian T tubule vesicles used in this study had a high density of dihydropyridine binding sites. Densities ≥80 pmol [\(^{3}H\)]nitrendipine/mg protein were routinely obtained, indicating the purity and junctional origin of the T tubule vesicles used in this work (9). In the presence of detergent (saponin) to unmask latent sites, specific [\(^{3}H\)]ouabain binding was 100–120 pmol/mg protein. In the absence of saponin, the values obtained were only 10–15% of the values measured in saponin, indicating that the vesicular preparations were at least 85% sealed. All sealed vesicles had the inside-out configuration (data not shown), as determined with the procedures described previously (19).

**Ca\(^{2+}\) influx in native T tubule vesicles.** As shown in Fig. 1, isolated T tubule vesicles incubated at 25°C in a loading solution containing 2 mM 45CaCl\(_2\) accumulated Ca\(^{2+}\) exponentially as a function of time, with a rate constant of 0.048 min\(^{-1}\). Lowering the temperature to 5°C decreased the rate constant of Ca\(^{2+}\) accumulation threefold to 0.015 min\(^{-1}\) (Fig. 1), indicating that Ca\(^{2+}\) influx had a higher temperature dependence than a passive diffusion process. On average and regardless of incubation temperature, vesicles incubated in 2 mM CaCl\(_2\), and extensively washed with 10 mM EGTA to remove contaminating external Ca\(^{2+}\), accumulated at equilibrium 32 ± 10 (SD) nmol/mg protein (n = 16). These values are higher than expected for simple equilibration. Previous evidence indicating that T tubule vesicles have luminal (extracellular) Ca\(^{2+}\) binding sites (18), with a dissociation constant (K\(_d\)) of 2.3 ± 0.3 mM and a maximal binding (B\(_{max}\)) of 70 ± 2 nmol/mg, was confirmed in the present work. Isolated T tubule vesicles incubated to equilibrium in solutions with varying CaCl\(_2\) concentration displayed a single class of intravesicular low-affinity Ca\(^{2+}\) binding sites with a K\(_d\) of 3.5 ± 1.1 mM and a B\(_{max}\) of 73 ± 10 nmol/mg (mean ± SD, n = 3).

Considering that after isolation T tubule vesicles contain Ca\(^{2+}\) in their lumen (10), Ca\(^{2+}/Ca^{2+}\) exchange may contribute to Ca\(^{2+}\) influx, originating its observed temperature dependence. To determine Ca\(^{2+}/Ca^{2+}\) exchange, vesicles were equilibrated for 3 h at 5°C in loading solution containing 2 mM CaCl\(_2\) (nonradioactive). After this time, a small aliquot of 45CaCl\(_2\) was added (see MATERIALS AND METHODS), and Ca\(^{2+}\) accumulation in the vesicles was followed at 5°C as a function of time. As shown in Fig. 2, Ca\(^{2+}\) preloaded vesicles rapidly accumulated about one-third the amount of Ca\(^{2+}\) taken up by control vesicles, reaching equilibrium in <20 min with a rate constant of 0.26 min\(^{-1}\). In contrast, at 5°C, total Ca\(^{2+}\) influx needed >2 h to reach equilibrium, with a 17-fold lower rate constant of 0.015 min\(^{-1}\) (Fig. 1). We conclude from these experiments that at 5°C Ca\(^{2+}/Ca^{2+}\) exchange represents only a fraction of the total Ca\(^{2+}\) influx measured in native vesicles, so that most Ca\(^{2+}\) influx would take place through slower pathways.
Passive $\text{Ca}^{2+}$ efflux. To study passive $\text{Ca}^{2+}$ efflux, T tubule vesicles were first incubated in 2 mM $^{45}\text{CaCl}_2$ for 3 h and were then diluted in $\text{Na}^+$-free solution plus 10 mM EGTA. These experimental conditions were chosen to prevent exchange of vesicular $\text{Ca}^{2+}$ with external (cytoplasmic) $\text{Na}^+$ or $\text{Ca}^{2+}$. After dilution, the vesicular $\text{Ca}^{2+}$ content decreased exponentially with time, with a rate constant of 0.009 min$^{-1}$ at 15°C (Fig. 3).

As expected for a simple diffusion process, changing temperature had a marginal effect on passive efflux. Increasing the temperature from 15 to 25°C produced a rate constant of $\text{Ca}^{2+}$ efflux of 0.010 min$^{-1}$, without changing the total amount of $\text{Ca}^{2+}$ lost from the vesicles. Likewise, at 5°C, the rate constant of passive $\text{Ca}^{2+}$ efflux was 0.012 ± 0.006 min$^{-1}$ ($n = 3$).

$\text{Na}^+$-dependent $\text{Ca}^{2+}$ efflux. To measure the effects of external $\text{Na}^+$ on the reverse NCX reaction, vesicles were passively equilibrated in 2 mM $\text{NaCl}$ and subsequently diluted in a solution containing 140 mM $\text{Na}^+$ plus 10 mM EGTA. In the experiment shown in Fig. 4, vesicles exponentially lost 40% of their vesicular $\text{Ca}^{2+}$ after dilution, with a rate constant of 2.8 min$^{-1}$. On average, at 5°C, the rate constant of $\text{Na}^+$-dependent $\text{Ca}^{2+}$ efflux was 4.2 ± 1.8 (SD) min$^{-1}$ ($n = 4$). Twofold higher rate constant values were obtained at 25°C (data not shown), but the process became too fast to collect accurate data manually (half-time = 5 s). For this reason, subsequent experiments were done at 5°C to improve the accuracy of data collection. At this temperature, the values for $\text{Na}^+$-dependent $\text{Ca}^{2+}$ efflux were in the range of 42 to 63 nmol·mg$^{-1}$·min$^{-1}$. Because by definition $\text{Na}^+$-dependent $\text{Ca}^{2+}$ fluxes represent $\text{Na}^+/\text{Ca}^{2+}$ exchange, these results confirm previous observations (18) indicating that the reverse mode of the exchanger operates in inside-out T tubule vesicles isolated from amphibian skeletal muscle.

To investigate the reverse NCX reaction in vesicles loaded with $\text{Ca}^{2+}$ through $\text{Ca}^{2+}/\text{Ca}^{2+}$ exchange, the following experiment was performed. Vesicles were equilibrated with 2 mM $\text{CaCl}_2$ for 3 h. After this time $^{45}\text{Ca}$ was added, and after a period of 15 min vesicles were diluted at time 0 in a solution containing 140 mM $\text{Na}^+$ and 10 mM EGTA. As shown in Fig. 4, at time 0, these vesicles had accumulated less $\text{Ca}^{2+}$ than control vesicles, in agreement with the $\text{Ca}^{2+}/\text{Ca}^{2+}$ exchange experiments shown in Fig. 2. After dilution in 140 mM $\text{Na}^+$ and 10 mM EGTA, the vesicles rapidly exchanged 63% of their intravesicular $\text{Ca}^{2+}$ for $\text{Na}^+$, with a rate constant of 2.5 min$^{-1}$ (Fig. 4). For comparison, in the parallel experiment, control vesicles (equilibrated with $^{45}\text{Ca}$ for 3 h) exchanged only 40% of their luminal $\text{Ca}^{2+}$ for $\text{Na}^+$ (Fig. 4).

Effect of extravesicular $\text{Na}^+$ on $\text{Na}^+$-dependent $\text{Ca}^{2+}$ efflux. To study the effect of cytoplasmic $\text{Na}^+$ on the reverse mode of the exchanger, $\text{Na}^+$-dependent $\text{Ca}^{2+}$ efflux was determined at 5°C at different extravesicular [Na$^+$] and 10 mM EGTA. In the absence of extravesicular $\text{Na}^+$, $\text{Ca}^{2+}$ efflux had a rate constant of 0.012 ± 0.006 (SD) min$^{-1}$ ($n = 3$). Increasing extravesicular [Na$^+$] produced a significant and nonlinear increase in the rate constant of exchange (Fig. 5), which reached its maximal value at ≈120 mM [Na$^+$]. Assuming a cooperative model for $\text{Na}^+$ activation of the reverse mode of the exchanger, a Hill equation was fitted to these data. The resulting analysis yielded a Hill number ($n_{\text{Hill}}$) of 2.8 and a Hill constant ($k_{\text{Hill}}$) of 55.9 mM [Na$^+$].
Effect of extravesicular \([Ca^{2+}]\) on \(Na^{+}\)-dependent \(Ca^{2+}\) efflux. To investigate the effect of extravesicular \(Ca^{2+}\), reverse \(Na^{+}/Ca^{2+}\) exchange was measured in extravesicular solutions containing 140 mM [Na\(^+\)] and different [Ca\(^{2+}\)]. As shown in Fig. 6, increasing extravesicular \([Ca^{2+}]\) from pCa 9 to pCa 5 produced a threefold decrease in the rate constants of \(Na^{+}\)-dependent \(Ca^{2+}\) efflux without affecting the total amount of \(Ca^{2+}\) exchanged. The IC\(_{50}\) for [Ca\(^{2+}\)] was 91 nM (pCa\(_{0.5}\) = 7.04).

Fiber Experiments

Single frog fibers were used to investigate the operation of NCX in muscle cells. Ionic currents and intracellular [Ca\(^{2+}\)] were measured simultaneously after either removing Na\(^+\) from the external solution or increasing intracellular [Ca\(^{2+}\)] by flash photolysis of a cytoplasmic caged Ca\(^{2+}\) compound. To eliminate contributions from changes in membrane potential, all experiments were done in muscle fibers voltage clamped at –90 mV.

Effect of Na\(^+\) removal from the external solution. In zero external Na\(^+\) and at –90 mV a functional NCX system should operate in the reverse mode, exchanging intracellular Na\(^+\) for extracellular Ca\(^{2+}\). This mode of operation should generate an outward Na\(^+\) current and a concurrent increase in intracellular [Ca\(^{2+}\)]. As shown in Fig. 7, replacement of extracellular Na\(^+\) by tetramethylammonium produced a net change in outward current of 10 nA. Replacement of external Na\(^+\) with Li\(^+\) or \(N\)-methylglucamine produced similar results (data not shown). Normalized current changes ranged from 0.18 to 0.65 \(\mu\)A/\(\mu\)F, giving an average value of 0.36 ± 0.17 (SD) \(\mu\)A/\(\mu\)F (n = 6). The outward current lasted the entire period while the fiber was perfused with zero Na\(^+\) solution. On reperfusing the fiber with normal Ringer, the current returned to the basal level (Fig. 7).
Effect of increasing intracellular [Ca\(^{2+}\)] on the reverse NCX reaction. The present results provide a characterization of the effects of varying cytoplasmic [Na\(^+\)] and [Ca\(^{2+}\)] on the reverse NCX reaction in T tubule vesicles isolated from amphibian skeletal muscle. In addition, this is to our knowledge the first description of the currents associated with the forward and reverse mode of the NCX in whole skeletal muscle fibers.

**Vesicular Experiments**

In the absence of external Na\(^+\) or Ca\(^{2+}\) to avoid operation of the NCX, T tubule vesicles equilibrated with millimolar [Ca\(^{2+}\)] displayed very low passive Ca\(^{2+}\) efflux, despite the large chemical gradient for Ca\(^{2+}\) present in these experiments. These results indicate that the isolated T tubule vesicles were tightly sealed and thus maintained after isolation the low Ca\(^{2+}\) permeability of resting muscle fibers (5).

The reverse NCX reaction engaged only a fraction of the luminal Ca\(^{2+}\). Of the total amount of Ca\(^{2+}\) equilibrated in the vesicles, most of it was bound to low-affinity sites. Because the T tubules used in this work were sealed only with the inside-out configuration, these sites, whose nature remains to be characterized, should correspond in vivo to extracellular Ca\(^{2+}\) binding sites present in the lumen of the T tubules. Only a fraction of the total luminal Ca\(^{2+}\), which varied from 25 to 43%, was available for fast exchange with Na\(^+\). Partial dissipation of the Na\(^+\) gradient may explain this limited exchange. The amount of Ca\(^{2+}\) exchanged, which on average was 12 nmol/mg, should produce a net Na\(^+\) entry of 36 nmol/mg. With a T tubule luminal volume of ≈0.5 μl/mg protein (P. Donoso and C. Hidalgo, unpublished observations), this Na\(^+\) entry should increase luminal [Na\(^+\)] and may produce a decrease in driving force, limiting the amount of Ca\(^{2+}\) exchanged for Na\(^+\). In addition, the NCX may be present in only a fraction of the vesicles, or all vesicles would have the exchanger, but a fraction of their luminal Ca\(^{2+}\) might be bound to sites that are not readily available for fast exchange with Na\(^+\). The present results do not allow a distinction between these last two options.

**Effects of external [Na\(^+\)] on the reverse NCX reaction.** Increasing extravesicular [Na\(^+\)] produced a significant and nonlinear increase in the rate constant of reverse exchange that reached its maximal value at ≈120 mM [Na\(^+\)]. Assuming a cooperative model for Na\(^+\) activation of the reverse mode of the exchanger, a Hill equation fitted to the data yielded $n_{Hill} = 2.8$ and $k_{Hill} = 55.9$ mM Na\(^+\). Although there are no other data available for amphibian muscle, similar values, with $n_{Hill} = 2.4$ and $k_{Hill} = 55$ mM Na\(^+\), were reported for Na\(^+\) activation of the reverse NCX currents in excised membrane patches from mammalian skeletal muscle (17). If
this Na\(^+\) dependence mirrors the physiological situation, at the resting cytoplasmic [Na\(^+\)] of 9 mM (15), the exchanger would operate in the reverse mode at ~1% of its maximal rate. Furthermore, the inhibition by cytoplasmic Ca\(^{2+}\) of the amphibian skeletal NCX (see below) should further decrease the reverse operation of this transporter in resting muscle.

**Effects of extravascular [Ca\(^{2+}\)] on the reverse NCX reaction.** Cytoplasmic Ca\(^{2+}\) influences the activity of all native NCX transporters examined to date. Increasing cytoplasmic Ca\(^{2+}\) stimulates the NCX of cardiac muscle, squid axons, and other cells (6, 34) but inhibits the exchanger present in Drosophila (23). Our previous experiments in amphibian T tubules indicated that addition of 20 μM [Ca\(^{2+}\)] to the extravesicular solution containing 140 mM Na\(^+\) decreased the reverse rate of the exchanger (18). This finding suggested that cytoplasmic Ca\(^{2+}\) had an inhibitory effect on the amphibian NCX, but a detailed characterization was not carried out.

In the present work, we found an IC\(_{50}\) for inhibition of the reverse NCX by [Ca\(^{2+}\)] of ~10\(^{-7}\) M, close to the resting [Ca\(^{2+}\)]. Thus increasing cytoplasmic Ca\(^{2+}\) in the submicromolar range should decrease the rate of operation of the reverse mode of the exchanger in amphibian muscle. This inhibition may operate as a negative feedback mechanism, reducing Ca\(^{2+}\) influx through the NCX during sustained stimulation. Whether cytoplasmic Ca\(^{2+}\) exerts a similar inhibition on the mammalian skeletal muscle NCX remains to be determined, but there is a distinct possibility that the NCX of amphibian and mammalian skeletal muscle are different, since the structure and properties of the cardiac NCX from frog heart differ from its mammalian counterpart (37). If this is the case, the inhibition by cytoplasmic Ca\(^{2+}\) may be a property of the insect and amphibian muscle NCX that would not be shared by mammalian muscle.

**Fiber Experiments**

This is the first description, to our knowledge, of NCX-mediated currents in whole muscle fibers from skeletal muscle. To measure NCX-mediated currents in skeletal muscle cells under voltage conditions that mimic those prevailing in whole muscle cells at rest, a constant membrane potential of ~90 mV was maintained in all experiments.

**Reverse NCX currents.** After Na\(^+\) withdrawal from the external solution, a reverse NCX current of 0.36 μA/μF, equivalent to 0.36 μA/cm\(^2\), was measured in amphibian fibers containing 10 mM internal [Na\(^+\)]. For comparison, in 90 mM [Na\(^+\)] and at 0 mV, reverse NCX currents of the order of 5 pA have been measured in isolated inside-out patches from mammalian skeletal muscle (17). The reverse currents measured in similar conditions in patches from mammalian cardiac muscle are about 10-fold higher (20).

To compare measurements, it is necessary first to correct for the differences in the [Na\(^+\)] used in patch-clamp experiments and our measurements. If the reverse NCX activity in whole fibers behaves toward Na\(^+\) as in isolated vesicles (see Fig. 5), the reverse NCX current of 0.36 μA/μF measured in 10 mM [Na\(^+\)] would correspond to 1% of the maximal current. In 90 mM [Na\(^+\)], the reverse current would be 80% of maximal, originating a density of ~30 μA/μF. Second, mammalian reverse currents, recorded with a patch pipette with an inner diameter of 10–20 μm (17), should be transformed into current density. From the corresponding patch area (3.1–12.6 × 10\(^{-6}\) cm\(^2\)), current densities ranging from 0.4 to 1.6 μA/cm\(^2\) can be estimated for mammalian skeletal muscle. Thus, despite the fact that a membrane potential of ~90 mV is less favorable to the reverse reaction than 0 mV, the reverse current density in amphibian muscle at ~90 mV would be severalfold higher than the current measured in mammalian skeletal muscle at 0 mV. It is possible that the conditions present in whole fibers better preserve the activity of the NCX, since regulatory factors may be lost from isolated membrane patches. Alternatively, these differences may reflect intrinsic differences between the activities of the mammalian and the amphibian skeletal NCX.

The reverse NCX reaction produced only a limited increase in cytoplasmic [Ca\(^{2+}\)]. In cardiac muscle, removal of external Na\(^+\) produces a significant increase of cytoplasmic Ca\(^{2+}\) that can reach the micromolar range in only a few seconds (1). In contrast, only a marginal increase in cytoplasmic Ca\(^{2+}\) was observed in single mammalian fibers after replacing external Na\(^+\) (3). Inhibition of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) pump or of mitochondrial Ca\(^{2+}\) uptake did not increase further cytoplasmic Ca\(^{2+}\), raising the possibility of a very limited reverse NCX operation in skeletal muscle (3).

The present results contribute to clarify this issue, since a significant reverse NCX current was recorded in amphibian skeletal muscle in zero external Na\(^+\), indicating that the NCX was effectively activated, yet [Ca\(^{2+}\)] increased only marginally, as observed in mammalian muscle (3), despite the fact that the increase in intracellular Ca\(^{2+}\) due to the measured reverse NCX current should have been much higher. Because the NCX moves three Na\(^+\) per one Ca\(^{2+}\), the reverse current of 0.36 μA/μF represents a coupled Ca\(^{2+}\) influx of 3.6 pmol · cm\(^{-2}\) · s\(^{-1}\) (assuming as above a membrane capacity of 1 μF/cm\(^2\)). Considering the fiber as a cylinder with an approximate diameter of 100 μm, this Ca\(^{2+}\) influx should have increased intracellular [Ca\(^{2+}\)] to 90 μM in 1 min.

Other than assuming that amphibian skeletal muscle has very efficient cytoplasmic Ca\(^{2+}\) buffer systems, we do not have at present an explanation as to why cytoplasmic Ca\(^{2+}\) did not increase as expected when bathing the fibers in zero Na\(^+\). Intracellular Ca\(^{2+}\)-binding proteins, such as parvalbumin, may bind Ca\(^{2+}\) entering the skeletal muscle cells via the NCX, since the SR or the mitochondria do not seem to fulfill this role, at least in mammalian skeletal muscle cells (3).

**Forward NCX current.** To study the forward operation of NCX in skeletal muscle fibers, the intracellular
[Ca$^{2+}$] was suddenly increased in the cytoplasm by flash photolysis of the caged Ca$^{2+}$ compound DM-nitrophen. This procedure has been successfully used in cardiac muscle to activate the direct mode of NCX, producing a net inward Na$^+$ current density of 0.7 μA/μF when measured at 20°C and 0 mV (33).

The results shown in Fig. 8 demonstrate that flash photolysis of DM-nitrophen is a robust method to force a change in [Ca$^{2+}$]. Theoretical calculations from a model of the flash photolysis reactions (12) suggest that 30% conversion of DM-nitrophen by a single UV flash would generate the fluorescence transient observed. In that case, the free [Ca$^{2+}$] is predicted to jump from the resting value of 0.1 μM to ~0.4 μM. At the same time, the predicted jump in free Mg$^{2+}$ concentration ([Mg$^{2+}$]) is from ~5 μM at rest to ~13 μM after the flash. Thus the current elicited in response to the UV flash is more likely associated with the change in [Ca$^{2+}$] than with the very small change in free [Mg$^{2+}$].

The inward current density of amphibian skeletal muscle measured at 15°C and ~90 mV after the flash was in the range of 0.3–1.0 μA/μF, with an average value of 0.6 μA/μF. The voltage dependence of the cardiac NCX indicated that at ~90 mV the cardiac inward current density would be 2.2-fold higher than the value of 0.7 μA/μF measured at 0 mV (33), giving a value of 1.54 μA/μF. Taking into account the differences in recording temperature, the inward current density measured in skeletal muscle would be about one-half that expected in cardiac muscle in similar conditions, suggesting that the NCX density in amphibian skeletal muscle is 50% lower than in cardiac muscle. In any case, the measured current density is high enough to ensure a role of the skeletal NCX in moving Ca$^{2+}$ out of cells after an increase of cytoplasmic [Ca$^{2+}$].

The results presented in this work indicate that, in amphibian skeletal muscle fibers, the NCX has the potential to function both in the direct or the reverse mode, producing outward or inward NCX currents, respectively. In T tubule vesicles, increasing extravesicular (intracellular) [Na$^+$] stimulated the reverse mode in a cooperative fashion, with $n_{HH} = 2.8$, but, in contrast to the stimulation of the mammalian cardiac exchanger, increasing extravesicular (intracellular) [Ca$^{2+}$] inhibited this reverse activity with half-maximal inhibition in the range of resting cytoplasmic [Ca$^{2+}$]. In single fibers, photolysis of a cytoplasmic caged Ca$^{2+}$ compound activated an inward current (forward mode), whereas external Na$^+$ withdrawal generated an outward current (reverse mode), yet Na$^+$ withdrawal increased cytosolic [Ca$^{2+}$] only marginally, suggesting that, in contrast to what happens in cardiac muscle, the reverse mode of the NCX does not contribute significantly to increase cytoplasmic [Ca$^{2+}$] in skeletal muscle. These results indicate that the main function of the skeletal muscle NCX is to move Ca$^{2+}$ out of the cells after muscle contraction.

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