Evidence for direct involvement of the sarcoplasmic reticulum Ca^{2+}-ATPase in a passive monovalent cation (K^+/Na^+) exchange

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Abstract A specific inhibitor of SERCA-pumps, thapsigargin (TG) was used to demonstrate the direct involvement of the SR Ca^{2+}-ATPase in passive K^+/Na^+ exchange. The K^+-potential variations across vesicle membranes were measured in the absence of ATP with a fluorescent probe: 3,3'-dipropylthiodicarbocyanine iodide. Addition of EGTA dissipates the K^+-potential whereas the presence of TG abolishes this effect. Our data prove that the Ca^{2+}-ATPase translocates monovalent cations at a rate similar to the $E_2 \rightarrow E_1$ conformational change.

Key words: Ca^{2+}-ATPase; Sarcoplasmic reticulum; K^+/Na^+ exchange; Thapsigargin; Potential sensitive dye; $DiSC_3(5)$

1. Introduction

Ca^{2+}-transport by the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is considered to be electrogenic [1–3] but evidence for a coupled countertransport of protons has been produced [3–5]. Participation of fluxes of other ions in the charge balance is still under debate.

Fluxes of monovalent ions, like K^+, have been reported during the Ca^{2+}-pumping activity [3,5,6]. The direct involvement of the Ca^{2+}-pump in these fluxes has never been demonstrated with native SR vesicles. The presence of channels within the membrane may hide the specific monovalent cation translocation mediated by the pump [7]. In fact, it has been estimated that two-thirds of the SR vesicles contain K^+/Na^+ channels which makes them highly permeable to K^+, Rb^+ and Na^+ [8] and is most probably responsible for the complete charge equilibration across the membrane.

Nevertheless, previous results have demonstrated the existence of K^+-countertransport which is not inhibited by typical channel blockers such as tetraethylammonium or 4-aminopyridine [5]. Furthermore, a passive monovalent cation exchange (K^+ or Na^+) has been observed in native SR vesicles in the absence of ATP. This exchange is inhibited by vanadate and seems to be regulated by pH and the extravesicular calcium concentration [9,11]. Consequently the authors have postulated that passive monovalent exchange was catalysed by the Ca^{2+}-ATPase.

In this study we have further investigated passive K^+/Na^+ exchange. Energy-independent monovalent fluxes were measured by monitoring changes in membrane potential with a fluorescent probe: $DiSC_3(5)$. The effect of a specific inhibitor, thapsigargin (TG), was tested to determine unequivocally if the translocation of monovalent ions is mediated by the Ca^{2+}-ATPase or due to passive diffusion through independent channels. The action of TG proves unambiguously that the Ca^{2+}-pump catalyses passive diffusion of monovalent cations (K^+ /Na^+) when the protein is switched to its E_2 conformational state.

These data suggest that during active uptake of Ca^{2+}, the Ca^{2+}-pump may be able to counter-transport a significant amount of positive charges in the form of K^+ or Na^+.

2. Materials and methods

2.1. Chemicals

$DiSC_3(5)$ was obtained from Molecular Probes and prepared as ethanolic solutions. All other products were purchased from Sigma. Valinomycin and gramicidin were prepared as ethanolic solutions (100%). TG was dissolved in DMSO (100%).

2.2. Preparation of sarcoplasmic reticulum vesicles

SR vesicles were prepared from rabbit skeletal muscle according to [12,13]. To obtain virtually Cl^--free preparations, the vesicular pellet was resuspended in a 20-fold volume of 20 mM KOH/MOPS pH 7.8 containing 5 mM Mg-gluconate. The suspension was centrifuged at $48{,}000 \times g$ for 60 min at 4°C rinsed using the above solution then recentrifuged. The final pellet was resuspended in 20 mM KOH/MOPS pH 7.8, 5 mM Mg-gluconate and 10% sucrose (w/v) and stored in liquid nitrogen.

Protein concentration was determined spectrophotometrically at 280 nm in the presence of 1% sodium dodecyl sulfate as decribed in [14].

Microsomal vesicles (4–5 mg/ml) loaded with 20 mM KOH/MOPS (pH 7.8) and 5 mM Mg-gluconate were preincubated for 1 min in 1 mM EGTA (pH 7.8), 5 mM Mg-gluconate, 20 mM KOH/MOPS (pH 7.8) in the presence of 80 μM TG. Controls were preincubated according to the preceding procedure using the same volume of DMSO but in the absence of TG. The aim of preincubation with EGTA is to bring the ATPase molecules into their E_2 conformation: under those conditions the binding of TG to the Ca^{2+}-ATPase is not perturbed by contaminant calcium. After incubation, a solution containing 1 mM Ca-gluconate, 5 mM Mg-gluconate and 20 mM KOH/MOPS (pH 7.8) was added to the reaction medium to stop the TG reaction and return the unreacted proteins to the E_1 state.

2.3. Fluorescence measurements

Fluorescence measurements were made at 4°C in a temperature-regulated fluorimeter cuvette (Bio-Logic, Claix, France) under continuous stirring. The fluorescence of the dye was excited at 622 nm and the emitted light was measured at 90°C through a 671 nm Balzers interference filter.

At $t = 0$, preincubated vesicles were diluted in 20 mM NaOH/MOPS (pH 7.8), 5 mM Mg-gluconate and 1 μM $DiSC_3(5)$ to yield a final concentration of 50 μg protein/ml. No TG was added to the dilution

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ATP, adenosine-5'-triphosphate; SR, sarcoplasmic reticulum; DMSO, dimethyl sulfoxide; $DiSC_3(5)$, 3,3'dipropylthiodicarbocyanine iodide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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medium so that the final TG concentration in the vesicles suspension was fixed at 0.9 μM. Prior to measurement, the vesicles were incubated for 3 min in the measuring cuvette with continuous stirring to stabilize the fluorescence signal.

The compositions of the reaction mixtures are described in the corresponding figure legends.

3. Results

To study the translocation of monovalent ions in native SR vesicles, we generated a transmembrane potential by increasing the K^+ permeability relatively to the Na^+ permeability. This was accomplished by adding a K^+-selective ionophore, valinomycin, to SR vesicles loaded with 20 mM of K^+. This suspension was then mixed with a large volume of buffer containing 20 mM Na^+. Fig. 1A demonstrates that the addition of valinomycin decreases the fluorescence signal from the probe $DiSC_3(5)$ which is consistent with the creation of a negative potential inside the vesicles. The K^+-diffusion potential is subsequently slowly dissipated, most likely by the passive influx of Na^+ ions.

The membrane potential rapidly collapses upon addition of the ionophore gramicidin which enhances the Na^+ influx. As reported in [9], the addition of 500 μM EGTA, which switches the Ca^{2+}-ATPase to E_2 state, prior to gramicidin dissipates most of the K^+ potential induced by valinomycin. This proves that EGTA has increased the Na^+-influx under conditions which switch the Ca^{2+}-ATPase to its E_2 conformation.

The pH dependence of the EGTA-induced Na^+-influx has been measured after dilution of K^+-loaded SR vesicles in a Na^+ medium buffered to the same pH as that used for K^+-loading, in order to avoid the formation of a transmembrane pH gradient.

The result shown in the inset of Fig. 1A demonstrates that the EGTA-induced Na^+ conductance is strongly pH dependent (pK~7.4 at 0°C) as previously reported [9]. For this reason TG inhibition and the pCa dependence of K^+-potential dissipation were studied at pH 7.8 in further experiments.

The fluorescence recording obtained with vesicles preincubated in TG is shown in Fig. 1B. Valinomycin injection, made 3 min after the SR vesicles dilution in the sodium medium creates a membrane potential similar to that observed in the control sample (Fig. 1A). Furthermore the total membrane potential released by gramicidin from the control and the sample containing TG are identical. These data indicate unambiguously that the passive permeability of the vesicles for monovalent cations is unaffected by TG in the conditions used.

TG, however, blocks the effect of EGTA, which no longer induces a collapse in the membrane potentiel. As TG is known to be a specific inhibitor of the Ca^{2+}-pump, this provides very strong evidence for the involvement of the Ca^{2+}-ATPase in the translocation of monovalent cations when switched to its calcium-free state (E_2).

In the experiments described above, the effect of EGTA was observed in the presence of valinomycin. As the latter ionophore causes fast K^+-outflux, this only demonstrates that the Ca^{2+}-ATPase has mediated a Na^+-influx. In order to find out if the Ca^{2+}-pump was responsible of both influx and outflux of monovalent ions when in its E_2 state, the experiment was redesigned as follows: we exposed the protein to a transient controlled Ca^{2+} concentration for a fixed time in the absence of valinomycin. After this transient incubation, the calcium concentration was adjusted to 130 μM to return the protein to the E_1 state and to stop exchange. The remaining K^+-gradient was then evaluated by the addition of valinomycin. Results shown in Fig. 2 confirm that a passive K^+/Na^+ exchange has occurred in the absence of valinomycin during the transient exposure of the protein at high pCa values.

In agreement with [9], the rate of dissipation of the K^+-potential depends on the value of the free calcium concentration with a $Ca_{1/2}$~0.2–0.15 μM. This is in good agreement with the calcium dependence of the E_1–E_2 transition and with the high affinity calcium binding measured at similar pH to that used in the present work [15,16].

When the vesicles have been incubated in TG, the remaining K^+-potential measured upon addition of valinomycin is high and constant when the pCa lies between 4 and 7. A decrease of the fluorescence signal relative to the development of the K^+-potential can be noticed when the pCa excedes 7. The significance of this drop remains unclear. Because of the presence of TG and the high values of pCa, the fall in the curve cannot be attributed to a specific effect on the Ca^{2+}-ATPase. It may be due to destabilization of the vesicle membranes that has been reported previously at 0°C in the presence of high concentrations of EGTA [17,18].

As already described [9], the addition of EGTA to SR vesicles in the absence of valinomycin induces a small fluorescence decrease (~1%) for pCa's ranging from 9 to 6.5. A possible explanation for this observation is that a fraction of the dye binds to the Ca^{2+}-ATPase to a degree that is sensitive to the conformation. This interpretation is confirmed by the disappearance of this signal when the vesicles have been incubated in TG. This, in turn, provides a useful control of the binding of TG to the protein.

By combining the ion flux and membrane potential experiments presented in previous work [9,10] and in this publication, we can give an evaluation of the turn-over rate of the monovalent cation exchange. The action of EGTA on vesicles loaded with 20 mM K^+ is to collapse the K^+-gradient with a half-life of about 10 s. Assuming an internal vesicle volume of 3.0 μl/mg protein [19], a molecular weight of 110,000 and a purity of 70% for the Ca^{2+}-ATPase, the turn-over rate of the ion flux is calculated to be around 0.5 s^{-1}. In spite of the uncertainties in this evaluation, it is interesting to note that the rate is similar to that of the $E_2 \rightarrow E_1$ transconformation measured at the same temperature [20].

4. Discussion

Previous reports suggest that the Ca^{2+}-pump from native SR vesicles is able to mediate an unspecific passive exchange of monovalent cations (K^+/Na^+) while in the E_2 state [9,10]. This hypothesis for K^+ or Na^+ exchange comes from the observation of a fast EGTA-induced K^+/Na^+ efflux from SR vesicles, which is sensitive to the concentration of protons, calcium and vanadate, an inhibitor of P-type ATPases [11]. This kind of study using native SR vesicles is generally complicated by the existence of a number of passive permeabilities in the native membrane. To overcome this difficulty and to demonstrate the implication of the Ca^{2+}-pump in the monovalent fluxes observed, we have measured the EGTA-induced (K^+/Na^+) exchange in the presence of TG, a highly specific inhibitor of the SERCA pumps.

It is remarkable that the K^+-diffusion potential created by valinomycin can be observed for up to 5 min. This seems to be

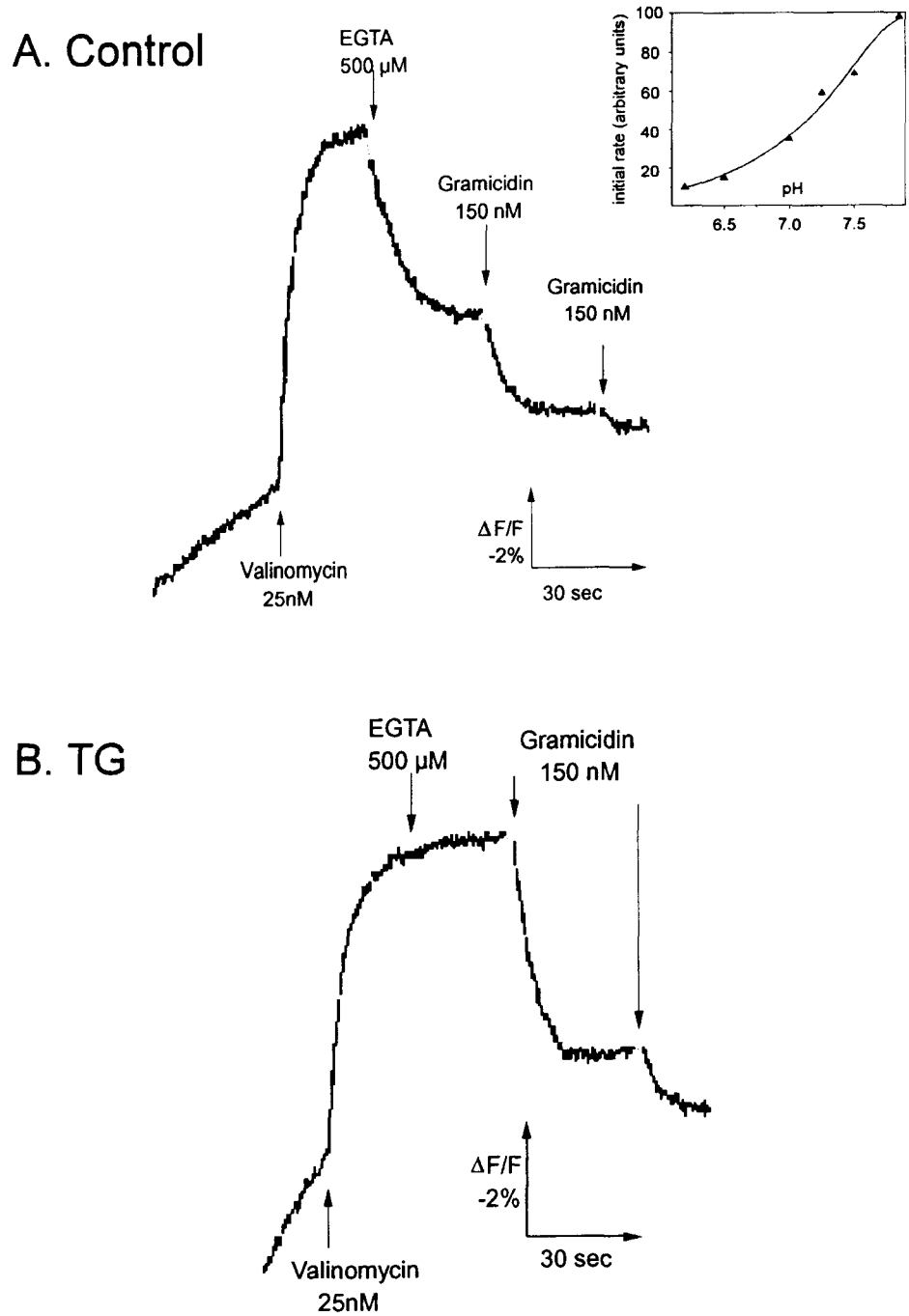

Fig. 1. Inhibition by TG of the EGTA-induced K^+-dissipation. (A) Control: K^+-potential dissipation by EGTA. Measurement of membrane potential changes with the fluorescent dye $DiSC_3(5)$. At $t = 0$, native K^+-loaded vesicles were suspended in 20 mM NaOH/MOPS (pH 7.8), 5 mM Mg-gluconate and 1 μM $DiSC_3(5)$ under continuous stirring. After injection of 25 nM valinomycin in the reaction medium, a K^+-transmembrane potential is developed. Addition of 500 μM EGTA in the medium induces a dissipation of the K^+-potential, which is completely reversed by the addition of 150 nM of gramicidin. The figure has been corrected for dilution and injection artefacts. (Inset) pH dependence of K^+-potential dissipation by EGTA. K^+-loaded vesicles were suspended in the same medium as described above, buffered at various pH. (B) Vesicles incubated with TG: inhibition of the EGTA effect. Same conditions as described in (A) except that the EGTA preincubation of K^+-loaded vesicles (4–5 mg/ml) is performed in the presence of 80 μM TG ([TG]/[Ca^{2+}-ATPase] = 2.5). The figure has been corrected for dilution and injection artefacts.

in contradiction with the existence of any ion channel in the vesicles that would be expected to collapse the K^+-gradient within a few seconds of dilution. According to [8], the density of channels within the SR vesicles is such that 2/3 of vesicles contain cation K^+/Na^+ channels while the remaining 1/3 does not. Consequently it seems clear that the membrane potentiel observed here is associated only to the fraction of vesicles (1/3) devoid of cation channels, the other pool (2/3) being entirely silent because of fast gradient equilibrium after vesicles dilution.

In the presence of TG the passive equilibration of K^+/Na^+ across the membrane is reduced compared to the control with maximal inhibition when the pCa lies between 4 and 7. The inhibition of this EGTA-induced monovalent flux by TG clearly demonstrates the direct involvement of the Ca^{2+}-pump in K^+ or Na^+ exchange. As described above, the rate of K^+/Na^+ exchange is reduced at acid pH with a pK near 7.4. Forge et al. [15] have proposed that the Ca^{2+}-binding involves the sequential release of at least 3 protons (with pK's 7 and 7.9). As these values are in the same range of pK as the K^+/Na^+ exchange, the protonation of the Ca^{2+}-sites at acid pH may inhibit the permeation of monovalent cations. The inhibition due to calcium ion binding to the translocation sites suggests a permeation of monovalent cations which may occur through the Ca^{2+}-sites embedded in the membrane domain of the protein [21].

Two models can now be proposed to explain the passive K^+/Na^+ exchange by the Ca^{2+}-ATPase. In model A (Fig. 3), the free unprotonated E_2 state is permeable to monovalent cations (K^+ or Na^+) which would cross the membrane through a channel like structure formed by the Ca^{2+}-ATPase containing the free calcium sites within helices. Inhibition of monovalent fluxes by calcium and protons would be explained by their occupancy of the acid residues sites in this channel. Vanadate and TG, which inhibit the K^+/Na^+ exchange, are known to bind exclusively on the E_2 state of the Ca^{2+}-ATPase. Vanadate simulates phosphorylation of the protein and its inhibitory effect on the exchange could be caused by a conformational change leading to a more tightly closed channel. In a similar way, the inhibition due to TG could be correlated either to a conformational change of the channel (different from the well-defined E_2 state) or to steric hindrance resulting from TG binding within the M_3 transmembrane span, i.e. in the vicinity of the putative Ca^{2+}-binding sites.

An alternative hypothesis is that the monovalent ion exchange occurs during the $E_2 \leftrightarrow E_1$ switch when the Ca^{2+}-sites are free (model B, Fig. 3). It has been reported in many instances that the rate constant (k^+) of the $E_2 \rightarrow E_1$ transition is much slower than the rate constant (k^-) of the $E_1 \rightarrow E_2$ transition so that, in the absence of ligands, most of the enzyme is in the E_2 state [22]. In the steady state without ligands, the interconversion between E_2 and E_1 may then occur continuously at a rate equal to k^+. It is therefore conceivable that during this continuous $E_2 \leftrightarrow E_1$ transconformation, the Ca^{2+}-ATPase transports monovalent cations down their gradient due to alternate exposure of the transport sites on opposite sides of the membrane. Inhibition of monovalent fluxes by calcium could be explained by complete stabilisation of the enzyme in its E_1 state. On the other hand, inhibition by vanadate and TG would result in locking the enzyme in a stable 'E_2' conformation.

Two characteristics of the E_1–E_2 equilibrium are in favor of this scheme: the rate of E_2–E_1 is accelerated at basic pH [23–25] and by monovalent cations [6,26,27]. In addition the evaluation of the K^+ or Na^+ flux rate gives a value of turn-over in the same range as the rate of the $E_2 \rightarrow E_1$ conformational change.

In conclusion the present results support the existence of a

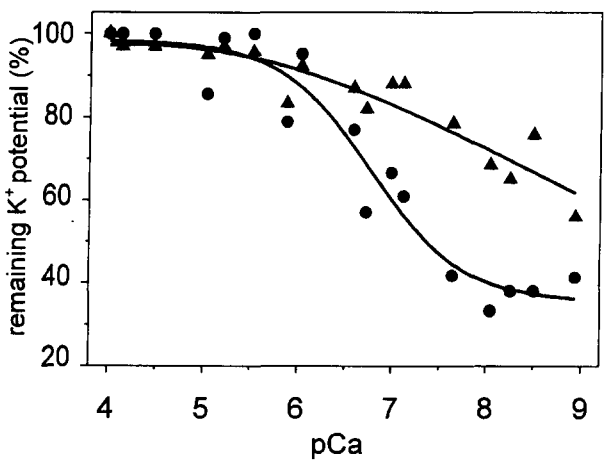

Fig. 2. pCa dependence of the K^+-potential dissipation by EGTA SR vesicles preincubated as in Fig. 1 (●, control; ▲, TG 80 μM) were diluted in 20 mM NaOH/MOPS (pH 7.8), 5 mM Mg-gluconate, 100 μM Ca-gluconate with 1 μM $DiSC_3(5)$. At t = 3 min varying amounts of EGTA were added to set a given pCa. Exposure to this pCa value was stopped at t = 3.5 min by addition of 130 μM Ca-gluconate. At t = 3.75 min, 25 nM valinomycin was injected to measure the remaining K^+-potential in the SR vesicles. The value of the fluorescent signal measured at pCa 4 is considered as 100%.

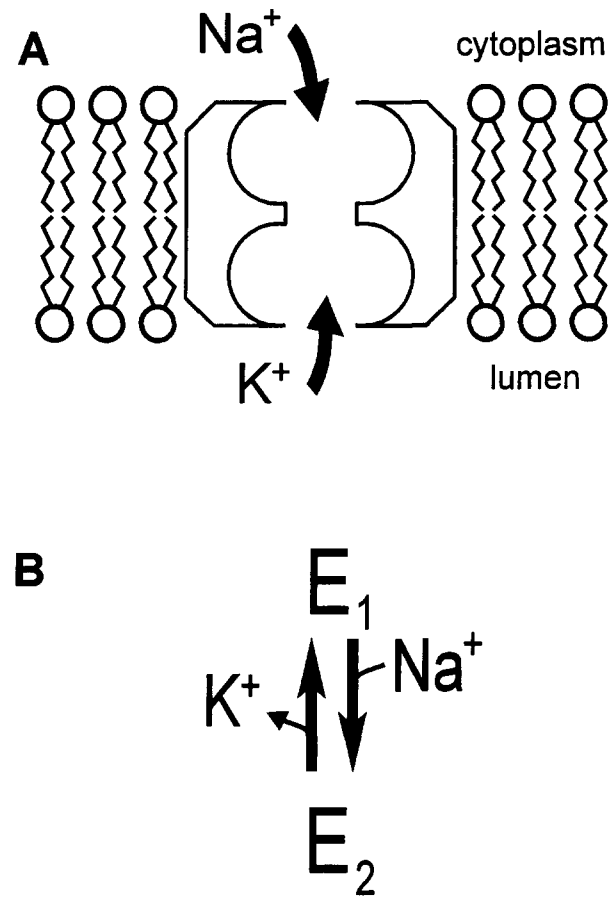

Fig. 3. Models describing the monovalent cation (Na^+/K^+) fluxes through the Ca^{2+}-ATPase. Model A depicts the translocation of Na^+/K^+ within the free Ca^{2+}-binding sites (E_2state) of the Ca^{2+}-pump. In Model B, the K^+/Na^+ translocation occurs during the $E_2 \leftrightarrow E_1$ switch within the free Ca^{2+}-binding sites. Subsequent redistribution of monovalent ions (H^+, Na^+, K^+) would depend on the available cations in the surrounding medium and would accelerate a new catalytic cycle of pumping. These models depict the translocation of monovalent cations K^+ and Na^+ as determined by our experimental conditions (i.e. K^+ inside of the vesicles and Na^+ outside the vesicles).

passive monovalent fluxes (K^+/Na^+) through the Ca^{2+}-ATPase. During part of its reaction cycle the pump is able to facilitate the permeation of monovalent ions. Using reconstituted proteoliposomes, it has been shown that at pH 7.2 protons are counter-transported during the Ca^{2+}-uptake [3,4]. If one tries to reconcile this observation with our results, the simplest explanation is that competition takes place between protons and monovalent cations for binding to translocation sites of the pump. This would have the effect of endowing the Ca^{2+}-ATPase with opportunist behaviour: namely the pump is able to translocate preferentially H^+ at acid pH and/or monovalent cations (K^+ or Na^+) at higher pH values. The monovalent cation exchange may be envisioned as a physiological way of cancelling and reequilibrating the ionic concentration in the SR vesicles during muscular relaxation.

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