The Squid Axon $\text{Na}^+/\text{Ca}^{2+}$ Exchanger Shows Ping Pong Kinetics only when the Ca_i -regulatory Site is Saturated

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Key Words

$\text{Na}^+/\text{Ca}^{2+}$ exchange • Transport kinetics • Transport regulation • Transport sites • Squid axons

Abstract

In a previous work we demonstrated that, in dialyzed squid axons, an impairment of the Ca^{2+} -regulatory site affected the apparent affinities for external Na^+ and Ca^{2+} in a way opposite to that predicted by the exiting (ping-pong) models for the exchangers. In the present work, we used model simulations and actual experiments where the Ca^{2+} -regulatory remained always saturated while $[\text{Ca}^{2+}]_i$ was either limiting or near saturating for the internal Ca^{2+} transport sites. Under these conditions, both the theoretical and experimental transport activation curves for external Na^+ and Ca^{2+} were those expected from the current kinetic schemes. These observations have two important implications: On the one hand, they confirm the ping-pong translocation schemes for $\text{Na}^+/\text{Ca}^{2+}$ exchange. On the other, they call for caution in interpreting kinetic data in membrane transport systems possessing intracellular ionic and/or metabolic regulation.

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Introduction

The existence of regulation in systems that move substances across biological membranes (channels and transporters) is well documented [1-5]. In these cases there are two simultaneous processes, with their own kinetics, at play: the regulatory mechanism and the substrate/s translocation. Obviously, regulation can affect the intra- and/or extracellular affinity for the substrate/s binding and/or the translocation rates; and this can complicate a transport kinetic analysis since the results may not be straightforward. A particular example is the squid nerve $\text{Na}^+/\text{Ca}^{2+}$ exchanger. All present models consider it as a ping pong system: acting as first substrate, Na^+ or Ca^{2+} binds to one side of the membrane (*cis*) and becomes translocated to the other (*trans*) side; however, it must be released, as first product, before Ca^{2+} or Na^+ on the *trans* side, acting as second substrate, binds to the empty carrier, is transported across the membrane and released (*cis* side) as a second product. Considering only substrates interactions with their binding sites this kinetic scheme predicts: a reduction in the concentration of a transported ligand in the *cis* side will decrease the maximal rate of translocation and will increase the apparent affinity

for a transported ligand located in the *trans* side; conversely, an increase in the concentration in the *cis* side will increase the maximal rate of translocation together with a decrease in the apparent affinity of the substrate in the *trans* side. [6].

However, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger has complex ionic and metabolic regulations [2, 7]. It has an intracellular non-transporting Ca^{2+} -regulatory site that must be occupied for transport to take place. The state of that site alters the apparent affinity of intra- and extracellular transport sites [8, 9]. Hampering the binding of Ca^{2+}_i (low pH_i , high $[\text{Na}^+]_i$, low $[\text{Ca}^{2+}]_i$, low ATP, high $[\text{Mg}^{2+}]_i$) diminishes the apparent affinity of intracellular transport sites. These effects agree with the overall kinetic reaction and are independent of the true affinity of the sites; they are quite noticeable for Na^+ , because of its synergism with H^+ inhibition, but much less marked for Ca^{2+}_i given its antagonism towards ($\text{H}^+_i + \text{Na}^+_i$) and Mg^{2+}_i inhibition.

In the squid, the state of the Ca_i -regulatory site also affects the affinity of the extracellular Na^+ and Ca^{2+} transport sites but it does so in a way that contradicts the prediction of all present kinetic models for the $\text{Na}^+/\text{Ca}^{2+}$ exchange [9]. For instances, the squid model predicts that increasing intracellular $[\text{Ca}^{2+}]$ from 0.5 to 50 μM should decrease the affinity of the extracellular sites both for Na^+ and Ca^{2+} while experimentally the reverse is observed [9]. These *trans* effects on the external affinities do not obey ping-pong transport kinetics and the underlying mechanism remains unknown. However, they address an important question: whether the actual translocation, aside from regulation, is intrinsically ping-pong. We decided to address this point by studying the effects of intracellular Ca^{2+} concentration on the exchange rate and on the apparent affinities for external Na^+ and Ca^{2+} , under conditions in which the Ca^{2+}_i regulation was saturated and the Ca^{2+}_i concentrations were at limiting and non-limiting concentrations for the intracellular Ca^{2+} -transport. The results are consistent with the notion that the actual translocation cycle of the squid nerve $\text{Na}^+/\text{Ca}^{2+}$ exchanger indeed follows ping-pong kinetics. Another important conclusion is that the kinetics of transporters that are subjected to regulatory mechanisms must be analyzed with caution.

Materials and Methods

Giant axons obtained from *Loligo pealei* (Marine Biological Laboratory, Woods Hole, MA) were dialyzed with highly permeable capillaries of regenerated cellulose fibers (210

μm OD; 200 μm ID; MWCO of 18 kDa; Spectrapor Number 132226; Spectrum, Houston, Texas). The standard dialysis medium had the following composition (mM): Tris-Mops, 385; NaCl , 40; MgCl_2 , 1; Glycine 285; Tris-EGTA (ethylene glycol-bis (β -aminoethyl ether)- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid), 1; pH 7.3. The standard external solution consisted of (mM): NaCl , 440; CaCl_2 , 0.3; MgCl_2 , 60; Tris-Cl, 10; pH 7.4. The osmolarity of all solutions was adjusted to 940 mosmols. In estimations of the Na^+_o -dependent Ca^{2+} efflux, the changes in external Na^+ concentrations were compensated with Li^+ ; for measurements of Ca^{2+}_e -dependent Ca^{2+} efflux, the external solutions were free of Na^+ and contained 440 mM LiCl . The estimation of $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$ was made with the WinMaxc computer program (Version 2.00, 1999; Chris Patton Hopkins Marine Station, CA, USA). Higher $[\text{Ca}^{2+}]_i$'s were taken as equal to the $[\text{CaCl}_2]$ added in excess to the concentrations of EGTA. The Ca^{2+} pump component of Ca^{2+} efflux and the Na^+/K^+ pump were eliminated by adding 100 μM vanadate. To stop any endogenous production of ATP, 1 mM NaCN was always present. Before including $^{45}\text{Ca}^{2+}$ in the dialysis solution, axons were routinely dialyzed for about 45 minutes with a standard medium containing 1 mM EGTA, free of calcium and ATP. In all cases each axon served as its own control, since steady-state fluxes were measured before and after a given experimental condition. All reagents were from SIGMA (St Louis, MO). The working temperature was between 17-18°C.

Results and Discussion

The approach to the problem must satisfy two requirements: on the one hand, to assess the translocation cycle isolated from possible influences of the regulatory process; on the other, to explore the effects of limiting and non-limiting concentrations of one of the transported ligands at a *cis* side on the overall rate of exchange and the apparent affinities for the transported substrates at a *trans* side. The selected *cis* side was intracellular and the ligand, Ca^{2+} ions. To that end, the axons were dialyzed without intracellular Na^+ at a pH of 8.0 that shows a $K_{0.5}$ for Ca^{2+} binding to its regulatory site of about 0.5 μM [10]. The two $[\text{Ca}^{2+}]_i$'s chosen for the *cis* side were 10 μM and 500 μM which will always be saturating for that site. For the intracellular Ca^{2+} transport sites, the K_m is 100-200 μM [9]. Therefore, 10 μM Ca^{2+}_i will be limiting and 500 μM Ca^{2+}_i about 70-80 percent saturating.

Additional information from this study concerns the predictive capability of the kinetic model proposed for the squid $\text{Na}^+/\text{Ca}^{2+}$ exchanger [8, 9, 10]. For that purpose we used the model proposed for the overall reaction of the squid $\text{Na}^+/\text{Ca}^{2+}$ exchanger [8] depicted in Figure 1. By this scheme, when the Ca^{2+}_i -regulatory is saturated, all the carrier complexes formed with intracellular H^+ and

Fig. 1. Reaction scheme for the squid nerve $\text{Na}^+/\text{Ca}^{2+}$ exchange. In the scheme the overall translocation cycle is represented by the solid lines linking the complexes of the carrier bound to Ca^{2+} at the regulatory site (CrE) with those binding Ca^{2+} (C) and Na^+ (N) at the intra- and extra-cellular transport sites. The dashed lines link the free carrier (Ei) with the carrier bound to Ca^{2+} at the regulatory site (CrE) and with the complexes formed with intracellular H^+ and Na^+ acting at their inhibitory sites. The rate constants for the complexes inter conversions are self explanatory. The values used for these constants are given in references [8] and [9].

Na^+ acting at their inhibitory sites cease to exist; i.e., one is left with the translocation cycle alone. For the simulation we used the complete scheme with the same unidirectional rate and affinity constants given before (see above references). The intracellular solutions were taken free of Na^+ and ATP, with a pH of 8.0; the extracellular solutions were free of Ca^{2+} for Na^+_{o} activation curves, and free of Na^+ when $\text{Ca}^{2+}_{\text{o}}$ was varied. The $\text{Na}^+/\text{Ca}^{2+}$ flux ratio was taken equal to 3. Solid lines represent the fluxes expressed as a percentage of the maximum flux in each condition; this permits a better visualization of the changes in apparent affinities. The long dashed line are the percentage of the fluxes $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ compared with those at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ allowing a comparison of the absolute changes in the predicted transport rates. Figure 2a shows the results on Na^+_{o} stimulated Ca^{2+} efflux (forward mode), where the $K_{0.5}$, equivalent to the Ks in a Hill equation, goes from 149 mM at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ to 80 mM at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$

Fig. 2. Simulation of the effects of $[\text{Ca}^{2+}]_{\text{i}}$ on $[\text{Na}^+]_{\text{o}}$ and $[\text{Ca}^{2+}]_{\text{o}}$ stimulation of Ca^{2+} efflux in axons with the Ca^{2+} -regulatory site saturated. The simulation conditions for intracellular solutions were: Na^+ -free, ATP-free, pH 8.0 at $[\text{Ca}^{2+}]_{\text{i}}$ of $10 \mu\text{M}$ and $500 \mu\text{M}$. The extracellular solutions were Ca^{2+} -free when Na^+ was the variable and Na^+ -free when Ca^{2+} was the variable. Solid lines express the fluxes as a percentage of the maximum flux under each condition; the long dashed lines are the percentage fluxes at $10 \mu\text{M}$ in relation to those at $500 \mu\text{M}$. (a) Extracellular Na^+ . Note: (i) An unmodified Hill coefficient of 3 was assumed; (ii) on those bases, the K_{s} for Na^+_{e} goes from 149 mM at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ to 80 mM at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$; (iii) the fluxes at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ are about 9 fold higher lower than at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$. (b) Extracellular Ca^{2+} . Note: (i) The $K_{0.5}$ for $\text{Ca}^{2+}_{\text{e}}$ goes from $233 \mu\text{M}$ at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ to $86 \mu\text{M}$ at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$; (ii) the fluxes at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ are about 3 fold higher than at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$.

mM at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$. The extracellular Ca^{2+} activation ($\text{Ca}^{2+}_{\text{o}}/\text{Ca}^{2+}_{\text{i}}$ exchange) (Figure 2b) displays a $K_{0.5}$ of $233 \mu\text{M}$ at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ falling to $86 \mu\text{M}$ at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$. An effect on the maximal rates of transport is also predicted by the model: the ratio between the fluxes at $500 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ over those at $10 \mu\text{M} \text{Ca}^{2+}_{\text{i}}$ is about 9 for $\text{Na}^+_{\text{o}}/\text{Ca}^{2+}_{\text{i}}$ exchange and 3 for $\text{Ca}^{2+}_{\text{o}}/\text{Ca}^{2+}_{\text{i}}$ exchange.

Fig. 3. Effects of $[Ca^{2+}]_i$ on Na^+ stimulation of Ca^{2+} efflux (forward Na^+_e/Ca^{2+}_i exchange) in a dialyzed squid axons with the Ca^{2+} -regulatory site saturated. The figure describes a typical experiment where the two $[Ca^{2+}]_i$ in μM are indicated by the horizontal bars on top of the figure. In addition, the intracellular medium was free of Na^+ and ATP and had a pH of 8.0. The different values of external $[Na^+]_o$ are indicated on the horizontal dashed lines. The total concentration of NaCl plus LiCl was 440 mM. The open circles refer to Na^+ -free Li $^+$ containing media. Ordinate: Ca^{2+} efflux in fmole. cm^{-2} . s^{-1} . Abscissa: time in minutes. Note: (i) The values of Ca^{2+} efflux at 440 mM Na^+_o are more than nine times lower at 10 μM than at 500 μM Ca^{2+}_i . (ii) The $K_{0.5}$ for Na^+_o is below 100 mM at 10 μM Ca^{2+}_i and around 150 mM at 500 μM Ca^{2+}_i .

Figure 3 shows a typical experiment on the effects of $[Ca^{2+}]_i$ on the Na^+_o stimulation of Ca^{2+} efflux with the Ca^{2+} -regulatory site saturated. Notice that, as always, each axon serves as its own control. After a steady efflux of about 1,400 fmole/ cm^2 s was obtained in the presence of 440 mM Na^+_o and no Ca^{2+}_o , external sodium was removed (replaced with Li $^+$); that induced a drop in the efflux to almost leak levels, indicating that all Ca^{2+} coming out from the cell in the presence of Na^+_e does so through the Na^+/Ca^{2+} exchanger. The external Na^+ concentration was then increased to 50 mM, 100 mM, 200 mM and returned, at the end, to the initial 440 mM; at each concentration sufficient time was allowed to reach steady state. Notice the similar final and initial values in full sodium medium. The dialysis solution was subsequently changed to one containing 500 μM Ca^{2+}_i and, after a steady efflux was obtained, the same protocol was applied. Qualitatively the behavior of the axon was similar to that before, with two major quantitative differences: (i) In this case, averaging the initial and final values, the Ca^{2+} coming out at 440 mM external Na^+ amounted to about 12,200 fmole/ cm^2 s; this is around nine times that observed with 10 μM Ca^{2+}_i . (ii) The $K_{0.5}$ for $[Na^+]_o$ was almost twice that at 10 μM Ca^{2+}_i . The changes in the apparent affinity for Na^+_o are best illustrated in Figure 4, which summarizes the results in three axons and where the fluxes are expressed as a percentage of the maximum in each condition. The data points are the mean $\pm$ SEM, while the lines through the points correspond to the simultaneous best fit to a Hill equation. The fitting parameters gave the

Fig. 4. Summarized data on the effects of $[Ca^{2+}]_i$ on the Na^+_e apparent affinity in the stimulation of Ca^{2+} efflux (forward Na^+_e/Ca^{2+}_i exchange) when the Ca^{2+} -regulatory site is saturated. This Figure 2b summarizes the results from three different axons. The data points are the mean $\pm$ SEM of the Na^+_o -dependent Ca^{2+} efflux from three different axons, expressed as a percentage of the maximum flux in each condition, 500 μM Ca^{2+}_i (open circles) or 10 μM Ca^{2+}_i (filled circles). The lines through the points correspond to the simultaneous best fit to a Hill equation with the following fitting parameters (mean $\pm$ SEM from the fit): 500 μM Ca^{2+}_i : K_s of 143 ± 11 mM and a Hill coefficient of 2.49 ± 0.37 . 10 μM Ca^{2+}_i : K_s of 84 ± 6 mM and a Hill coefficient of 2.57 ± 0.33 . Ordinate: Percentage of Na^+_e -dependent Ca^{2+} efflux. Abscissa: extracellular $[Na^+]_o$ in mM. The difference between the two K_s 's is statistically significant ($P < 0.01$). See text for details.

Fig. 5. Effects of $[Ca^{2+}]_i$ on Ca^{2+} stimulation of Ca^{2+} efflux (Ca^{2+}_o/Ca^{2+}_i exchange) in a dialyzed squid axon with the Ca^{2+} -regulatory site saturated. This figure describes a representative experiment where the two $[Ca^{2+}]_i$'s in μM are indicated by the horizontal bars on top of the figure. In addition, the intracellular medium was free of Na^+ and ATP and had a pH of 8.0. The different values of external $[Ca^{2+}]_o$ are indicated on the horizontal dashed lines. The external solution was free of Na^+ and contained 440 mM LiCl. Open circles refer to Ca^{2+} -free media. Ordinate: Ca^{2+} efflux in fmoles. $cm^{-2} \cdot s^{-1}$. Abscissa: time in minutes. Note: (i) The values of Ca^{2+} efflux at 2 mM Ca^{2+}_o are about three times lower at 10 μM than at 500 μM Ca^{2+}_i . (ii) The $K_{0.5}$ for Ca^{2+}_o goes from about 100 μM at 10 μM to more than 200 μM at 500 μM Ca^{2+}_i .

following values (mean $\pm$ SEM from the fit): At 500 μM Ca^{2+}_i : K_s of 143 ± 11 mM and a Hill coefficient of 2.49 ± 0.37 . At 10 μM Ca^{2+}_i : K_s of 84 ± 6 mM and a Hill coefficient of 2.57 ± 0.33 . The difference between the K_s 's is statistically significant ($P < 0.01$). Therefore, we can conclude that, with the Ca^{2+} -regulatory site saturated, a fifty-fold increase in the intracellular Ca^{2+} concentration increases the maximal rate of the forward Na^+/Ca^{2+} exchange nine fold while it reduces the affinity for extracellular Na^+ to about one half. This concurs with the predictions in the simulations depicted in Figure 2a.

Figure 5 illustrates results with approaches similar to those seen above, investigating the effects of the same $[Ca^{2+}]_i$ on the external Ca^{2+} stimulation of Ca^{2+}/Ca^{2+}_i exchange. As before, each axon served as its own control. This typical experiment shows the same features described above for external Na^+ : (i) The efflux of Ca^{2+} in Ca^{2+} -free Li^+ sea water is practically leak level; therefore, all Ca^{2+} -stimulated Ca^{2+} efflux must go through the Ca^{2+}_o/Ca^{2+}_i mode of the Na^+/Ca^{2+} exchanger. (ii) The maximum efflux at 500 μM Ca^{2+}_i is about three times that observed with 10 μM Ca^{2+}_i ; (iii) The $K_{0.5}$ for Ca^{2+}_o is higher at 500 μM than at 10 μM Ca^{2+}_i . This last result can be clearly seen in Figure 6 which, like Figure 4, summarizes the normalized fluxes from three different axons. Again, the data points represent the mean $\pm$ SEM of the Ca^{2+}_o -dependent Ca^{2+} efflux, at 500 μM (open circles) or 10 μM (filled circles) Ca^{2+}_i . The lines through the points correspond to the simultaneous best fit to a Michaelian function. The estimated K_m 's had a value of 239 ± 15 μM at 500 μM mM Ca^{2+} and 87 ± 6 μM at 10 μM Ca^{2+} with a statistical significance of $P < 0.001$.

Fig. 6. Summarized data on the effects of $[Ca^{2+}]_i$ on the Ca^{2+} apparent affinity in the stimulation of Ca^{2+} efflux (Ca^{2+}_o/Ca^{2+}_i exchange) with the Ca^{2+} -regulatory site saturated. The data points represent the mean $\pm$ SEM of the Ca^{2+}_o -dependent Ca^{2+} efflux from three different axons, expressed as a percentage of the maximum flux in each condition, 500 μM Ca^{2+}_i (open circles) or 10 μM Ca^{2+}_i (filled circles). The lines through the points correspond to the simultaneous best fit to a Michaelian function. The estimated K_m 's had a value of 239 ± 18 μM at 500 μM mM Ca^{2+} and 87 ± 8 μM at 10 μM Ca^{2+} . Ordinate: Ca^{2+}_o -dependent Ca^{2+} efflux in pmoles. $cm^{-2} \cdot s^{-1}$. Abscissa: extracellular Ca^{2+} in μM . The difference between the two $K_{0.5}$'s is statistically significant ($P < 0.001$). See text for details.

Also, it should be noticed that the maximal rate of Ca^{2+} efflux is smaller for the Ca^{2+}_o/Ca^{2+}_i exchange than for the forward Na^+/Ca^{2+}_i exchange mode. This reproduces previous observations [11] and has been taken into account

when building the kinetic model. Also, notice the good agreement with the simulations in Figure 2b.

The results presented here demonstrate that the transport reactions of the squid $\text{Na}^+/\text{Ca}^{2+}$ exchanger indeed follow ping-pong sequential kinetics. According to all available models for $\text{Na}^+/\text{Ca}^{2+}$ exchangers [9, 10, 12, 13], hampering the Ca^{2+}_i -regulatory site is comparable to a reduction of the Ca^{2+} available for the intracellular transport sites; conversely activation of the Ca^{2+}_i -regulatory site is equivalent to an increase in the Ca^{2+}_i accessible to its transport sites. And the predictions are ping-pong kinetics. However, the experimental results contradict that prediction [9], indicating other still unknown properties of regulation in these transporters. As this may very well also be the case for other regulated transporters,

caution must be used when performing kinetics studies in those systems. Finally, the excellent agreement, not just qualitative but quantitative, seen in this work between the predictions of the squid $\text{Na}^+/\text{Ca}^{2+}$ exchange model and the experimental results, must be stressed.

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