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In squid nerve fibers monovalent activating cations are not cotransported during Na^+/Ca^{2+} exchange

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Squid axons display a high activity of Na^+/Ca^{2+} exchange which is largely increased by the presence of external K^+, Li^+, Rb^+ and NH_4^+. In this work we have investigated whether this effect is associated with the cotransport of the monovalent cation along with Ca^{2+} ions. $^{86}Rb^+$ influx and efflux have been measured in dialyzed squid axons during the activation (presence of Ca_i^{2+}) of Ca_o^{2+}/Na_i^+ and Ca_i^{2+}/Ca_o^{2+} exchanges, while $^{86}Rb^+$ uptake was determined in squid optic nerve membrane vesicles under equilibrium Ca^{2+}/Ca^{2+} exchange conditions. Our results show that although K_o^+ significantly increases Na_i^+-dependent Ca^{2+} influx (reverse Na^+/Ca^{2+} exchange) and Rb_i^+ stimulates Ca_o^{2+}-dependent Ca^{2+} efflux (Ca^{2+}/Ca^{2+} exchange), no sizable transport of rubidium ions is coupled to calcium movement through the exchanger. Moreover, in the isolated membrane preparation no $^{86}Rb^+$ uptake was associated with Ca^{2+}/Ca^{2+} exchange. We conclude that in squid axons although monovalent cations activate the Na^+/Ca^{2+} exchange they are not cotransported.

Intracellular free Ca^{2+} ions play a fundamental second messenger role in numerous physiological processes including muscle contraction, regulation of membrane excitability and phototransduction. One of the most important mechanisms responsible for calcium translocation across the plasma membrane is the Na^+/Ca^{2+} exchange. A knowledge of the stoichiometry of the exchange system is critical for a mechanistic understanding of this transport process. During the past few years data obtained from flux measurements in vesicles and cells [1–5] and from electrophysiological experiments [6–9] had led to the consensus that the stoichiometry of the exchange process is 3 Na^+ for 1 Ca^{2+}. However, recent evidence obtained from rod outer segments suggests that K^+ ions are cotransported during the Na^+/Ca^{2+} exchange cycle and that the exchanger coupling ratio is 4 Na^+ : 1 Ca^{2+} + 1K^+ [10,11].

Experimental data obtained is different cell types and membrane vesicles preparations indicate that monovalent cations interact with the Na^+/Ca^{2+} exchanger [12–14]. In squid axons, external K^+, Li^+ and Rb^+ strongly stimulate the Na_i^+/Ca_o^{2+} and Ca_i^{2+}/Ca_o^{2+} exchanges through occupancy of an external activating site [15–17]. The fact that internal K^+ (at constant membrane potential) also activates the Na_o^+/Ca_i^{2+} exchange raises the question of whether an exchange of Na^+ for $Ca^{2+} + K^+$ could be a general feature of the Na^+/Ca^{2+} exchanger.

In the present work we explored this possibility by measuring rubidium ($^{86}Rb^+$) fluxes under conditions of maximal activation of Ca_o^{2+}/Na_i^+ and Ca_o^{2+}/Ca_i^{2+} exchange, using both dialyzed squid axons and squid optic nerve membrane vesicles. Our data are not consistent with transport of the monovalent cation involved in the activation of the Na^+/Ca^{2+} exchanger, thus suggesting that the stoichiometry reported in retinal rods is not a general property of the Na^+/Ca^{2+} exchange.

The experiments were carried out either in dialyzed giant axons from the squids *Loligo pealei* at the Marine Biological Laboratory in Woods Hole, MA, U.S.A., and *Loligo plei* at IVIC, Caracas, Venezuela, or in optic nerve membrane vesicles from the squid *Sepiotheutis sepioidea*. The experimental chamber for dialyzing and voltage clamping the freshly dissected axons has been previously described [18,19]. The compositions of the dialysis solutions as well as those of the external media

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NMG, *N*-methyl-D-glucamine; TEA, tetraethylammonium; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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are given in the legend of Figs. 1 and 2. All external solutions contained: tetrodotoxin ($2 \cdot 10^{-7}$ M), ouabain ($1 \cdot 10^{-4}$ M) and cyanide ($1 \cdot 10^{-3}$ M). In order to reduce the calcium buffering capacity of the axoplasm, 20 μM of ruthenium red was added to the internal medium [20]. Nerve membrane vesicles were isolated from optic nerves using the method described previously [21]. At the end of the isolation procedure the membranes were resuspended at a concentration of approximately 1 mg protein/ml in different media whose compositions are indicated in the legends of Figs. 3 and 4. Membranes were quickly frozen in solid CO_2/acetone and stored at $-60\,^{\circ}$C. To measure $^{45}Ca^{2+}$ and $^{86}Rb^{+}$ uptakes, vesicles were thawed at room temperature and aliquots were diluted 20-fold in media containing either $^{45}Ca^{2+}$ or $^{86}Rb^{+}$.

The compositions of the media appear in the legend of Figs. 3 and 4. The reaction was terminated after an appropriate period of time by filtration of 100-μl aliquots of the mixture through Millipore filters (type HA, 0.45 μm). The filters were subsequently washed twice with 5 ml of ice-cold 0.3 M KCl, 1 mM EGTA, 10 mM Tris-HCl (pH 7.4) and were dried. The radioactivity retained was counted after dissolving the filters in 5 ml Aquasol. All experiments were conducted at 25 °C. $^{45}CaCl_2$ and $^{86}RbCl$ were obtained from New England Nuclear.

The experiment of Fig. 1A shows the well known activating effect of external potassium on the Na_i^+-dependent Ca^{2+} influx (reverse Na^+/Ca^{2+} exchange). For this, the axon was dialyzed with saturating concentrations of internal Na^+ and enough Ca_i^{2+} to fully activate the regulatory intracellular Ca^{2+} site [22]. The membrane potential was held constant (0 mV) during the entire course of the experiment. After a steady-state Ca^{2+} influx was reached (about 4 pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}), addition of 20 mM K_o^+ increased Ca^{2+} influx to 8 pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}. This effect was reversed upon withdrawal of K_o^+. Subsequent removal of internal Ca^{2+} decreased the influx to very low values, indicating that most of the Ca^{2+} influx observed under these conditions occurs through the reversal of the Na^+/Ca^{2+} exchange.

In order to test whether the stimulating monovalent cation is transported by the exchanger while performing Ca_o^{2+}/Na_i^+ exchange, rubidium influx was measured during activation by intracellular Ca^{2+} of reverse Na^+/Ca^{2+} exchange in the presence of 20 mM K_o^+. The experiment of Fig. 1B shows that addition of 100 μM Ca_i^{2+} causes no effect on the steady-state level of ^{86}Rb influx measured in the absence of internal Ca^{2+}. If Ca_o^{2+}/Na_i^+ exchange were associated with a cotransport of rubidium with a 1 Rb^+ : 1 Ca^{2+} stoichiometry, a significant increase (about 4 pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}) in the $^{86}Rb^+$ influx would be expected upon activation of the exchanger by Ca_i^{2+} (see Fig. 1A). The presence of 50 mM external K^+ in this experiment should not affect the expected Rb^+ influx since the affinities of the exchanger for K^+ and Rb^+ are similar [12,13,23].

It has previously been shown that in the absence of Na^+ ions external monovalent cations (Li^+, K^+, Rb^+, NH_4^+) induce a sizable Ca_o^{2+}/Ca_i^{2+} exchange through the Na^+/Ca^{2+} exchanger [12,16]. Moreover, this exchange can be further activated by internal monovalent cations [17]. Fig. 2A shows that in the absence of Na^+ ions and in the presence of 440 mM Li_o^+, 50 mM internal rubidium stimulates the efflux of Ca^{2+}. This increase in Ca^{2+} efflux is completely dependent on the presence of Ca_o^{2+} thus indicating that the internal monovalent cation effect is on the Ca_o^{2+}/Ca_i^{2+} ex-

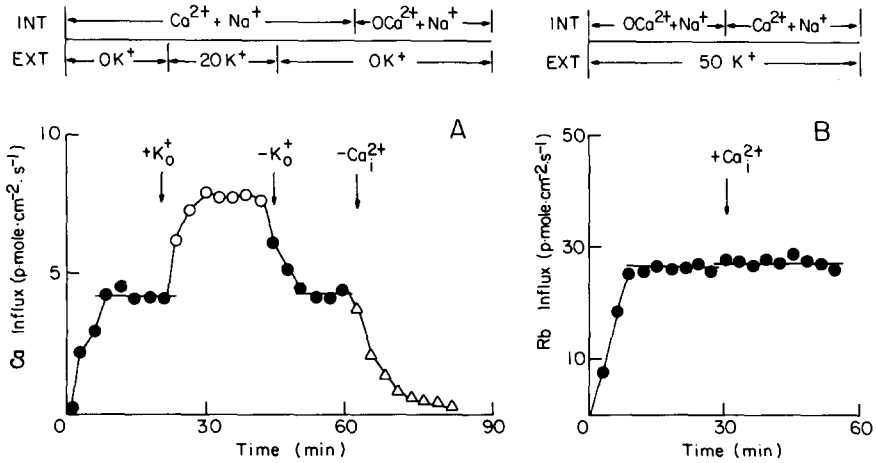

Fig. 1. (A) The effect of external potassium on the Na_i^+-dependent Ca^{2+} influx (reverse Na^+/Ca^{2+} exchange) at constant membrane potential (0 mV). Ordinate: Ca^{2+} influx in pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}. Abscissa: time in minutes. The internal dialysis solution had the following composition (mM): KCl, 250; NaCl, 200; Tris-Mops (pH 7.4), 50; TEA-Cl, 50; EGTA, 1; $CaCl_2$, 1.1. The external solution had the following composition (mM): Tris-Mops, 230; Tris-HCl, 216; KCl, 20; $MgCl_2$, 50; $CaCl_2$, 11; EGTA, 1 (pH 7.8). The osmolarity was adjusted to 1000 mosM. (B) The effect of internal Ca^{2+} on the $^{86}Rb^+$ influx in the presence of 50 mM external K^+. Ordinate: Rb^+ influx in pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}. Abscissa: time in minutes. The internal medium had the same composition as in Fig. 1A. The external medium contained 50 mM K^+. The temperature was 18 °C.

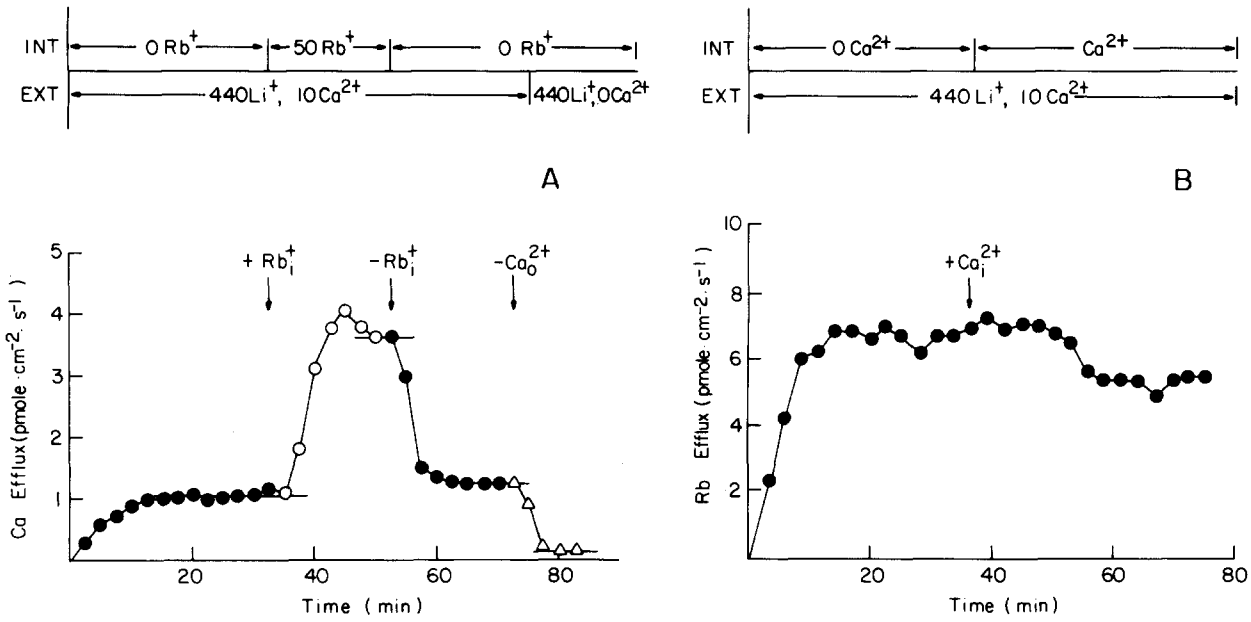

Fig. 2. (A) The effect of internal rubidium on the Ca_o^{2+}-dependent Ca^{2+} efflux (Ca^{2+}/Ca^{2+} exchange). Ordinate: Ca^{2+} efflux in pmol·cm^{-2}·s^{-1}. Abscissa: time in minutes. The internal dialysis solution had the following composition (mM): Tris-Mops, 200 (pH 7.4); TEA-Cl, 50; RbCl, 0 or 50; $MgCl_2$, 5: EGTA, 1; $CaCl_2$, 1.1; glycine was added to adjust the osmolarity to 1000 mosM. The external solution had the following composition (mM): LiCl, 440; TEA-Cl, 50; $CaCl_2$, 10; $MgCl_2$, 50; Tris-Mops, 10 (pH 7.8). (B) The effect of internal Ca^{2+} (100 μM) on $^{86}Rb^+$ efflux. The internal dialysis medium contained 50 mM RbCl with or without Ca^{2+}. The external medium had the same composition as in Fig. 2A. The temperature was 18° C.

change component. To test whether Ca_o^{2+}/Ca_o^{2+} exchange is associated with an efflux of the stimulating internal rubidium, $^{86}Rb^+$ efflux was measured in the absence (zero Ca_i^{2+}) and in the presence (100 μM Ca_i^{2+}) of a large Ca_o^{2+}/Ca_i^{2+} exchange component. Fig. 2B clearly shows that addition of internal Ca^{2+} does not stimulate the efflux of Rb^+ but actually reduces it below the level obtained in the absence of Ca_i^{2+}.

Membrane vesicles from squid nerve fibers are a suitable preparation to explore further the effect of monovalent cations in stimulating Ca^{2+}/Ca^{2+} exchange activity and their possible cotransport during exchange activity. It has previously been shown in cardiac sarcolemmal vesicles that K^+, Li^+, Rb^+ and Cs^+ activate Ca^{2+}/Ca^{2+} exchange under equilibrium conditions. However, the stimulating monovalent cations do not appear to be cotransported with Ca^{2+} ions during Ca^{2+}/Ca^{2+} exchange [13]. We first explored the effect of monovalent cations (100 mM) on the $^{45}Ca^{2+}$ uptake under equilibrium conditions. The effectiveness of Li^+, K^+ and Rb^+ in stimulating equilibrium Ca^{2+}/Ca^{2+} exchange activity in squid nerve membrane vesicles is depicted in Fig. 3. Our results confirm previous reports of this phenomenon in cardiac sarcolemmal vesicles and synaptosomes [13,14,24].

In order to test if the stimulating monovalent cation is cotransported during the operation of Ca^{2+}/Ca^{2+} exchange in nerve membrane vesicles we adopted a procedure similar to that described previously by Slaughter et al. [13]. The isolated membrane was resuspended in either 300 mM RbCl, 0.5 mM EGTA plus 1 mM $CaCl_2$ or 300 mM RbCl plus 0.5 mM EGTA, and was quickly frozen in solid CO_2/acetone. After thawing

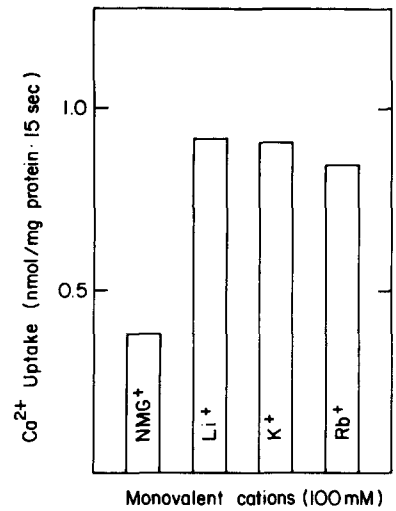

Fig. 3. Stimulation of Ca^{2+}/Ca^{2+} exchange by various monovalent cations. Squid optic nerve membranes were resuspended in different mixtures containing the Cl^- salts of the cations indicated (290 mM NMG^+ or 190 mM NMG^+ + 100 mM Li^+, K^+ or Rb^+) and 100 μM $CaCl_2$, were quickly frozen in solid CO_2/acetone and stored at −60° C. $^{45}Ca^{2+}$ uptake was measured under equilibrium conditions in each case, diluting 10 μl of thawed vesicles to 200 μl with the corresponding medium which contained $^{45}Ca^{2+}$. 100-μl aliquots were filtered after 15 s through Millipore filters (type HA, 0.45 μm). The filters were washed twice with 5 ml of ice-cold 0.3 M KCl, 1 mM EGTA. All solutions used were buffered to pH 7.4 with 10 mM Mops-NMG.

at room temperature, the vesicles were diluted 20-fold into a medium containing 30 mM RbCl (final Rb^+ concentration was approximately 43.5 mM) and either 0.5 mM EGTA plus 1 mM $CaCl_2$ or 1 mM EGTA. $^{86}Rb^+$ influx was determined under conditions in which Ca^{2+}/Ca^{2+} exchange was maximally activated. The results of this experiment are shown in Fig. 4. In Fig. 4A, in the presence of external Ca^{2+}, EGTA-containing vesicles show only a basal level of passive $^{45}Ca^{2+}$ uptake, while Ca^{2+}/Ca^{2+} exchange takes place in the calcium-containing vesicles. The rate of $^{45}Ca^{2+}$ uptake by calcium-containing vesicles minus that corresponding to EGTA-containing vesicles provides a measure of the Ca^{2+}/Ca^{2+} exchange activity. This Ca^{2+}/Ca^{2+} exchange attains a steady state after 5 min and shows an initial rate of approx. 0.25 nmol/mg protein per s. If the exchange process were associated with Rb^+ transport we should expect a noticeable stimulation of $^{86}Rb^+$ uptake during Ca^{2+}/Ca^{2+} exchange. As shown in Fig. 4B, external $^{86}Rb^+$ rapidly equilibrates with internal unlabelled Rb^+ in the presence of valinomycin. In the absence of the ionophore, $^{86}Rb^+$ uptake is slower and, similarly to the results obtained in cardiac sarcolemmal vesicles, the presence of extravesicular Ca^{2+} does not stimulate $^{86}Rb^+$ uptake, but slightly inhibits it with respect to that observed in the presence of EGTA. The experiment indicates that Rb^+ is not cotransported during Ca^{2+}/Ca^{2+} exchange. We must stress that although the passive Rb^+ permeability is relatively high compared to that of Ca^{2+}, the experiment is sensitive enough to detect, if present, any Rb^+ movement associated to Ca^{2+}/Ca^{2+} exchange. At an initial rate of Ca^{2+}/Ca^{2+} exchange of about 0.25 nmol/mg protein per s, after 1 min, approx. 15 nmoles of $^{86}Rb^+$ should have entered the vesicles if $Rb^+ : Ca^{2+}$ stoichiometry is 1 : 1. On the contrary, we actually observed a decrease in Rb^+ uptake during Ca^{2+}/Ca^{2+} exchange.

The experiments presented in this paper were directed to reexamine the issue of the stoichiometry of the Na^+/Ca^{2+} exchange in squid axons in view of the recent interesting finding that in rod outer segments the exchanger also transports K^+ ions, with a consequent stoichiometry of 4 Na^+ : 1 Ca^{2+} + 1 K^+. Our results clearly demonstrate that in squid axon the monovalent activating cations are not cotransported during Na_i^+/Ca_o^{2+} or Ca_i^{2+}/Ca_o^{2+} exchange.

The activating effect of monovalent cations on Na^+/Ca^{2+} exchange was first noted in early studies in squid axons [12]. Since then, this phenomenon has been reported in a variety of intact cells as well as in mem-

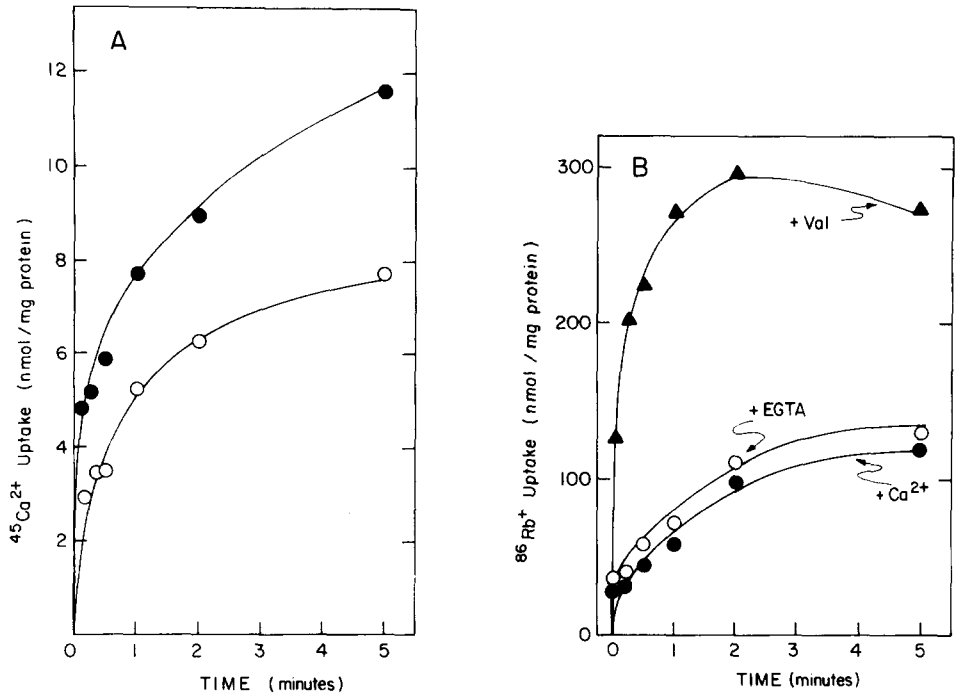

Fig. 4. (A) Time course of Ca^{2+} uptake by squid optic nerve membrane vesicles in the presence (●) or in the absence (○) of intravesicular Ca^{2+}. The membranes were resuspended in either 300 mM RbCl, 0.5 mM EGTA and 1 mM $CaCl_2$ (●) or in 300 mM RbCl and 0.5 mM EGTA (○) and were frozen as indicated in the legend of Fig. 3. To be assayed for Ca^{2+}/Ca^{2+} exchange the vesicles were thawed and 40 μl of the appropriate batch were diluted 20-fold in a medium containing 30 mM RbCl, 270 mM NMG-Cl, 0.5 mM EGTA and 1 mM $^{45}CaCl_2$. All solutions used were buffered to pH 7.4 with 10 mM Mops-NMG. (B) $^{86}Rb^+$ uptake by vesicles performing Ca^{2+}/Ca^{2+} exchange. Membranes were resuspended in 300 mM RbCl, 0.5 mM EGTA, 1 mM $CaCl_2$ and 10 mM Mops-NMG (pH 7.4) and were quickly frozen as indicated. Aliquots of vesicles (40 μl) were mixed with 760 μl of 30 mM ^{86}RbCl, 270 mM NMG-Cl plus 10 mM Mops-NMG (pH 7.4) and either 1 mM EGTA (○), 0.5 mM EGTA and 1 mM $CaCl_2$ (●) or 1 mM EGTA and 2 μM valinomycin (▲). The points represent average of duplicate determinations for three different membrane preparations.

brane vesicles preparations. Although it appears to be a general feature of the Na^+/Ca^{2+} exchange system, the monovalent cations effect in squid axons seems to be markedly different from that reported in retinal rods: (i) in squid axons although monovalent cations activate the exchanger, they are not absolutely required for Na^+/Ca^{2+} countertransport as is the case in the retinal rod outer segments [10,11]; (ii) in squid axons the apparent K_m of activation by monovalent cations is approx. 100 mM [12] while an apparent K_m of 1 mM has been reported in retinal rods for this effect [11]; (iii) Na^+/Ca^{2+} exchange in retinal rod outer segments requires K^+ ions but not with a fixed sidedness, K^+ must be present on the same side of the membrane as Ca^{2+} [11]. On the contrary, in squid axons the activating monovalent cation site appears to be located at the extracellular face of the carrier. In fact, no activation by internal Li^+ was observed unless the external monovalent cation site was occupied [17]. The fact that the squid data closely resemble those obtained in cardiac membrane vesicles may indicate a higher analogy between the Na^+/Ca^{2+} exchange molecule of squid nerves and cardiac sarcolemma as compared to the retinal rod exchanger.

Furthermore, recent purification studies have identified the retinal rod Na^+/Ca^{2+} exchanger as a polypeptide of 215 kDa in contrast to the 120/70 kDa polypeptide suggested to be the cardiac sarcolemmal ex changer [25–27]. The above considerations lead to the conclusion that the Na^+/Ca^{2+} exchange appears to be a versatile transport system which in the case of the retinal rod outer segments is able to utilize both Na^+ and K^+ gradients for Ca^{2+} extrusion in order to overcome the absence of an ATP-dependent Ca^{2+} pump.

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References

1 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232–6235.
2 Reeves, J.P. and Hale, C.C. (1984) J. Biol. Chem. 259, 7733–7739.
3 Wakabayashi, S. and Goshima, K. (1981) Biochim. Biophys. Acta 648, 311–317.
4 Bridge, J.H.B. and Bassingthwaighte, J.B. (1983) Science 219, 178–180.
5 Rasgado-Flores, H., Santiago, E.M. and Blaustein, M.P. (1989) J. Gen. Physiol. 93, 1219–1241.
6 Kimura, J., Miyamae, S. and Noma, A. (1987) J. Physiol. 384, 199–222.
7 Caputo, C., Bezanilla, F. and DiPolo, R. (1988) Biophys. J. 53, 224a.
8 Yau, K.W. and Nakatani, K. (1984) Nature 311, 661–663.
9 Ehara, T., Matsuoka, S. and Noma, A. (1989) J. Physiol. 410, 227–249.
10 Cervetto, L., Lagnado, L., Perry, R.J. and McNaughton, P.A. (1989) Nature 337, 740–743.
11 Schnetkamp, P.P.M., Basu, D.K. and Szerencsei, R.T. (1989) Am. J. Physiol. 257, C153–C157.
12 Baker, P.F., Blaustein, M.P., Hodgkin, A.L. and Steinhardt, R.A. (1969) J. Physiol. 200, 431–458.
13 Slaughter, R.S., Sutko, J.L. and Reeves, J.P. (1983) J. Biol. Chem. 258, 3183–3190.
14 Coutinho, O.P., Carvalho, A.P. and Carvalho, C.A.M. (1983) J. Neurochem. 41, 670–676.
15 Allen, T.J.A. and Baker, P.F. (1986) J. Physiol. 378, 53–76.
16 Blaustein, M.P. (1977) Biophys. J. 20, 79–111.
17 DiPolo, R. and Beaugé, L. (1990) J. Gen. Physiol. (in press).
18 DiPolo, R. (1979) J. Gen. Physiol. 73, 91–113.
19 DiPolo, R., Bezanilla, F., Caputo, C. and Rojas, H. (1985) J. Gen. Physiol. 86, 457–478.
20 Baker, P.F. and Umbach, J.A. (1987) J. Physiol. 383, 369–394.
21 Condrescu, M., Gerardi, A. and DiPolo, R. (1988) Biochim. Biophys. Acta 946, 289–298.
22 DiPolo, R. and Beaugé, L. (1988) Biochim. Biophys. Acta 947, 549–569.
23 Blaustein, M.P. and Russell, J.M. (1975) J. Membr. Biol. 22, 285–312.
24 Blaustein, M.P. and Ector, A.C. (1976) Biochim. Biophys. Acta 419, 295–308.
25 Cook, N.J. and Kaupp, U.B. (1988) J. Biol. Chem. 263, 11382–11388.
26 Nicoll, D.A. and Applebury, M.L. (1989) J. Biol. Chem. 264, 16207–16213.
27 Philipson, K.D., Longoni, S. and Ward, R. (1988) Biochim. Biophys. Acta 945, 298–306.