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The sodium–calcium exchanger of bovine rod photoreceptors: K^+-dependence of the purified and reconstituted protein

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The K^+-dependence of the rod photoreceptor sodium–calcium exchanger was investigated using the Ca^{2+}-sensitive dye arsenazo III after reconstitution of the purified protein into proteoliposomes. The uptake of Ca^{2+} by Na^+-loaded liposomes was found to be greatly enhanced by the presence of external K^+ ($EC_{50} \approx 1$ mM) in a Michaelis-Menten manner, suggesting that one K^+ ion is involved in the transport of one Ca^{2+} ion. We also found a minimal degree of Ca^{2+} uptake in the total absence of K^+. Other alkali cations, notably Rb^+ and, to a lesser extent, Cs^+, were also able to stimulate Na^+–Ca^{2+} exchange. We also investigated the K^+-dependence of the photoreceptor Na^+–Ca^{2+} exchanger by determining the effects of electrochemical K^+ gradients on the Na^+-activated Ca^{2+} efflux from proteoliposomes. We found that, under conditions of membrane voltage clamp with FCCP, inwardly directed electrochemical K^+ gradients (i.e., $K_0^+ > K_i^+$) inhibited, whereas an outwardly directed electrochemical K^+ gradient (i.e., $K_i^+ > K_0^+$) enhanced, Na^+-dependent Ca^{2+} efflux, consistent with the notion that K^+ is cotransported in the same direction as Ca^{2+}. The investigation of the reconstituted exchanger at physiological (i.e. $K_i^+ = 110$ mM, $K_0^+ = 2.5$ mM) potassium concentrations revealed that the Na^+-dependence of Ca^{2+}-efflux was highly cooperative ($n = 3.01$ from Hill plots), indicating that at least three, but possibly four, Na^+ ions are exchanged for one Ca^{2+} ion. Under these conditions the reconstituted exchanger showed a K_m for Na^+ of 26.1 mM, and a turnover number of 115 $Ca^{2+} \cdot s^{-1}$ per exchanger molecule. Our results with the purified and reconstituted sodium–calcium exchanger from rod photoreceptors are therefore consistent with previous reports (Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W. and McNaughton, P.A. (1989) Nature 337, 740–743; Schnetkamp, P.P.M., Basu, D.K. and Szerencsei, R.T. (1989) Am. J. Physiol. 257, C153—C157) that the sodium–calcium exchanger of rod photoreceptors cotransports K^+ under physiological conditions with a stoichiometry of 4 Na^+ : 1 Ca^{2+}, 1 K^+.

Introduction

The sodium–calcium exchanger of bovine rod photoreceptors is a 220 kDa glycoprotein specifically located in the plasma membrane of the rod outer segment [1–5]. It is responsible for the extrusion of calcium ions entering the ROS cytosol through cGMP-gated cation channels, also present in the plasma membrane, under conditions of darkness. It was originally believed that the driving force for calcium-extrusion by the exchanger was exclusively constituted by an inwardly directed electrochemical sodium gradient, and that the exchange process occurred with a stoichiometry of 3 Na_0^+ : 1 Ca_i^{2+}, as is believed to be the case for other tissues [6]. Recent evidence [7–9] has however shown that, at least in rod photoreceptors, the sodium–calcium exchanger is potassium-dependent and that potassium itself may be transported. The outwardly directed electrochemical K^+ gradient present in these cells would thereby provide a further driving force for sodium–calcium exchange. In other systems, notably cardiac sarcolemma and squid axon, it has been difficult to clearly demonstrate potassium-cotransport with sodium–calcium exchange [10, 11]. This has led to speculation [10] that either (i) the sodium–calcium exchangers of rod photoreceptors and cardiac sarcolemma are different processes carried out by different molecules, or (ii) that the sodium–calcium exchanger in ROS is associated with a distinct K^+-transport process.

Abbreviations: ROS, rod outer segment; cGMP, guanosine 3′,5′-cyclic monophosphate; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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We have recently succeeded in isolating and functionally reconstituting the sodium–calcium exchanger of bovine rod outer segments [12]. The availability of the exchanger in its purified form permits the direct investigation of the ROS sodium–calcium exchanger in the complete absence of other transport processes, thereby allowing the direct demonstration of whether or not particular transport properties are intrinsic properties of the exchanger molecule itself. Furthermore, the reconstitution procedure employed [13] allows the imposition of completely defined ionic conditions on both sides of the liposome membrane, thereby eliminating the possibility of ionic contamination which may have complicated the interpretation of results from other studies. In this report we have investigated the potassium dependence of the ROS sodium–calcium exchanger in its purified and reconstituted form. The results demonstrate a clear K^+-dependence of the purified protein, and are consistent with the notion that K^+ is cotransported during the sodium–calcium exchange process.

Experimental

Materials

Arsenazo III was purchased from Fluka (Ulm, F.R.G.) and treated by passage over a Chelex-100 chelating column before use. All other chemicals and reagents were of the highest quality available, and were from the suppliers described in previous publications [12–14].

Preparation of bovine ROS

ROS membranes were prepared from dark-adapted bovine retinas by modifying the procedure of Uhl et al. [15]. Briefly, retinas from 60–80 eyes were excised and suspended in (final volume) 108 ml 600 mM sucrose solution (10 mM Hepes-KOH (pH 7.4), 1 mM $CaCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 mM glucose and 600 mM sucrose). After briefly vortexing (30–60s), the extract was filtered through a nylon mesh (150 μm mesh) and 9-ml aliquots of filtrate were applied on top of 25 ml cushions of the same solution containing 34.2% (w/v) sucrose. After centrifugation in a SW-28 rotor (20000 rpm for 20 min), the ROS bands at the interface between the two solutions were collected by aspiration and diluted to (final volume) 180 ml with 10 mM Hepes-KOH (pH 7.4) containing 600 mM sucrose and 1 mM DTT. After centrifugation (SS-34 rotor, 17000 rpm for 30 min) the ROS pellets were resuspended in dilution buffer and stored at −70°C until use. ROS prepared in this manner typically showed an $A_{280/500}$ ratio of 2.3–2.8 when determined in the presence of 1% (v/v) LDAO (lauryldimethylamine oxide).

Purification and reconstitution of the ROS Na^+/Ca^{2+} exchanger

The sodium–calcium exchanger was purified from stripped ROS membranes by a combination of anion-exchange chromatography, AF-Red affinity chromatography and lectin-affinity chromatography as previously described [12]. This procedure yielded essentially homogeneous 220 kDa protein. Purified exchanger was desalted and the buffer was exchanged by gel-filtration on PD-10 columns (Pharmacia) equilibrated with the appropriate buffer followed by concentration using a Centricon-30 microconcentator (Amicon). The concentration of the exchanger protein was determined by SDS-electrophoresis of purified extract or proteoliposomes and a standard curve of bovine serum albumin (0.1–1 μg) using the method of Laemmli [16]. After staining with Coomassie blue R-250, gels were scanned using a Hoefer GS-300 gel scanner and protein bands were integrated and quantitated using the Hoefer GS-360 data system.

Reconstitution into calcium-containing asolectin liposomes was carried out essentially as previously described [13]. Briefly, a concentrated asolectin solution was added to exchanger extract to give the following concentrations: 10 mM Hepes-arginine (pH 7.4), 1 mM DTT, 0.6% (w/v) CHAPS, 10 $mg \cdot ml^{-1}$ asolectin and KCl, choline chloride and $CaCl_2$ as described in the figure legends. After dialysis (three changes, total time 48 h) against 10 mM Hepes-arginine containing the appropriate salt concentrations, the liposomes were dialysed against calcium-free buffer in order to establish a transmembrane calcium gradient.

Reconstitution into sodium-loaded liposomes was carried out using the same dialysis procedure except that the reconstitution and dialysis buffers contained the appropriate sodium concentration and were calcium-free. Dialysis was carried out for a total of 36–48 h with three changes. In all cases where potassium-free conditions were desired, the potassium concentration was below the limit of detection (about 20 μM) of atomic absorption procedures. In all reconstitution procedures, the concentration of the ROS sodium–calcium exchanger was 5–20 $\mu g \cdot ml^{-1}$ liposomes.

Spectroscopic measurements

All spectroscopic measurements were carried out using an Aminco DW-2000 spectrophotometer and the calcium-sensitive metallochromic dye arsenazo III at an extraliposomal concentration of 50 μM. The cuvette was connected to a magnetic stirring block and contained a sample volume of 2 ml. Reagents were added to the cuvette by injection through an orifice in the lid of the cuvette-holder. In the case of Ca^{2+} efflux measurements, the cuvette contained 0.4 ml liposomes (except in Fig. 6, where 46 μl liposomes were present in the

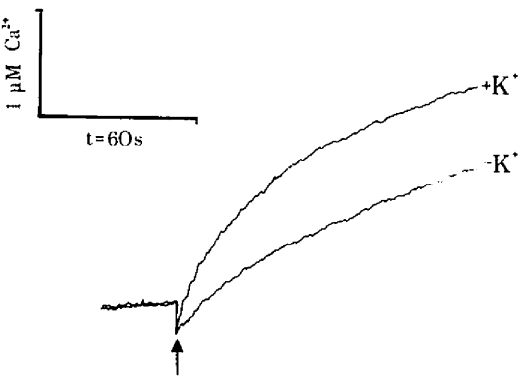

Fig. 1. The effects of symmetrical K^+ on Na^+-activated Ca^{2+} release. Proteoliposomes were prepared and assayed in the presence (upper trace) or absence (lower trace) of symmetrical KCl (100 mM). In the absence of K^+, 100 mM choline chloride was used to establish the appropriate ionic strength. Liposomes contained 2 mM $CaCl_2$, and at the time-point indicated by the arrow Ca^{2+} efflux was initiated by the addition of 50 mM NaCl to the cuvette.

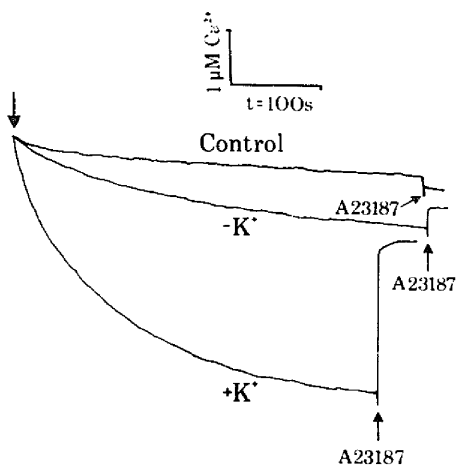

Fig. 2. The effects of K^+ on Na_i^+-activated Ca^{2+} uptake. Proteoliposomes (100 μl) prepared in the presence of symmetrical (150 mM) NaCl were added to a cuvette containing 50 μM arsenazo III and 30 μM $CaCl_2$ in the presence (lower trace) or absence (middle trace) of 50 mM KCl. Ionic strength was maintained at the appropriate concentration by choline chloride. In the control experiment (upper trace), proteoliposomes were added to the cuvette to yield 150 mM Na^+ both intra- and extraliposomally. Ca^{2+} taken up could be rereleased by the addition of the calcium ionophore A23187 to the cuvette at the time points indicated by the arrows.

cuvette) and efflux was activated by the addition of NaCl. In all experiments the protonophore FCCP was added at a concentration of 2 μM to prevent the build-up of an unfavourable membrane potential during the exchange process [3]. In the case of Ca^{2+}-uptake measurements, the cuvette contained a buffered Na^+-free Ca^{2+}-arsenazo III solution and uptake was initiated by the addition of 100 μl liposomes prepared in the presence of symmetrical (150 mM) NaCl. This resulted in dilution of the extraliposomal Na^+ concentration, thereby providing the Na^+ gradient necessary for Ca^{2+} uptake. Spectral data were recorded in the dual wavelength mode using the wavelength pair 650–730 with a slit of 3 nm. Therefore, calcium-release signals exhibited a positive absorbance change whereas calcium-uptake signals exhibited a negative change in absorbance. Signals were calibrated by injecting known concentrations of Ca^{2+} or EGTA into the cuvette and were analysed for initial rates of uptake or efflux on an IBM PS/2 personal computer using the Aminco DW-2000 software.

Results

In Fig. 1, Na^+-activated Ca^{2+} efflux from Ca^{2+}-loaded liposomes in the presence or absence of symmetrical (100 mM) K^+ is shown. The presence of K^+ was found to yield a significantly (at least 5-fold) faster initial rate of Ca^{2+} efflux. A significant degree of Na^+-dependent Ca^{2+} release was also observed in the absence of K^+, thereby alluding to the existence of a slow K^+-independent Na^+–Ca^{2+} exchange mode.

To confirm the results in Fig. 1, we examined the uptake of Ca^{2+} by Na^+-loaded proteoliposomes (Fig. 2). The results demonstrate that when K^+ is present extraliposomally, i.e., on the same side of the membrane as Ca^{2+}, the rate of Na^+-dependent Ca^{2+} uptake is greatly enhanced. We also found significant Ca^{2+} uptake in the absence of K^+, thereby confirming the results of Fig. 1 which indicate the presence of a slow K^+-independent Na^+–Ca^{2+} exchange activity.

In Fig. 3, the alkali cation dependence of Na^+-dependent Ca^{2+}-uptake is investigated. Not unexpectedly,

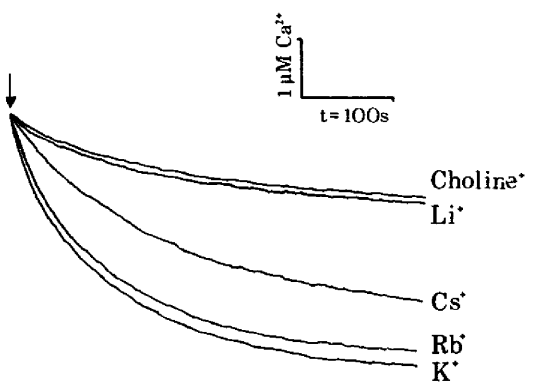

Fig. 3. The effects of various monovalent cations on Na_i^+-activated Ca^{2+}-uptake. Spectroscopy was carried out as described in the legend to Fig. 2, except that various monovalent cations were present extraliposomally at a concentration of 50 mM.

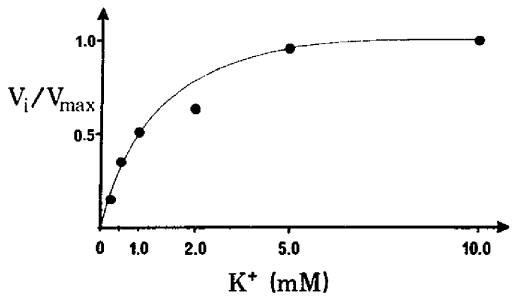

Fig. 4. The K^+-concentration dependence of Na_i^+-activated Ca^{2+} uptake. Ca^{2+} uptake of proteoliposomes was measured as described in the legend to Fig. 2, except that different K^+ concentrations were present extraliposomally. Normalised initial rates of Ca^{2+} uptake are plotted against the extraliposomal K^+ concentration.

Rb^+ was also found to activate Na^+–Ca^{2+} exchange to a level almost comparable to the activity observed in the presence of K^+. Cs^+ was also found to enhance Ca^{2+} uptake significantly, whereas activity in the presence of Li^+ or choline was minimal.

In Fig. 4, the rate of Ca^{2+} uptake is plotted as a function of the K_0^+ concentration. The K^+ dependence was found to follow Michaelis-Menten kinetics (thereby suggesting that one K^+ ion is involved in the transport of one Ca^{2+} ion), with an EC_{50} value of about 1 mM K^+. This correlates well with the values recently reported by Schnetkamp et al. [9].

Although the above results demonstrate a clear K^+ dependence of the purified and reconstituted Na^+–Ca^{2+} exchanger, they do not demonstrate whether or not K^+ is transported during the exchange process. We therefore investigated the effects of electrochemical K^+ gradients, where K^+ was present at saturating concentrations on both sides of the liposome membrane, on Na^+-activated Ca^{2+} efflux. Should K^+ ions activate

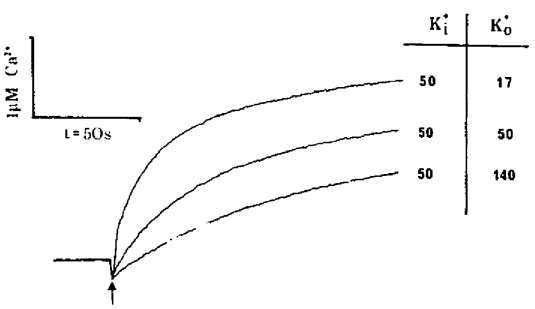

Fig. 5. The effects of electrochemical K^+ gradients on Na_0^+-activated Ca^{2+} release. Proteoliposomes containing 2 mM $CaCl_2$ were prepared in the presence of 50 mM symmetrical KCl, and added to a cuvette to give the indicated external K^+ concentrations. At the time-point indicated by the arrow 50 mM NaCl was added to the cuvette. The respective K_i^+ and K_0^+ concentrations are given in mM at the right-hand side of the traces.

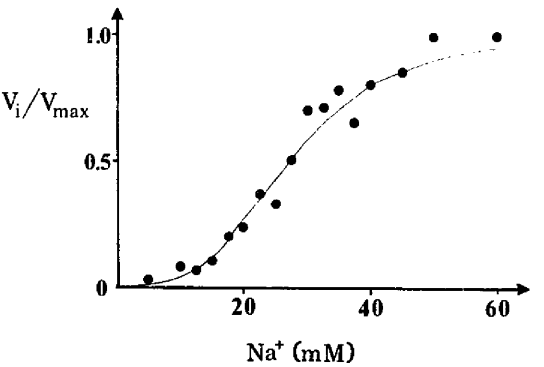

Fig. 6. The Na^+-dependence of Ca^{2+}-release at physiological K^+ concentrations. Proteoliposomes containing 5 mM $CaCl_2$ were prepared in the presence of 110 mM symmetrical KCl, and added to a cuvette to give an external K^+ concentration of 2.5 mM. Normalised initial rates of Ca^{2+} efflux are plotted as a function of the Na^+ concentration added to the cuvette. The continuous line represents normalised efflux rates for a cooperative process where $n = 3.4$ and $K_m = 27$ mM Na^+, and was calculated as previously described [12].

Na^+–Ca^{2+} exchange by, say, simply binding to the exchange protein without being transported, then such gradients should have no effect on Na^+-dependent Ca^{2+} efflux. The results (Fig. 5) demonstrate that an electrochemical K^+ gradient imposed against the direction of Ca^{2+}-transport decreased the rate of Na^+/Ca^{2+} exchange, whereas an electrochemical K^+ gradient imposed in the same direction as Ca^{2+} transport greatly enhanced the rate of Ca^{2+} efflux. These results clearly support the notion [8,9] that K^+ is cotransported in the same direction as Ca^{2+}.

In order to further characterise the kinetic properties of the purified and reconstituted ROS exchanger, we investigated the sodium-dependence of the Na^+–Ca^{2+} exchanger at physiological (i.e., 110 mM K_i^+, 2.5 mM K_0^+) K^+ concentrations. A typical experiment is shown in Fig. 6. We found that, under these conditions, the

TABLE I

Kinetic properties of the Na^+-dependence of the ROS Na^+–Ca^{2+} exchanger at physiological K^+ concentrations

Data represent the results from four different kinetic experiments (see Fig. 6).

	Mean ± S.D.	Range
K_m (Na^+)	26.1 ± 2.0	24.0 – 28.0
Cooperativity, n [a]	3.01 ± 0.34	2.50 – 3.42
V_{max} [b]	31.1 ± 1.6	29.7 – 33.6
Turnover number [c]	115 ± 6	110 – 123

[a] Determined from Hill plots.

[b] Units are μmol Ca^{2+}/min per mg protein.

[c] Calculated from the values for V_{max} assuming a molecular mass of 220 kDa for the exchanger molecule. Units are Ca^{2+} ions transported per exchanger per s.

Na^+ dependence of Ca^{2+} release was highly sigmoidal, suggesting that the transport of one Ca^{2+} ion is coupled to the transport of several Na^+ ions. A summary of the results from four such experiments is given in Table I.

Discussion

The K^+-dependence of the purified and reconstituted ROS Na^+/Ca^{2+}-exchanger

Our results demonstrate a clear K^+-dependence of the purified and reconstituted sodium–calcium exchanger from bovine ROS. Although we found that sodium–calcium exchange could occur to some degree even in the absence of potassium, this observation is probably of no physiological significance, since it is hardly likely that K^+ would ever be present intracellularly at concentrations which would not influence the Na^+–Ca^{2+} exchanger (i.e., below 1 mM, see Fig. 4). The existence of a K^+-independent form of the ROS exchanger may, however, be of mechanistic interest. This could imply that the K^+-binding site is of relatively low specificity and other ions such as choline (the ion we have used to maintain ionic strength in the absence of K^+) or even Ca^{2+} itself can substitute for K^+, albeit very poorly, at this site.

Our results concerning the effects of electrochemical K^+-gradients on the reconstituted ROS Na^+–Ca^{2+} exchanger are in compliance with previous reports [7–9] that K^+ is cotransported during ROS Na^+–Ca^{2+} exchange. Since these results were obtained using the purified exchanger, we were able to unequivocally demonstrate that this property is intrinsic to the exchanger molecule itself and is not due to the action of an associated K^+-transport process.

Na^+–Ca^{2+} exchange at physiological K^+ concentrations

It was of interest to investigate the kinetics of the Na^+-dependence of the purified and reconstituted exchanger, since when identifying and purifying the exchange protein from ROS membranes [12] we performed Ca^{2+}-efflux measurements in the presence of symmetrical (100 mM) K^+. In such experiments the purpose of the reconstitution procedure was simply to monitor the exchange protein during chromatographic purification, whereas an attempt to optimise the conditions for Na^+–Ca^{2+} exchange was not undertaken.

From Fig. 6 and Table I it can be seen that at physiological K^+ concentrations the ROS Na^+–Ca^{2+} exchanger exhibits a slightly lower K_m for Na^+ than in the presence of symmetrical 100 mM KCl [12]. This may indicate that extraliposomal K^+ is capable of competing with Na^+ for its binding site. Furthermore, the Na^+ dependence for Ca^{2+} transport under these conditions was found to be highly sigmoidal, indicative of a cooperative process. From Hill plots ($n = 3.01$, range 2.50–3.42) the number of Na^+ ions needed to activate the transport of one Ca^{2+} is at least three but, given that Hill plots tend to underestimate the degree of cooperativity, more likely four. Given that one K^+ ion appears to be necessary for the transport of one Ca^{2+} ion (see Fig. 4, above), this is consistent with an exchange stoichiometry of 4 Na^+ : 1 Ca^{2+}, 1 K^+ [8]. In comparison, the analysis of initial rates of Na^+-dependent Ca^{2+} efflux for the cardiac Na^+–Ca^{2+} exchanger [27], where only three Na^+ ions are believed to be exchanged per Ca^{2+} ion, yielded values for n in the range 2.30–3.17 (i.e., lower than those for the ROS exchanger).

At physiological K^+ concentrations the turnover number of the exchange protein was found to be about 115 $Ca^{2+} \cdot s^{-1}$ per exchanger molecule. This is significantly higher than our value of 30 for the exchanger in the presence of 100 mM symmetrical KCl. This lower turnover value is probably due not only to the absence of an extra driving force (i.e., an outwardly directed electrochemical K^+ gradient) for Ca^{2+} efflux, but also to an inhibitory effect of K^+ on Na^+–Ca^{2+} exchange [17], possibly through antagonism of Na^+-binding sites. A turnover number of 115 is nevertheless a lower estimate, and is relatively low compared to values (up to 1000 $Ca^{2+} \cdot s^{-1}$ per exchanger molecule) calculated for the exchanger from cardiac sarcolemma [18]. By various procedures [3,12] we have estimated the density of the Na^+–Ca^{2+} exchanger in the ROS plasma membrane to lie in the range of 200–600 μm^{-2}. Given that the exchanger has been reported to transport 25 pmol $Ca^{2+} \cdot s^{-1}\, cm^{-2}$ in intact bovine ROS [17], this would indicate a turnover number of 250–750. The reconstitution conditions employed may account for a significant part of this discrepancy [6], thereby implying that it is simply not possible to attain maximal transport rates in a reconstitution system.

K^+-dependence of the Na^+/Ca^{2+} exchanger in other cells

Although K^+ ions have been reported to have a stimulatory effect on some modes of Na^+–Ca^{2+} exchange [19–21], recent studies [10,11] have shown that the cotransport of K^+ is not a general property of Na^+–Ca^{2+} exchange in other cells. This may suggest that the ROS exchanger is constituted by a different protein than that reponsible for Na^+–Ca^{2+} exchange in other systems. Studies directed at the molecular identification of Na^+–Ca^{2+} exchangers from nonphotoreceptor sources have yielded inconclusive results. Although the intact protein of heart sarcolemma has been reported to have a molecular mass of 220 kDa (i.e., similar to that of ROS) [22], other reports have described Na^+–Ca^{2+} exchange activity attributable to proteins of lower molecular mass [23–26]. Indeed, preliminary studies in our laboratory using antibodies [4]

directed against the ROS exchanger (Müller, H., et al., unpublished results) have failed to demonstrate the presence of a high-molecular-weight cross-reactive protein in both cardiac sarcolemma and synaptosome membranes. It is therefore conceivable that the K^+-transporting Na^+–Ca^{2+} exchanger of rod photoreceptors is a unique system which has evolved to meet the requirement for rapidly attaining and maintaining extremely low calcium concentrations in the cytosol of these cells by utilising an outwardly directed electrochemical K^+ gradient as an extra driving force for Ca^{2+} extrusion.

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