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Na^+–Ca^{2+},K^+ exchange in bovine retinal rod outer segments: quantitative characterization of normal and reversed mode

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Abstract

Ca^{2+} homeostasis of bovine retinal rod outer segments is maintained through Na^+–Ca^{2+},K^+ exchangers and cGMP-gated channels in the plasma membrane. It has recently been demonstrated that both proteins are associated. This novel finding allowed us to investigate quantitatively normal and reversed mode Na^+–Ca^{2+},K^+ exchange in rod outer segment membrane vesicles and reconstituted proteoliposomes both containing exchangers in rightside-out and inside-out orientations. Addition of Na^+ activated both normal and reversed mode exchange; if, however, initially Ca^{2+} from vesicles containing inside-out oriented exchangers has been released by activation of the associated channels, only normal mode exchange was observed upon addition of Na^+. Using this approach, the fractions of vesicles containing rightside-out and inside-out oriented exchangers were about similar in these vesicle preparations. Normal and reversed mode exchange had similar Na^+ concentrations of about 70 mM for half maximal activation (in the presence of 115 mM K^+) and cooperativity parameters, n_{Hill}, of about 2.0. Furthermore, both modes were electrogenic, and showed only little Na^+–Ca^{2+},K^+ exchange in the absence of K^+. The two modes of exchange differed, however, in the maximal exchange rate, the normal mode being about twice as fast as the reversed mode.

Key words: Sodium–calcium ion exchange; Photoreceptor; Rod outer segment; Protein association; Vesicle sidedness; (Bovine)

1. Introduction

In vertebrate photoreceptors, Ca^{2+} entering the outer segment through cGMP-gated cation channels is extruded by Na^+–Ca^{2+} exchange (reviewed in Refs. 1 and 2). The intracellular Ca^{2+} concentration of rod outer segments (ROS) is largely controlled by Na^+–Ca^{2+} exchange [3,4] and by Ca^{2+} buffering [5,6]. Moreover, both cGMP-gated channels and Na^+–Ca^{2+} exchangers are localized exclusively in the plasma membrane of ROS [7–9] and not in the disk membrane where their occurrence would be detrimental for the cell function [10].

Considerable effort has been directed towards elucidation of the mechanism of Na^+–Ca^{2+} exchange in ROS. After photoactivation of the ROS, i.e., after closure of the cGMP-gated channels, Na^+–Ca^{2+} exchange could readily be investigated without any interfering conductivities [11,12]. Comparison of the Na^+–Ca^{2+} exchange in ROS with the Na^+–Ca^{2+} exchange in cardiac muscle cells or in the giant axon [13] exhibits two similarities: (a) Na^+–Ca^{2+} exchange is always electrogenic [11,14], and (b) Na^+–Ca^{2+} exchange operates in either direction depending on the ion gradients. However, there are also remarkable peculiarities: (a) the retinal Na^+–Ca^{2+} exchange is about one order of magnitude slower [11] than Na^+–Ca^{2+} exchange in the cardiac muscle [15], (b) the amino acid sequence of the retinal Na^+–Ca^{2+} exchanger shows very little homology with that of the cardiac Na^+–Ca^{2+} exchanger [16], and (c) the retinal Na^+–Ca^{2+} exchange cotransports

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Abbreviations: CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphonate; DTT, dithiothreitol; FCCP, carbonylcyanide-*p*-(trifluoromethoxy)phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; PC, soybean phosphatidylcholine; ROS, rod outer segment.

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K^+ along with Ca^{2+} [17–21]. Therefore, the retinal Na^+–Ca^{2+} exchange will be denoted in the following more correctly as Na^+–Ca^{2+},K^+ exchange.

Although normal and reversed Na^+–Ca^{2+},K^+ exchange have been clearly demonstrated [17,22], only the normal mode of Na^+–Ca^{2+},K^+ exchange has yet been quantitatively investigated in intact ROS [12,23]. We report here a comparative study of both modes of Na^+–Ca^{2+},K^+ exchange as measured in ROS membrane vesicles and ROS proteoliposomes which contain inside-out and rightside-out oriented Na^+–Ca^{2+},K^+ exchangers. Discrimination between the two modes of exchange is based on the recent experimental finding from our laboratory that Na^+–Ca^{2+},K^+ exchangers and cGMP-gated channels are associated [24,25].

Preliminary accounts of this work had been given at the 19th Göttingen Neurobiology Conference [26], at the 46th Meeting of the Society of General Physiology [27] and at the 101st Conference of the German Society of Biochemistry [28].

2. Materials and methods

2.1. Fused rod outer segment membrane vesicles

Retinae were taken from cattle eyes enucleated in a local slaughter house shortly after the animal had been killed. All preparation steps were carried out in dim red light. Preparation and storage of purified ROS were performed as previously described [7] with the modifications given in Ref. 25. ROS membranes (containing 5 mg rhodopsin) were washed twice (centrifugation at 27 100 × *g* for 30 min) in the hypotonic buffer 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), adjusted to pH 7.4 with Tris, 1 mM EDTA, 1 mM dithiothreitol (DTT) and resuspended mostly in 0.8 ml of 10 mM Hepes adjusted to pH 7.4 with KOH, 100 mM KCl, 1 mM DTT (KCl buffer) and if K^+ had to be omitted, in 0.8 ml of 75 mM Hepes adjusted with Tris to pH 7.4, 1 mM DTT; the latter buffer was iso-osmolar with the KCl buffer. The suspension was shock-frozen in liquid nitrogen and thawed to room temperature to achieve membrane fusion [25], then suspension buffer (KCl buffer containing 10 mM $CaCl_2$, or 75 mM Hepes adjusted with Tris to pH 7.4, 1 mM DTT, 10 mM $CaCl_2$) was added to obtain 2 mg rhodopsin per ml, overlayered with argon and equilibrated overnight at 4°C. Ca^{2+} containing vesicles (fused ROS membrane vesicles) were prepared from this fused ROS membrane suspension by pipetting 1 ml of suspension in a 2 ml Eppendorf vial immediately before each experiment, adding 1 ml of suspension buffer and sonicating for 60 s as described [25].

2.2. Rod outer segment proteoliposomes

Solubilization in 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphonate (CHAPS) and reconstitution in soybean phosphatidylcholine (PC) of ROS membrane proteins (ROS proteoliposomes) was performed in dim red light as described [25]. The lipid to rhodopsin ratio was 4:1 (mg/mg) throughout since under this condition the association of channel and exchanger is virtually complete [25]. In experiments where the influence of K^+ on the Na^+–Ca^{2+},K^+ exchange was examined, KCl buffer was replaced either with 75 mM Hepes adjusted with Tris to pH 7.4, 1 mM DTT, 10 mM $CaCl_2$, or with 10 mM Hepes adjusted with Tris to pH 7.4, 1 mM DTT, 10 mM $CaCl_2$, 100 mM XCl where X stands for Li, guanidine, or choline.

2.3. Purification and reconstitution of cGMP-gated channel and Na^+–Ca^{2+},K^+ exchanger

Purification of cGMP-gated channels and Na^+–Ca^{2+},K^+ exchangers from bovine rod outer segments was carried out as described [29,30]. Protein concentrations were determined according to Bradford [31] using ovalbumin as a standard. Electrophoresis of purified proteins was carried out on 7.5% (w/v) sodium dodecylsulfate-polyacrylamide gels (Pharmacia, Phast-system).

Activity of the purified proteins was assayed after reconstitution into Ca^{2+} containing liposomes. Purified channel or exchange proteins were solubilized in 15 mM CHAPS, 4 mg/ml PC, 1 mM DTT, 10 mM $CaCl_2$, 10 mM Hepes/KOH, pH 7.4 and 500 mM KCl or 100 mM KCl, respectively. Since elution of the channel from the AF-Red column employed a KCl gradient, the indicated KCl concentration of 500 mM was only approximate. Solutions (10 ml) were dialysed against KCl buffer containing 10 mM $CaCl_2$ as described [25].

2.4. Ca^{2+} release measurements

Extravesicular Ca^{2+} was removed by ion exchange as described [7]. Using Arsenazo III (always 60 μM) as an indicator, changes in Ca^{2+} concentrations were monitored at 652 nm with a spectrophotometer (SLM-Aminco DW 2000). Kinetic studies were carried out in the dual wavelength mode (wavelength pair: 652 nm, 700 nm) with a sampling rate of 0.1 s (time base: 1 min). Total Ca^{2+} releases were examined in the dual beam mode (sampling rate 1.8 s) against a reference sample to compensate the baseline slope due to passive Ca^{2+} release. No electronic filter was used for the measurements. The cuvette has been continuously stirred with a magnetic bar; mixing was complete 0.7 s after an addition. A 2 μM Ca^{2+} pulse was added after

50 s for calibration of the absorption signal. All measurements were carried out at 25°C.

2.5. Evaluation of Ca^{2+} fluxes and estimation of turnover numbers

Initial Ca^{2+} fluxes were determined by linear regression of the absorption values measured 0.7 s after an addition in a time interval of about 1 s. The slope of this straight line was converted into Ca^{2+} per s using the Ca^{2+} calibration step at the end of each experiment.

The vesicle fractions containing inside-out and rightside-out oriented exchangers were determined, as described in Results, from the Na^+ releasable fractions of Ca^{2+} before and after a saturating cGMP induced Ca^{2+} release. Both fractions were almost equal in ROS proteoliposomes but in fused ROS membrane vesicles, the ratio of inside-out to rightside-out oriented exchangers was about 57:43. For reasons given earlier [32] the rhodopsin density in bovine ROS is likely to be similar as in amphibian ROS [33], namely about 25 000 rhodopsin molecules per μm^2. Since the density of exchanger in the plasma membrane is in the range of 300–450 exchanger molecules per μm^2 [9,25], we estimate the ratio of exchanger to rhodopsin in bovine ROS to be about 1:1000. Based on this value and after correction for the ratio of inside-out to rightside-out oriented exchangers, turnover numbers were calculated for fused ROS membrane vesicles and ROS proteoliposomes as Ca^{2+}/s per 1000 rhodopsin molecules.

2.6. Intravesicular Ca^{2+} and vesicle shape

From fused ROS membrane vesicles, 0.99 ± 0.23 ($n = 4$) Ca^{2+} per rhodopsin (mol/mol) could be released by Na^+–Ca^{2+},K^+ exchange which was about 35% of the totally releasable Ca^{2+} (i.e., the Ca^{2+} released upon addition of the ionophore A23187). In contrast, 2.61 ± 0.55 ($n = 8$) Ca^{2+} per rhodopsin (mol/mol) could be released by Na^+–Ca^{2+},K^+ exchange from ROS proteoliposomes which was about 27% of the totally releasable Ca^{2+}. With the above estimate of 1 exchanger per 1000 rhodopsin molecules this means that in ROS proteoliposomes about 2600 Ca^{2+} are released per exchanger molecule whereas in fused ROS membrane vesicles only about 1000 Ca^{2+} are released per exchanger.

For the following reason, the Na^+-releasable Ca^{2+} pool of 2600 Ca^{2+} per exchanger molecule measured for ROS proteoliposomes is in agreement with a spherical vesicle shape. The exchanger molecules are likely to be isolated on different vesicles in ROS proteoliposomes [25]. Since the intravesicular Ca^{2+} concentration is 10 mM the volume containing 2600 Ca^{2+} is $4.3 \cdot 10^{-19}$ l. This is the volume of a spherical vesicle with a diameter of 94 nm; in fact, electron microscopic investigations of negatively stained proteoliposomes yield diameters of about 90 nm (data not shown). On the other hand, the Ca^{2+} release from fused ROS membrane vesicles mediated by Na^+–Ca^{2+},K^+ exchange suggests a collapsed vesicle shape since the diameter of these vesicles was determined to about 120 nm [25]. This conclusion is further supported by atomic force microscopy which showed that fused ROS membrane vesicles are collapsed whereas ROS proteoliposomes are spherical (Drechsler, personal communication).

3. Results

3.1. Discrimination between normal and reversed mode Na^+–Ca^{2+},K^+ exchange in vesicles

Two different vesicle preparations were used viz. (a) vesicles prepared from fused ROS membranes (fused ROS membrane vesicles) and (b) vesicles prepared by dialysis of ROS membranes solubilized in CHAPS in the presence of 4 mg soybean PC per mg of rhodopsin (ROS proteoliposomes). In both preparations the membrane proteins have been evenly distributed either by membrane fusion induced by freeze-thawing, or by detergent solubilization and subsequent reconstitution in lipid vesicles. For both preparations the mean exchanger density was below 0.4 exchanger per vesicle [25].

The orientation of the Na^+–Ca^{2+},K^+ exchanger was either rightside-out or inside-out, i.e., the cytoplasmic moiety of the exchanger was either intra- or extravesicular, respectively. Since Na^+–Ca^{2+},K^+ exchange could take place in either case [7,25], Ca^{2+} was released from all vesicles containing a Na^+–Ca^{2+},K^+ exchanger and will be denoted as normal or reversed mode Na^+–Ca^{2+},K^+ exchange, respectively. Experimentally, discrimination between the two modes of exchange was feasible because of the high affinity between Na^+–Ca^{2+},K^+ exchanger and cGMP-gated channel leading to the association of these proteins [25]. For this reason, vesicles with inside-out oriented exchangers contained most probably also inside-out oriented cGMP-gated channels, i.e., channels with extravesicular cGMP-binding sites, the intravesicular Ca^{2+} of which could be released by addition of cGMP. Thus, Na^+–Ca^{2+},K^+ exchange after a saturating cGMP induced Ca^{2+} release was due only to normal mode Na^+–Ca^{2+},K^+ exchange.

The total Na^+ induced Ca^{2+} release from ROS proteoliposomes was about twice as great as the Na^+ induced Ca^{2+} release after a saturating cGMP induced Ca^{2+} release (Fig. 1). The latter one was due to normal mode Na^+–Ca^{2+},K^+ exchange; the reversed mode Na^+–Ca^{2+},K^+ exchange was given by the difference

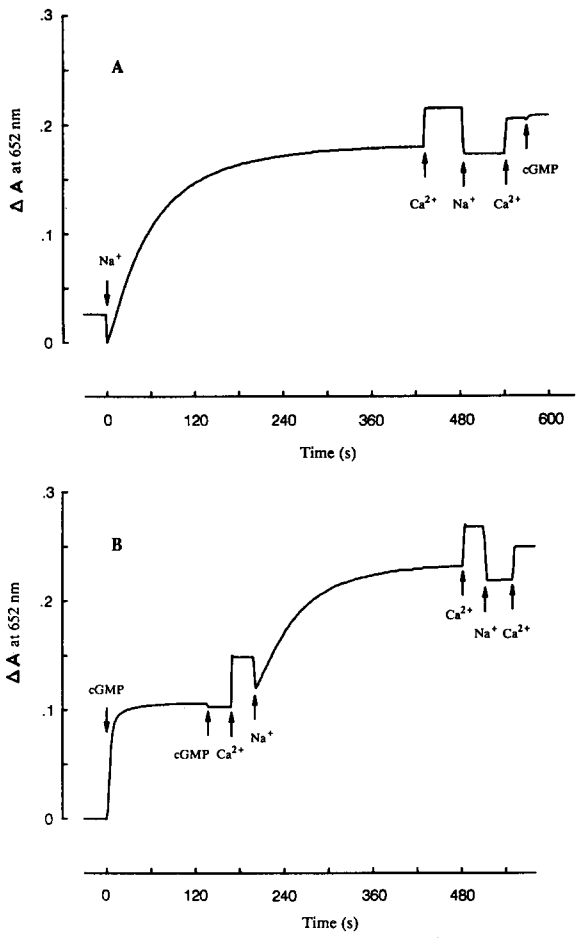

Fig. 1. Ca^{2+} releases from ROS proteoliposomes (4 mg PC per mg rhodopsin). The absorbance was monitored at 652 nm versus a reference sample (split beam mode) to compensate the baseline drift; 60 μM Arsenazo III was used as a Ca^{2+} indicator. The decreases in absorbance after the Na^+ additions were due to decreases in the Ca^{2+} affinity of Arsenazo III [34]. The experiments were carried out in KCl buffer. (A) Ca^{2+} releases upon consecutive additions of 100 mM Na^+ followed by a Ca^{2+} release upon addition of 200 μM cGMP. After each Na^+ addition a 2 μM Ca^{2+} pulse was applied to calibrate the Ca^{2+} sensitivity. (B) Ca^{2+} releases upon consecutive additions of 200 μM cGMP followed by 100 mM Na^+. Ca^{2+} calibration pulses (2 μM) were applied after the second cGMP addition and after each Na^+ addition.

between the total Na^+ induced Ca^{2+} release (Fig. 1A) and the Ca^{2+} release due to normal mode Na^+–Ca^{2+},K^+ exchange (Fig. 1B). The ratio of the cGMP- and Na^+-induced Ca^{2+} releases shown in Fig. 1B corresponded to the ratio of vesicle fractions containing inside-out and rightside-out exchangers; this ratio was near one suggesting that the exchangers were randomly oriented. The cGMP induced Ca^{2+} release after a saturating Na^+ induced Ca^{2+} release was minimal (Fig. 1A) in keeping with the fact that each cGMP-gated channel was associated with a Na^+–Ca^{2+},K^+ exchanger. Similar results were also obtained for fused ROS membrane vesicles [25].

The Na^+ releasable Ca^{2+} pool accessible to the exchanger in fused ROS membrane vesicles was 0.99 ± 0.23 ($n = 4$) whereas, in ROS proteoliposomes, it was 2.61 ± 0.55 ($n = 8$) Ca^{2+} per rhodopsin (mol/mol). The latter value is in agreement with a spherical vesicle with a diameter of about 94 nm whereas the former value indicates a collapsed shape of fused ROS membrane vesicles (see Materials and methods).

3.2. Electrogenicity of the Na^+–Ca^{2+},K^+ exchange

Na^+–Ca^{2+},K^+ exchange in ROS has been shown to be electrogenic since one positive electric charge is moved oppositely to Ca^{2+} [11,14]. The electrogenicity of the Na^+–Ca^{2+},K^+ exchange was probed by adding an ionophore which dissipates the transmembrane voltage gradient. As shown in Fig. 2, an enhanced rate of Na^+–Ca^{2+},K^+ exchange was obtained for ROS proteoliposomes in the presence of either carbonylcyanide-*p*-(trifluoromethoxy)phenylhydrazone (FCCP) or valinomycin; the titration curves with and without ionophore differed in amplitude by a factor of about 2, being otherwise similar. An ionophore-mediated increase of the rate of Na^+–Ca^{2+},K^+ exchange was also observed for fused ROS membrane vesicles (Table 1). The Na^+–Ca^{2+},K^+ exchange measured in the absence of a shunting ionophore was probably due to a small leak current compensating part of the electrogenicity.

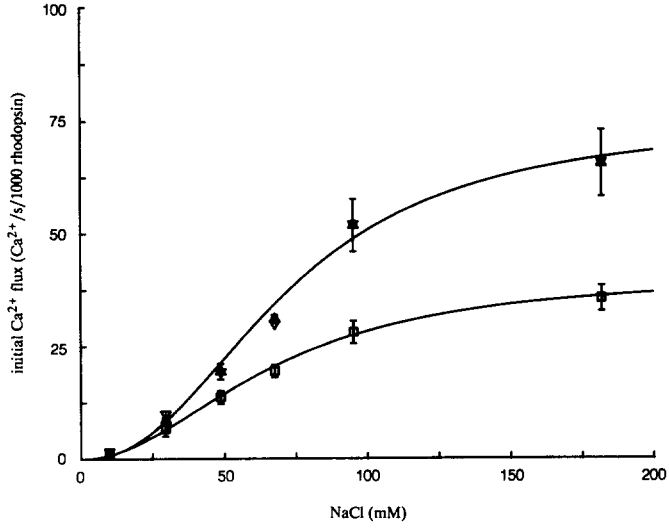

Fig. 2. Initial Ca^{2+} efflux from ROS proteoliposomes due to Na^+–Ca^{2+},K^+ exchange upon adding increasing Na^+ concentrations. The Ca^{2+} efflux is due to normal and reversed mode Na^+–Ca^{2+},K^+ exchange. Data were obtained in the absence of an ionophore (□) and in the presence of 2 μM FCCP (△) or 2 μM valinomycin (▽). The plotted curves for the data in the absence and the presence of ionophore correspond to all-or-nothing processes with half-maximal Na^+ concentrations, EC_{50}, of 67 mM and 73 mM, Hill coefficients of 2.03 and 2.30 and plateau values of 41 and 75 Ca^{2+}/s per 1000 rhodopsin, respectively. The experiments were carried out in KCl buffer and 60 μM Arsenazo III. Error bars are standard deviations.

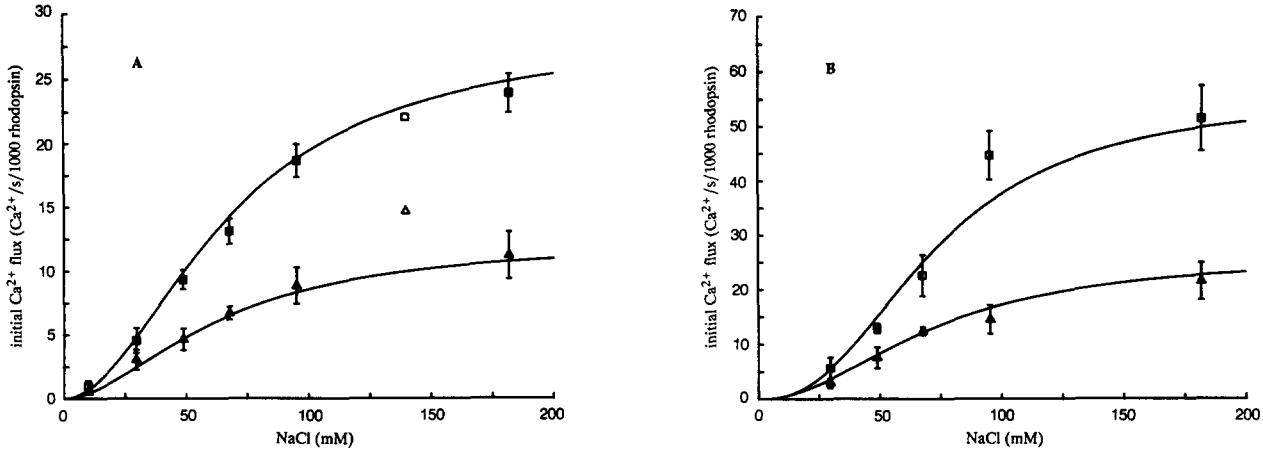

Fig. 3. Normal (□) and reversed (△) mode Na^+–Ca^{2+},K^+ exchange from ROS proteoliposomes. Discrimination between normal and reversed mode was based on the finding that each exchanger molecule was associated with a cGMP-gated channel [25] and evaluation was carried out as described in Materials and methods. (A) Na^+ titration in the absence of an ionophore. The titration curves (all-or-nothing processes) were plotted with half-maximal Na^+ concentrations, EC_{50}, of 68 mM and 63 mM, Hill coefficients, n_{Hill}, of 1.93 and 1.83 and plateau values of 28.6 and 12.3 Ca^{2+}/s per 1000 rhodopsin for the normal and reversed mode, respectively. (B) Na^+ titration as in (A), but in the presence of 2 μM FCCP. Parameters of the drawn curves are: EC_{50}, 73 mM and 72 mM, n_{Hill}, 2.42 and 2.03; plateau values, 55.2 and 26.1 Ca^{2+}/s/1000 rhodopsin for the normal and reversed mode, respectively. Error bars are standard deviations. The experiments were carried out in KCl buffer and 60 μM Arsenazo III. Mind the difference in ordinate scaling between Fig. 3A and Fig. 3B.

3.3. Na^+ dependence of normal and reversed mode exchange

Analysis of normal and reversed mode Na^+–Ca^{2+},K^+ exchange was carried out as described above. Fig. 3 shows that in ROS proteoliposomes the reversed mode Na^+–Ca^{2+},K^+ exchange was about half as fast as the normal mode. Both normal and reversed mode Na^+–Ca^{2+},K^+ exchange were enhanced after addition of FCCP suggesting that both mechanisms were electrogenic (Fig. 3A,B). Similar results were obtained for fused ROS membrane vesicles (Table 1) except for the fact that the maximal exchange rates, V_{max}, were considerably smaller than for ROS proteoliposomes (see Discussion). Apart from V_{max}, comparison of the results for both vesicle systems (Table 1) shows that the Na^+ concentrations for half-maximal Na^+–Ca^{2+},K^+ exchange (EC_{50}) were reasonably constant and similar for normal and reversed mode. The same notion applies also for the Hill coefficients (n_{Hill}).

3.4. K^+ dependence of the Na^+–Ca^{2+},K^+ exchange

Characteristically, Na^+–Ca^{2+},K^+ exchange in the presence of closed cGMP-gated channels requires K^+ [17,19–21]. This property was also evident in ROS proteoliposomes (Fig. 4): replacement of K^+ by Hepes, Li^+, guanidine, or choline reduced the total exchange

Table 1
Na^+ titration of normal and reversed mode Na^+–Ca^{2+},K^+ exchange

	Na^+–Ca^{2+},K^+ exchange from fused ROS membrane vesicles; exchange mode		Na^+–Ca^{2+},K^+ exchange from ROS proteoliposomes; exchange mode	
	normal	reversed	normal	reversed
EC_{50} (+FCCP)			73.2 ± 7.9 (n = 6)	72.2 ± 6.8 (n = 6)
EC_{50} (−FCCP) (mM Na^+)	67.1 ± 2.0 (n = 4)	69.6 ± 8.3 (n = 4)	67.7 ± 2.0 (n = 7)	63.1 ± 6.2 (n = 6)
n_{Hill}(+FCCP)			2.42 ± 0.26 (n = 6)	2.03 ± 0.19 (n = 6)
n_{Hill}(−FCCP)	2.00 ± 0.06 (n = 4)	2.36 ± 0.28 (n = 4)	1.93 ± 0.30 (n = 7)	1.83 ± 0.33 (n = 7)
V_{max} (+FCCP)	18.9 ± 3.3(n = 3)	9.3 ± 2.9 (n = 3)	55.2 ± 6.4 (n = 13)	26.1 ± 4.2 (n = 13)
V_{max} (−FCCP) (Ca^{2+}/s/1000 rhodopsin)	13.2 ± 0.8 (n = 4)	6.8 ± 1.3 (n = 4)	28.6 ± 1.8 (n = 8)	12.3 ± 2.0 (n = 8)

Fused ROS membrane vesicles and ROS proteoliposomes were prepared as described (see Materials and methods). Experiments were carried out in KCl buffer and either in the presence or in the absence of 2 μM FCCP (labelled + FCCP or −FCCP, respectively). Normal and reversed mode Na^+–Ca^{2+},K^+ exchange were evaluated, based on the association of channel and exchanger, from the Na^+–Ca^{2+},K^+ exchange measured after and before a saturating cGMP-induced Ca^{2+} release (see Materials and methods). The Na^+ concentrations for half-maximal activation, EC_{50}, and the Hill coefficients, n_{Hill}, were obtained by linear regression from Hill plots, the errors being standard deviations. The values, V_{max}, are estimates of the maximal turnover numbers based on a ratio of exchanger to rhodopsin of 1:1000 (mol/mol) in ROS.

by a factor of at least 7; this means that both normal and reversed mode of exchange were K^+ dependent. Titration of this K^+ dependence yielded an EC_{50} of about 3 mM for half-maximal activation, in reasonable agreement with published data [19,21].

Considering this low value, it is unclear whether the small Na^+–Ca^{2+},K^+ exchange observed in the absence of any added K^+ was due to K^+ independent, electroneutral Na^+–Ca^{2+} exchange [19] or to some minor contaminating K^+ in the preparation which cannot be excluded.

3.5. Reconstitution of the purified proteins

Purified Na^+–Ca^{2+},K^+ exchangers reconstituted in soybean PC showed a definite Na^+ induced Ca^{2+} release with similar Na^+ titration parameters as above (Fig. 5). However, in these experiments no discrimination between normal and reversed mode of exchange was possible.

Purified cGMP-gated channels and Na^+–Ca^{2+},K^+ exchangers together were also reconstituted in soybean PC. However, the cGMP induced Ca^{2+} release was about 20-times smaller than the Na^+ induced Ca^{2+} release, an observation which may be due to aggregation of purified channel (see Discussion). In spite of this complication, competition experiments carried out with these proteoliposomes suggested again an association of the Na^+–Ca^{2+},K^+ exchanger with the cGMP-gated channel (data not shown).

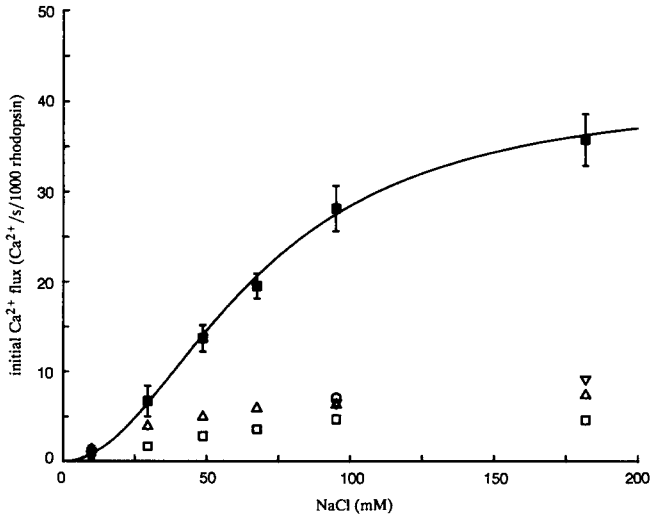

Fig. 4. K^+ dependence of the Na^+–Ca^{2+},K^+ exchange. Na^+ titration of the Na^+–Ca^{2+},K^+ exchange in ROS proteoliposomes in the presence of 115 mM K^+ (■), or upon substitution of K^+ with Li^+ (□), guanidine (▽), choline (○), or in 75 mM Hepes adjusted with Tris to pH 7.4, 1 mM DTT (△). The titration curve (all-or-nothing process) was plotted with the parameters: EC_{50}, 67 mM; n_{Hill}, 2.03; plateau value, 41 Ca^{2+}/s per 1000 rhodopsin. No FCCP was present in these experiments.

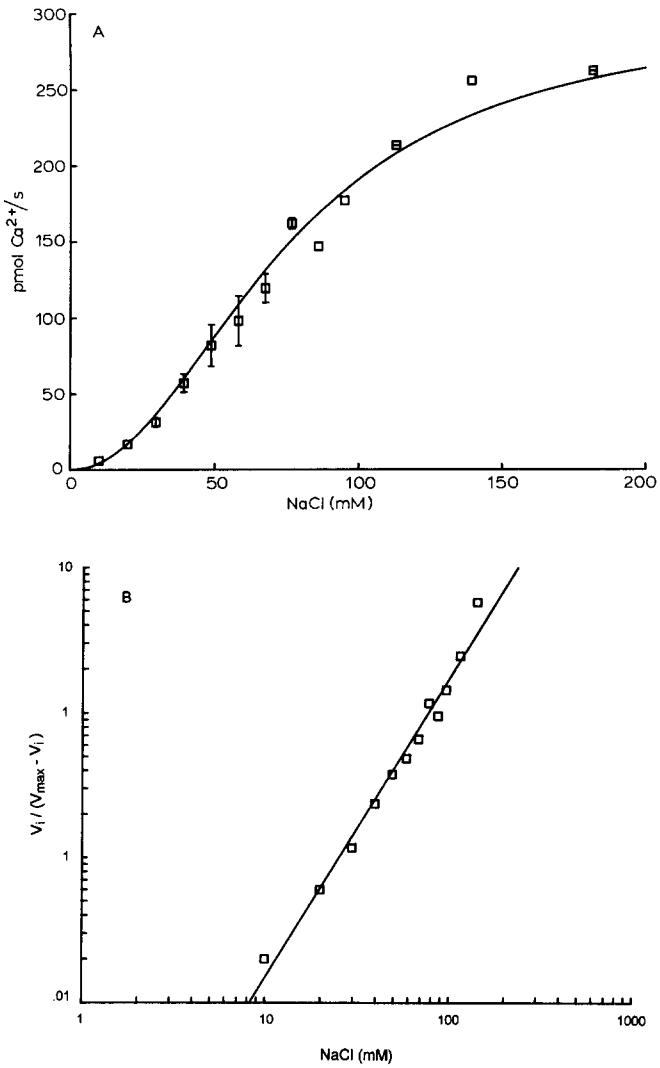

Fig. 5. Na^+ titration of the Na^+–Ca^{2+},K^+ exchange obtained with proteoliposomes containing purified exchangers. Experiments were carried out in KCl buffer, 60 μM Arsenazo III and in the presence of 2 μM FCCP. (A) The plotted curve corresponds to an all-or-nothing process with the values of EC_{50} and n_{Hill} determined in the Hill plot (B) and with a plateau value of 300 pmol Ca^{2+}/s. Error bars are standard deviations ($n = 3$). (B) Hill plot of the data shown in the Na^+ titration. The linear regression yields the values for n_{Hill} of 2.07 ± 0.09 and for EC_{50} of 76.4 ± 10.8 mM Na^+.

4. Discussion

cGMP-gated channels and Na^+–Ca^{2+},K^+ exchangers of ROS are restricted to the plasma membrane, the density of both proteins being in bovine ROS about 300 μm^{-2} [25,35]. Therefore, Ca^{2+} containing ROS membrane vesicles obtained by hypotonic lysis of ROS were not suited for investigation of normal and reversed mode Na^+–Ca^{2+},K^+ exchange because each plasma membrane vesicle contains many exchangers [7]. In this report we use fused ROS membrane vesicles and ROS proteoliposomes to investigate Na^+–Ca^{2+},K^+ exchange; in both preparations each vesicle contains at

most one exchanger, the probability of accidental occurrence of two exchangers in one vesicle being negligible.

Compared with whole ROS, these vesicle systems appear at first sight less suited to study Na^+–Ca^{2+},K^+ exchange because inside-out and rightside-out orientations of the exchangers occur with about equal probability. This obvious problem could, however, be overcome since exchangers are associated with cGMP-gated channels [25], thus allowing to discriminate between the two orientations (see Results). The association of channel and exchanger outlasted membrane fusion induced by freeze-thawing and was even fully existent when, after solubilization in CHAPS, the ROS membrane proteins were reconstituted in soybean PC at low lipid-to-protein ratios [25].

Using this approach, the values obtained for the normal mode exchange (Table 1) agree reasonably with published data [12,23]. In this study, Na^+ gradients were the only driving forces used for Na^+–Ca^{2+},K^+ exchange; the greater exchange rate found by one group [20] may be due to a K^+-gradient acting as an additional driving force. It should be qualified, however, that the values of the exchange rate given in Table 1 rely on an estimated ratio of exchanger to rhodopsin of 1:1000 (mol/mol) as well as on lipid interactions (see below).

No quantitative study of the reversed mode exchange in a vesicle system has yet been published. Comparing normal and reversed mode of Na^+–Ca^{2+},K^+ exchange reveals that the values of both modes are conspicuously similar. Both modes of exchange are not distinguishable regarding (a) the Na^+ concentration, EC_{50}, for half-maximal activation, (b) the cooperativity parameter, n_{Hill}, (c) the electrogenicity and (d) the K^+ dependence.

As opposed to these parameters, we observed a conspicuous difference in the maximal turnover numbers, V_{max}, in that the normal mode Na^+–Ca^{2+},K^+ exchange was found to be about twice as fast as the reversed mode. If the orientation of channel and exchanger were random one would not expect to observe two different kinetics of exchange depending on a preceding cGMP induced Ca^{2+} release. The finding that the normal mode is about a factor of two faster than the reversed mode suggests that after association the orientation of channel and exchanger with respect to each other is specific.

Interestingly, we generally found about three times greater values of V_{max} for both normal and reversed mode exchange in ROS proteoliposomes as compared to fused ROS membrane vesicles (Table 1). The origin of this observation is possibly due to a change in the lipid–protein interaction, much similar to the lipid influence reported for the activity of the cardiac Na^+–Ca^{2+} exchanger [13,21]. It should be noted that membrane fusion between disk and plasma membrane entails almost certainly a distribution of the different lipids of disk and plasma membrane [36,37]. Therefore, with fused ROS vesicles, we measured essentially the exchanger activity in disk membrane lipids which might differ from the exchanger activity measured in intact ROS.

Previously, we reported that the channels distribute randomly in both fused ROS membrane vesicles and ROS proteoliposomes [25]. In both systems the cGMP-gated channel is associated with (at least) one exchanger. Purified channels, however, have a distinct tendency to aggregate (Kaupp and Vetter, personal communication). The low cGMP-induced Ca^{2+}-fluxes observed after reconstitution of purified channels into liposomes may, therefore, be explained by localization of this protein to a small vesicle fraction. Alternatively, functional impairment of the channel due to the biochemical purification procedure cannot be excluded.

The photoreceptor cell has been considered to be the most favourable cell to study Na^+–Ca^{2+},K^+ exchange because the only Ca^{2+} flux in the ROS interfering with Na^+–Ca^{2+},K^+ exchange is due to the cGMP-gated channels which can be readily closed by a flash of light. Therefore, all studies on Na^+–Ca^{2+},K^+ exchange published thus far were carried out during channel closure. Since cGMP-gated channels and Na^+–Ca^{2+},K^+ exchangers are associated, it would be of interest whether there is a functional coupling of these two proteins. Experiments are in progress to study Ca^{2+} fluxes when both channel and exchanger are activated simultaneously.

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