Optical Measurements of Na-Ca-K Exchange Currents in Intact Outer Segments Isolated from Bovine Retinal Rods

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ABSTRACT The properties of Na-Ca-K exchange current through the plasma membrane of intact rod outer segments (ROS) isolated from bovine retinas were studied with the optical probe neutral red. Small cellular organelles such as bovine ROS do not offer an adequate collecting area to measure Na-Ca-K exchange currents with electrophysiological techniques. This study demonstrates that Na-Ca-K exchange current in bovine ROS can be measured with the dye neutral red and dual-wavelength spectrophotometry. The binding of neutral red is sensitive to transport of cations across the plasma membrane of ROS by the effect of the translocated cations on the surface potential of the intracellular disk membranes (1985. *J. Membr. Biol.* 88: 249–262). Electrogenic Na^+ fluxes through the ROS plasma membrane were measured with a resolution of 10^5 Na^+ ions/ROS per s, equivalent to a current of ~0.01 pA; maximal electrogenic Na-Ca-K exchange flux in bovine ROS was equivalent to a maximal exchange current of 1–2 pA. Electrogenic Na^+ fluxes were identified as Na-Ca-K exchange current based on a comparison between electrogenic Na^+ flux and Na^+-stimulated Ca^{2+} release with respect to flux rate, Na^+ dependence, and ion selectivity. Neutral red monitored the net entry of a single positive charge carried by Na^+ for each Ca^{2+} ion released (i.e., monitored the Na-Ca-K exchange current). Na-Ca-K exchange in the plasma membrane of bovine ROS had the following properties: (*a*) Inward Na-Ca-K exchange current required internal Ca^{2+} (half-maximal stimulation at a free Ca^{2+} concentration of 0.9 μM), whereas outward Na-Ca-K exchange current required both external Ca^{2+} (half-maximal stimulation at a free Ca^{2+} concentration of 1.1 μM) and external K^+. (*b*) Inward Na-Ca-K exchange current depended in a sigmoidal manner on the external Na^+ concentration, identical to Na^+-stimulated Ca^{2+} release measured with Ca^{2+}-indicating dyes. (*c*) The neutral red method was modified to measure Ca^{2+}-activated K^+ fluxes (half-maximal stimulation at 2.7 μM free Ca^{2+}) via the Na-Ca-K exchanger in support of the notion that the rod Na-Ca exchanger is in effect a Na-Ca-K exchanger. (*d*) Competitive interactions between Ca^{2+} and Na^+ ions on the exchanger protein are described.

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/91/09/0555/19 $2.00
Volume 98 September 1991 555–573

INTRODUCTION

The plasma membrane of the outer segments of vertebrate rod photoreceptors (ROS) exhibits large Na-Ca exchange fluxes (Schnetkamp, 1980, 1986; Schnetkamp and Bownds, 1987) or Na-Ca exchange currents (Yau and Nakatani, 1984; Hodgkin, McNaughton, and Nunn, 1987; Lagnado, Cervetto, and McNaughton, 1988); the Na-Ca exchange protein has been identified and purified as a 230-kD single polypeptide (Cook and Kaupp, 1988; Nicoll and Applebury, 1989). The rod Na-Ca exchanger appears to differ from Na-Ca exchangers in other systems. It requires and transports both a single Ca^{2+} ion and a single K^+ ion in exchange for four Na^+ ions (Schnetkamp, Szerencsei, and Basu, 1988; Cervetto, Lagnado, Perry, Robinson, and McNaughton, 1989; Schnetkamp, Basu, and Szerencsei, 1989). Typical mammalian ROS such as bovine ROS (commonly used for biochemical studies) are small cylindrical structures (1×20 μm), and despite the fact that Na-Ca-K exchange fluxes can change total intracellular Ca^{2+} by as much as 0.5 mM/s (Schnetkamp, 1986), maximal Na-Ca-K exchange currents can be calculated to amount to only 1–2 pA and are difficult to measure with electrophysiological techniques that have been applied so successfully in the much larger amphibian ROS (Yau and Nakatani, 1984; Hodgkin et al., 1987; Lagnado et al., 1988). Also, the plasma membrane of bovine ROS could contain electrogenic transporters that could cause rapid and significant changes in internal ion concentration and yet remain undetected with electrophysiological techniques.

In earlier studies we used the pH-indicating dye phenol red to measure an electrogenic efflux of protons that electrically compensated an inward electrogenic Na-Ca-K exchange flux and was carried by the added electrogenic protonophore FCCP (Schnetkamp, 1989; Schnetkamp et al., 1989). The electrogenicity of Na-Ca-K exchange in bovine ROS was found to be one positive charge carried by Na^+ for each Ca^{2+} released. In the remainder of this paper I will use the term Na-Ca-K exchange current to indicate electrogenic Na-Ca-K exchange flux in bovine ROS, although no direct current measurements were made. Measurement of Na-Ca-K exchange current with pH-indicating dyes has practical limitations, especially when free Ca^{2+} concentrations need to be stabilized with the use of Ca^{2+} chelators. The dye neutral red has been used to measure electrogenic cation fluxes in a suspension of small cells or cellular organelles (Schnetkamp, 1985*a*, *b*); a resolution equivalent to a current of 0.01 pA was obtained for cGMP-induced cation fluxes in ROS membrane vesicles (Schnetkamp, 1990). In this study, neutral red and dual-wavelength spectroscopy are applied to measure Na-Ca-K exchange currents in bovine ROS with a resolution equivalent to a current of 0.01 pA. Properties of Na-Ca-K exchange currents were measured that are not easily obtained by other more direct methods, including the internal and external Ca^{2+} binding constants of the exchanger protein obtained from Ca^{2+}-activated K^+ and Na^+ fluxes.

METHODS

Bovine ROS with an intact plasma membrane were isolated and purified as described before; bovine ROS were purified as either Ca^{2+}-depleted ROS containing no measurable Ca^{2+}, or as Ca^{2+}-enriched ROS containing ~8 mol Ca^{2+}/mol rhodopsin (Schnetkamp, Klompmakers, and

Daemen, 1979; Schnetkamp, 1986). Bovine ROS were stored as a concentrated suspension (200–300 μM rhodopsin) at 4°C in a medium containing 600 mM sucrose, 5% wt/vol Ficoll 400, and 20 mM HEPES (adjusted to pH 7.4 with arginine), and were used within 3 h. All experiments were carried out in dim red illumination. Na^+-induced Ca^{2+} release was measured with arsenazo III as described before (Schnetkamp, 1986). Ionophore-induced Na^+ and K^+ release from ROS was measured with atomic absorption spectroscopy as described (Schnetkamp, Szerencsei, and Basu, 1991).

Optical recordings of electrogenic cation fluxes in ROS with the dye neutral red were performed in an SLM DW2C dual-wavelength spectrophotometer (SLM Instruments, Urbana, IL) with the wavelength pair of 540 and 650 nm as described (Schnetkamp, 1985*b*, 1990). Temperature was controlled to 25°C with a circulating waterbath and the suspension was mixed with a magnetic spinbar. Intact bovine ROS were diluted to a final overall rhodopsin concentration in the cuvette of 7–10 μM; the medium contained 600 mM sucrose, 50 μM neutral red, 20 mM HEPES (adjusted to pH 7.4 with arginine), and other constituents as indicated. Ionophores (FCCP, valinomycin, A23187, gramicidin) were added to the suspension from concentrated (1–2 mM) ethanolic solutions; addition of ethanol alone had no effect. Optical recordings of Na^+-induced proton release from ROS were performed on the above instrument with the dye phenol red as described (Schnetkamp et al., 1989).

Binding of Neutral Red as a Quantitative Indicator for Electrogenic Na^+ Transport

Binding of the dye neutral red to the intracellular disk membranes is a simple function of the internal cation concentration, probably due to the effect of internal cations on the electrostatic potential at the disk membrane/water interface (Schnetkamp, 1985*a*, 1990). Changes in light absorption occur due to unbinding of neutral red upon an increase of the internal cation concentration (for example, upon an increase of internal Na^+); the internal Na^+ concentration can be controlled by application of the nonselective alkali cation channel ionophore gramicidin. In the absence of other ionic conductances in the plasma membrane of intact bovine ROS, the inward current of Na^+ via gramicidin is compensated by an equally large outward current of protons via gramicidin, very similar to compensation of the inward Na-Ca-K exchange current in isolated intact ROS by an outward current of protons carried by the electrogenic protonophore FCCP (Schnetkamp et al., 1989). The notion that cation fluxes via electrogenic ionophores such as gramicidin or valinomycin are electrically compensated by equally large proton fluxes via gramicidin or via the electrogenic protonophore FCCP in the case of valinomycin, was put to a quantitative test. Addition of the ionophore gramicidin (1 μM) caused the rapid release of both Na^+ and K^+ from bovine ROS as measured with atomic absorption spectroscopy, whereas addition of both valinomycin (1 μM) and FCCP (1 μM) caused the release of only K^+. Proton uptake induced by addition of the above ionophores was measured under exactly the same experimental conditions with the pH-indicating dye phenol red. The coupling ratio between proton uptake and cation release was obtained in seven cases and an average coupling ratio of 1.00 (SD = 0.08) was observed; this result indicates that cation-induced proton fluxes via ionophores such as FCCP or gramicidin are a quantitative indicator for cation fluxes in bovine ROS.

In Fig. 1, the Na^+-induced changes in binding of neutral red ($-\Delta A_{540-650}$, Fig. 1 *A*) are compared with Na^+-induced proton release (measured with the extracellular pH–indicating dye phenol red as $-\Delta A_{570-650}$, Fig. 1 *B*). Na^+-induced changes in light absorption due to unbinding of neutral red were linearly related to outward proton flux, and, in view of the above discussion, were linearly related to Na^+ influx. As discussed elsewhere, essentially all the internal neutral red and a large amount of protons in bovine ROS are bound to a cation exchange matrix formed by the fixed negatively charged residues on the surface of the internal disk membranes (Schnetkamp, 1985*a*, 1990). Both protons and neutral red carry a single positive charge and

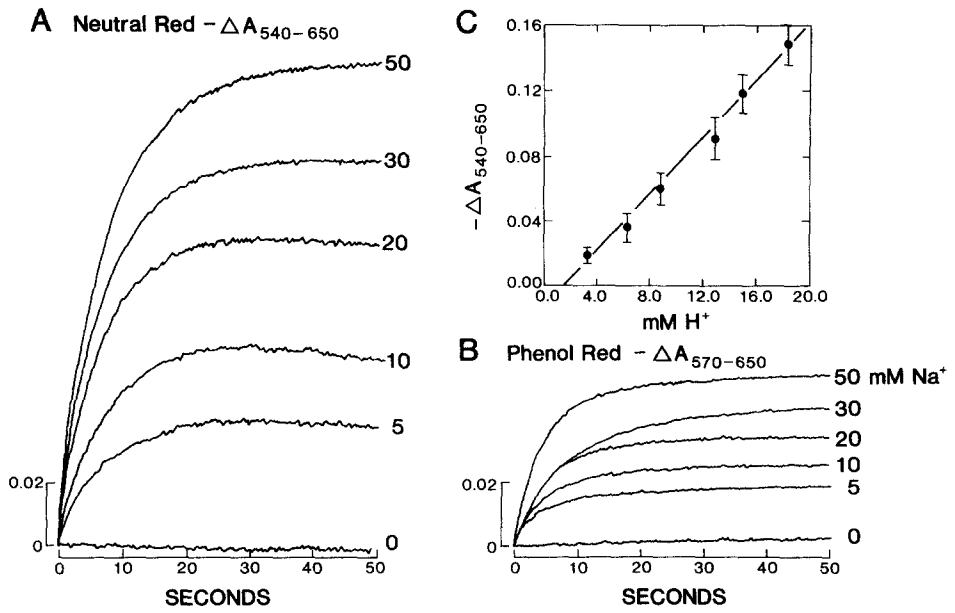

FIGURE 1. Calibration of neutral red signals into cation fluxes. (*A*) Intact Ca^{2+}-rich bovine ROS were incubated for 2 min in 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 200 μM BAPTA, 5 mM KCl, 50 μM neutral red, 1 μM gramicidin, and 1 μM FCCP; the suspension contained ROS to a final rhodopsin concentration of 7 μM. Changes in light absorption were monitored at the wavelength pair of 540 and 650 nm in the dual-wavelength mode. Na^+ uptake into ROS was initiated at time zero by addition of NaCl to the indicated final concentrations. Calibration bar is in absorbance units. (*B*) Intact Ca^{2+}-rich bovine ROS were incubated in 600 mM sucrose, 0.5 mM HEPES (adjusted to pH 7.4 with arginine), 200 μM BAPTA, 5 mM KCl, 40 μM phenol red, 1 μM gramicidin, and 1 μM FCCP; the suspension contained ROS to a final rhodopsin concentration of 7 μM. Changes in light absorption were monitored at the wavelength pair of 570 and 650 nm in the dual-wavelength mode. Na^+-induced proton release was initiated at time zero by addition of NaCl to the indicated final concentrations. Calibration bar is in absorbance units. (*C*) For each Na^+ concentration the Na^+-induced change in absorption observed with neutral red was plotted against Na^+-induced proton release; proton release was obtained by comparing Na^+-induced absorption changes of phenol red with those observed upon addition of a calibration pulse of HCl and by assuming that the total intracellular rhodopsin concentration in ROS amounts to 3 mM. Average values ± SD are shown representing four different ROS preparations. Temperature, 25°C.

respond to changes of the electrostatic potential at the surface of the ion exchange membrane in an identical fashion. Combined, the coupling ratio observed for cation fluxes via electrogenic ionophores (see above) and the experiment illustrated in Fig. 1 suggest that the rate of changes in light absorption ($-\Delta A_{540-650}$) can be used as a quantitative measure for electrogenic Na^+ transport across the ROS plasma membrane.

RESULTS

In the Methods an optical technique is described to measure electrogenic cation fluxes in bovine ROS with a resolution equivalent to a current of 0.01 pA. Na-Ca-K

exchange in bovine ROS is an electrogenic, but complicated cation exchange process and in the first part of this study an empirical approach is taken to examine the quantitative relationship between electrogenic Na^+ entry measured with the neutral red signals and Na^+-induced Ca^{2+} release measured with the Ca^{2+}-indicating dye arsenazo III in a separate aliquot of the same suspension of ROS. In subsequent sections some properties of Na-Ca-K exchange currents in bovine ROS are described.

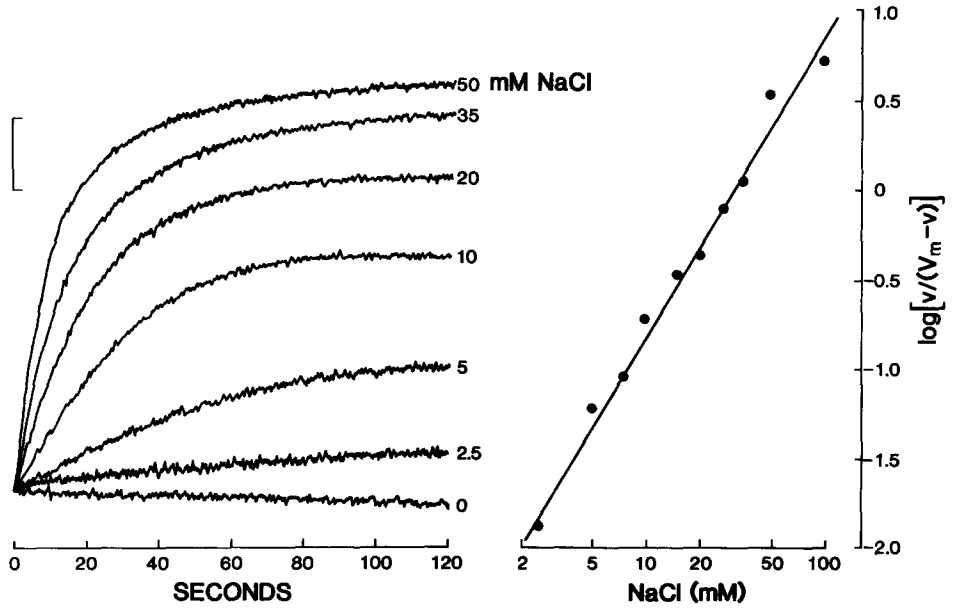

FIGURE 2. Optical recordings of Na-Ca-K exchange current in bovine ROS. (*Left*) Intact Ca^{2+}-rich bovine ROS were incubated for 5 min in 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 5 mM KCl, 50 μM neutral red, and 2 μM FCCP; the suspension contained rhodopsin to a final concentration of 9.0 μM. Changes in light absorption were monitored at the wavelength pair of 540 and 650 nm in the dual-wavelength mode; signals indicating an increase in internal cation concentration are plotted upward. The calibration bar indicates a change in light absorption by 0.02 absorbance units. EDTA was added to final concentration of 500 μM and Na-Ca-K exchange was initiated at time zero by addition of NaCl to the indicated final concentration. Temperature, 25°C. (*Right*) Hill plot of the data illustrated in *A*, v represents the initial rate of changes in light absorption, V_m is the initial rate at saturating Na^+ concentration.

Na^+-induced Changes in Neutral Red Binding Measure Na-Ca-K Exchange Current

Addition of Na^+ to a suspension of Ca^{2+}-rich intact bovine ROS in a sucrose medium containing pH buffer, neutral red, and the electrogenic protonophore FCCP caused unbinding of neutral red from the internal disk membranes; these changes can be followed by changes in light absorption at the wavelength pair of 540 and 650 nm, and indicated an increase in the internal Na^+ concentration (Fig. 2). The initial rate

of Na^+-induced changes in light absorption was obtained with high resolution and was converted to net Na^+ influx with the calibration curve shown in Fig. 1. To establish the relationship between net Na^+ entry (indicated by neutral red) and Ca^{2+} release (measured with arsenazo III), I compared in the same set of 11 ROS preparations both the initial rate and amount of electrogenic Na^+ entry with the initial rate and amount of Na^+-induced Ca^{2+} release observed upon addition of 50 mM NaCl. Experimental conditions in both assays were identical except for the different dyes used and a slight difference in the external free Ca^{2+} concentration (<0.01 μM in the neutral red assay as compared with a few micromolar in the arsenazo assay). External Ca^{2+} concentrations <10 μM have very little effect on inward Na-Ca-K exchange current at an external Na^+ concentration of 50 mM (see Fig. 4 *B*). The average initial rate of net Na^+ entry amounted to 5.5×10^6 (SD = 1.7×10^6) Na^+/ROS per s, while the rate of Na^+-induced Ca^{2+} release amounted to 6.2×10^6 (SD = 1.2×10^6) Ca^{2+}/ROS per s; the cumulative amount of net Na^+ uptake during the first 2 min after addition of Na^+ was 3.9 (SD = 0.7) mol Na^+/mol rhodopsin, while total Ca^{2+} release during the same period amounted to 4.2 (SD = 0.6) mol Ca^{2+}/mol rhodopsin. These results suggest that the neutral red signals indicate the electrogenic entry of one Na^+ for each Ca^{2+} released, similar to the electrogenicity of Na-Ca-K exchange reported before both in bovine ROS (Schnetkamp, 1989; Schnetkamp et al., 1989) and in amphibian ROS (Yau and Nakatani, 1984; Lagnado et al., 1988).

The drift in light absorption observed in Fig. 2 in the absence of external Na^+ indicated an outward current of 0.008 pA. The initial rate of changes in light absorption observed upon addition of 2, 5, 10, 20, 35, and 50 mM NaCl, indicated the equivalent of inward Na-Ca-K exchange currents of 0.016, 0.10, 0.29, 0.57, 1.05, and 1.62 pA, respectively. The Na-Ca-K exchange currents observed in Fig. 2 displayed a sigmoidal dependence on the external Na^+ concentration and yielded a linear Hill plot with a Hill coefficient of 1.7 and a K_m for Na^+ of 35 mM (Fig. 2, *right*). For 11 different ROS preparations the average Hill coefficient was 1.94 (SD = 0.22) and the average K_m for Na^+ ions was 36 mM (SD = 10). Fig. 3 compares, in the same ROS preparation, the Na^+ concentration dependence of both the initial rate and amplitude of (*a*) net Na^+ entry or Na-Ca-K exchange current (indicated by neutral red), (*b*) Na^+-induced Ca^{2+} release (measured with arsenazo III), and (*c*) the electrogenicity of Na-Ca-K exchange as measured by the Na^+-induced proton countercurrent via the electrogenic protonophore FCCP (indicated by phenol red). A very similar dependence on the external Na^+ concentration is observed for both parameters of Na-Ca-K exchange with all three methods used.

The above results suggest that neutral red can be used to measure Na-Ca-K exchange current in bovine ROS (see Discussion), and in the remainder of this paper Na^+- and Ca^{2+}-induced optical signals will be referred to as inward and outward Na-Ca-K exchange current, respectively.

Inhibition of Inward Na-Ca-K Exchange Current by External Ca^{2+}

The compatibility of the neutral red technique with a full range of external Ca^{2+} concentrations constitutes one of the major advantages of this method as compared with the use of ^{45}Ca or Ca^{2+}-indicating dyes. The Na-Ca(-K) exchanger is generally

believed to have a common binding site for Na^+ and Ca^{2+} (e.g., Reeves, 1985; Schnetkamp and Szerencsei, 1991). Inhibition of inward Na-Ca-K exchange current by external Ca^{2+} is illustrated in Fig. 4 at two different external Na^+ concentrations. Half-maximal inhibition of current (as judged from the initial rate of changes in light absorption) was typically observed at ~40 μM Ca^{2+} (20 mM Na^+) and at 200 μM Ca^{2+} (50 mM Na^+), respectively. A separate effect of an increase in external Ca^{2+} concentration was to reduce the amount of charge translocated (amplitude of change in light absorption 2 min after Na^+ addition). The latter effect suggests that the equilibrium free internal Ca^{2+} concentration established by the Na-Ca-K exchanger

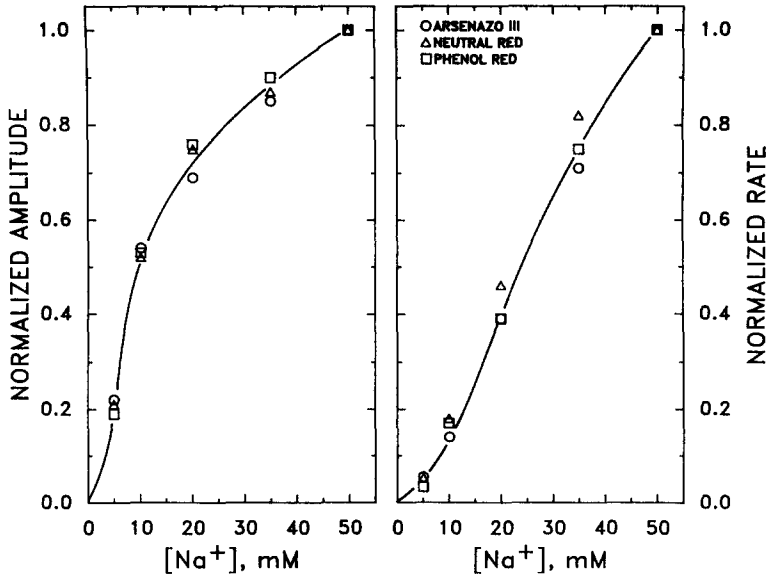

FIGURE 3. A comparison between Na^+-induced Ca^{2+} release, Na^+-induced proton release, and Na-Ca-K exchange current. Na^+-induced Ca^{2+} release and Na^+-induced proton release were measured as described under Methods; Na-Ca-K exchange current was measured as illustrated in Fig. 2. Signals obtained from the different assays were normalized by dividing the initial rate (*right*) or amplitude (*left*) observed for each Na^+ concentration by the rate or amplitude observed at a Na^+ concentration of 50 mM. Amplitude refers to the signal observed 120 s after addition of NaCl.

steadily increases with increasing external Ca^{2+} concentration, and, hence, the amount of Ca^{2+} that needs to be extruded by Na-Ca-K exchange steadily decreases.

A competitive interaction between Ca^{2+} and Na^+ for a common binding site is suggested by the higher Ca^{2+} concentration required for half-maximal inhibition of inward Na-Ca-K exchange current at 50 mM Na^+ as compared with 20 mM Na^+. Another way of analyzing the effect of external Ca^{2+} concentration on inward Na-Ca-K exchange current is illustrated in Fig. 5. An increase in external Ca^{2+} concentration caused a parallel shift of the Hill plot of inward Na-Ca-K exchange current as a function of external Na^+ concentration (i.e., the Hill coefficient was not affected by external Ca^{2+}), while the K_m of the exchanger protein for Na^+ was

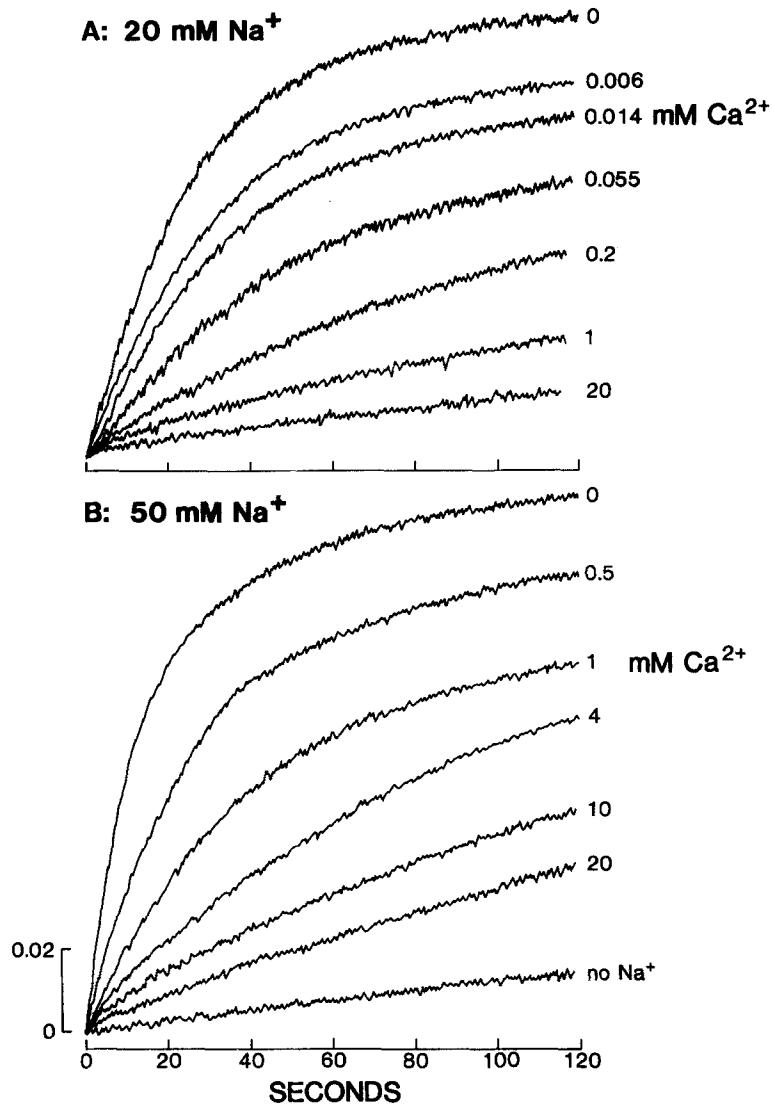

FIGURE 4. Inhibition of inward Na-Ca-K exchange current by external Ca^{2+}. Inward Na-Ca-K exchange current was measured as described in the legend of Fig. 2 at the indicated external Na^+ concentrations. Zero external Ca^{2+} indicates the presence of 1 mM EDTA. Free Ca^{2+} concentrations <0.1 mM were obtained with mixtures of NTA and CaNTA (total NTA concentration, 5 mM). The CaNTA dissociation constant was calculated to be 55 μM at pH 7.4. The calibration bar indicates a change in light absorption by 0.02 absorbance units. Temperature, 25°C.

progressively increased as the external Ca^{2+} concentration was increased. For the experiment illustrated in Fig. 5, the Hill coefficients ranged between 1.85 and 2.05; the K_m values of the exchanger protein for Na^+ were 47, 93, and 147 mM at external Ca^{2+} concentrations of 1.5 μM, 250 μM, and 1 mM, respectively.

Reverse Na-Ca-K Exchange Currents

The Na-Ca-K exchanger in ROS can mediate both Ca^{2+} efflux (dependent on external Na^+) as well as Ca^{2+} influx or reverse Na-Ca-K exchange (dependent on internal Na^+) (Schnetkamp, 1986; Cervetto et al., 1989; Schnetkamp et al., 1989). Reverse Na-Ca-K exchange currents were first measured in Ca^{2+}-rich ROS after preincubation with Na^+ and EDTA. Subsequent addition of Ca^{2+} to the indicated free Ca^{2+} concentrations resulted in neutral red signals, indicating a net efflux of Na^+ due to outward Na-Ca-K exchange current (Fig. 6). The outward Na-Ca-K exchange current (initial rate of change in light absorption upon addition of Ca^{2+}) increased as the external Ca^{2+} concentration increased until the equivalent of a maximal outward current of ~0.23 pA was observed. After the outward Na-Ca-K exchange current had saturated, the amplitude of the signals indicating the total amount of Na^+ released still increased with increasing external Ca^{2+} concentration to reflect the continuously changing equilibrium conditions of the exchanger. The outward Na-Ca-K exchange

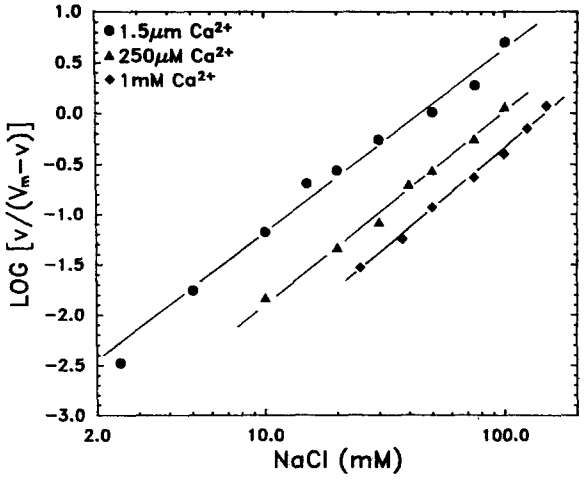

FIGURE 5. Effect of external Ca^{2+} on Hill plots of Na-Ca-K exchange currents. Inward Na-Ca-K exchange currents were obtained from the initial rate of Na^+-induced changes in light absorption. Experimental conditions were as described in the legend of Fig. 2. A free Ca^{2+} concentration of 1.5 μM was obtained by the presence of 500 μM CaHEDTA and 500 μM HEDTA.

current reached a maximum at a Ca^{2+} concentration of 1 mM (20 mM Na^+) or 5 mM (50 mM Na^+) and decreased as the external Ca^{2+} concentration was further increased, although the total amount of Na^+ release kept increasing (one example is illustrated in Fig. 6 *B* by the smooth trace). The maximal outward Na-Ca-K exchange current observed was very similar for the two different Na^+ loading concentrations used; in seven different preparations, the average maximal rate of changes in light absorption indicated the equivalent of an outward Na-Ca-K exchange current of 0.23 pA (SD = 0.03) or the equivalent of a Ca^{2+} influx rate of 1.4×10^6 Ca^{2+}/ROS per s.

I examined three properties of the Ca^{2+}-induced outward current to corroborate its identification as reverse Na-Ca-K exchange current: (*a*) Sr^{2+}, but not Mg^{2+}, Mn^{2+}, or Ba^{2+} could replace Ca^{2+}, identical to the ion selectivity of the Ca^{2+} site of the Na-Ca-K exchanger determined by ^{45}Ca flux experiments (Schnetkamp, 1980). (*b*) The Ca^{2+}-induced outward current required external K^+ (half-maximal activation between 1 and 2 mM K^+), similar to the K^+ requirement observed for Ca^{2+} uptake via reverse Na-Ca-K exchange (Schnetkamp et al., 1989). (*c*) Addition of the Na^+

ionophores such as monensin and gramicidin (but not addition of the K^+-selective ionophore valinomycin) abolished the Ca^{2+}-induced neutral red signal, suggesting that this signal was caused by Ca^{2+}-induced Na^+ efflux; monensin and gramicidin equilibrate internal and external Na^+ concentration and as a result prevent changes in internal Na^+ via Na-Ca-K exchange.

The Ca^{2+} activation constant of reverse Na-Ca-K exchange in the absence of external Na^+ was determined by measuring the outward Na-Ca-K exchange current

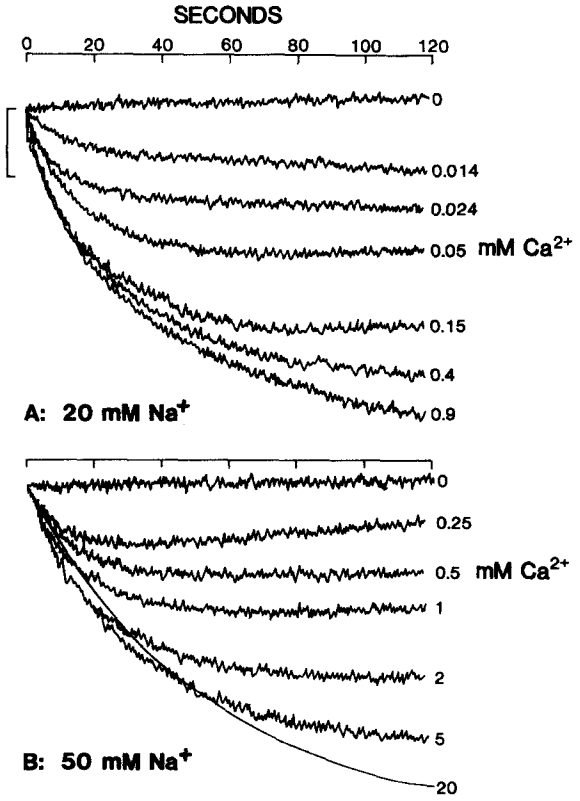

FIGURE 6. Reverse Na-Ca-K exchange current at high external Na^+. Intact Ca^{2+}-rich bovine ROS were preincubated for 5 min in 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 100 μM BAPTA, 5 mM KCl, 20 mM NaCl (*A*) or 50 mM NaCl (*B*), 50 μM neutral red, and 2 μM FCCP; the suspension contained rhodopsin to a final concentration of 9 μM. Reverse Na-Ca-K exchange was initiated at time zero by addition of $CaCl_2$ to the indicated final free concentration. Changes in light absorption indicating a decrease in internal Na^+ concentration are plotted downward. The calibration bar indicates a change in light absorption by 0.01 absorbance unit. Free Ca^{2+} concentrations <0.1 mM were obtained by using mixtures of NTA and CaNTA (total NTA concentration, 5 mM). The CaNTA dissociation constant was calculated to be 55 μM at pH 7.4. The outward exchange current in *B*, induced by addition of 20 mM $CaCl_2$, is presented by a smooth line to prevent confusion with the other traces. Temperature, 25°C.

in Ca^{2+}-depleted ROS (Fig. 7 *A*). Addition of external free Ca^{2+} in the 1 μM range activated outward Na-Ca-K exchange current; a Scatchard plot of the data yielded a straight line with a K_m of 0.8 μM (Fig. 7 *B*). In six different preparations the average maximal rate of Ca^{2+}-induced optical signals was equivalent to an average outward current of 0.17 pA (SD = 0.03), or the equivalent of a Ca^{2+} influx rate of 1.0×10^6 Ca^{2+}/ROS per s. In the same set of Ca^{2+}-depleted ROS preparations the average K_m was 1.1 μM (SD = 0.2). The optical signals observed for the Ca^{2+}-induced outward

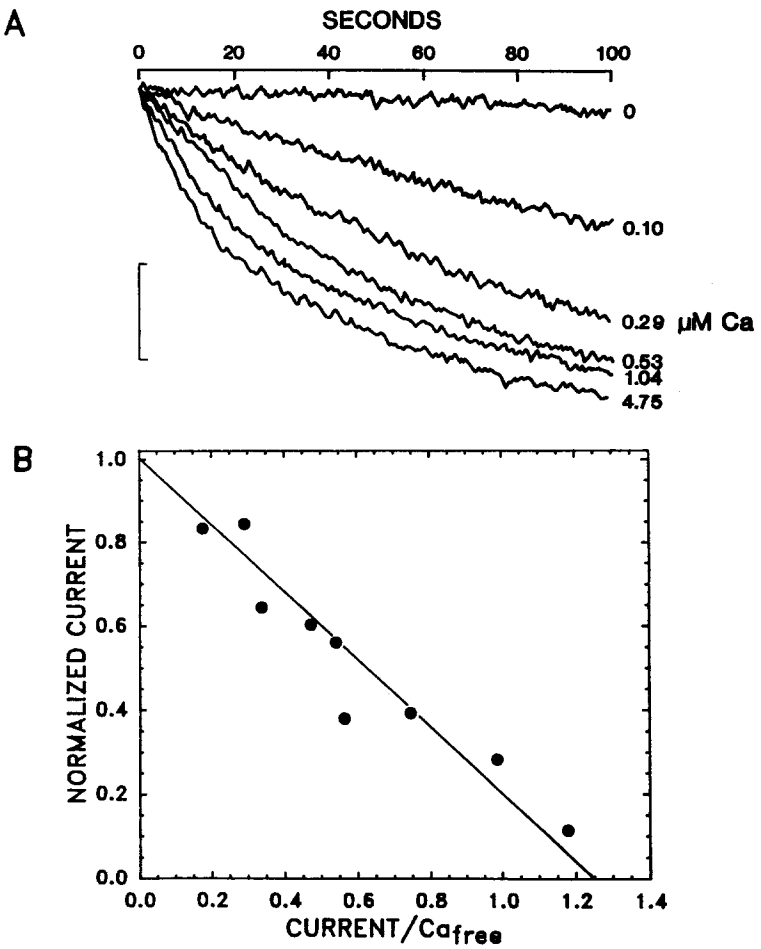

FIGURE 7. (*A*) Reverse Na-Ca-K exchange current in the absence of external Na^+. Intact Ca^{2+}-depleted bovine ROS were preincubated for 5 min in 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 0.1 mM EDTA, HEDTA (as described below), 10 mM KCl, 50 μM neutral red, and 1 μM FCCP; the suspension contained rhodopsin to a final concentration of 10 μM. Changes in light absorption were monitored at the wavelength pair of 540 and 650 nm in the dual-wavelength mode. Signals indicating a decrease in internal cation concentration are plotted downward. The calibration bar indicates a change in light absorption by 0.01 absorbance unit. Total HEDTA plus CaHEDTA was 5 mM. Reverse Na-Ca-K exchange was initiated at time zero by addition of different concentrations of CaHEDTA to obtain the indicated final free Ca^{2+} concentrations. An apparent dissociation constant of 1.5 μM (pH 7.4) for the CaHEDTA complex was used. Temperature, 25°C. (*B*) Scatchard plot of Ca^{2+} activation of reverse Na-Ca-K exchange. The initial rates of reverse Na-Ca-K exchange current observed in the experiment illustrated in *A* were transformed into a Scatchard plot. The observed currents were normalized to the maximal current extrapolated from the linear Scatchard plot.

Na-Ca-K exchange current in Ca^{2+}-depleted ROS were rather small. When the experiment illustrated in Fig. 7 was carried out in the presence of the Ca^{2+} ionophore A23187, the amplitude of the Ca^{2+}-induced signals was increased about twofold since the net Ca^{2+} flux was shunted by the Ca^{2+} ionophore, whereas the net Na^+ flux was not. In five experiments with A23187 present, the average K_m of Ca^{2+}-induced outward current was 1.1 μM (SD = 0.1).

Ca^{2+} uptake via reverse Na-Ca-K exchange is stimulated by external K^+ and inhibited by external Na^+ (Schnetkamp, 1986; Schnetkamp et al., 1989). Likewise, Ca^{2+}-induced outward currents indicated by neutral red were stimulated by external K^+ and inhibited by external Na^+ (Fig. 8).

Internal Ca^{2+} Requirement of Inward Na-Ca-K Exchange Currents

Measurement of the internal Ca^{2+} requirement of inward Na-Ca-K exchange currents necessitated in addition to FCCP the use a Ca^{2+} ionophore to control intracellular

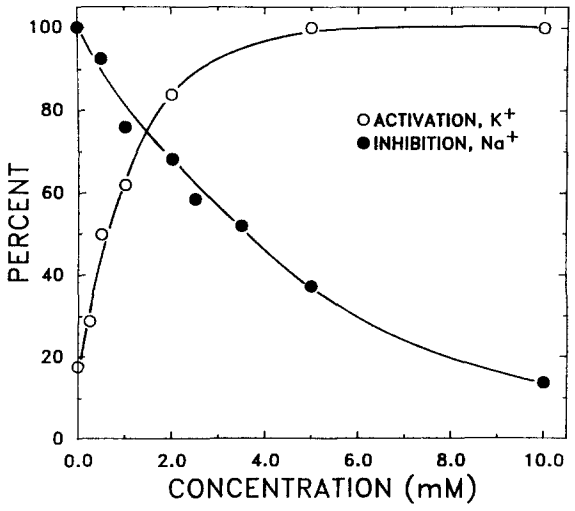

FIGURE 8. Reverse Na-Ca-K exchange is activated by K^+ and inhibited by Na^+ in the external medium. The initial rate of reverse Na-Ca-K exchange was measured at a free Ca^{2+} concentration of 2.25 μM as described in the legend of Fig. 7 *A*. The K^+ concentration was varied between 0 and 10 mM with no added Na^+; the Na^+ concentration was varied between 0 and 10 mM with 10 mM KCl present. The observed rate for a particular K^+ and Na^+ concentration was normalized by dividing this rate by the rate observed at 10 mM KCl and zero NaCl.

Ca^{2+} concentration. The Ca^{2+} ionophore A23187 can be utilized to equilibrate the internal and external free Ca^{2+} concentration in ROS (Kaupp, Schnetkamp, and Junge, 1979). However, A23187 disturbs the coupling between net Ca^{2+} and Na^+ fluxes and, thus, the amplitude of the neutral red signal. Here, I assume that A23187 increases the amplitude of signals due to Na-Ca-K exchange current independent of the Ca^{2+} concentration. This assumption was tested by measuring the dependence of outward Na-Ca-K exchange current on external Ca^{2+} concentration with and without A23187. At free Ca^{2+} concentrations < 10 μM the above assumption appeared valid and the measured K_m of the exchanger for external Ca^{2+} was not affected by the presence of A23187 (see above).

In addition to the inclusion of A23187, it proved useful to add the electrogenic K^+ ionophore valinomycin as well. Valinomycin abolished a rapid transient neutral red

signal, indicating a rapid transient Na^+ influx in Ca^{2+}-depleted ROS even in the presence of EDTA. Although the amplitude of this transient signal was < 10% of the Na^+ influx observed in the presence of Ca^{2+}, it made measurements of the initial rate of changes in light absorption (Na-Ca-K exchange current) more difficult. Addition of valinomycin in the presence of 5–10 mM external KCl otherwise had little effect on the Na-Ca-K exchange currents observed in this study. Fig. 9 illustrates a typical

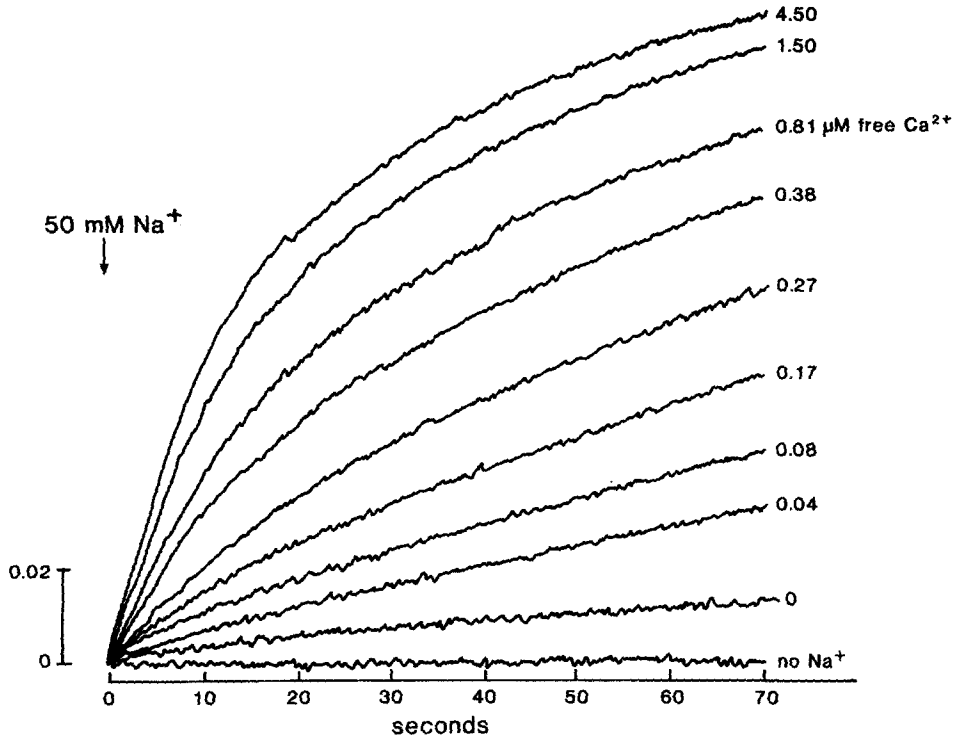

FIGURE 9. Requirement of forward Na-Ca-K exchange for internal Ca^{2+}. Internal Ca^{2+} was removed from Ca^{2+}-rich ROS by preincubation for 5 min with 1 mM EDTA, 2 μM A23187, and 20 mM ammonium acetate; ammonium acetate and EDTA were removed by washing. ROS were resuspended and preincubated for 5 min in 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 5 mM KCl, 50 μM neutral red, 2 μM FCCP, 2 μM A23187, 2 μM valinomycin, and 5 mM CaHEDTA/HEDTA to control free Ca^{2+} concentration as described in the legend of Fig. 7 *A*. Zero Ca^{2+} indicates the presence of 5 mM EDTA instead of HEDTA. The suspension contained rhodopsin to a final concentration of 6.0 μM. Signals indicating an increase in internal cation concentration are plotted upward. Forward Na-Ca-K exchange was initiated at time zero by addition of 50 mM NaCl. Temperature, 25°C.

experiment on the internal Ca^{2+} dependence of inward Na-Ca-K exchange current. Na^+-induced inward current was very small in the absence of internal Ca^{2+}, but was greatly stimulated when the internal free Ca^{2+} concentration was increased to the 1 μM level, indicating activation of inward Na-Ca-K exchange current. The Na-Ca-K exchange current observed at a free Ca^{2+} concentration of 4.5 μM was typically

20-fold greater than that observed at zero Ca^{2+}. Data such as those shown in Fig. 9 were transformed into Lineweaver-Burke plots to determine the Ca^{2+} concentration at which half-maximal activation was observed. For 15 different plots from 9 different ROS preparations (4 with FCCP and 11 with FCCP plus valinomycin), half-maximal activation of inward Na-Ca-K exchange current was observed at a free Ca^{2+} concentration of 0.9 μM (SD = 0.7). The fairly large standard deviation is probably indicative of the efficiency of the Ca^{2+} shunt applied in the form of A23187.

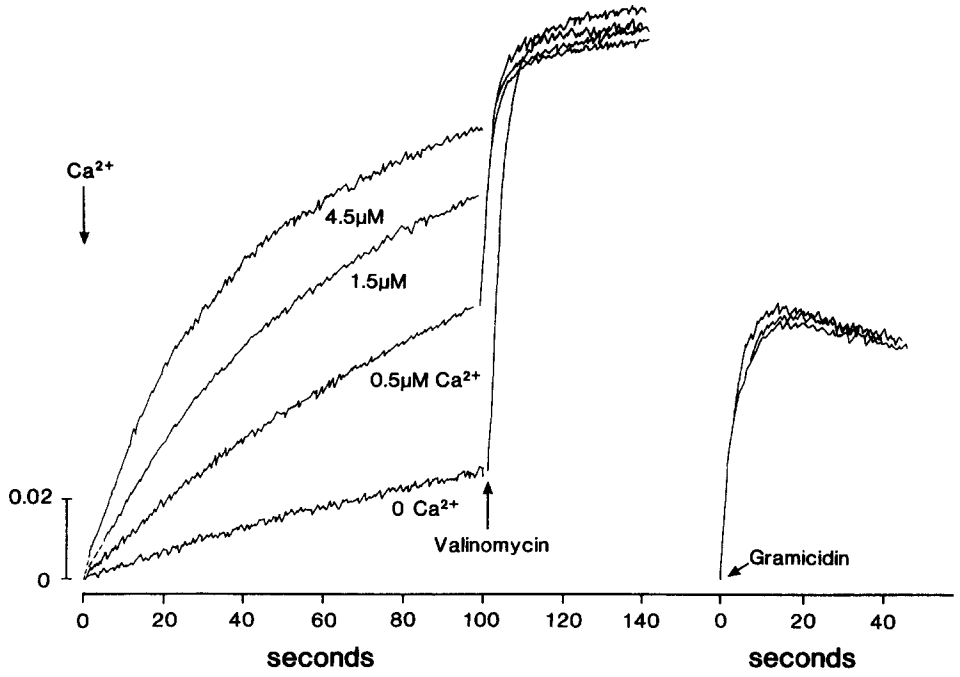

FIGURE 10. Ca^{2+} activation of ammonium-K exchange in ROS. Intact Ca^{2+}-rich bovine ROS were preincubated for 1 min in 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 100 μM HEDTA, 2 mM ammonium acetate, 50 μM neutral red, and 2 μM FCCP; the suspension contained rhodopsin to a final concentration of 10 μM. Changes in light absorption were monitored at the wavelength pair of 540 and 650 nm in the dual-wavelength mode. Signals indicating a decrease in internal cation concentration are plotted upward. Ammonium-K exchange was initiated at time zero by addition of HEDTA-CaHEDTA mixtures to a final concentration of 5 mM. Different CaHEDTA/HEDTA ratios were used to obtain the indicated free Ca^{2+} concentrations as described in the legend of Fig. 7 *A* . The second arrow at ~100 s indicates addition of 1.5 μM valinomycin; subsequent addition of 1.5 μM gramicidin gave rise to the signals illustrated on the far right. Temperature, 25°C.

Ca^{2+} Activation of K^+ Fluxes through the Na-Ca-K Exchanger

The Ca-Ca self-exchange mode of the Na-Ca-K exchanger in bovine ROS is accompanied by K-K exchange as demonstrated by Ca^{2+}-dependent ^{86}Rb fluxes (Schnetkamp et al., 1991). The self-exchange mode is an electroneutral process and does not produce a signal with the neutral red technique. However, with the

application of ammonium ions as a K^+ substitute in the external medium, it is possible to convert the Ca^{2+}-dependent ammonium-K exchange process into a Ca^{2+}-dependent net K^+ efflux that can be monitored with neutral red. Ammonium ions can replace K^+ in activating Na-Ca-K exchange (Schnetkamp and Szerencsei, 1991); the concentration of ammonium ions (added as acetate salt) is rapidly (< 1 s) equilibrated between external medium and intracellular space due to the permeation of the neutral species ammonia and acetic acid (Schnetkamp, 1985*b*). The (Ca + ammonium):(Ca + K) exchange process is expected to equilibrate the existing outward K^+ gradient and cause a nearly complete loss of internal K^+. (K^+ release does not increase the very low external K^+ concentration due to the fact that ROS are in a very dilute suspension.) The above protocol is illustrated in Fig. 10. Addition of 2 mM ammonium acetate caused an instantaneous neutral red signal, indicating the rapid rise of the internal cation concentration by 2 mM due to the appearance of 2 mM ammonium acetate in the cytoplasm. This rapid signal was followed by a slow drift in the opposite direction when external free Ca^{2+} concentration was very low in the presence of 5 mM HEDTA. Addition of CaHEDTA to raise the external free Ca^{2+} concentration to the micromolar range caused neutral red signals, indicating Ca^{2+}-induced cation release, probably K^+ and/or Na^+. In five different preparations the average activation constant for external Ca^{2+} was 2.7 μM (SD = 1.3) as determined from Lineweaver-Burke plots. To ascertain which cation was released by the above Ca^{2+}-dependent process, different ionophores were applied. Addition of the K^+-selective ionophore valinomycin caused a rapid and complete release of internal K^+ due to ammonium-K^+ exchange via valinomycin. Subsequent addition of the nonspecific channel ionophore gramicidin caused the release of internal Na^+. The ion selectivity of ionophore-induced cation release was confirmed by atomic absorption spectroscopy (not illustrated). The Ca^{2+}-dependent cation release observed as an intrinsic property of the ROS plasma membrane reduced the subsequent release induced by valinomycin, but not that induced by gramicidin. This demonstrates that Ca^{2+} activated ammonium-K exchange, but not ammonium-Na exchange. The maximal rate of Ca^{2+}-activated changes in light absorption were equivalent with a rate of K^+ release of 3.8×10^6 K^+/ROS per s (SD = 1.0×10^6).

DISCUSSION

Neutral Red as an Optical Probe to Measure Na-Ca-K Exchange Current in Intact Isolated Bovine ROS

In this study I have used changes in binding of the dye neutral red to the internal disk membranes to measure electrogenic Na^+ fluxes across the plasma membrane of bovine ROS. Cation-induced screening of the interfacial potential at the membrane/water interface of the intracellular disk membranes is the most likely mechanism of cation-induced release of membrane-bound neutral red (Schnetkamp, 1985*a*, 1990). The main advantages of the neutral red technique are its good resolution (e.g., Fig. 2), its quick and simple execution, and its compatibility with a full range of external Na^+ and Ca^{2+} concentrations, whereas its major drawback lies in the absence of a

direct calibration of changes in light absorption into cation flux. In this and a previous study (Schnetkamp, 1990) electrogenic cation fluxes were quantitated by comparing cation-induced changes in neutral red binding with cation-induced proton fluxes in the presence of an electrogenic protonophore such as FCCP or gramicidin. On the assumption of overall charge neutrality of transport across the ROS plasma membrane, electrogenic cation fluxes should be electrically compensated by equally large proton currents in the opposite direction. In this study, the assumption of charge neutrality was tested and found to be valid (see Methods): cation-induced proton fluxes across the ROS plasma membrane (measured with phenol red) were stoichiometrically coupled to oppositely directed Na^+ or K^+ fluxes carried by electrogenic ionophores such as gramicidin and valinomycin (measured with atomic absorption spectroscopy). Similarly, Na^+-induced proton efflux (carried by FCCP) showed a kinetic and stoichiometric correlation with Na^+-induced Ca^{2+} release (carried by Na-Ca-K exchange) consistent with the notion that charge neutrality couples proton efflux (via FCCP) to Na-Ca-K exchange current (Schnetkamp, 1989; Schnetkamp et al., 1989).

Na-Ca-K exchange is the only functional and electrogenic cation transporter present in the plasma membrane of isolated bovine ROS (Schnetkamp, 1989; Schnetkamp et al., 1991). The cGMP-dependent channel in the plasma membrane of isolated bovine ROS is probably closed due to lack of cGMP, but its presence can be readily demonstrated in the same preparation of isolated bovine ROS, either in membrane vesicles with the use of the neutral red technique (Schnetkamp, 1990) or in excised patches of plasma membrane (Quandt, Nicol, and Schnetkamp, 1991). Identification of Na^+- and Ca^{2+}-induced optical neutral red signals as Na-Ca-K exchange current is based on a quantitative correlation between the Na-Ca-K exchange currents deduced from these signals and Na^+-induced Ca^{2+} and proton fluxes. The electrogenicity of Na-Ca-K exchange in bovine ROS under our measuring conditions is one positive charge carried by Na^+ for each Ca^{2+} released (Schnetkamp, 1989; Schnetkamp et al., 1989). The average inward Na^+ current at 50 mM NaCl represented a net Na^+ flux ($5.5 \pm 1.7 \times 10^6$ Na^+/ROS per s) of similar magnitude as the average Na^+-induced Ca^{2+} efflux ($6.2 \pm 1.2 \times 10^6$ Ca^{2+}/ROS per s) or the average Na^+-induced proton efflux of $6.5 \pm 1.2 \times 10^6$ protons/ROS per s (Schnetkamp, 1989), suggesting that the neutral red technique registered the inward Na-Ca-K exchange current of one positive charge carried by Na^+ for each Ca^{2+} released. A similar conclusion can be drawn by comparing the Ca^{2+}-induced outward Na-Ca-K exchange current (Fig. 7) and Ca^{2+} influx in Ca^{2+}-depleted (Na^+-rich) ROS. The average outward Na-Ca-K exchange current amounted to $1.03 \pm 0.18 \times 10^6$ Na^+/ROS per s, similar to Ca^{2+} influx measured in the same preparation under very similar conditions with either arsenazo III ($1.1 \pm 0.2 \times 10^6$ Ca^{2+}/ROS per s; Schnetkamp et al., 1989) or with ^{45}Ca influx ($1.0 \pm 0.4 \times 10^6$ Ca^{2+}/ROS per s; Schnetkamp and Szerencsei, 1991). The neutral red technique was adapted to measure Ca^{2+}-activated K^+ exchange fluxes of 3.8×10^6 K^+/ROS per s (SD = 1.0×10^6) (Fig. 10), comparable with Ca^{2+}-activated ^{86}Rb-K exchange fluxes of $4.4 \pm 2.2 \times 10^6$ K^+/ROS per s (Schnetkamp et al., 1991).

We have previously reported that Na-Ca-K exchange in isolated bovine ROS could also operate in an electroneutral 3Na:(1 Ca + 1 K) mode when no ionophores were

present to shunt the Na-Ca-K exchange current. This conclusion was based on (*a*) the absence of Na^+-induced proton or Mg^{2+} release under such conditions (to compensate for the inward current; Schnetkamp, 1989; Schnetkamp et al., 1989), and (*b*) the observation that FCCP increased the ratio of ^{22}Na uptake/^{45}Ca release by one-third, consistent with a switch of the Na:Ca coupling ratio from 3 to 4 upon addition of FCCP (Schnetkamp et al., 1991). No Na^+-induced neutral red signals were observed in the the absence of FCCP, suggesting that the ionic changes due to the electroneutral mode of Na-Ca-K exchange cancel each other out and corroborating the idea that neutral red signals monitor only the Na-Ca-K exchange current. Electroneutral cation fluxes in general are not registered by the neutral red technique, with the exception of cation-proton exchange (Schnetkamp, 1985*a*); no evidence was found for the presence of a Na-H exchanger in the ROS plasma membrane.

Internal and External Ca^{2+} Dependence of Na-Ca-K Exchange and K-K Exchange

The major advantage of the neutral red technique over other methods of measuring Na-Ca-K exchange fluxes rests in its potential to provide high-resolution kinetic measurements of Na-Ca-K exchange currents that can encompass a range of Ca^{2+} and Na^+ concentrations not accessible to other more direct methods for measuring Na^+-induced Ca^{2+} fluxes such as the Ca^{2+}-indicating dye arsenazo III or application of ^{45}Ca. The dependence on Ca^{2+} concentration of three transport modes of the bovine ROS Na-Ca-K exchanger (forward Na-Ca-K exchange, reverse Na-Ca-K exchange, and Ca^{2+}-activated ammonium-K exchange) was measured in this study. All three transport modes displayed single-site Michaelis-Menten kinetics with respect to Ca^{2+} as judged by linear Scatchard plots (e.g., Fig. 7 *B*). Half-maximal activation of both inward and outward Na-Ca-K exchange current was observed at 0.9 ± 0.7 and 1.1 ± 0.2 μM free Ca^{2+}, respectively, whereas half-maximal activation of ammonium-K exchange by external Ca^{2+} occurred at 2.7 ± 1.3 μM. The above values can be compared with values between 1 and 3 μM obtained from ^{45}Ca and ^{86}Rb fluxes in bovine ROS activated by external Ca^{2+} (Schnetkamp, 1980; Schnetkamp et al., 1991), and with a value of 2.3 μM for activation of forward Na-Ca-K exchange current by internal Ca^{2+} in tiger salamander ROS (Lagnado et al., 1988). For the above measurements of Ca^{2+}-activated fluxes via the Na-Ca-K exchanger, the presence of competing cations was minimized. Under these conditions the Na-Ca-K exchanger appears symmetrical with respect to the half-activation by internal and external Ca^{2+}, respectively. In a previous study the Na-Ca-K exchanger was found to be symmetrical with respect to activation by internal (1.5 mM) and external K^+ (1.2 mM), respectively (Schnetkamp et al., 1989).

Interactions between Na^+ and Ca^{2+}

Several observations suggest a competitive interaction between Ca^{2+} and Na^+ for a common transport site on the exchanger protein (e.g., Figs. 4–6 and 8). When analyzed with a simple Michaelis-Menten equation describing sequential binding of two Na^+ ions or one Ca^{2+} ion to a common transport site, a quantitative problem emerges. The inhibitory K_i's do not appear to match the transport K_m values. The inhibitory K_i for Ca^{2+} is at least in the tens of micromolar as compared with a transport K_m of ~1 μM, whereas the inhibitory K_i for Na^+ appears to be ~5 mM

compared with a transport K_m of ~35 mM. The data presented in this study suggest that the neutral red technique can be applied to obtain precise and more complete data on the interactive effects of Na^+ and Ca^{2+} on Na-Ca-K exchange currents that would provide tests for the more complex kinetic models of Na-Ca exchange (Hilgemann, 1988, and references therein).

This research was financially supported by grants from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada.

Original version received 18 June 1990 and accepted version received 18 March 1991.

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