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Ionic Permeabilities of the Plasma Membrane of Isolated Intact Bovine Rod Outer Segments as Studied with a Novel Optical Probe

Paul P.M. Schnetkamp*
Jules Stein Eye Institute, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

Summary. The permeability properties of the plasma membrane of intact rod outer segments purified from bovine retinas (ROS) were studied with the aid of the optical probe neutral red as described in the companion paper. The following observations were made: (1) Electrical shunting of ROS membranes greatly stimulated Na^+ and K^+ transport, suggesting that this transport reflects Na^+ and K^+ currents, respectively. The dissipation of a Na^+ gradient across the plasma membrane occurred with a half-time of 30 sec at 25°C. (2) The Na^+ permeability was progressively inhibited when the external Ca^{2+} concentration was raised from 1 μM to 20 mM. A similar Ca^{2+} dependence was observed for H^+ and Li^+ transport. The Na^+ permeability was not affected when the total internal Ca^{2+} content of ROS was varied between 0.1 mol Ca^{2+}/mol rhodopsin and 7 mol Ca^{2+}/mol rhodopsin, or when the free internal Ca^{2+} concentration was varied between 0.1 and 50 μM. (3) The K^+ permeability was progressively stimulated when the external Ca^{2+} concentration was raised from 0.001 to 1 μM, whereas a further increase to 20 mM was without effect. A similar Ca^{2+} dependence was observed for Rb^+ and Cs^+ transport. (4) At an external Ca^{2+} concentration in the micromolar range the rate of transport decreased in the order: $Na^+ > K^+ = H^+ > Cs^+ > Li^+$. (5) Na^+ fluxes depended in a sigmoidal way on the external Na^+ concentration, suggesting that sodium ions move in pairs. The concentration dependence of uniport Na^+ transport and that of Na^+-stimulated Ca^{2+} efflux (exchange or antiport transport) were very similar.

Key Words vision · rod photoreceptors · ionic channels

Introduction

The ion selectivity of the light-sensitive conductance in vertebrate rod photoreceptors has recently gained considerable attention. Earlier studies suggested that the light-sensitive conductance was selective to Na^+ since replacement of Na^+ by any other cation in the presence of normal Ca^{2+} abolished the light-sensitive current (Sillman, Ito & Tomita, 1969; Brown & Pinto, 1974; Hagins & Yoshikami, 1975). In later experiments ion substitution was carried out also in low Ca^{2+} solutions and it was concluded that the light-sensitive conductance is permeable to alkali cations and a number of divalent cations (Fain & Lisman, 1981; Bastian & Fain, 1982; Torre, 1982; Woodruff, Fain & Bastian, 1982; Capovilla, Caretta, Cervetto & Torre, 1983; Hodgkin, McNaughton, Nunn & Yau, 1984; Yau & Nakatani, 1984*a*). One explanation for the apparent discrepancy in ion selectivity is that the light-sensitive conductance is controlled by the internal Ca^{2+} concentration (as originally proposed by Yoshikami & Hagins, 1971). The internal Ca^{2+} concentration in turn could be controlled by Na-Ca exchange (Fain & Lisman, 1981; Schnetkamp, 1981). Na-Ca exchange in rods has an absolute selectivity for Na^+ over other alkali cations (Schnetkamp, 1980; Yau & Nakatani, 1984*b*), and when Na^+ is replaced by other cations in the presence of Ca^{2+} the cell fills up with Ca^{2+} by reverse Na-Ca exchange. The increase in intracellular Ca^{2+} then closes the light-sensitive conductance.

Relatively few studies have been carried out on the ion permeability of isolated intact rod outer segments (ROS). The studies on frog ROS (Korenbrot & Cone, 1972; Bownds & Brodie, 1975; Wormington & Cone, 1978) focused on the light-sensitive Na^+ permeability. In bovine ROS the plasma membrane was found to be permeable to uniport transport of alkali cations, but not of divalent cations (Schnetkamp, 1980). All these studies used the osmotic properties of ROS to measure ion permeabilities.

In the companion paper a novel optical method was introduced to measure ionic permeabilities in biological membranes as demonstrated in liposomes and ROS. This method employs the dye neutral red and allows real-time measurements of ionic currents across the outer envelope of small particles in suspension. In this study the ionic permeabilities of

* *Present address:* Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Drive, Madison, WI 53706

the plasma membrane of intact ROS isolated from bovine retinas were measured. In this preparation the integrity of the plasma membrane is maintained during various experimental manipulations, and the cytoplasm contains a normal complement of soluble proteins and small solutes such as ATP, GTP, cGMP, and reducing agents (Schnetkamp, Klompmakers & Daemen, 1979; Schnetkamp, 1981, 1985*b*). The plasma membrane of intact bovine ROS showed Ca^{2+}-sensitive ionic permeabilities for alkali cations, whereas aspecific leakage was virtually absent. A Ca^{2+}-inhibited Na^+ permeability is described and compared with the light-sensitive permeability in rods in the functioning retina. Some of these experiments have been presented before in preliminary form (Schnetkamp & Hubbell, 1983; Schnetkamp; 1984).

Materials and Methods

The preparations of intact and leaky bovine ROS used in this study were described in the companion paper. In most experiments Ca^{2+}-enriched ROS were used containing 5–7 mol Ca^{2+}/mol rhodopsin. In some experiments the properties of Ca^{2+}-enriched ROS were compared with those of Ca^{2+}-depleted ROS containing less than 0.1 mol Ca^{2+}/mol rhodopsin. ROS were suspended and stored in a solution containing 600 mM sucrose, 5% Ficoll 400, 20 mM HEPES, adjusted to pH 7.4 with 8.8 mM arginine. All experiments were carried out within 6 hr after isolation of ROS. During this period the permeability properties of ROS did not change. All experiments were carried out in darkness or under dim red illumination.

Optical Measurements of the Internal Ionic Milieu in ROS

Changes in the ionic milieu in ROS were measured by the salt-dependent adsorption of the dye neutral red to the surface of the disk membranes as described earlier (Schnetkamp et al., 1981; Schnetkamp, 1985*a*).

Changes in the adsorption of neutral red were monitored in two ways: First, the amount of dye adsorbed to ROS membranes was determined by sedimenting ROS from a suspension incubated with neutral red (30 sec, 12,000 rpm in a table top centrifuge). The amount of dye remaining in the supernatant was measured in a solution containing 1% SDS and 20 mM HEPES, adjusted to pH 7.4 with 8.8 mM arginine. The kinetics of cation uptake by ROS indicate that sedimentation was complete within 5 sec. The amount of dye adsorbed to ROS membranes before and after uptake of added cations was determined. The partition coefficient (ratio of bound/free dye) was calculated and the value prior to uptake of cations was set at unity. When cations were taken up by ROS the internal cation concentration increased, the surface potential became less negative, and some of the bound and positively charged neutral red was released. As a result the partition coefficient decreased. The precision of the measurement of the partition coefficient was determined by pipetting accuracy and was routinely within 5%. A change in the partition coefficient of about 20% was observed for a change in the internal concentration of monovalent cations by 1 mM, while an increase by 25 mM changed the partition coefficient by four- to sevenfold, depending on the internal ionic milieu in ROS at the start of the incubation (e.g. Ca^{2+}-enriched ROS or Ca^{2+}-depleted ROS).

Second, the protonated form of neutral red predominates when adsorbed to the disk membrane, whereas at neutral pH the unprotonated form predominates in solution. This makes it possible to monitor the binding and release of neutral red from disk membranes by following the light absorption at the absorption maximum (540 nm) of the protonated form (Schnetkamp et al., 1981; Schnetkamp, 1985*a*). In earlier experiments single wavelength measurements were made with a DW2 Aminco spectrophotometer. Electrolytes were added by taking the cuvette out of the spectrophotometer. This took about 10 sec and a small artifact due to light scattering changes caused by the mixing was visible. In later experiments dual-wavelength recordings were made with a DW2C SLM-Aminco spectrophotometer equipped with a cuvette holder having a magnetic stirrer. Measurements were made at the wavelength pair of 540 and 650 nm with a bandwidth of 3 nm. At 650 nm light absorption was completely due to light scattering by ROS particles. All experiments were carried out with thermostated cuvettes.

Osmotic Experiments

Solute transport across the plasma membrane of ROS was also monitored by an osmotic assay. The osmolarity of a suspension of intact ROS was reduced by dilution in a medium containing 200 mM sucrose and 20 mM HEPES (adjusted to pH 7.4 with 8.8 mM arginine). The apparent light absorption at 700 nm was due to light scattering and was followed in a thermostated cuvette equipped with a magnetic stirrer. The cuvette was placed in a DW2C SLM-Aminco spectrophotometer. Addition of solutes increased the osmolarity of the external medium and resulted in a shrinkage of the ROS. This caused an increase in apparent light absorption. A return to the original level of light scattering was observed when the solute was able to cross the plasma membrane and equilibrate in the cytoplasm. Salt transport requires the permeation of both the anion and the cation. In order to measure the permeability for cations a permeable anion was created by using acetate salts in the presence of the protonophore FCCP as described before (Schnetkamp, 1980).

Results

Measuring Ionic Permeabilities in Isolated ROS

In the companion paper the dye neutral red was used as a measure of the internal cation concentration in ROS or in simple model systems made up of phosphatidylcholine and acidic phospholipids such as phosphatidylserine. The first section of this paper outlines the general procedure of permeability measurements with the use of this dye. When a step change of 25 mM K^+ was made in the external medium the dye registered the equilibration of the K^+ gradient established across the outer envelope of

ROS or liposomes (unless indicated otherwise cations were added as chloride salts). The experimental protocol is illustrated by the experiment shown in Fig. 1. The partition parameter used in Fig. 1 is defined as the change in the partition coefficient of neutral red between the disk membranes and the external medium. A value of unity indicates no change in the internal ionic milieu (no net cation movements), while a value of n indicates an n-fold change of the partition coefficient. Values less than unity indicate more neutral red binding caused by an efflux of internal cations. Values greater than unity indicate a release of neutral red caused by an uptake of cations. When the alkali cation ionophore gramicidin was added to intact ROS, the distribution of neutral red as a function of the alkali cation concentration could be calibrated (*see* Fig. 3 in companion paper (Schnetkamp, 1985*a*)). From such calibration curves the free internal alkali cation ($Na^+ + K^+$) concentration in intact ROS isolated in sucrose solutions was determined to be about 3 mM. The total concentration of alkali cations was 15 mM as determined with X-ray microanalysis (Schnetkamp et al., *in preparation*[1]). As shown in the companion paper, at low ionic strength membrane-bound cations outnumber osmotic cations by severalfold.

1) When 25 mM choline$^+$ was added to the suspension medium the partition parameter did not change for up to 1 hr after its addition as compared with a control sample to which no choline$^+$ was added. The same result was obtained when 25 mM of either arginine$^+$, tetramethylammonium$^+$, tetraethylammonium$^+$, or lysine$^+$ was added. The plasma membrane of intact ROS appeared virtually impermeable to these organic cations as indicated by their lack of effect on the binding of neutral red. In contrast, addition of 25 mM ammonium acetate caused an immediate change in the partition parameter by more than fourfold. NH_4OAc is a permeant electrolyte due to the permeation of the neutral species ammonia and acetic acid. On the time scale of our experiments addition of NH_4OAc instantaneously increased the electrolyte concentration by the same amount in all internal and external compartments.
2) In order to equilibrate a K^+ gradient the plasma membrane must be electrically shunted. If no electrical shunting occurs transport of charged K^+ will build up an inside-positive membrane potential and little mass transport of K^+ will take place (so-called backpressure effect). As discussed in the companion paper, the combination of a K^+ channel or carrier (such as valinomycin) and the electrogenic proton carrier FCCP allowed equilibration of a K^+ gradient across the outer membrane of ROS or liposomes. This means that FCCP electrically shunts the membrane and the rate of K^+ uptake should be limited by the K^+ current carried by channels or carriers native to ROS. Addition of FCCP indeed increased the K^+-induced rate of neutral red release indicating an increased rate of K^+ uptake (*compare* open circles with open diamonds in Fig. 1).
3) Another way to electrically shunt the plasma membrane was achieved by addition of 1 mM NH_4OAc, which increased the rate of K^+ uptake by the same amount as observed for FCCP (Fig. 1, crosses). This can be understood as follows: ammonia freely crosses membranes and is protonated by intracellular buffers. The ammonium ion, thus formed, relieves the back-pressure effect by carrying an outward current of NH_4^+

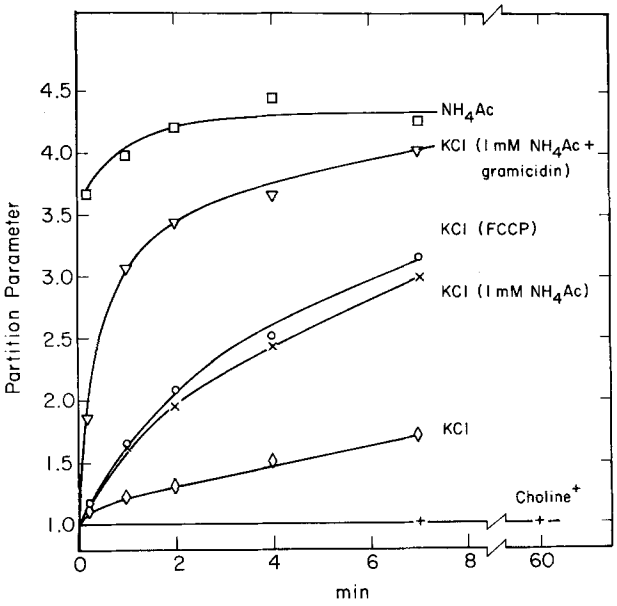

Fig. 1. K^+ transport in intact ROS as indicated by the redistribution of neutral red. Ca^{2+}-enriched intact ROS were suspended in 600 mM sucrose, 40 mM HEPES, 17.6 mM arginine, 10 mM nitrilotriacetic acid, 2.5 mM $CaCl_2$ (free Ca^{2+} concentration: 18.3 μM), 75 μM neutral red, 17.6 μM rhodopsin (adjusted to pH 7.4 with arginine). To this suspension was added: 25 mM choline chloride (—+—), 25 mM KCl (— ◇ —), 25 mM KCl and 1 mM NH_4OAc (—×—), 25 mM KCl and 2 μM FCCP (—○—), 25 mM KCl, 1 mM NH_4OAc, and 2 μM gramicidin (—▽—), 25 mM NH_4OAc (—□—). After incubation with these salts for the indicated times the neutral red distribution was measured by the sedimentation method. The partition parameter is defined in the text and reflects the change of the partition coefficient. T = 22°C

[1] X-ray microanalysis of purified intact bovine rod outer segments. P.P.M. Schnetkamp, J. Hill, and J. McD. Tormey, *in preparation*.

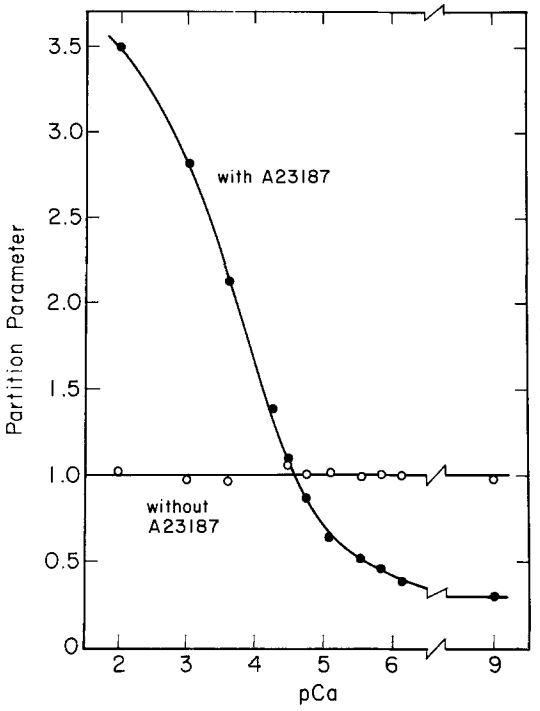

Fig. 2. Net Ca^{2+} transport in intact ROS as indicated by the redistribution of neutral red. Ca^{2+}-enriched intact ROS were incubated for 10 min in 600 mM sucrose, 40 mM HEPES, 17.6 mM arginine, 25 mM choline chloride, 1 mM NH_4OAc, 2 μM FCCP, 85 μM neutral red, 18.2 μM rhodopsin, and 10 mM of different Ca^{2+} buffers (10 mM nitrilotriacetic acid and varying amounts of Ca^{2+}; 10 mM EDTA was used for pCa of 9). Internal Ca^{2+} was accounted for in calculating free Ca^{2+} concentrations. These calculations used an apparent pK of 4.26 for Ca^{2+} binding to nitrilotriacetic acid at pH 7.4. The final pH was adjusted to 7.4 with arginine. The neutral red distribution was measured by the sedimentation method. The partition parameter is defined in the text discussing Fig. 1. T = 22°C

through the same channel or carrier that carried the inward K^+ current. The net effect is K-H exchange. The rate of K^+ uptake was not dependent on the anion present (acetate, sulfate, chloride, or thiocyanate), even in the absence of FCCP. For all potassium salts used the partition parameter could be extrapolated to within 10% of the value of unity at time zero.

4) Addition of gramicidin (a nonselective channel for alkali cations and protons) greatly accelerated the uptake of K^+ (Fig. 1, triangles). The partition parameter assumed a value close to that when NH_4OAc was added. The partition parameter was approximately linearly related to the concentration of monovalent cations in the range used (Fig. 3 in companion paper).
5) In the companion paper it was shown that neutral red is predominantly located in the intradiskal space and thus reports on cations arriving in the intradiskal space. Notwithstanding, the rate of cation uptake in intact ROS as reported by the redistribution of neutral red never showed a lag phase when ROS membranes were electrically shunted. This indicates that transport across either the plasma membrane or the disk membrane was rate limiting. Experiments described in the remainder of this paper show that the plasma membrane is the rate-limiting step and that the redistribution of neutral red in intact ROS measures the permeability properties of the plasma membrane.

Effect of Ca^{2+} on the Cation Permeabilities in Intact ROS

In this section the permeability of the plasma membrane of intact ROS to different alkali cations and the effect of Ca^{2+} on these permeabilities are described. The experimental protocol has the same format as that illustrated in Fig. 1. The partition coefficient of neutral red in ROS membranes was obtained by the sedimentation method.

First, the effect of Ca^{2+} on the neutral red distribution was investigated. When intact ROS were exposed for 10 min to external Ca^{2+} concentrations ranging from 10^{-2} to 10^{-9} M, the partition parameter showed little deviation from unity (Fig. 2, open symbols). This indicates that no change occurred in internal Ca^{2+}. When ROS membranes were made permeable to Ca^{2+} by addition of the ionophore A23187 the same range of external Ca^{2+} concentrations caused rather large changes in the partition parameter (Fig. 2, filled symbols). Ca^{2+} was 10- to 25-fold more effective in releasing neutral red from ROS membranes as compared with monovalent cations. This is in accordance with the electrostatic nature of the process underlying the redistribution of neutral red (McLaughlin, 1977). The experiment shown in Fig. 2 was carried out with a preparation of Ca^{2+}-enriched intact ROS. The curves with and without A23187 intersected at a free Ca^{2+} concentration of about 25 μM: a similar value as was obtained earlier from an analysis of Ca^{2+} binding with and without A23187 (Schnetkamp, 1979). This value probably reflects the internal free Ca^{2+} concentration in Ca^{2+}-enriched ROS (containing 15–20 mM total Ca^{2+}). When Ca^{2+}-depleted ROS were used, a result similar to that shown in Fig. 2 was obtained with the exception that the two curves (with and without A23187) intersected at a much lower free Ca^{2+} concentration (less than 1 μM, *not illustrated*).

The above experiment was repeated (no A23187 present) with different cations substituting for the impermeable $choline^+$. Three different patterns

were consistently observed in seven preparations tested. The results of one experiment are illustrated in Fig. 3. First, the only organic cation found to be permeable in ROS was glucosamine$^+$, and external Ca^{2+} had no effect on its permeability (the stereoisomer galactosamine$^+$ was much less permeable than glucosamine$^+$). Second, Ca^{2+} progressively stimulated transport of K^+ as the free external Ca^{2+} concentration was raised from 10^{-9} to 10^{-6} M, whereas higher Ca^{2+} concentrations were only slightly inhibitory (transport of Rb^+ and Cs^+ was similar to that of K^+). Third, Ca^{2+} progressively inhibited both Li^+ and H^+ transport as the free external Ca^{2+} concentration increased from 1 μM to 20 mM. The Na^+ permeability in ROS was inhibited by external Ca^{2+} in a similar fashion (*see* Fig. 10). The proton permeability was measured by making a step change of the external K^+ concentration in the presence of the electrogenic K^+ carrier valinomycin. As discussed in the context of Fig. 1, the equilibration of a K^+ gradient was independent of the anion present and electrical shunting of the membrane could be provided by the protonophore FCCP. Conversely, K^+ transport is rate limited by the H^+ permeability of the plasma membrane when FCCP is replaced by valinomcyin (*see also* Fig. 4 in companion paper).

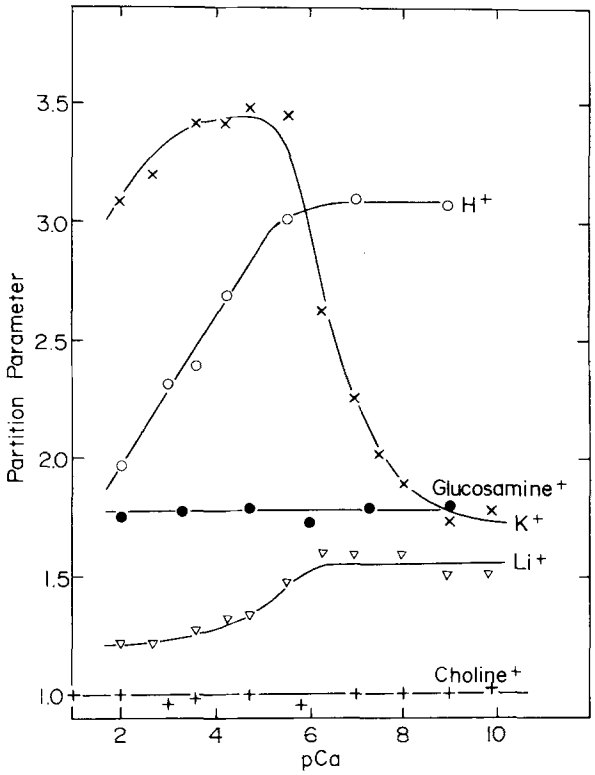

Fig. 3. Effect of Ca^{2+} on cation transport in intact ROS. Ca^{2+}-enriched intact ROS were suspended in 600 mM sucrose, 40 mM HEPES, 17.6 mM arginine, 70 μM neutral red, 17.6 μM rhodopsin, 2 μM FCCP (for measurements of H^+ transport FCCP was replaced by 2 μM valinomycin), and 10 mM Ca^{2+} buffer (pH = 7.4). Intact ROS were incubated for 10 min in this medium containing in addition 25 mM choline chloride (—+—); 25 mM LiCl (—▽—); 25 mM glucosamine HCl adjusted to pH 7.4 with arginine (—●—); 25 mM KCl (—×—, —○—). Ca^{2+} buffers were based on EGTA (between pCa of 6 and 10; apparent pK = 7.47 at pH = 7.4) and nitrilotriacetic acid (between pCa of 4 and 6; apparent pK = 4.26 at pH = 7.4). The neutral red distribution was measured by the sedimentation method. The partition parameter is defined in the text discussing Fig. 1. T = 22°C

Real-Time Measurements of Ionic Permeabilities in Isolated ROS

In the remainder of this paper real-time measurements of the ionic permeabilities in ROS are presented. The light absorption of a suspension of ROS containing neutral red was monitored at 540 nm, the absorption maximum of the membrane-adsorbed dye. The absorption changes observed were dependent only on the cations added and not on the anions. In part this is a consequence of a surface potential caused by negative charges, which is essentially screened by cations (McLaughlin, 1977). When 50 mM choline$^+$ (chloride) was added to a suspension of intact ROS containing 20 μM neutral red, the absorption at 540 nm showed an instantaneous drop with little further decrease in time (Fig. 4). An absorption decrease at 540 nm is plotted upward. The instantaneous drop in absorption could be accounted for by the dilution caused by the salt addition. When 50 mM ammonium$^+$ (actetate) was added, a much larger instantaneous decrease in absorption was noted indicating that, in addition to the dilution artifact, a release of membrane-bound neutral red from ROS had occurred. This was caused by the step change in the internal ionic strength due to the addition of the permeant NH_4OAc. When other cations were tested, the absorption at 540 nm initially assumed the same value as observed in the case of choline$^+$. This was followed by a time-dependent decrease towards the value observed when ammonium$^+$ was added, suggesting that the internal concentration of the respective cation increased at a rate indicated by the rate of the absorption change:

$$Na^+ > H^+ = K^+ > Cs^+ > Li^+ > \text{choline}^+.$$

All time-resolved cation permeabilities found in intact ROS were strongly dependent on the temperature as illustrated for the Na^+ and K^+ permeabilities (Fig. 5). At 0°C the specific resistance of the plasma membrane for alkali cations was estimated to have the very high value of 9 MΩ cm^2 (*see* Ap-

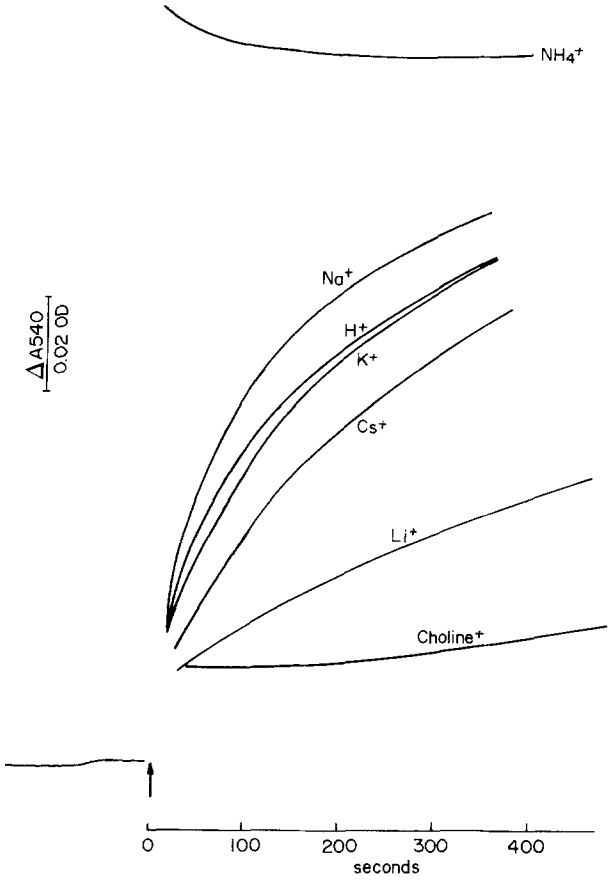

Fig. 4. Kinetics of cation transport in intact ROS as measured by the redistribution of neutral red. Intact ROS were suspended in 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 1 mM NH_4OAc, 5 μM FCCP (in the case of H^+ transport FCCP was replaced by 5 μM valinomycin and 1 mM NH_4OAc was omitted), 5 mM nitrilotriacetic acid, 0.25 mM $CaCl_2$ (free Ca^{2+} concentration 2.9 μM), 20 μM neutral red, 8.4 μM rhodopsin. At time zero 50 mM of the indicated chloride salts was added (except for ammonium acetate). The H^+ permeability was measured by addition of 50 mM KCl in the presence of valinomcyin. The traces show absorption changes at 540 nm, the absorption maximum of neutral red bound to ROS, measured in the split-beam mode. The bandwidth was 3 nm. Negative absorption changes are plotted upward. $T = 24°C$

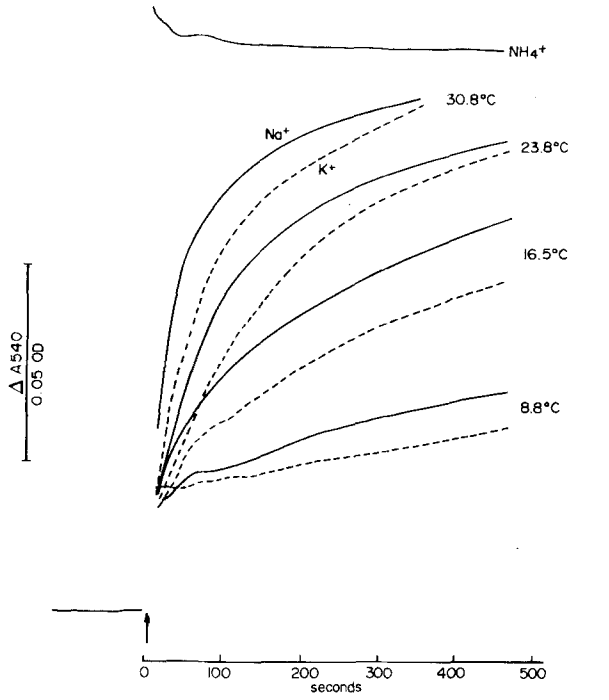

Fig. 5. Temperature dependence of the Na^+ and K^+ permeabilities in intact ROS. Preparation and experimental conditions were identical to those described in the legend of Fig. 4 except for the temperature (temperatures were as indicated). At time zero, 50 mM NaCl (solid lines) or KCl (broken lines) was added. In the case of the trace labeled NH_4^+ 50 mM ammonium acetate was added at 23.8°C

pendix). No temperature dependence was observed for permeant electrolytes such as amines. Even at 0°C amines caused a redistribution of neutral red, which was instantaneous on the time scale of our experiments (i.e., the redistribution was completed in less than 1 sec).

Osmotic Assay for Ion Permeabilities in ROS

Figure 6 illustrates the use of an osmotic assay to measure the ionic permeabilities in intact ROS. This offers an independent set of data and addresses the question which membrane in the neutral red assay is rate limiting for the observed transport of alkali cations. To a suspension of intact ROS 50 mM of the indicated salts were added at time zero. The addition of choline chloride resulted in an increase of the apparent absorption at 700 nm. This increase in absorption indicates a shrinkage of ROS, due to the increase in the osmolarity of the external medium. In time, little recovery was observed, indicating that no transport of choline chloride across the plasma membrane occurred. In contrast, addition of ammonium acetate was accompanied by an instantaneous recovery of light absorption (the small decrease in absorption can be accounted for by the dilution due to the salt addition). This recovery is due to the permeability of the neutral species ammonia and acetic acid and the recombination to ammonium acetate in the cytoplasm. The permeabilities of the different alkali cations in the osmotic experiment were indicated by the rate of the absorption decrease from the value when choline chloride was added to that when ammonium acetate was added. After 5 min and at the second arrow the ionophore gramicidin (a nonselective channel for alkali cations and protons) was added. When alkali cations were present this caused a rapid decrease of light absorption to the value observed when ammo-

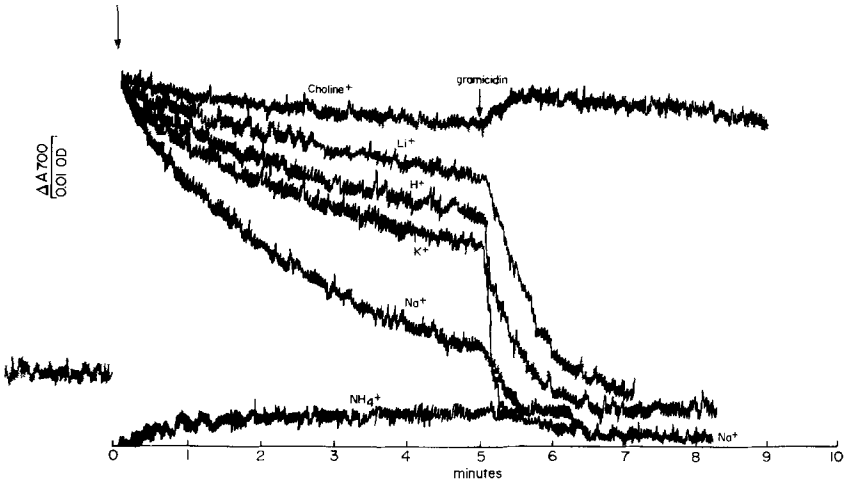

Fig. 6. Kinetics of cation transport in intact ROS as indicated by light-scattering measurements. Ca^{2+}-enriched intact ROS were suspended in 200 mM sucrose, 20 mM HEPES, 8.8 mM arginine, 1 μM FCCP (in the case of H^+ transport FCCP was replaced by 1 μM valinomycin), 5.1 μM rhodopsin (pH = 7.4). Light scattering was measured by the apparent absorption at 700 nm (10-nm band width) in the split beam mode. The noise was caused by the stirring in the cuvette. At time zero, 50 mM of the indicated acetate salts were added (except for choline chloride). Acetate is a permeable anion when FCCP is present. Acetic acid freely permeates biological membranes. In the cytoplasm it dissociates and the proton leaves the cell via FCCP, when the cation enters the cell. The net effect is cation acetate transport. The H^+ permeability was measured by addition of KOAc in the presence of 1 μM valinomycin. At the second arrow after about 5 min 1 μM gramicidin was added. T = 26°C

nium acetate was added, indicating a rapid equilibration of the respective cation gradient across the plasma membrane. The equilibration of the different cation gradients without gramicidin occurred with a similar time course as observed with the neutral red method (*compare* Figs. 4 and 6), and the same sequence of permeabilities was observed. Other properties of cation transport in ROS that were observed with the neutral red method were observed with the osmotic assay as well. These included the stimulation of K^+ and Cs^+ transport by external Ca^{2+}, and the strong temperature dependence of alkali cation transport. These results indicate that the permeability properties of the plasma membrane control the access of cations to the neutral red containing compartment. Therefore, the redistribution of neutral red is a proper indicator for the permeability properties of the plasma membrane. Light-scattering measurements did not reveal any osmotically active compartment in leaky ROS. The light-scattering properties of leaky ROS (stacked disks), therefore, appear not to be determined by the intradiskal spacing.

Na^+ Permeability in Intact ROS: Concentration Dependence

The experiments shown in Figs. 4 and 5 used single wavelength recordings. The irregularities observed immediately after the addition of electrolytes were caused by light scattering changes due to the mix-

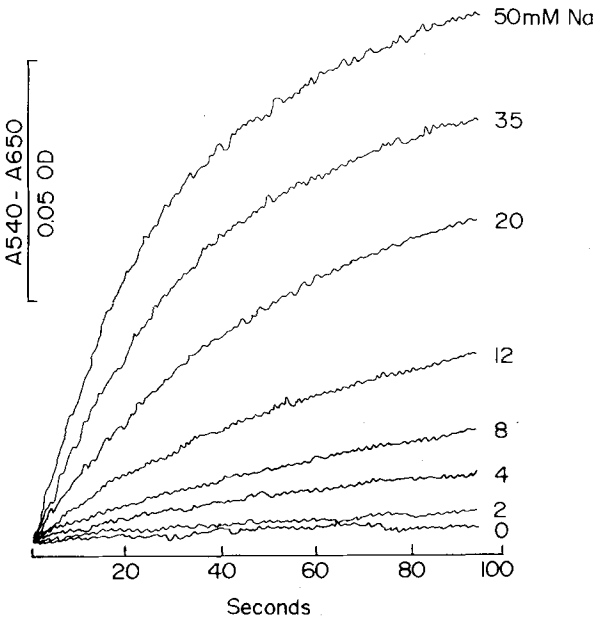

Fig. 7. The dependence of the Na^+ permeability in intact ROS on the external Na^+ concentration. Ca^{2+}-enriched intact ROS were suspended in 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 2 mM KCl, 2 μM FCCP, 2 μM valinomycin, 20 μM neutral red, 9.0 μM rhodopsin (pH = 7.4). The recordings were initiated by addition of the indicated concentrations of NaCl. The rates of Na^+ transport were indicated by the time-dependent absorption changes. Time-dependent decreases in (A540–A650) were measured in these dual-wavelength recordings that indicated a release of neutral red from ROS membranes due to the increase in the internal Na^+ concentration. Negative absorption changes are plotted upward. T = 26°C

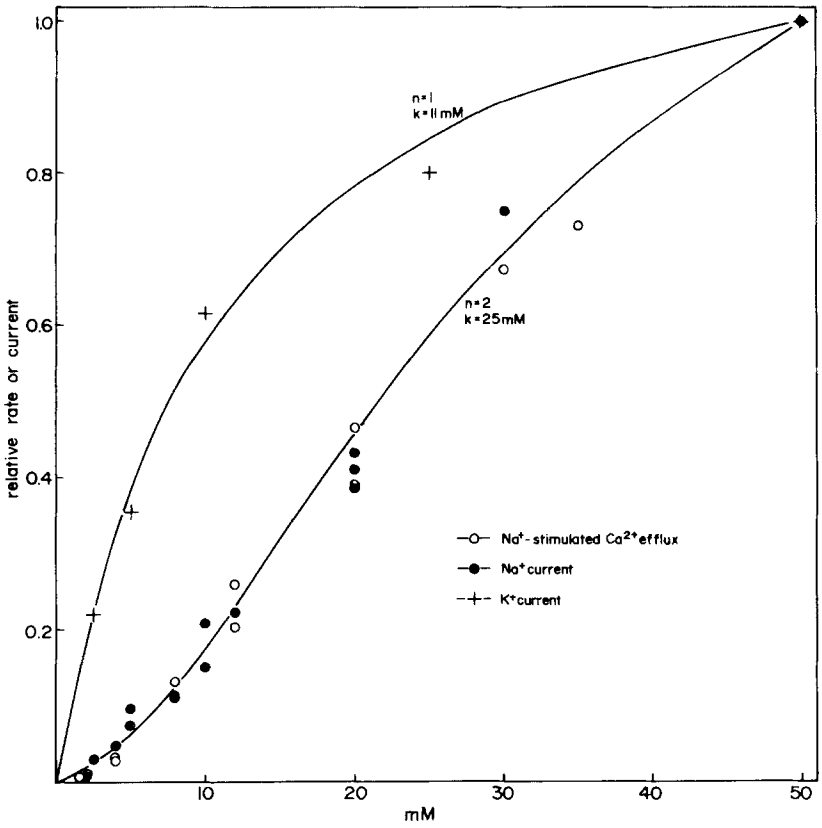

Fig. 8. Comparison of the Na^+-dependence of antiport Na^+ fluxes (Na-Ca exchange) and uniport Na^+ fluxes (Na^+ currents) in intact ROS. The data of the Na^+ currents (—●—) were taken from experiments as shown in Fig. 7; the data of Na-Ca exchange (—○—) were taken from experiments as described in Fig. 9. The suspension medium contained 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 2 mM KCl, 2 μM FCCP, 2 μM valinomycin (pH = 7.4) and either arsenazo III (Na-Ca exchange) or neutral red (Na^+ currents). K^+ currents (—+—) were measured as described for Na^+ currents except for the omission of 2 mM KCl and valinomycin. The solid lines were calculated according to the Michaelis-Menten equations

$$\frac{v}{V} = \frac{[(Na^+)^2/K^2]}{[1 + (Na^+)/K + (Na^+)^2/K^2]}$$

or

$$\frac{v}{V} = \frac{[(K^+)/K]}{[1 + (K^+)/K]}$$

ing. This affected in particular measurements of the Na^+ permeability, which caused the most rapid absorption changes. During the final stages of this study a dual-wavelength spectrophotometer could be used and experiments concentrated on measurements of the Na^+ permeability of the plasma membrane.

In eight preparations tested the rate of Na^+ transport in intact ROS depended in a sigmoidal way on the external Na^+ concentration. The results of one such experiment are illustrated in Fig. 7. The Na^+-induced and time-dependent absorption changes are depicted. A sigmoidal dependence on the external Na^+ concentration was previously noted for Na-Ca exchange in ROS (Schnetkamp, 1985*b*), and the data shown in Fig. 8 compare the Na^+-dependence of both the Na^+ current (uniport; filled circles) and Na-Ca exchange (antiport; open circles). Both modes of Na^+ transport showed a very similar dependence on the external Na^+ concentration. The solid line was calculated with a model that assumes that transport was rate limited by the binding of 2 sodium ions. The two modes of Na^+ transport observed in intact ROS are illustrated in Fig. 9; Na-Ca exchange (as indicated by Na^+-stimulated Ca^{2+} efflux, Fig. 9*A*) and an inward Na^+ current (as indicated by the redistribution of neutral red, Fig. 9*B*). The two modes of Na^+ transport can be distinguished by the effect of electrical shunting by the protonophore FCCP. The absence of FCCP had only a minor effect on the antiport process of Na-Ca exchange (Fig. 9*A*), whereas the uniport process of Na^+ current became very slow (Fig. 9*B*). The latter is probably due to the back-pressure effect (the Na^+ current builds up an inside-positive membrane potential). When the Na^+ ionophore monensin was added, the change in the absorption of neutral red was independent of FCCP (Fig. 9*B*; this was, of course, not observed in the arsenazo measurement because monensin is not a Ca^{2+} ionophore). The absorption level observed in the presence of monensin indicates the equilibration of Na^+ in internal and external compartments.

In contrast to Na^+ transport, the dependence uniport K^+ transport did not show a sigmoidal relation between the rate of transport and the external K^+ concentration (Fig. 8, crosses). The solid lines shown in Fig. 8 were calculated with the equations given in the figure legend and with the use of the kinetic parameters indicated.

Na^+ Permeability in Intact ROS: Effect of External Ca^{2+}

The effect of the external Ca^{2+} concentration on the Na^+ permeability is illustrated in Fig. 10. ROS were exposed for 5 min to the indicated Ca^{2+} concentra-

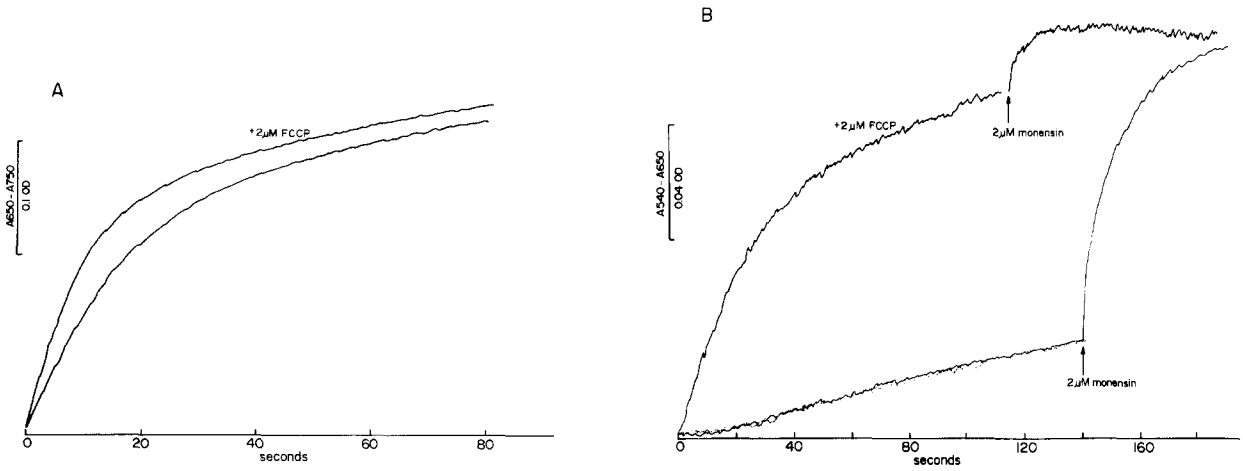

Fig. 9. A comparison between Na-Ca exchange (*A*) and the uniport Na^+ transport (*B*) in intact ROS. (*A*) Ca^{2+}-enriched intact ROS were suspended in 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 50 μM arsenazo III, 2 mM KCl, 6.4 μM rhodopsin (pH = 7.4). FCCP was added as indicated. Na^+-stimulated Ca^{2+} efflux was initiated by addition of 50 mM NaCl and was indicated by an increase in (A650–A750) in this dual-wavelength recording. In 60 sec 2.6 mol Ca^{2+}/mol rhodopsin was released. The initial rate amounted to 5.7×10^6 Ca^{2+}/outer segment/sec when FCCP was present. T = 26°C. (*B*) Ca^{2+}-enriched intact ROS were suspended in 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 2 mM KCl, 2 μM valinomycin, 20 μM neutral red, 9 μM rhodopsin (pH = 7.4). FCCP and monensin were added as indicated. At time zero 50 mM NaCl (solid traces) or NaSCN (dotted trace) was added. Na^+ transport was indicated by the absorption changes as described in the legend of Fig. 7. T = 26°C

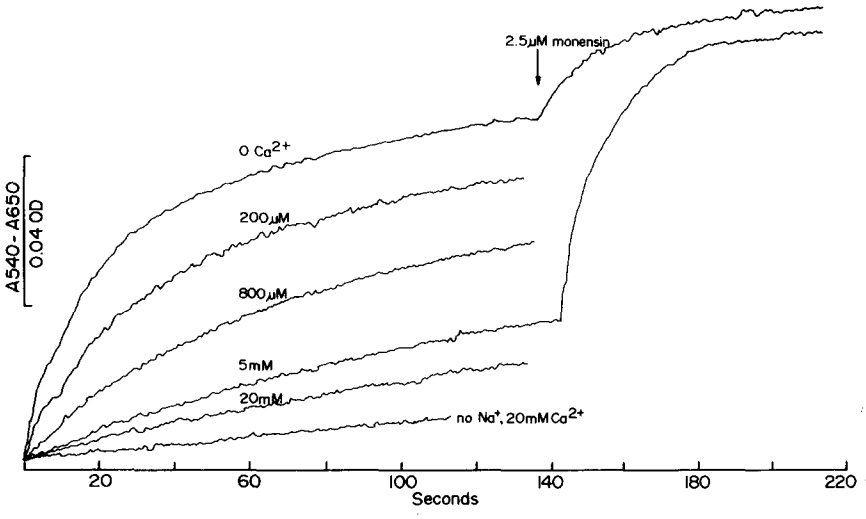

Fig. 10. The effect of Ca^{2+} on the Na^+ permeability in intact ROS. Ca^{2+}-enriched intact ROS were preincubated for 5 min in 600 mM sucrose, 20 mM HEPES, 8.8 mM arginine, 2 mM KCl, 2 μM FCCP, 2 μM valinomycin, 20 μM neutral red, 8.0 μM rhodopsin (pH = 7.4), and the indicated Ca^{2+} concentrations (*0 Ca^{2+}* indicates the presence of 0.5 mM EDTA). At time zero the recordings were started by addition of 50 mM NaCl and the time-dependent absorption changes were measured as described in the legend of Fig. 7. At the arrow 2.5 μM monensin was added. T = 26°C

tions prior to the addition of Na^+. Increasing the external Ca^{2+} concentration progressively blocked the Na^+ permeability as measured when a step change in the external Na^+ concentration from 0 to 50 mM was made. At 20 mM Ca^{2+} the Na^+ permeability was reduced to less than 5% of the value observed at zero Ca^{2+}. The Ca^{2+} additions themselves did not cause a release of neutral red except for the case of 20 mM Ca^{2+}. Addition of 20 mM Ca^{2+} caused a rapid release of neutral red, amounting to about 20% of the release observed after addition of 50 mM Na^+. This release was followed by a slow drift in absorption (trace labeled *no Na^+, 20 mM Ca^{2+}*), which was independent of the Ca^{2+} concentration. The small transient release of neutral red may reflect a small transient Ca^{2+} current, but further experiments are needed to confirm this. When the Na^+ ionophore monensin was added a rapid absorption change was noted in all cases when Na^+ was present. External Ca^{2+} did not affect the rate of Na^+ equilibration induced by monensin. The total absorption change induced by 50 mM Na^+ in the presence of monensin was largely independent of the external Ca^{2+} concentration (illustrated for two cases in Fig. 10).

When the Ca^{2+} ionophore A23187 was present changes of the external Ca^{2+} concentration gave rise to large absorption changes (Fig. 2). This limits the range of Ca^{2+} concentrations that allow a meaningful measurement of the Na^+ permeability in the

presence of A23187. Under conditions of a Ca^{2+} clamp (in the presence of A23187) the Na^+ permeability was not noticeably different when the Ca^{2+} concentration was varied between 0.1–50 μM free Ca^{2+} (results not illustrated). These results suggest that the Na^+ permeability in intact ROS is not particularly sensitive to the internal Ca^{2+} concentration, but can be inhibited by external Ca^{2+}.

Discussion

Neutral Red Distribution as an Assay for Ionic Permeabilities in Intact ROS

The distribution of the dye neutral red was used to investigate the cation transport pathways native to ROS. The method is based on the binding of neutral red to intracellular membranes and its dependence on the internal cation concentration as demonstrated in the companion paper. This dependence can be used for measuring the permeability properties of the plasma membrane of intact ROS as illustrated in Figs. 1 and 9. The equilibration of a K^+ or a Na^+ gradient was accelerated considerably when ROS membranes were electrically shunted by the protonophore FCCP. In contrast, the antiport transport of Na-Ca exchange was only slightly affected by FCCP. These results suggest that Na^+ and K^+ transport monitored in ROS by the redistribution of the dye neutral red reflects electrogenic transport of these cations and that true currents for Na^+ or K^+ can be measured in this way.

The experimental protocol worked best when the internal ionic strength in ROS was lowered by isolation of ROS in sucrose-Ficoll solutions. The reason for this is that surface potentials and changes in surface potentials are largest at low ionic strength. These conditions are a major departure from physiological conditions and may be the cause for the failure to detect light-induced blockage of ionic currents in this preparation. This protocol, however, has the advantage that it allows unambiguous measurements of individual ionic permeabilities and their properties including those of the H^+ permeability. This information is less easily extracted from current measurements in the functioning rod cell, where the sum is obtained from the various inward and outward currents contributed by the different extracellular and intracellular ions. The usefulness of intact bovine ROS and the demonstration of specific ion transport pathways in this preparation also lies in the ready availability of large quantities (tens of mg) of purified intact ROS offering a good starting point for the purification of rod ion transporters. It is unlikely that the ionic permeabilities observed in this study originate from particles other than ROS. The neutral red method as well as the osmotic assay rely on bulk properties of membrane-rich particles. The ionic permeabilities caused a rapid equilibration of ionic gradients in the major fraction of particles in the suspension, i.e., ROS. The isolation procedure for ROS involves vigorous shaking of the retina. This results in very few ROS with the inner segment still attached.

Selectivity of Ionic Permeabilities in Isolated ROS

Intact bovine ROS contained specific ionic permeabilities for alkali cations, protons, and glucosamine$^+$; most organic cations tested such as choline$^+$ and tetramethylammonium$^+$ were virtually impermeable (Figs. 3–6). Preliminary results suggest that small transient Ca^{2+} currents were observed under certain conditions. Large Ca^{2+} fluxes by the antiport processes of Ca-Ca and Na-Ca exchange are observed in intact bovine ROS (Schnetkamp, 1979, 1980; Fig. 9*A*). After a step change of the external cation concentration by 25–50 mM at an external Ca^{2+} concentration in the micromolar range, the initial transport rates decreased in the order:

$$Na^+ > K^+ = > Rb^+ = H^+ > Cs^+ > Li^+.$$

This result was found independently by two different methods, the "neutral red" method (Fig. 4) and the "osmotic" method (Fig. 6). Since the same electrochemical driving force was used in all experiments the above sequence also applies to the permeability coefficient, except for protons (experiments were carried out at pH 7.4). The selectivity for alkali cations (e.g. the permeability to Cs^+) and the lack of permeability to organic cations such as choline and tetramethylammonium is in general agreement with the selectivity of the light-sensitive conductance (Woodruff et al., 1982; Hodgkin et al., 1984; Yau & Nakatani, 1984*a*). In bovine ROS the selectivity sequence was dependent on the Ca^{2+} concentration and on the concentration of the alkali cations used (Figs. 3, 8 and 10). The results of the osmotic experiments show that the permeability properties of the plasma membrane are the rate-limiting permeability barrier in intact bovine ROS. The permeability of the plasma membrane for anions was investigated with the osmotic method (data not illustrated). Chloride, sulfate, and acetate were found to be impermeable, whereas thiocyanate and, in the presence of FCCP, acetate were permeable anions.

Effect of Ca^{2+} on the Cation Permeabilities in ROS

Changes of the external Ca^{2+} concentration affected the ionic permeabilities in intact ROS in three distinct patterns: Ca^{2+} had *no effect* on the permeability of glucosamine$^+$; Ca^{2+} *decreased* the permeability of Li^+, H^+ and Na^+; and Ca^{2+} *increased* the permeability of K^+, Rb^+ and Cs^+ (Figs. 3 and 10). Half-maximal inhibition of Na^+ transport by Ca^{2+} increased with an increase of the external Na^+ concentration. Half-maximal stimulation of K^+ transport occurred at about 0.5 μM Ca^{2+}. Ca^{2+}-enriched ROS were used for the experiments illustrated, and in this preparation changes of external Ca^{2+} did not seem to result in changes in internal Ca^{2+}. A very similar pattern of ionic permeabilities was observed in Ca^{2+}-depleted ROS. The free internal Ca^{2+} concentration ranged from less than 1 μM in Ca^{2+}-depleted ROS to about 25 μM in Ca^{2+}-enriched ROS (Fig. 2). These results suggest that the effects of Ca^{2+} on the ionic permeabilities were due to the action of Ca^{2+} on external site(s) of the plasma membrane and that the internal Ca^{2+} level of ROS did not control ionic permeabilities in any direct way. A similar conclusion is reached from observations of the Na^+ permeability under a Ca^{2+} clamp in the presence of the ionophore A23187.

Comparison with Electrophysiological Results

A direct comparison between the ion permeabilities, described in this study on isolated intact ROS, and electrophysiological measurements on ionic currents in the outer segment of the functioning rod cell is compromised by the lack of light-sensitive permeabilities in the isolated ROS. Nevertheless, it seems likely that the permeability properties of isolated intact ROS are, at least in part, due to the properties of the light-sensitive conductance for two reasons. First, it has been demonstrated recently that isolated patches of ROS membrane contain a cGMP-dependent conductance very similar to the light-sensitive conductance (Fesenko, Kolesnikov & Lyubarski, 1985). This suggests that the light-sensitive conductance survives isolation procedures. Intact bovine ROS contained sufficient cGMP (30 ± 10 μM; SD of six preparations) to activate this cGMP-dependent conductance. This value for the cGMP content of bovine ROS is similar to that observed in physiologically functioning frog ROS with the ellipsoid part of the inner segment still attached (Cote, Biernbaum, Nicol & Bownds, 1984). Second, Baylor and coworkers have shown that the light-sensitive conductance is the only conductance present in the ROS plasma membrane and that the specific resistance in the light is very high (>4 MΩ cm^2; Baylor, Lamb & Yau, 1979; Baylor & Lamb, 1982). At 0°C all ionic permeabilities in isolated ROS were abolished, and the specific resistance of the plasma membrane assumed the very high value of 9 MΩ cm^2 (Appendix). It seems unlikely that conductances would be expressed in isolated ROS that are not expressed in functioning rod cells.

Dissipation of the Na^+ gradient in rods in the living retina most likely occurs when the Na^+ pump located in the inner segment is blocked.. This treatment results in a disappearance of the dark current within one to a few minutes (Yoshikami & Hagins, 1973; Woodruff et al., 1982). The Na^+ permeability of the plasma membrane of isolated bovine ROS is sufficient to equilibrate large Na^+ gradients within 1 min (Figs. 7 and 9). The internal Ca^{2+} content of isolated ROS had no major direct effect on their ionic permeabilities. Internal Ca^{2+} is generally believed to control the light-sensitive conductance in rods (*see*, e.g. Fain & Lisman, 1981; Hodgkin et al., 1984; Yau & Nakatani, 1984*a*). On the other hand, large Ca^{2+} currents through the light-sensitive conductance have been observed in toad rods (Yau & Nakatani, 1984*b*). These currents persisted despite the fact that the cell filled up with millimolar amounts of Ca^{2+}. The cGMP-dependent conductance in isolated patches of rod membrane is not affected by varying the Ca^{2+} concentration on the cytoplasmic side (Fesenko et al., 1985). These results suggest that the internal Ca^{2+} concentration can be dissociated from the blocking of the light-sensitive conductance and that internal Ca^{2+} may not affect the light-sensitive conductance in a direct way.

Competition between External Na^+ and Ca^{2+} Controls the Dark Current?

The dark current in toad rods is constant as long as the external $(Na^+)^2/Ca^{2+}$ is kept constant (Hodgkin et al., 1984). One way to explain the current-voltage characteristics of the light-sensitive conductance is by assuming that the main charge carriers (i.e., sodium ions) are moved in pairs (Baylor & Nunn, 1983). The sigmoidal dependence of the Na^+ permeability on the external Na^+-concentration suggests that in bovine ROS sodium ions move in pairs. If the Na^+ current is dependent on the simultaneous binding of 2 Na^+, and if Ca^{2+} competes with Na^+ for these sites, the Na^+ current will depend on the external Na^+ and Ca^{2+} concentrations according to the Michaelis-Menten equation given in the legend of Fig. 11. The data for the dark current shown in

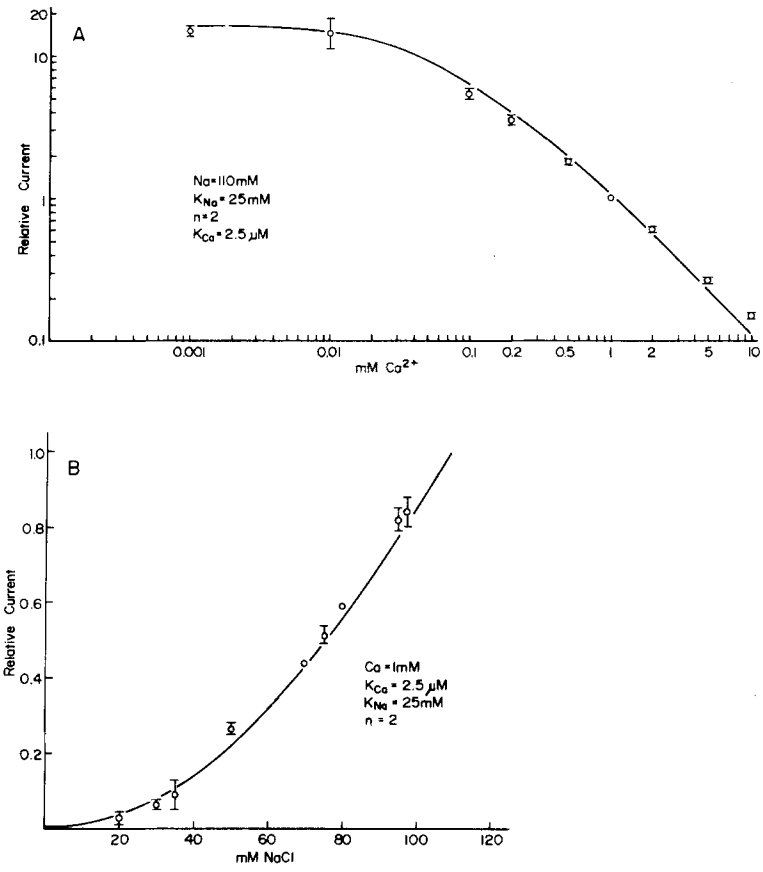

Fig. 11. Calculations of the variation of the dark current upon variations of the external Na^+ and Ca^{2+} concentration. The data on the dark current in toad rods as a function of the external Na^+ and Ca^{2+} concentration (open symbols with error bars) were taken from Hodgkin et al. (1984). The solid lines were calculated on the assumption that the dark current is controlled by the binding of 2 sodium ions to sites that are shared with Ca^{2+} and competed for by Ca^{2+}. The corresponding Michaelis-Menten equation is

$$\frac{v}{V} = \frac{[(Na^+)^2/(K_{Na})^2]}{[1 + (Na^+)/K_{Na} + (Na^+)^2/(K_{Na})^2 + (Ca^{2+})/K_{Ca}]}.$$

The dissociation constant for Na^+ and the simultaneous occupancy by 2 Na^+ were taken from the observations on Na^+ permeabilities in isolated intact ROS in this study (Fig. 8)

Fig. 11 are taken from Hodgkin et al. (1984). The solid lines were calculated with the Michaelis-Menten equation and with the use of the indicated parameters. The dissociation constant for Na^+ is taken from the data on the Na^+ permeability shown in Fig. 8. The only free parameter used is the dissociation constant for Ca^{2+} ions. The best fit between calculations and experimental observations was observed when this dissociation constant was taken to be 2.5 μM. According to this model, the large changes in the dark current upon changing the external Ca^{2+} concentration are due to competition between external Na^+ and Ca^{2+} for common sites. A peculiar coincidence is that the binding sites of the Na^+ conductance for Na^+ and Ca^{2+} may be very similar to those of the Na-Ca exchanger in ROS (Fig. 8; Schnetkamp, 1985*b*); the dissociation constant of Na-Ca exchange for Ca^{2+} is 0.2–1.2 μM as determined from Ca-Ca exchange (Schnetkamp, 1980). This leaves open two possible interpretations: First, the effects of external Ca^{2+} on the dark current are due to competition between external Na^+ and external Ca^{2+} for common sites on the light-sensitive conductance facing the external side of the plasma membrane. Second, the magnitude of the dark current is controlled by the Na-Ca exchanger (e.g. by regulating the free internal Ca^{2+} concentration), and the similarity between the Na^+-dependence of Na-Ca exchange and Na^+ current, respectively, is fortuitous.

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Appendix

In order to calculate the specific resistance of the plasma membrane, bovine ROS are taken to be cylinders of 1×20 μm containing 3×10^7 rhodopsin molecules. At 0°C it took 1 hr to increase the free internal Na^+ concentration by 2 mM upon a step change in the external Na^+ concentration from 0 to 25 mM. The plasma membrane was electrically shunted in the presence of FCCP, and presumably no transmembrane voltage was present. The free internal Na^+ concentration at the start of the incubation was estimated to be 2 mM. This value is calculated from the free internal (Na^+ + K^+) concentration as determined from the calibration of neutral red binding in the presence of gramicidin and from the total Na^+ and K^+ concentrations as determined by X-ray microanalysis[2]. From this it follows that the electrochemical driving force for Na^+ was about 60 mV. A change in the free Na^+ concentration by 2 mM reflected a change in the total Na^+ concentation by about 3 mol/mol rhodopsin. At low ionic strength membrane-bound cations outnumber free cations in ROS due to the high concentration of negatively charged groups confined to the disk membrane (companion paper). The total amount of charge transport in one hour amounted to 1.4×10^{-11} C, reflecting a current of 4.0 fA. From this the specific resistance of the plasma membrane at 0°C can be calculated, and an extremely high value of 9 MΩ cm^2 is obtained. This suggests that the plasma membrane of intact bovine ROS contained only the temperature-sensitive conductance(s).

[2] *See* footnote 1, p. 265.