J. Physiol. (1986), **373**, *pp.* 25–45
With 10 *text-figures*
Printed in Great Britain

SODIUM–CALCIUM EXCHANGE IN THE OUTER SEGMENTS OF BOVINE ROD PHOTORECEPTORS

BY PAUL P. M. SCHNETKAMP

From the Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, CA 90024, U.S.A.

(*Received 16 April 1985*)

SUMMARY

1. Intact rod outer segments (r.o.s.) isolated from bovine retinas were used to measure net Ca^{2+} fluxes using the optical Ca^{2+} indicator Arsenazo III. Ca^{2+} fluxes were observed, which could change the internal Ca^{2+} content of isolated r.o.s. by as much as 0·5 mM s^{-1}.

2. The Ca^{2+} content of isolated intact r.o.s. was strongly dependent on the Na/Ca ratio in the isolation medium, and could be made less than 0·1 mol Ca^{2+} mol^{-1} rhodopsin (zero Ca^{2+} in isolation medium) or up to 7 mol Ca^{2+} mol^{-1} rhodopsin (zero Na^+ in isolation medium).

3. Ca^{2+} efflux from r.o.s. rich in Ca^{2+} was observed only when Na^+ was added to the external medium (as opposed to any other alkali cation); in Ca^{2+}-depleted r.o.s. Ca^{2+} uptake required the presence of internal Na^+ and was inhibited selectively by external Na^+. These results suggest that Na–Ca exchange across the plasma membrane operated freely in both directions and controlled the internal Ca^{2+} concentration in r.o.s.

4. Na^+-stimulated Ca^{2+} efflux depended on the external Na^+ concentration in a sigmoidal way. This suggests that the simultaneous binding of two Na ions is rate limiting for transport.

5. In Ca^{2+}-depleted r.o.s. and in the absence of external Na^+, 1 mol Ca^{2+} mol^{-1} rhodopsin (or 3 mM-total Ca^{2+}) could be taken up within 1 min by intact r.o.s. at a free external Ca^{2+} concentration of about 1 μM.

6. Only part of the internal Ca^{2+} was available for Na–Ca exchange. The external Na^+ and K^+ concentration as well as the temperature were factors controlling the accessibility of internal Ca^{2+} to participate in Na–Ca exchange.

7. Ca^{2+} fluxes in r.o.s. with a permeabilized plasma membrane but intact disk membranes were very similar to those observed in intact r.o.s.; Na–Ca exchange could operate in both directions across the disk membrane.

8. In addition to Na–Ca exchange, leaky r.o.s. also showed a guanosine 3′, 5′-cyclic monophosphate (cyclic GMP)-induced Ca^{2+} release that was about $\frac{1}{20}$ of the rate of

Present address: Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Drive, Madison, WI 53706, U.S.A.

Na–Ca exchange. Na–Ca exchange could release 1·5 mol Ca^{2+} mol^{-1} rhodopsin from disks as compared with a cyclic-GMP-induced release of 0·15 mol Ca^{2+} mol^{-1} rhodopsin.

INTRODUCTION

Ca ions play an important role in the physiology of vertebrate rod photoreceptor cells (for reviews, see Fain & Lisman, 1981; Kaupp & Schnetkamp, 1982; Korenbrot, 1985). Changes in the external Ca^{2+} concentration result in rapid and large changes in a Na^+ current flowing into the outer segment in the dark (Yau, McNaughton & Hodgkin, 1981; Hodgkin, McNaughton, Nunn & Yau, 1984; MacLeish, Schwartz & Tachibana, 1984), and in apparent changes in the range of cations able to carry the dark current (Bastian & Fain, 1982; Woodruff, Fain & Bastian, 1982; Yau & Nakatani, 1984*a*; Hodgkin, McNaughton & Nunn, 1985). These effects are generally thought to be mediated by changes in the intracellular Ca^{2+}. The internal Ca^{2+} concentration has been proposed to control the light-sensitive conductance (Yoshikami & Hagins, 1971), while it has been suggested that the internal Ca^{2+} concentration is controlled by Na–Ca exchange (Fain & Lisman, 1981; Schnetkamp, 1981).

Intact rod outer segments (r.o.s.) isolated from bovine retinas contain about 9 mM-total Ca^{2+}, and the transport of Ca^{2+} through the plasma membrane takes place by Na–Ca and Ca–Ca exchange (Schnetkamp, 1980). For rod cells in the toad retina, Yau & Nakatani (1984*b*) have observed currents, which they attribute to electrogenic Na–Ca exchange. These currents have properties very similar to Na–Ca exchange measured in isolated bovine r.o.s. This study employs the optical Ca^{2+} indicator Arsenazo III to investigate in more detail the kinetic and steady-state properties of Na–Ca exchange in isolated r.o.s. The preservation of intact isolated r.o.s. and the unambiguous measurement of Ca^{2+} fluxes in r.o.s. requires manipulation of the internal and external ionic conditions in a rather unphysiological manner. The use of Arsenazo III as Ca^{2+} indicator limits in particular the range of external Ca^{2+} concentrations that can be used. Nevertheless, the properties of Ca^{2+} fluxes through the plasma membrane of r.o.s. under a wide range of internal and external ionic conditions are consistent with the operation of Na–Ca exchange; the direction of Ca^{2+} transport simply reflects the equilibrium conditions of the Na–Ca exchanger. No evidence was found for a unidirectional Ca^{2+} pumping activity. Part of these results have been presented previously in abstract form (Schnetkamp, 1984).

METHODS

Preparations

Bovine eyes were collected in a light-tight box from a local abbatoir. Retinas were dissected and r.o.s. were isolated according to procedures described before (Schnetkamp, Klompmakers & Daemen, 1979; Schnetkamp & Daemen, 1982; Schnetkamp & Kaupp, 1985). R.o.s. were isolated with a sealed plasma membrane (intact r.o.s.) or with a plasma membrane leaky to small solutes (Schnetkamp, 1981; Schnetkamp & Daemen, 1982); both r.o.s. preparations can be made Ca^{2+} enriched or Ca^{2+} depleted by varying the Na/Ca ratio in the isolation medium. The basic constituents of the isolation medium are: 600 mM-sucrose, 5 % (w/v) Ficoll 400, 10 mM-glucose, and 20 mM-Tris HCl at pH 7.4. The necessity and rationale for the different medium components has been described elsewhere (Schnetkamp *et al.* 1979; Schnetkamp, 1979); they are required for the

purification and preservation of a homogeneous population of intact r.o.s. under the various manipulations imposed on r.o.s.

Ca^{2+}-enriched (5–7 mol Ca^{2+} mol^{-1} rhodopsin) r.o.s. were prepared by isolation in standard medium containing 1 mM-$CaCl_2$, 0·2 mM-EDTA and zero NaCl. Ca^{2+}-depleted (less than 0·1 mol Ca^{2+} mol^{-1} rhodopsin) r.o.s. were prepared by isolation in standard medium containing 50 mM-NaCl and 0·5 mM-EGTA. An intermediate Ca^{2+} content (2–3 mol Ca^{2+} mol^{-1} rhodopsin) was obtained with an isolation medium containing 0·6 mM-$CaCl_2$, 0·1 mM-EDTA and 50 mM-NaCl. The Ca^{2+} content was measured with Arsenazo III after solubilization of r.o.s. in 0·1 % (v/v) Triton X-100 (see below). The rhodopsin concentration was measured by difference spectroscopy in the detergent ammonyx LO (1 % (v/v)), using a molar extinction coefficient of 40000 at 500 nm. The Ca^{2+} content (converted from mol Ca^{2+} mol^{-1} rhodopsin to total Ca^{2+} concentration in mM) is probably somewhat over-estimated due to the presence of opsin in the preparations. The rhodopsin concentration in r.o.s. was assumed to be 3 mM (Daemen, 1973).

For the preparation of leaky r.o.s., retinas were freshly dissected and placed overnight at −20 °C prior to the isolation procedure. It should be emphasized that the frozen retinas commercially available in the U.S.A. (American Stores, Hormel, Lawson) are not suitable. The specific ion permeabilities observed in leaky r.o.s. (e.g. Na–Ca exchange described in this paper) are lost in leaky r.o.s. prepared from these retinas, whereas the disk membranes become very leaky to H^+ and small alkali cations such as Li^+.

Experiments were performed within 6 h after the end of the isolation procedure. During this period the properties of Na–Ca exchange in r.o.s. did not change. The isolation procedure and all experimental procedures were carried out in darkness or under dim red illumination.

Ca^{2+} measurements

Ca^{2+} fluxes in r.o.s. were monitored with the Ca^{2+}-indicating dye Arsenazo III. The Ca^{2+} contamination present in the dye was reduced by passage over a standard 200 mesh cation exchange column (H^+ form). The Arsenazo Ca^{2+} assay was calibrated by adding known amounts of Ca^{2+} or the chelators EDTA or EGTA to the cuvette. This assay allowed the detection of changes in total Ca^{2+} in the cuvette by as little as 0·01 μM when the dual-wave-length mode was used (see below). The presence of intact r.o.s. did not affect the absorption changes when a known amount of Ca^{2+} was added to the cuvette unless the Ca^{2+} ionophore A23187 was present. In the presence of A23187, Ca^{2+} buffering by intracellular components of r.o.s. was significant (see Fig. 8). In earlier experiments Arsenazo III spectra in the presence of r.o.s. were recorded in the split-beam mode of an Aminco DW2 spectrophotometer. Small changes in the light-scattering properties of the suspension during the course of an experiment were corrected by adjusting the beam balance at 750 nm. At this wave-length, absorption was due completely to light scattering. The Arsenazo III concentration used was 40 μM. In later experiments dual-wave-length recordings were made with an SLM-Aminco DW2C spectrophotometer. The wave-length pair was 650 and 750 nm, and the band width was 3 nm. The Arsenazo III concentration in these experiments was 110 μM and the starting Ca^{2+} concentration was such that less than 10 % of the dye was bound to Ca^{2+}. In this way the absorption changes induced by Na^+-stimulated Ca^{2+} release in r.o.s. were linear with the amount of Ca^{2+} released.

Na^+-stimulated Ca^{2+} release was initiated by adding small amounts of concentrated stock solutions of different Na salts to a suspension of r.o.s. Corrections were made for the small amounts of Ca^{2+} contamination introduced with the different salts, and for the small changes in sensitivity of Arsenazo III for Ca^{2+} caused by changes in ionic strength. These corrections were determined from Arsenazo spectra without r.o.s. present. Different ionophores (FCCP, valinomycin, gramicidin, A23187) were added from concentrated stock solutions (1 mM) in ethanol or methanol. Additions of ethanol or methanol had no effect. None of the above procedures affected the integrity of the plasma membrane or the homogeneity of r.o.s. as judged by the criteria described earlier (Schnetkamp, 1979; Schnetkamp & Daemen, 1982).

Nucleotide analysis by high-pressure liquid chromatography

Small solutes were extracted from r.o.s. with 60 % methanol at −20 °C according to Donofrio, Colman, Hutton, Daoud, Lampkin & Dyminski (1978). The rhodopsin concentration in the r.o.s. suspension used for the extraction was about 200 μM. The denatured proteins were sedimented in a Brinkman table-top centrifuge (2 min at 12000 revs min^{-1}) and the methanol evaporated under

N_2. The samples were injected onto an Ultrasil AX 10 μm (4·6 × 250 mm) column and eluted following the procedure of Liebes, Kuo, Krigel, Pelle & Silber (1981). Identification and quantification was done by applying samples of known nucleotide composition to the column. Amounts of the different nucleotides were converted into over-all concentrations in r.o.s. by assuming that the rhodopsin concentration in r.o.s. was 3 mM (Daemen, 1973).

Abbreviations

EGTA, ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulphonic acid; cyclic GMP, guanosine-3′,5′-cyclic monophosphate; GTP, guanosine-5′-triphosphate; ATP, adenosine-5′-triphosphate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

RESULTS

Na^+-stimulated Ca^{2+} efflux

Na^+-stimulated Ca^{2+} efflux from r.o.s. can be studied most accurately in intact r.o.s., enriched in Ca^{2+} to unphysiologically high levels. When intact r.o.s. are suspended in a solution containing sucrose and some inert buffer, addition of Na^+ to

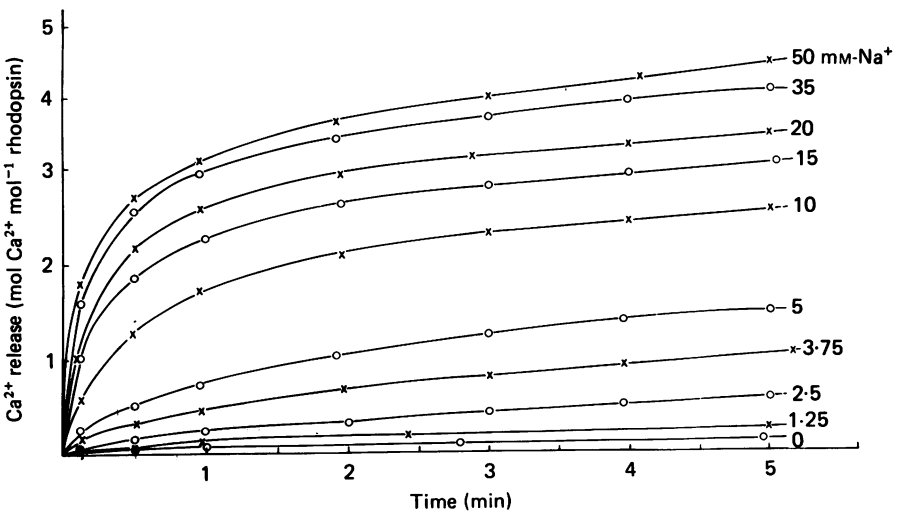

Fig. 1. Na^+-stimulated Ca^{2+} efflux in Ca^{2+}-enriched intact r.o.s. Intact r.o.s. were suspended in 600 mM-sucrose, 20 mM-HEPES, 8·8 mM-arginine, 2·5 mM-KCl, 5 μM-FCCP, 40 μM-Arsenazo III (pH = 7·4). The over-all rhodopsin concentration in the suspension was 4·8 μM. Ca^{2+} efflux was started by addition of the indicated NaCl concentrations (in mM) at time zero. The data were corrected for the small changes in Ca^{2+} sensitivity of Arsenazo III caused by the differences in ionic strength. Split-beam measurements were made of A650–A750. Absorption changes were converted to Ca^{2+} release in mol Ca^{2+} mol^{-1} rhodopsin by back-titration with EGTA or EDTA. The occupancy of Arsenazo III by Ca^{2+} varied from 20 to 42 %. Temperature = 25 °C.

the medium causes the release of part of the internal Ca^{2+} by Na–Ca exchange (Schnetkamp, 1980). The use of the optical Ca^{2+} indicator Arsenazo III allows a precise analysis of Na–Ca exchange and the data shown in Fig. 1 illustrate the typical pattern of Na^+-stimulated Ca^{2+} efflux in intact Ca^{2+}-enriched r.o.s. Ca^{2+}-enriched intact r.o.s. contained about 7 mol Ca^{2+} mol^{-1} rhodopsin (21 mM-total Ca^{2+}). The rate of Ca^{2+} leakage from Ca^{2+}-enriched intact r.o.s. was very slow, even when subsequent

addition of the Ca^{2+} ionophore A23187 caused a nearly complete discharge of internal Ca^{2+} into the external medium (not shown). Addition of Na^+ to the medium greatly enhanced the rate of Ca^{2+} efflux. At Na^+ concentrations greater than 10 mM, Ca^{2+} efflux appeared to have two components; a fast phase lasted about 30 s and subsided in a persistent slow phase. At 50 mM-Na^+ the initial efflux rate was more than 400 times the leakage rate. Addition of 50 mM-K^+, choline$^+$ or tetramethylammonium$^+$ did not increase the leakage rate, while 50 mM-Li^+ increased it slightly (see Fig. 4). In all cases chloride salts were used. The addition of 50 mM-Na^+ caused within 5 min the release of 60–70 % of the total amount of internal Ca^{2+} present in Ca^{2+}-enriched r.o.s. (at 25 °C and in the presence of 2 mM-K^+), while lower Na^+ concentrations caused the release of less Ca^{2+}. This suggests that the external Na^+ concentration controls both the rate of Ca^{2+} efflux and the amount of Ca^{2+} released. The limited amount of release is probably not due to equilibrium conditions of Na–Ca exchange (i.e. the rise in external free Ca^{2+}; external Ca^{2+} was buffered by Arsenazo III preventing large changes in the external free Ca^{2+} concentration). A similarly incomplete Ca^{2+} release (as measured with ^{45}Ca) has been observed when the external medium contains a large excess of the Ca^{2+} chelator EGTA (Schnetkamp, 1980). Under these conditions a complete Ca^{2+} release would be expected.

In the above experiments the electrogenic protonophore FCCP (Benz & McLaughlin, 1983) was present. At the concentrations used (2–5 μM) FCCP was the dominant conductance in r.o.s. membranes. Both the interior of r.o.s. and the external medium are heavily buffered with respect to protons. Under these conditions FCCP is expected to clamp the membrane potential to the prevailing Nernst potential for protons, i.e. r.o.s. membranes are electrically shunted in the presence of FCCP (as demonstrated elsewhere, Schnetkamp, 1985*b*). Na–Ca exchange in other systems has been reported to be electrogenic, that is more than two Na ions are exchanged for each Ca^{2+} (Baker & McNaughton, 1976; Blaustein, 1977; Pitts, 1979; Reeves & Sutko, 1980; Philipson & Nishimoto, 1980). Therefore, the effect of different ionophores on the rate and amount of Na–Ca exchange was investigated (Fig. 2). The presence of FCCP increased the rate of Na–Ca exchange, but not the amount of Ca^{2+} released. This result is qualitatively consistent with the notion that Na–Ca exchange in r.o.s. operates electrogenically (FCCP relieves the back-pressure effect of the Na–Ca exchange current). Na–Ca exchange in the absence of FCCP could be either due to electroneutral Na–Ca exchange or to the electrical shunting by conductance(s) endogenous to r.o.s. In a separate experiment, gramicidin was added. Gramicidin forms channels in bilayer membranes, which are relatively non-selective for alkali cations and protons (Hladky & Haydon, 1972; Myers & Haydon, 1972). When added to r.o.s., gramicidin acted as observed in bilayer membranes; it caused rapid transport of alkali cations across the membrane (Schnetkamp, 1985*a*). Addition of gramicidin to a suspension of intact r.o.s. caused a reduction in the rate of Na^+-stimulated Ca^{2+} efflux. This result is consistent with a rapid rise of internal Na^+ competing with internal Ca^{2+} for occupation of common internal sites of the Na–Ca exchanger, as observed in other systems (Blaustein & Russell, 1975; Reeves & Sutko, 1983). Gramicidin did not cause a reduction in the amount of Ca^{2+} released. Apparently, the amount of Ca^{2+} released is a function of the external Na^+ concentration rather than of the Na^+ gradient (cf. Fig. 1). It should be noted that these experiments can only analyse the total internal

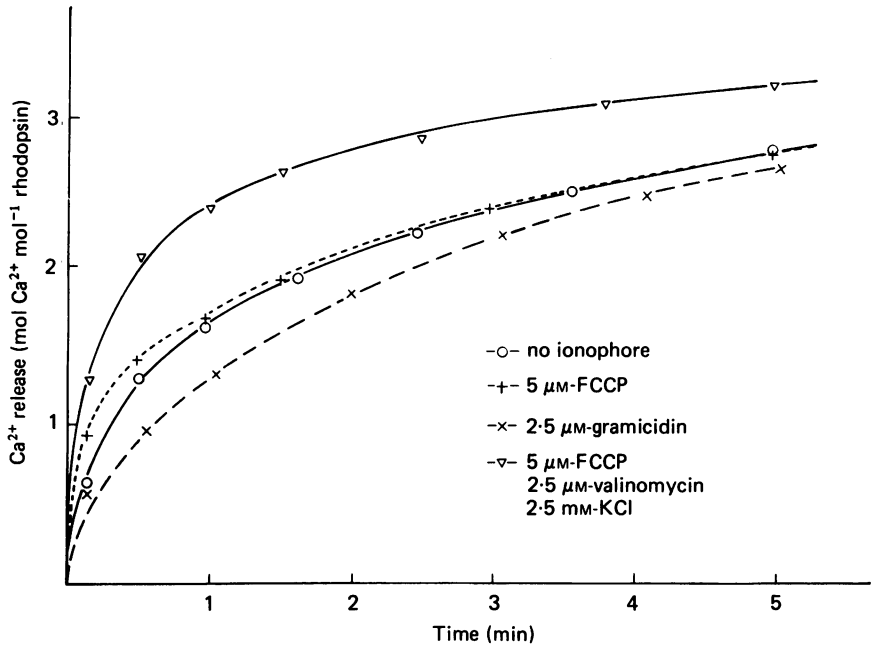

Fig. 2. Effect of different ionophores on Na^+-stimulated Ca^{2+} efflux in Ca^{2+}-enriched intact r.o.s. Intact r.o.s. were suspended in 600 mM-sucrose, 20 mM-HEPES, 8·8 mM-arginine, 40 μM-Arsenazo III (pH = 7·4). The rhodopsin concentration in the suspension was 5·1 μM. Ca^{2+} efflux was initiated by the addition of 50 mM-NaCl. Ionophores were present as indicated. Other conditions were as described in the legend of Fig. 1.

Ca^{2+} content of r.o.s. and not the free internal Ca^{2+} concentration nor the compartmentalization of internal Ca^{2+} (cytosolic or intradiskal). The large majority of internal Ca^{2+} in intact bovine r.o.s. is bound to intradiskal binding sites in equilibrium with a free Ca^{2+} concentration of 10–20 μM (Schnetkamp, 1979; Schnetkamp & Kaupp, 1985).

In addition to the effects of FCCP and gramicidin, Na–Ca exchange was affected by K^+ concentration as well. The Na–Ca exchanger in other systems possesses a non-selective alkali cation site that is not involved directly in transport but must be occupied before transport can occur. The effect of this site is most clearly noted for Ca–Ca exchange, which is stimulated by alkali cations (Blaustein & Russell, 1975; Philipson & Nishimoto, 1981; Slaughter, Sutko & Reeves, 1983). Ca–Ca exchange in r.o.s. does not require the presence of alkali cations, but K^+ has complex effects on both the rate and amount of Ca–Ca exchange (Schnetkamp, 1980). K^+ increased both the rate and the amount of Ca^{2+} release stimulated by addition of 50 mM-external Na^+ (Fig. 2). The optimum K^+ concentration for this effect was 2–2·5 mM. Increasing the K^+ concentration further has a slightly inhibitory effect on Na–Ca exchange, but prolonged exposure to high K^+ has deleterious effects (Schnetkamp, 1979, 1980).

Accessibility of internal Ca^{2+} to exchange with external Na^+

Measurements of ^{45}Ca–^{40}Ca exchange in isolated bovine r.o.s. (Schnetkamp, 1980)

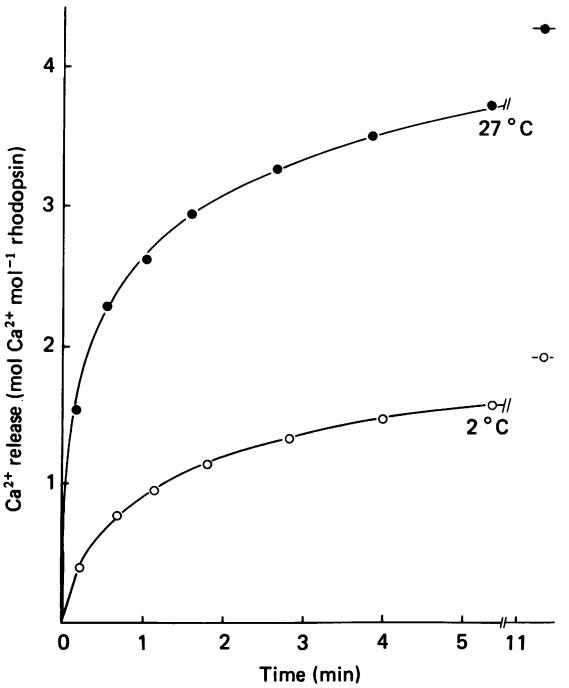

Fig. 3. Effect of temperature on Na^+-stimulated Ca^{2+} efflux in Ca^{2+}-enriched intact r.o.s. Ca^{2+} efflux was initiated by the addition of 50 mM-NaCl at the indicated temperature. Other conditions were as described in the legend of Fig. 1.

or ^{44}Ca–^{40}Ca exchange in r.o.s. in the toad retina (Schröder & Fain, 1984) show that only part of the internal Ca^{2+} is available for rapid exchange and that the size of the accessible Ca^{2+} pool is dependent on the ionic composition of the external medium. The data shown in Figs. 1 and 2 suggest that only part of the total intracellular Ca^{2+} pool in r.o.s. could participate in rapid Na–Ca exchange as both Na^+ and K^+ could affect the size of this accessible pool. Another way to influence the accessibility of the internal Ca^{2+} pool to rapid Na–Ca exchange is illustrated in Fig. 3. The relative rate (the fraction of Ca^{2+} released with respect to the total Ca^{2+} release) was not greatly affected by lowering the temperature by 25 °C, but the amount of Ca^{2+} accessible to rapid release was greatly reduced. This result could be interpreted as some sort of on/off regulation of Na–Ca exchange. The Ca^{2+} content of r.o.s. was not affected by temperature.

Analysis of the rate of Na^+-stimulated Ca^{2+} efflux

In this section the initial rate of Na–Ca exchange in intact r.o.s. is analysed. Fig. 4 shows the dependence of Na^+-stimulated Ca^{2+} efflux on the external Na^+ concentration. R.o.s. membranes were electrically shunted by inclusion of the protonophore FCCP. In addition, the effect of 2 mM-K^+ on the rate of Na–Ca exchange was investigated. To ensure control of both the internal and external K^+ concentration, the experiment was carried out under a K^+ clamp (with the K^+ ionophore valinomycin and the protonophore FCCP present).

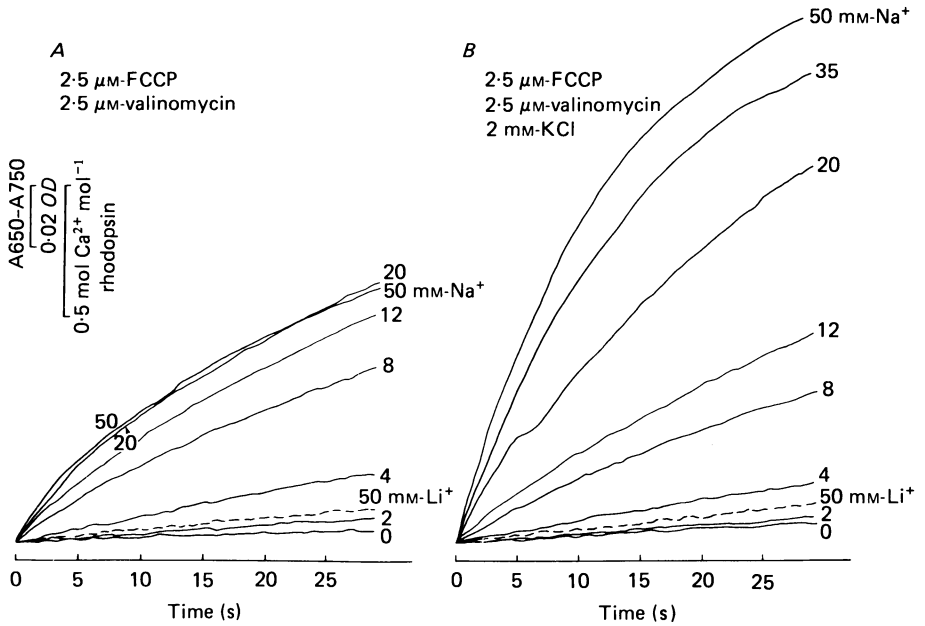

Fig. 4. Dependence of Na–Ca exchange on the external Na^+ concentration in Ca^{2+}-enriched intact r.o.s. Intact r.o.s. were suspended in 600 mM-sucrose, 30 mM-HEPES, 13·2 mM-arginine, 2 mM-KCl (Fig. 4*B* only), 110 μM-Arsenazo III, 2·5 μM-FCCP, 2·5 μM-valinomycin (pH = 7·4). The rhodopsin concentration in the suspension was 3·5 μM. At time zero NaCl or LiCl (dashed line) was added to the indicated final concentration (in mM) and Ca^{2+} release was monitored in the dual-wave-length mode by the increase in A650–A750. The calibration bar applies to the case where 50 mM-NaCl or LiCl was added; the sensitivity of Arsenazo III to Ca^{2+} increased by about 20% when no NaCl was added. The occupancy of Arsenazo III by Ca^{2+} varied from 10% to 16%. Temperature = 26 °C. 1 *OD* is 1 absorbance unit.

K^+ (at 2 mM) had marked effects on the rate and pattern of Na–Ca exchange and the direction of this effect depended on the external Na^+ concentration. At Na^+ concentrations lower than 15 mM, the rate of Na–Ca exchange was inhibited by the presence of 2 mM-K^+, whereas at Na^+ concentrations greater than 15 mM both the rate and amount of Na–Ca exchange were increased by 2 mM-K^+ (compare Fig. 4*A* and *B*). As a result, plots of the initial rate of Na–Ca exchange against the external Na^+ concentration showed distinctly different shapes, both displaying a prominent sigmoidicity (Fig. 5). This indicates that more than one Na^+ is transported against each Ca^{2+}, as is commonly observed for Na–Ca exchange. In ten different preparations, the relation between the initial rate of Na–Ca exchange and the external Na^+ concentration was always sigmoidal, and the degree of sigmoidicity varied in a systematic fashion with the presence of K^+, FCCP or valinomycin.

The data were analysed according to the Michaelis–Menten equation (Dixon & Webb, 1964):

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

where v is the observed rate, V is the maximal rate, (Na^+) is the external Na^+ concentration, K is the dissociation constant of the Na–Ca exchanger for Na^+, and the exponent n is the number of Na ions that determines the rate of Na–Ca exchange. This Michaelis–Menten equation is derived from a model in which sequential binding of more than one Na^+ is required for Ca^{2+} efflux. The Na–Ca exchange rates in the presence of 2 mM-K^+ could be reasonably well fitted when n was equal to 2 and K

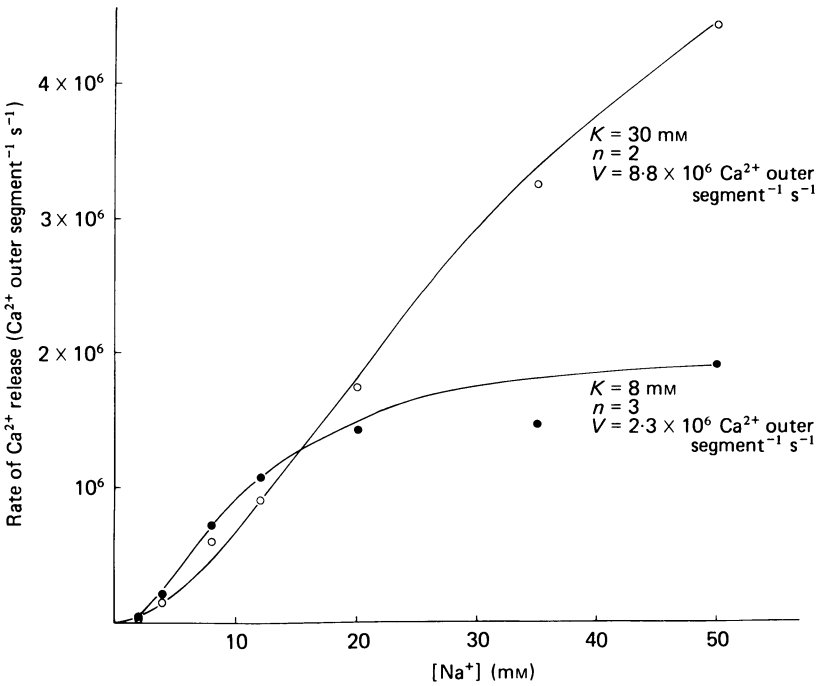

Fig. 5. Dependence of the initial rate of Na–Ca exchange on the external Na^+ concentration. The data shown in Fig. 4 were used for this plot. ○ represent Ca^{2+} release when 2 mM-KCl was present; ● represent Ca^{2+} release without KCl. The continuous lines were calculated according to the Michaelis–Menten equations given in the text. The parameters were used as indicated.

was between 25 and 30 mM (Fig. 5, continuous line through open symbols). A much less satisfactory fit was obtained, when n was assumed to be 3 (not shown). These results are quite similar to those obtained for the Na–Ca exchanger in heart sarcolemma (Kadoma, Froelich, Reeves & Sutko, 1982; Reeves & Sutko, 1983). The Na–Ca exchange rates in the absence of K^+ gave a better fit, when n was equal to 3 and K was 8 mM (Fig. 5 continuous line through filled symbols).

The rate of Na–Ca exchange at 50 mM-Na^+ (2 mM-K^+ present) ranged between 4 and 6×10^6 Ca^{2+} outer segment $^{-1}$ s^{-1}. In the Michaelis–Menten model this observed rate represents a maximal rate V of 10^7 Ca^{2+} outer segment $^{-1}$ s^{-1}.

Ca^{2+} uptake in Ca^{2+}-depleted r.o.s. by reverse Na–Ca exchange

Intact r.o.s. were isolated under more physiological conditions in a medium that included 50 mM-NaCl and 0·5 mM-$CaCl_2$. Under these conditions r.o.s. contained

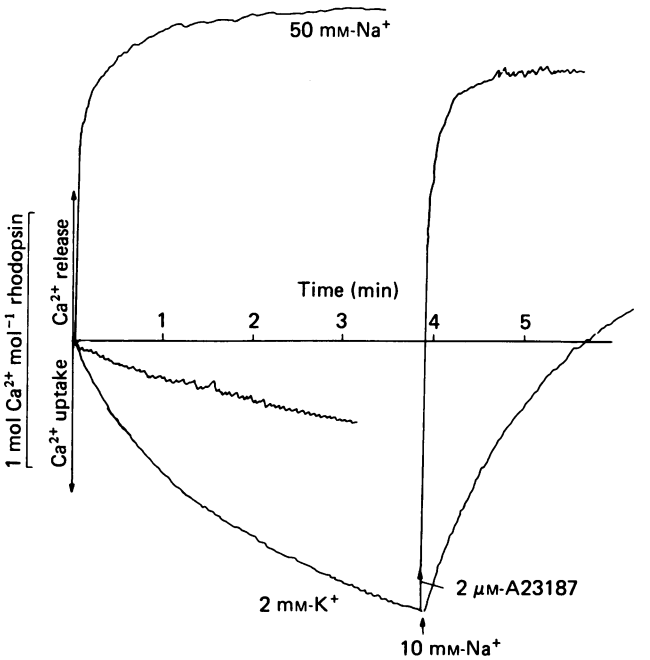

Fig. 6. Ca^{2+} uptake and Ca^{2+} release in intact 'normal' Ca^{2+} r.o.s. Intact r.o.s. were suspended in 600 mM-sucrose, 30 mM-HEPES, 13·2 mM-arginine, 2 μM-FCCP, 110 μM-Arsenazo III (pH = 7·4). The rhodopsin concentration in the suspension was 3·2 μM. Ca^{2+} fluxes were monitored in the dual-wave-length mode (A650–A750) and the occupancy of Arsenazo III with Ca^{2+} was less than 5% (free Ca^{2+} concentration was less than 1 μM). Na^+-stimulated Ca^{2+} release was initiated by adding 50 mM-NaCl (2 mM-KCl present). Ca^{2+} uptake was started by addition of 8 μM-Ca^{2+} with or without 2 mM-KCl as indicated. After a little less than 4 min 10 mM-NaCl or 2 μM-A23187 was added as indicated. Temperature = 26 °C.

2–3 mol Ca^{2+} mol^{-1} rhodopsin. After resuspension in the inert sucrose medium r.o.s. released Ca^{2+} upon addition of Na^+, and also accumulated Ca^{2+} upon addition of Ca^{2+} (Fig. 6). The rate of Na^+-stimulated Ca^{2+} release was similar to that observed in Ca^{2+}-enriched r.o.s. ($3·9 \times 10^6$ Ca^{2+} outer segment^{-1} s^{-1} after addition of 50 mM-NaCl).

Ca^{2+} uptake was initiated by adding 8 μM-Ca^{2+} to the r.o.s. suspension at time zero (the absorption change due to Ca^{2+} addition is not shown). The suspension now contained 100 μM-free Arsenazo III and 10 μM-Ca^{2+}–Arsenazo III. The dissociation constant for the Arsenazo–Ca^{2+} complex is about 3 μM under these conditions (Kaupp, Schnetkamp & Junge, 1979) and, accordingly, the free external Ca^{2+} concentration was about 0·3 μM. Ca^{2+} uptake was stimulated by addition of K^+ (optimum K^+ concentration 2–2·5 mM). The initial rate of uptake was $3·3 \times 10^5$ Ca^{2+} outer segment^{-1} s^{-1}, and uptake amounted to 1·1 mol Ca^{2+} mol^{-1} rhodopsin after 5 min. Subsequent addition of 10 mM-Na^+ caused a Ca^{2+} efflux that reversed the preceding Ca^{2+} uptake. In a separate sample, addition of the Ca^{2+} ionophore A23187 caused a large and fast release of Ca^{2+}.

The mechanism underlying the Ca^{2+} uptake by intact r.o.s. was investigated in

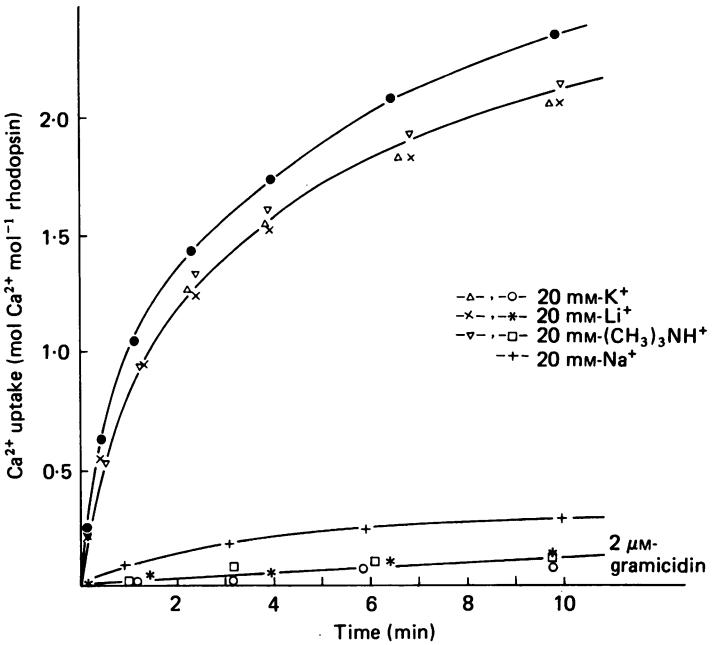

Fig. 7. Ca^{2+} uptake in Ca^{2+}-depleted intact r.o.s. Intact r.o.s. were suspended in 600 mM-sucrose, 30 mM-HEPES, 13·2 mM-arginine, 40 μM-Arsenazo III, 50 μM-EDTA, 5 μM-FCCP, 2·5 mM-KCl (pH = 7·4). The rhodopsin concentration in the suspension was 6·0 μM. EDTA was added to the suspension to prevent uptake of Ca^{2+} present as contamination in the Arsenazo. To initiate Ca^{2+} uptake by r.o.s., Ca^{2+} was added at time zero to give about 50% occupancy of the Arsenazo with Ca^{2+} (about 68 μM-total Ca^{2+}: 50 μM to chelate the EDTA, 15 μM-Ca^{2+} chelated to Arsenazo, and 3 μM-free Ca^{2+}). ●: no further addition to the suspension medium. Chloride salts were present as indicated. Gramicidin was present at 2 μM (○, *, □). Ca^{2+} uptake was measured in the split-beam mode by recording spectra of Arsenazo III plus r.o.s. at the indicated times. Temperature = 26 °C.

detail with the preparation of Ca^{2+}-depleted r.o.s. The experiment shown in Fig. 7 examined whether Ca^{2+} uptake in Ca^{2+}-depleted r.o.s. occurred by reverse Na–Ca exchange. Uptake of Ca^{2+} was strongly inhibited when Na^+ was added to the medium, whereas addition of either K^+, Li^+, or trimethylamine$^+$ had little effect. This result is consistent with Ca^{2+} uptake via reverse Na–Ca exchange in view of the competition between Na^+ and Ca^{2+} for common sites on the Na–Ca exchanger. Such competition has been observed for Na–Ca exchangers in other systems as well (Blaustein & Russell, 1975; Reeves & Sutko, 1983). The maximal rate of uptake amounted to $7{\cdot}4 \times 10^5$ Ca^{2+} outer segment^{-1} s^{-1}, and 2·3 mol Ca^{2+} mol^{-1} rhodopsin was taken up in 10 min. The initial rate was observed at a free Ca^{2+} concentration of about 3 μM.

If Ca^{2+} uptake occurred via reverse Na–Ca exchange, it should be abolished when intracellular Na^+ is allowed to leak out by the ionophore gramicidin, especially when the outward movement of Na^+ is electrically compensated by an inward movement of alkali cations or protons. Ca^{2+} uptake in Ca^{2+}-depleted r.o.s. was abolished when gramicidin was present and the external medium contained either 20 mM-K^+,

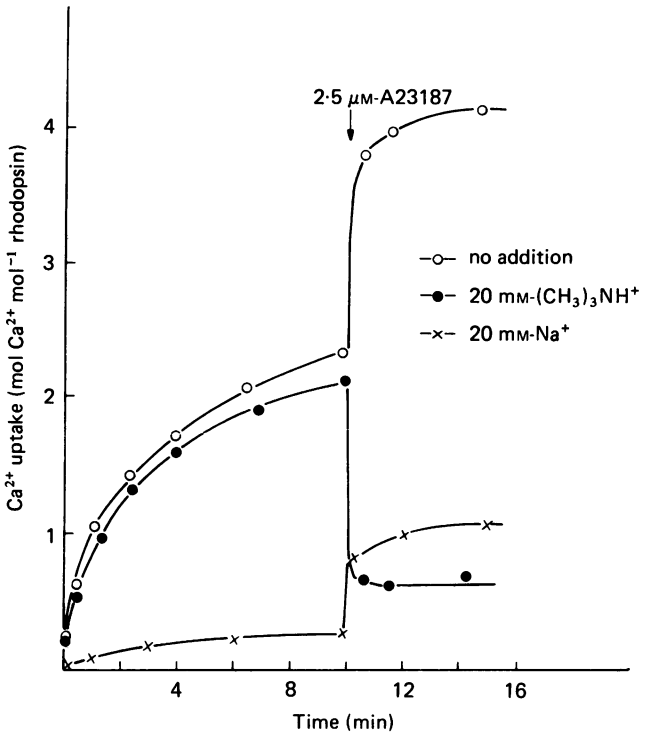

Fig. 8. Ca^{2+} uptake in Ca^{2+}-depleted intact r.o.s. with and without A23187. Experimental conditions are as described in the legend of Fig. 7. At 10 min 2·5 μM-A23187 was added.

20 mM-Li^+, or 20 mM-trimethylamine$^+$. Trimethylamine is an intrinsically permeant 'cation' due to the fact that the neutral form readily crosses biological membranes in a similar manner as observed for NH_3 (Roos & Boron, 1981; see below). The Na^+ efflux is electrically compensated by a proton influx (both Na^+ and proton flux via gramicidin). The net effect is an exchange between internal Na^+ and protonated trimethylamine. Taken together, the properties of Ca^{2+} uptake in r.o.s. such as stimulation by K^+, inhibition by external Na^+, and dependence on internal Na^+ suggest that Ca^{2+} uptake reflects reverse Na–Ca exchange.

The experiments described to this point suggest that the Na–Ca exchanger in isolated r.o.s. freely operates in both directions and that Ca^{2+} movements in r.o.s. are controlled by Na^+. To illustrate this point the direction of Ca^{2+} movements through the Na–Ca exchanger is compared with those through the Ca–H exchanger A23187 (Fig. 8). In the presence of A23187 the free Ca^{2+} concentration in r.o.s. is clamped to that in the external medium (Schnetkamp, 1979; Kaupp, Schnetkamp & Junge, 1981; Schnetkamp & Kaupp, 1985). Under these conditions Ca^{2+} uptake and release is dependent only on the properties of Ca^{2+} binding sites in r.o.s. For the present argument it suffices to note that Ca^{2+} binding is strongly affected by permeant electrolytes such as salts of weak bases (the mechanism for this is described in detail elsewhere, Schnetkamp, 1985*a*). During the first 10 min of the incubation Ca^{2+} transport proceeded via transporters native to r.o.s. (Fig. 8). Ca^{2+} uptake was inhibited

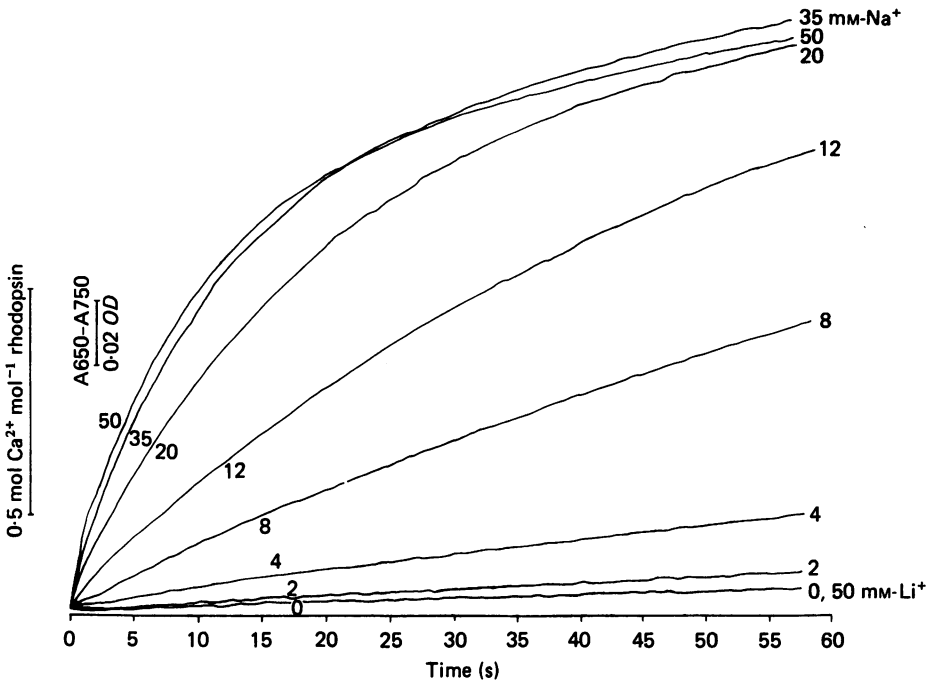

Fig. 9. Na^+-stimulated Ca^{2+} release in Ca^{2+}-enriched leaky r.o.s. Leaky r.o.s. were suspended in 300 mM-sucrose, 30 mM-HEPES, 13·2 mM-arginine, 2 mM-KCl, 110 μM-Arsenazo III, 2 μM-FCCP, 2 μM-valinomycin (pH = 7·4). The rhodopsin concentration in the suspension was 7·0 μM. External Ca^{2+} was titrated with 20 μM-EDTA until the occupancy of Arsenazo III with Ca^{2+} was 8%. Maximal Ca^{2+} release increased this occupancy to 16%. Ca^{2+} release was monitored in the dual-wave-length mode (A650–A750). The sensitivity of the Arsenazo towards Ca^{2+} was decreased by 20% when Na^+ was increased from zero to 50 mM. Temperature = 26 °C. 1 *OD* is 1 absorbance unit.

by Na^+, whereas trimethylamine had little effect. After 10 min the addition of 2·5 μM-A23187 caused a rapid redistribution of Ca^{2+} according to the equilibrium conditions of the ionophore. This illustrates two points. First, the concentration of A23187 used was amply sufficient to overcome the Ca^{2+} fluxes (and the equilibrium conditions) maintained by the Na–Ca exchanger. Secondly, the equilibrium conditions for A23187 are easily distinguished from those of the Na–Ca exchanger. The addition of A23187 caused a further uptake of 1·7 mol Ca^{2+} mol^{-1} rhodopsin when trimethylamine was not present; 1·5 mol Ca^{2+} mol^{-1} rhodopsin was released when trimethylamine was present.

Na–Ca exchange in leaky r.o.s.

Na^+-stimulated Ca^{2+} efflux was also observed in leaky r.o.s. (Fig. 9). The pattern was very similar to intact r.o.s.; a fast efflux phase lasted about 15 s and subsided into a much slower efflux phase. The selectivity for Na^+ as compared with Li^+ or K^+ was the same as noted for intact r.o.s. Neither Li^+ nor K^+ stimulated Ca^{2+} efflux from leaky r.o.s., although K^+ (2 mM) had similar effects on Na–Ca exchange in leaky

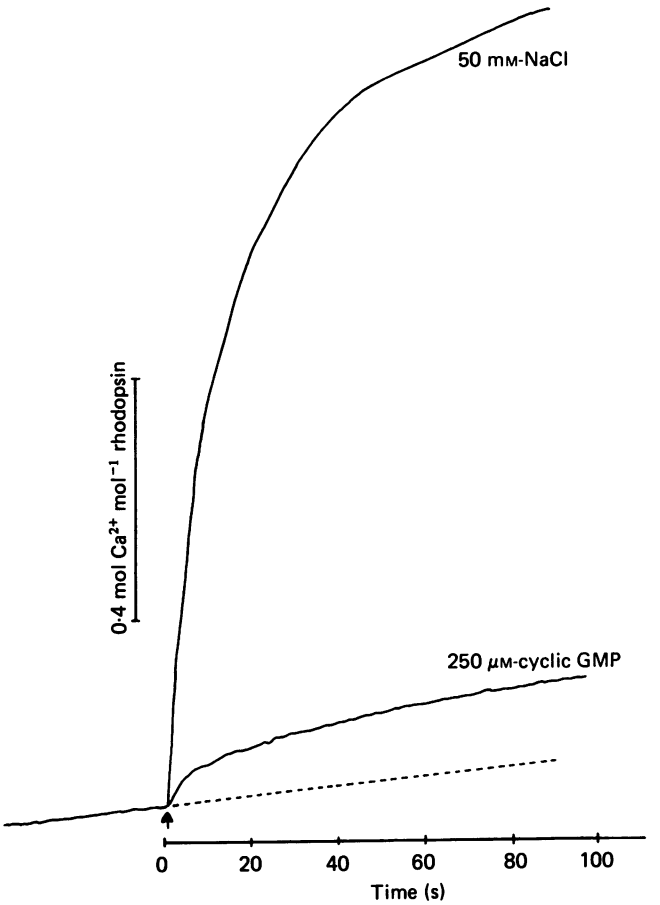

Fig. 10. Comparison of Na^+-induced and cyclic-GMP-induced Ca^{2+} release in Ca^{2+}-enriched leaky r.o.s. Experimental conditions were as described in the legend of Fig. 9. Ca^{2+} efflux was initiated by addition of 50 mM-NaCl or 250 μM-cyclic GMP as indicated. Cyclic-GMP-induced Ca^{2+} release required the presence of alkali cations. Therefore, 50 mM-KCl was added to the cuvette, in which cyclic-GMP-induced Ca^{2+} release was measured. KCl by itself did not cause Ca^{2+} release.

r.o.s. as noted above for intact r.o.s. (Fig. 4). The initial rate at 50 mM-Na^+ ranged between 2 and 4×10^6 Ca^{2+} outer segment^{-1} s^{-1}, which is comparable with that observed in intact r.o.s. The initial rate of Na–Ca exchange depended in a sigmoidal way on the external Na^+ concentration, although a precise kinetic analysis was more difficult in leaky r.o.s. because of the non-linear nature of the initial absorption changes.

Ca^{2+} uptake in Ca^{2+}-depleted leaky r.o.s. was compared with that observed in intact r.o.s. Results similar to those shown in Figs. 7 and 8 were obtained when using leaky r.o.s. (not shown).

Leaky r.o.s., but not intact r.o.s., showed a cyclic-GMP-induced Ca^{2+} release in addition to Na–Ca exchange. This cyclic-GMP-induced Ca^{2+} release is similar to that described by others (Caretta & Cavaggioni, 1983; Kaupp & Koch, 1984). The

experiment illustrated in Fig. 10 compared the Na^+-induced Ca^{2+} release with the cyclic-GMP-induced Ca^{2+} release. The initial rates were 2×10^6 and 10^5 Ca^{2+} outer segment^{-1} s^{-1} for Na^+- and cyclic-GMP-induced Ca^{2+} release, respectively, while the amount of Ca^{2+} released was 1·5 and 0·15 mol Ca^{2+} mol^{-1} rhodopsin, respectively.

Metabolic requirements of Ca^{2+} fluxes

The Ca^{2+} fluxes in intact r.o.s. described in this study and in previous studies (Schnetkamp, 1979, 1980) can be accounted for by Na–Ca exchange operating in both directions or operating as Ca–Ca exchange. When Na–Ca exchange was prevented from operating, evidence for a Ca^{2+} pump in r.o.s. was not obtained. This might be caused by insufficient high-energy phosphates to fuel such a pump. However, Ca^{2+}-enriched intact r.o.s. did contain sufficient high-energy phosphates as judged from methanol extracts analysed by high-pressure liquid chromatography. Intact r.o.s. contained about 3 mM-high-energy phosphates, $1{\cdot}10 \pm 0{\cdot}38$ mM-ATP, $0{\cdot}76 \pm 0{\cdot}15$ mM-GTP, $0{\cdot}95 \pm 0{\cdot}51$ mM-phosphocreatine (average ± standard deviation from six different preparations). These values for the ATP and GTP content are comparable with those obtained from frozen sections of rabbit and monkey retinas (Berger, DeVries, Carter, Schulz, Passonneau, Lowry & Ferendelli, 1980), and with those obtained from frog r.o.s. preparations that contain part of the inner segment and show normal photocurrents (Biernbaum & Bownds, 1985). The above values are over-all concentrations based on a rhodopsin concentration of 3 mM in r.o.s. The cytoplasmic space occupies probably only half of the total r.o.s. volume, and, accordingly, the cytoplasmic concentrations were probably twice as high. Incubations for 30 min under conditions that permitted either Na–Ca exchange (high Na^+, low Ca^{2+}) or Ca–Ca exchange did not cause a significant drop in ATP or GTP levels (not shown). Incubation for 30 min with the mitochondrial uncoupler FCCP (frequently used in this study to short-circuit r.o.s. membranes) had no significant effect on ATP and GTP levels. Another indicator for 'metabolic health' commonly used is the ATP/ADP and GTP/GDP ratio. Typically, very little ADP or GDP was extracted by the methanol extraction procedure (less than 0·05 mM). However, after a 5 min exposure to low Ca^{2+} in the dark as much as 0·5 mM-ADP and 0·35 mM-GDP could be extracted despite the fact that the ATP and GTP levels did not change. One possible explanation for these preliminary results is that most of the ADP and GDP was normally bound (e.g. to the G-protein and the so-called '48K' protein, cf. Godchaux & Zimmerman, 1979; Fung & Stryer, 1980; Zuckerman, Buzdygon & Liebman, 1984) and apparently released at low Ca^{2+}.

DISCUSSION

Na–Ca exchange was found to be the dominant Ca^{2+} transporter in the plasma membrane of isolated intact bovine r.o.s. This preparation allows precise measurements of the properties of the Na–Ca exchanger and four aspects, reported in this study, are noteworthy:

1. Na–Ca exchange can change the total internal Ca^{2+} concentration by as much as 0·5 mM s^{-1} at observed rates of 5×10^6 Ca^{2+} outer segment^{-1} s^{-1}.

2. Within 30 s Na–Ca exchange can cause the release of as much as 7·5 mM-total

Ca^{2+} (Fig. 1) or the uptake of as much as 2 mM-total Ca^{2+} (Fig. 7). The large majority of this Ca^{2+} is bound to Ca^{2+} buffer sites inside disks in equilibrium with free internal Ca^{2+} concentrations in the micromolar range (Schnetkamp, 1979; Schnetkamp & Kaupp, 1985). Disk membranes contain at least two pathways to account for rapid transport of Ca^{2+} across the disk membrane: Na–Ca exchange (Schnetkamp, Daemen & Bonting, 1977; Biernbaum & Bownds, 1985; Fig. 9), and a cyclic-GMP-dependent pathway (Caretta & Cavaggioni, 1983; Kaupp & Koch, 1984; Fig. 9).

3. Na–Ca exchange could occur in both directions (Ca^{2+} uptake and Ca^{2+} release), and in r.o.s. with widely varying internal Ca^{2+} levels (Figs. 1, 6 and 7). Ca^{2+} fluxes through the Na–Ca exchanger appeared to flow freely according to the prevailing gradients for Na^+ and Ca^{2+}.

4. Ca^{2+} storage in isolated r.o.s. is compartmentalized. Not all Ca^{2+} is accessible to Na–Ca exchange (Figs. 2 and 3) or, in leaky r.o.s., to the cyclic-GMP-dependent pathway (Fig. 9). This suggests the presence of different Ca^{2+} pools, perhaps reflecting different types of disks. These results cannot be accounted for by the properties of Ca^{2+} binding sites, since all internal Ca^{2+} in r.o.s. is freely accessible and exchangeable in the presence of the ionophore A23187 (Schnetkamp, 1979; Caretta & Cavaggioni, 1983; Kaupp & Koch, 1984; Schnetkamp & Kaupp, 1985).

The above results were obtained with isolated intact r.o.s. exposed to rather unphysiological conditions. Several observations are consistent with the notion that Na–Ca exchange similarly operates in the outer segments of physiologically functional rod cells both in the dark as well as in the light. The rapid changes of the dark current in functioning rod cells upon changes of the external Na/Ca ratio are consistent with the notion that the internal Ca^{2+} concentration in these cells is controlled by Na–Ca exchange (Fain & Lisman, 1981; Hodgkin *et al.* 1984; Yau & Nakatani, 1984*a*). Similarly, the Na^+ dependence of the light-induced Ca^{2+} release from r.o.s. in the retina suggests that this Ca^{2+} release is mediated by Na–Ca exchange (Yoshikami, George & Hagins, 1980; Gold & Korenbrot, 1980). In isolated r.o.s. the maximal Ca^{2+} flux through the plasma membrane by Na–Ca exchange amounted to 26 pmol $cm^{-2}\ s^{-1}$; among the highest Ca^{2+} fluxes reported for biological systems (Kaupp & Schnetkamp, 1982). Yau & Nakatani (1984*b*) recently reported a maximal current of 30 pA in illuminated toad rod outer segments, which the authors attribute to electrogenic Na–Ca exchange operating at a stoicheiometry of 3 Na/Ca. If the dimensions of a toad rod outer segment are taken to be $6{\cdot}5 \times 60\ \mu m$ (Hodgkin *et al.* 1984) a Ca^{2+} flux of 25 pmol $cm^{-2}\ s^{-1}$ can be calculated. It appears that the maximal Ca^{2+} flux of Na–Ca exchange is quite similar in isolated bovine r.o.s. as compared with functioning toad r.o.s. when expressed per unit of surface area. These numbers show that the maximal capacity of Na–Ca exchange is about two orders of magnitude larger as compared with the light-induced Ca^{2+} efflux observed from r.o.s. in the functioning rod cell (Yoshikami *et al.* 1980; Gold & Korenbrot, 1980).

Ca^{2+} fluxes through a conductance mechanism were not detected at the low external Ca^{2+} concentrations (μM) typical for the experiments in this study. Na^+ and K^+ fluxes through a conductance mechanism were observed in the preparation of isolated intact r.o.s., but Ca^{2+} fluxes through a conductance mechanism were only detected at high (20 mM) external Ca^{2+} concentrations (Schnetkamp, 1985*b*). Under physiological conditions part of the dark current through the light-sensitive con-

ductance may be carried by Ca^{2+} (Hodgkin *et al.* 1984). The maximal Ca^{2+} flux through the Na–Ca exchanger would be of comparable magnitude as that due to a Ca^{2+} current of 60 pA. The Ca^{2+} flux through the Na–Ca exchanger would be difficult to detect in current measurements, if it was due to electroneutral Ca–Ca exchange or to reverse Na–Ca exchange.

Properties of Na–Ca exchange in different systems

Na–Ca exchange has been described in a number of other systems. Some of its properties as have emerged from studies on squid axon (Baker & McNaughton, 1976; Blaustein, 1977) and cardiac sarcolemma vesicles (Pitts, 1979; Philipson & Nishimoto, 1980; Trosper & Philipson, 1983; Reeves & Sutko, 1983; Slaughter *et al.* 1983; Reeves & Hale, 1984) will be summarized and compared with findings on Na–Ca exchange in bovine r.o.s. as reported in this and previous studies (Schnetkamp, 1979, 1980):

1. Na–Ca exchange and Ca–Ca exchange have comparable transport rates and are most likely two transport modes of the same transporter. In all three systems Na–Ca exchange can operate freely in both directions as measured by Ca^{2+} influx and Ca^{2+} efflux; Na^+ is the only alkali cation that can promote counter-transport with Ca^{2+}, and Na^+ and Ca^{2+} compete for common sites on the Na–Ca exchanger.

2. Na–Ca exchange operates passively (i.e. it is not activated) in the direction set by the prevailing electrochemical gradients of Na^+ and Ca^{2+}. Na–Ca exchange does not operate as a unidirectional Ca^{2+} pump. Ca–Ca exchange without net flux component has been demonstrated under steady-state conditions.

3. Most divalent and trivalent cations inhibit both Na–Ca and Ca–Ca exchange, although only Sr^{2+} and (sometimes) Ba^{2+} (as opposed to Mg^{2+}, Mn^{2+} and La^{3+}) can replace Ca^{2+} in actual transport.

4. Both in squid axons and in sarcolemma vesicles Ca–Ca exchange is activated by alkali cations such as Li^+ and K^+. In r.o.s. Ca–Ca exchange does not require alkali cations; both Na–Ca exchange and Ca–Ca exchange are affected in a complex way by K^+, but not by Li^+.

5. Na–Ca exchange depends on the membrane potential in a direction consistent with the notion that more than 2 Na^+ are exchanged against each Ca^{2+}. Although external Ca^{2+} appears to compete with 2 Na^+ for common binding sites, probably 3 Na^+ are transported against each Ca^{2+} (evidence for this is reviewed recently by Reeves & Hale, 1984).

Kinetics of Na^+-stimulated Ca^{2+} efflux

When Ca^{2+} efflux is accompanied by the uptake of more than 2 Na^+ polarization of the plasma membrane will inhibit the rate of Ca^{2+} efflux. This back-pressure effect will be relieved by electrically shunting the membrane. The observation that the rate of Na^+-stimulated Ca^{2+} efflux was enhanced by the protonophore FCCP (Fig. 2) is qualitatively consistent with the notion that Na–Ca exchange in r.o.s. is electrogenic, but does not prove this point or imply that electrogenicity is obligatory.

Another approach to assess the number of Na^+ transported against each Ca^{2+} is to analyse the dependence of the initial rate of Na–Ca exchange on the external Na^+ concentration (Figs. 4 and 5). When external K^+ (2 mM) was present the data were consistent with a model in which the binding of 2 Na^+ was required for Ca^{2+} efflux

to occur (actual transport could involve 3 Na^+ but the binding of 2 Na^+ is rate limiting). In this analysis a Michaelis–Menten equation was used (Fig. 5). This equation is derived from a model in which Na^+ binding is sequential and with the same binding constant. The sigmoidal dependence of the initial rate of Na–Ca exchange on the external Na^+ concentration suggests that at least two of the binding constants are not dissimilar. The dissociation constant of the Na–Ca exchanger for Na^+ was 30 mM. The value for the dissociation constant of Na–Ca exchange for Na^+ in r.o.s. is comparable with that obtained for Na–Ca exchange in sarcolemma vesicles (Philipson & Nishimoto, 1981; Kadoma *et al.* 1982; Reeves & Sutko, 1983), but is lower than that reported for squid axon. In the latter system the dissociation constants depend on the presence of ATP in the axon, 50–60 mM in the presence of ATP and 100–120 mM in its absence (Baker & McNaughton, 1976; Blaustein, 1977).

Yau & Nakatani (1984*b*) have determined a stoicheiometry of 3 Na/Ca for Na–Ca exchange in toad rods. Their calculation is based on a comparison of an inward Ca^{2+} current that loads the cell with Ca^{2+} with a Na^+-dependent outward current removing Ca^{2+} from the cell, thought to be Na–Ca exchange. This calculation ignores the possibility that substantial Ca^{2+} loading occurs not only by a Ca^{2+} current through the light-sensitive conductance, but also by reverse Na–Ca exchange. In this study, Ca^{2+} uptake via reverse Na–Ca exchange could increase the Ca^{2+} content of r.o.s. by as much as 2 mM in 30 s (Fig. 8). If reverse Na–Ca exchange in toad rods occurs at a similar rate as forward Na–Ca exchange (Na^+-stimulated Ca^{2+} efflux), then the maximal Ca^{2+} influx through the Na–Ca exchanger could be equivalent to the flux of a Ca^{2+} current of 60 pA.

Na–Ca exchange in leaky r.o.s.

Ca^{2+} transport across the disk membrane was studied in r.o.s. with a plasma membrane leaky to small solutes. Ca^{2+}-enriched and Ca^{2+}-depleted leaky r.o.s. were prepared following the same principles as found for intact r.o.s. This suggests that Ca^{2+} transport across the disk membrane is also mediated by Na–Ca exchange. Na–Ca exchange operated in both forward and reverse mode. The rate of Na–Ca exchange in leaky r.o.s. was of comparable magnitude as observed in intact r.o.s. when expressed in number of Ca^{2+} outer segment^{-1} s^{-1}. This is a peculiar coincidence considering that in intact r.o.s. the plasma membrane was rate limiting and in leaky r.o.s. the disk membrane was rate limiting. The observation that cyclic-GMP-induced Ca^{2+} release was only observed in leaky r.o.s. and not in intact r.o.s. is consistent with a proper assessment of the rate-limiting barrier in both preparations. The most obvious way to interpret these results is that both the disk and plasma membrane contain a very similar Na–Ca exchanger. Most of the internal Ca^{2+} in intact r.o.s. is stored in the disks (Schnetkamp, 1979; Schnetkamp & Kaupp, 1985), and it is peculiar that Na^+-stimulated Ca^{2+} efflux in intact r.o.s. never showed a lag phase indicating the time required for Na^+ to cross the plasma membrane and for Na–Ca exchange to transport Ca^{2+} from the intradiskal space to the cytoplasmic space.

I thank Drs G. L. Fain, G. D. Nicol and M. D. Bownds for reading earlier drafts of the manuscript. I am especially grateful to Dr W. L. Hubbell for generously providing facilities and financial support. This research was supported by NIH grant EY 00729 (to W. L. Hubbell).

REFERENCES

BAKER, P. F. & MCNAUGHTON, P. A. (1976). Kinetics and energetics of calcium efflux from intact squid giant axons. *Journal of Physiology* **259**, 103–144.

BASTIAN, B. L. & FAIN, G. L. (1982). The effects of sodium replacement on the responses of toad rods. *Journal of Physiology* **330**, 331–347.

BENZ, R. & MCLAUGHLIN, S. (1983). The molecular mechanism of action of the proton ionophore FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). *Biophysical Journal* **41**, 381–398.

BERGER, S. J., DEVRIES, G. W., CARTER, J. G., SCHULZ, D. W., PASSONNEAU, P. N., LOWRY, O. H. & FERENDELLI, J. A. (1980). The distribution of the components of the cyclic GMP cycle in retina. *Journal of Biological Chemistry* **255**, 3128–3133.

BIERNBAUM, M. S. & BOWNDS, M. (1985). Frog rod outer segments with attached inner segment elipsoids as an in vitro model for photoreceptors on the retina. *Journal of General Physiology* **85**, 83–105.

BLAUSTEIN, M. P. (1977). Effects of internal and external cations and of ATP on sodium-calcium exchange in squid axons. *Biophysical Journal* **20**, 79–111.

BLAUSTEIN, M. P. & RUSSELL, J. M. (1975). Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. *Journal of Membrane Biology* **22**, 285–312.

CARETTA, A. & CAVAGGIONI, A. (1983). Fast ionic flux activated by cyclic GMP in the membrane of cattle rod outer segments. *European Journal of Biochemistry* **132**, 1–8.

DAEMEN, F. J. M. (1973). Vertebrate rod outer segment membranes. *Biochimica et biophysica acta* **300**, 255–288.

DIXON, M. & WEBB, E. C. (1964). *The Enzymes*. London: Longmans.

DONOFRIO, J., COLMAN, M. S., HUTTON, J. J., DAOUD, A., LAMPKIN, B. & DYMINSKI, J. (1978). Overproduction of adenine deoxynucleosides and deoxynucleotides in adenosine deaminase deficiency with severe combined immunodeficiency disease. *Journal of Clinical Investigation* **62**, 884–887.

FAIN, G. L. & LISMAN, J. E. (1981). Membrane conductances of photoreceptors. *Progress in Biophysical and Molecular Biology* **37**, 91–147.

FUNG, B. K. & STRYER, L. (1980). Photolyzed rhodpsin catalyzes the exchange of GTP for bound GDP in retinal rod outer segments. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 2500–2504.

GODCHAUX III, W. & ZIMMERMAN, W. F. (1979). Membrane-dependent guanine nucleotide binding and GTPase activities of soluble protein from bovine rod cell outer segments. *Journal of Biological Chemistry* **254**, 7874–7884.

GOLD, G. H. & KORENBROT, J. I. (1980). Light-induced calcium release by intact retinal rods. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 5557–5561.

HLADKY, S. B. & HAYDON, D. A. (1972). Ion transfer across lipid membranes in the presence of gramicidin A. I. studies of the unit conductance channel. *Biochimica et biophysica acta* **274**, 294–312.

HODGKIN, A. L., MCNAUGHTON, P. A. & NUNN, B. J. (1985). The ion selectivity and calcium dependence of the light-sensitive pathway in toad rods. *Journal of Physiology* **358**, 447–468.

HODGKIN, A. L., MCNAUGHTON, P. A., NUNN, B. J. & YAU, K.-W. (1984). Effect of ions on retinal rods from *Bufo marinus*. *Journal of Physiology* **350**, 649–680.

KADOMA, M., FROELICH, J., REEVES, J. & SUTKO, J. (1982). Kinetics of sodium induced calcium ion release in calcium ion loaded sarcolemmal vesicles: determination of initial velocities by stopped-flow spectrophotometry. *Biochemistry* **21**, 1914–1918.

KAUPP, U. B. & KOCH, K.-W. (1984). Cyclic GMP releases calcium from leaky rod outer segments. *Vision Research* **24**, 1477–1479.

KAUPP, U. B. & SCHNETKAMP, P. P. M. (1982). Calcium metabolism in vertebrate photoreceptors. *Cell Calcium* **3**, 83–112.

KAUPP, U. B., SCHNETKAMP, P. P. M. & JUNGE, W. (1979). Flash-spectrophotometry with arsenazo III in vertebrate photoreceptor cells. In *Detection and Measurement of Free Ca^{2+} in Cells*, ed. ASHLEY, C. C. & CAMPBELL, A. K., pp. 287–308. Amsterdam: Elsevier/North Holland.

KAUPP, U. B., SCHNETKAMP, P. P. M. & JUNGE, W. (1981). Rapid calcium release and proton uptake at the disk membranes of isolated cattle rod outer segments. 1. Stoichiometry of light-stimulated calcium release and proton uptake. *Biochemistry* **20**, 5500–5510.

KORENBROT, J. I. (1985). Signal mechanisms of phototransduction in retinal rods. *Critical Reviews in Biochemistry* **17**, 223–256.

LIEBES, L. F., KUO, S., KRIGEL, R., PELLE, E. & SILBER, R. (1981). Identification and quantitation of ascorbic acid in extracts of human lymphocytes by high-performance liquid chromatography. *Analytical Biochemistry* **118**, 53–57.

MACLEISH, P. R., SCHWARTZ, E. A. & TACHIBANA, M. (1984). Control of the generator current in solitary rods of the *Ambystoma tigrinum* retina. *Journal of Physiology* **348**, 645–664.

MYERS, V. B. & HAYDON, D. A. (1972). Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. *Biochimica et biophysica acta* **274**, 313–322.

PHILIPSON, K. D. & NISHIMOTO, A. Y. (1980). Na–Ca exchange is affected by membrane potential in cardiac sarcolemma vesicles. *Journal of Biological Chemistry* **255**, 6880–6882.

PHILIPSON, K. D. & NISHIMOTO, A. Y. (1981). Efflux of Ca^{2+} from cardiac sarcolemma vesicles. Influence of external Ca^{2+} and Na^{+}. *Journal of Biological Chemistry* **256**, 3698–3702.

PITTS, B. J. R. (1979). Stoichiometry of sodium–calcium exchange in cardiac sarcolemma vesicles. *Journal of Biological Chemistry* **254**, 6232–6235.

REEVES, J. P. & HALE, C. C. (1984). The stoichiometry of the cardiac sodium–calcium exchange system. *Journal of Biological Chemistry* **259**, 7733–7739.

REEVES, J. P. & SUTKO, J. L. (1980). Sodium–calcium exchange activity generates a current in cardiac membrane vesicles. *Science* **208**, 1461–1464.

REEVES, J. P. & SUTKO, J. L. (1983). Competitive interactions of sodium and calcium with the sodium–calcium exchange system of cardiac sarcolemmal vesicles. *Journal of Biological Chemistry* **258**, 3178–3182.

ROOS, A. & BORON, W. F. (1981). Intracellular pH. *Physiological Reviews* **61**, 296–434.

SCHNETKAMP, P. P. M. (1979). Calcium translocation and storage of isolated intact cattle rod outer segments in darkness. *Biochimica et biophysica acta* **554**, 441–459.

SCHNETKAMP, P. P. M. (1980). Ion selectivity of the cation transport system of isolated intact cattle rod outer segments. Evidence for a direct communication between the rod plasma membrane and the rod disk membranes. *Biochimica et biophysica acta* **598**, 66–90.

SCHNETKAMP, P. P. M. (1981). Metabolism in the cytosol of intact isolated cattle rod outer segments as indicator for cytosolic calcium and magnesium ions. *Biochemistry* **20**, 2449–2456.

SCHNETKAMP, P. P. M. (1984). Sodium and calcium transport in outer segments isolated from rod photoreceptors. *Biophysical Society Annual Meeting Abstracts* **45**, 295a.

SCHNETKAMP, P. P. M. (1985*a*). Ca^{2+} buffer sites in intact bovine rod outer segments: introduction to a novel probe to measure ionic permeabilities in suspensions of small particles. *Journal of Membrane Biology* (in the Press).

SCHNETKAMP, P. P. M. (1985*b*). Ionic permeabilities of the plasma membrane of isolated intact bovine rod outer segments as studied with a novel optical probe. *Journal of Membrane Biology* (in the Press).

SCHNETKAMP, P. P. M. & DAEMEN, F. J. M. (1982). Isolation and characterization of osmotically sealed bovine rod outer segments. *Methods in Enzymology* **81**, 110–116.

SCHNETKAMP, P. P. M., DAEMEN, F. J. M. & BONTING, S. L. (1977). Calcium accumulation in cattle rod outer segments: evidence for a calcium–sodium exchange carrier in the rod sac membrane. *Biochimica et biophysica acta* **468**, 259–270.

SCHNETKAMP, P. P. M. & KAUPP, U. B. (1985). Calcium–Hydrogen exchange in isolated bovine rod outer segments. *Biochemistry* **24**, 723–727.

SCHNETKAMP, P. P. M., KLOMPMAKERS, A. A. & DAEMEN, F. J. M. (1979). The isolation of stable cattle rod outer segments with an intact plasma membrane. *Biochemica et biophysica acta* **552**, 379–389.

SCHRÖDER, W. H. & FAIN, G. L. (1984). Light-dependent calcium release from photoreceptors measured by laser micro mass analysis. *Nature* **309**, 268–270.

SLAUGHTER, R. S., SUTKO, J. L. & REEVES, J. P. (1983). Equilibrium calcium–calcium exchange in cardiac sarcolemmal vesicles. *Journal of Biological Chemistry* **258**, 3183–3190.

TROSPER, T. L. & PHILIPSON, K. D. (1983). Effects of divalent and trivalent cations on Na^{+}–Ca^{2+} exchange in cardiac sarcolemmal vesicles. *Biochimica et biophysica acta* **731**, 63–68.

WOODRUFF, M. L., FAIN, G. L. & BASTIAN, B. L. (1982). Light-dependent ion influx into toad photoreceptors. *Journal of General Physiology* **80**, 517–536.

YAU, K.-W., MCNAUGHTON, P. A. & HODGKIN, A. L. (1981). Effect of ions on the light-sensitive current in retinal rods. *Nature* **292**, 502–505.

YAU, K.-W. & NAKATANI, K. (1984*a*). Cation selectivity of light-sensitive conductance in retinal rods. *Nature* **309**, 352–354.

YAU, K.-W. & NAKATANI, K. (1984*b*). Electrogenic Na–Ca exchange in retinal rod outer segment. *Nature* **311**, 661–663.

YOSHIKAMI, S., GEORGE, J. S. & HAGINS, W. A. (1980). Light-induced calcium fluxes from outer segment layer of vertebrate retinas. *Nature* **286**, 395–398.

YOSHIKAMI, S. & HAGINS, W. A. (1971). Light, calcium and the photocurrent in vertebrate rods and cones. *Biophysical Society Annual Meeting Abstracts* **11**, 47a.

ZUCKERMAN, R., BUZDYGON, B. & LIEBMAN, P. (1984). Photolyzed rhodopsin catalyzes ATP/ADP exchange on the 48 kilodalton protein of retinal rod outer segments. *Biophysical Society Annual Meeting Abstracts* **45**, 292a.