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ELEMENTAL DISTRIBUTION IN *RANA PIPIENS* RETINAL RODS: QUANTITATIVE ELECTRON PROBE ANALYSIS

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SUMMARY

1. The composition of dark-adapted and illuminated retinal rod outer and inner segments and mitochondria was determined with electron probe X-ray micro-analysis of cryosections.

2. The concentration of Ca in the outer segment was 0·4 mmol/kg dry wt. (0·1 Ca/rhodopsin) and did not measurably change upon illumination with saturating light for 5 min.

3. The non-mitochondrial regions of the inner segment contained the highest concentrations (up to 13 mmol/kg dry wt.) of Ca in rods; these regions probably represent the endoplasmic reticulum.

4. The equilibrium potentials estimated from the measured elemental concentrations and the known water content of dark-adapted outer segments were (mV): $E_{Na} = +17$, $E_K = -83$, $E_{Cl} = -27$. The respective values in the inner segment were: $E_{Na} = +20$, $E_K = -89$, $E_{Cl} = -26$. The above values were obtained in frog rods bathed in 0·18 mM-Ca Ringer solution. In the outer segment of toad rods bathed in 1·8 mM-Ca Ringer, $E_{Na} = +33$ mV.

5. The Mg content of the rods was high. The (computed) concentration in the dark-adapted retinae was 11 mM in the outer segment and 24 mM in the inner segment. Illumination caused a reduction in Mg to 9 mM (outer segment) and 16 mM (inner segment).

6. Illumination caused a highly significant reduction in Na and Cl concentrations, and an increase in K concentration in both outer and inner segments.

7. Exposure to Na-free (choline Ringer) solution resulted in reduction in Na to just-detectable levels (3 ± 1 mmol/kg dry wt.) in the outer segment and to 5 ± 1 mM in the inner segment. This was associated with a significant loss of Cl and decrease in E_{Cl} to -50 mV.

8. The low Na content of the outer segment in the Na-depleted rods is not compatible with an extracellular concentration (105 mM) of inexchangeable Na in the intradiskal space.

9. Mitochondrial Na and Mg paralleled the changes in the cytoplasmic concentra-

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tions: both mitochondrial Na and Mg were significantly decreased in illuminated, compared to dark-adapted rods. There was no detectable Ca ($0 \pm 0{\cdot}2$ mmol/kg dry wt.) in mitochondria of dark-adapted rods containing high concentrations of Na; mitochondrial Ca was slightly higher ($0{\cdot}5 \pm 0{\cdot}2$ mmol/kg dry wt.) in the mitochondria that contained low Na following illumination.

INTRODUCTION

The perception of light by the photoreceptor cells in the vertebrate retina is signalled by hyperpolarization resulting from a light-induced reduction of a 'dark current' (Penn & Hagins, 1969; Hagins, Penn & Yoshikami, 1970). The dark current is generally thought to be carried by an inward Na flux into the rod or cone outer segment, and, according to one of the major hypotheses of visual transduction (for review, see Hagins, 1972), it is turned off by the light-induced release of Ca ions from disks located in the rod outer segments. This hypothesis is strongly dependent on the content and exchangeability of Ca in the disk lumen (intradiskal space), while the ionic composition (Na, K, Cl) determines the electromotive forces that drive the dark current and other, voltage-activated currents. Previous attempts to measure these quantities have led to variable, and often conflicting results (Liebman, 1975; Szuts, 1980; Kaupp & Schnetkamp, 1982), with reported Ca contents varying from 0·1 Ca/rhodopsin to 12 Ca/rhodopsin and K concentrations of 28 mM (Hagins & Yoshikami, 1975) to 96 mM (Owen & Torre, 1981; Torre, 1982). Information about Na and Cl concentrations is even more limited.

The development of quantitative electron probe analysis in conjunction with rapid freezing and cryoultramicrotomy now permits the measurement of *in situ* concentrations of less than 1 mmol/kg dry wt. (for Ca) at spatial resolutions down to approximately 10 nm (for review, see Somlyo & Shuman, 1982; Hall & Gupta, 1983). Therefore, we have used these methods to determine the elemental composition of the major cellular compartments in dark-adapted and illuminated frog retinal rods. We show here that the Ca content of the disks is at the lower limits of compatibility with the intradiskal Ca-release hypothesis, and that the total Ca content of the rod outer segment does not change upon prolonged illumination. We also show that the highest concentration of Ca in rods is in the region of the endoplasmic reticulum in the rod inner segment, contrary to a recent report (Schroder & Fain, 1984) claiming that the Ca content is high in the outer segment and could not be detected by laser mass spectroscopy in the inner segment. The possible reasons for this discrepancy will be discussed.

A reduction in rod Mg after illumination was an unexpected result of this study, in which we also present the first direct measurements of a light-induced change in cytoplasmic K/Na ratio, Cl concentration and mitochondrial Na content. Finally, a very small difference in mitochondrial Ca between dark-adapted and illuminated rods is consistent with *in situ* Na-induced mitochondrial Ca release (Carafoli & Crompton, 1978).

A preliminary account of some of these results has been presented at the International Congress of Biophysics (Walz & Somlyo, 1984).

METHODS

Leopard frogs (*Rana pipiens*) and toads (*Bufo marinus*) (from West Jersey Biological, Wenonah, NJ, U.S.A.) were kept in an aquarium, at ambient laboratory illumination. The animals were kept in complete darkness for 8–12 h before the experiments. Decapitation and all subsequent manipulations until cryofixation were done under infra-red illumination with the aid of an infra-red-to-visible light image converter. The retinae were isolated in frog Ringer solution (pH 7·7) containing (mM): NaCl, 100; $NaHCO_3$, 5; KCl, 3·5; $CaCl_2$, 0·18; $MgCl_2$, 2·0; glucose, 20·0; Tris, 20·0 (bubbled with 95% O_2, 5% CO_2). Na-free frog Ringer solution was prepared by substituting 105 mM-choline chloride for NaCl and $NaHCO_3$. This solution was oxygenated with pure O_2. 0·18 mM-Ca solutions were used for the majority of experiments to replicate, as closely as possible, the experimental conditions described (0·2 mM-external Ca) in Gold & Korenbrot's (1980) experiments. The external Ca concentration used by George & Hagins (1983) for the demonstration of light-induced Ca release was 10–50 μM, and even this low Ca did not cause a loss of the light-induced Ca signal detectable by a Ca electrode. A change of free Ca from 1 to 0·18 mM would give rise to an approximately twofold increase in the dark current (Fig. 12, Hodgkin, MacNaughton, Nunn & Yau, 1984). For comparison, in solutions having a Ca activity, a_{Ca}, of 10^{-3}, the Na content of rat rods is 40 ± 5 mM (Yoshikami, Foster & Hagins, 1983). Two experiments (one frog and one toad) were done with a 1·8 mM-$CaCl_2$ frog Ringer solution, to test whether or not an increase of Ca in the Ringer solution affects the Ca concentration in the rod outer segment and whether toad rod outer segments contain higher concentrations of Ca (cf. Schroder & Fain, 1984). All dissections were done in the same solutions as used for the respective experiments (i.e. 0·18 mM-Ca, 1·8 mM-Ca, Na-free).

The isolated retinae remained in frog Ringer solution for about 1 h at room temperature (21–23 °C) until cryofixation. Each retina was bisected. One half was frozen in the dark, then a tungsten filament light source delivering about $1{\cdot}5 \times 10^{14}$ photons/cm^2.s was turned on, and the remaining half of the retina was frozen after 5 min of illumination.

For freezing, the retinae were drawn on small pieces of Millipore filter (3 × 3 mm), cautiously removed from the bath, slightly blotted from below and rapidly frozen by plunging the mounted retina into Freon 22, supercooled to −165 °C with liquid N_2 (Somlyo & Silcox, 1979; Somlyo, Shuman, 1979; Karp, Silcox & Somlyo, 1982).

Cryosections (100–200 nm thick) were cut on a LKB cryoultramicrotome, modified to maintain an ambient temperature of −130 °C in the cryochamber, a specimen temperature of −110 °C and a glass knife temperature of −100 °C (Somlyo *et al.* 1977). The cryosections were dried at 10^{-5} Torr while they were still below −80 °C, and carbon coated (Somlyo *et al.* 1977; Somlyo, Somlyo & Shuman, 1979; Karp, Silcox & Somlyo, 1982).

Electron probe micro-analysis was done on a liquid-N_2-cooled specimen holder at −100 °C (with partial water pressure in the column of $\sim 3{\cdot}5 \times 10^{-9}$ torr) in a Philips EM400T electron microscope operated at 80 kV with an LaB_6 gun and equipped with a 30 mm^2 Kevex energy dispersive detector, connected to a Kevex multichannel analyser and PDP 11/34 computer (Kitazawa, Somlyo & Somlyo, 1984). In order to obtain the precision necessary for the Na and Ca measurements, each individual analysis was performed for 500 s. Probe diameters were 250–500 nm and currents were 2–3 nA.

The principle (Hall, 1971) of the method used for quantitation of elements with atomic numbers ~10–20 in biological thin sections and details of the methods used for calibration, obtaining the number of characteristic and continuum X-ray counts, and estimates of statistical and systematic errors of the method have been published elsewhere (for references, see Kitazawa *et al.* 1984). For the analyses of mitochondria and endoplasmic reticulum, small (~50–80 nm) and astigmated electron beams were used.

RESULTS

An electron micrograph of an unstained cryosection of rapidly frozen retinal rods is shown in Pl. 1 and illustrates the preparations analysed. The measured elemental concentrations in mmol/kg dry wt. were normally distributed and are shown in Table 1

for rod outer segments, rod inner segments and mitochondria of dark-adapted and illuminated retinae. The concentrations of Na, K, Mg, Cl, K and Ca are higher, on a mmol/kg dry wt. basis, in the inner segment than in the outer segment. However, the fraction of dry mass/volume is considerably higher in the outer segment than in the inner segment, a fact that is reflected in the different number of continuum counts obtained with identical probe parameters from these two regions (Table 1). Therefore, comparison of inner and outer segment concentrations of solutes on the basis of amount/dry wt. is not meaningful, because the less-hydrated region contains a relatively higher amount of dry solid/unit microvolume analysed. However, the relative degree of hydration of the inner segment can be calculated from the ratio of X-ray continuum counts (Somlyo *et al.* 1979) obtained from the outer and inner segment, respectively. Since the rod outer segment water content has been determined very accurately by independent methods to be 57% (Sidman, 1957; Blaurock & Wilkins, 1969), rod inner segment water can be computed as

$$H_2O_{r.i.s.} = 100 \times 1 - 0{\cdot}43(cont_{r.i.s.}/cont_{r.o.s.}),$$

where $cont_{r.i.s.}$ and $cont_{r.o.s.}$ are, respectively, the continuum counts originating from the inner segments and outer segments. The percentage of water in the inner segment was calculated in this manner, and is given in Table 2, together with the calculated concentrations of Na, K, and Cl in cell water and the calculated Nernst equilibrium potentials. The calculations have been done under the assumption that these ionic species are in solution.

The concentrations of P and S were not changed upon illumination, and presumably represent largely P bound to phospholipids (outer and inner segments), ribosomes (inner segments), adenyl and guanyl nucleotides, S-containing proteins and, possibly, taurine.

Rod Ca and Mg

The very low Ca content of the rod outer segment ($0{\cdot}4 \pm 0{\cdot}1$ mmol/kg dry wt.) did not change detectably during prolonged, intense illumination. The highest concentrations of Ca ($3{\cdot}8 \pm 0{\cdot}4$ mmol/kg dry wt.) measured with large-diameter probes in rods were in the inner segments. Analyses with focused and astigmated probes (~ 75 nm diameter) in the myoid region revealed localized areas containing high concentrations (13 ± 3 mmol/kg dry wt., mean $\pm$ S.D., $n = 7$) of Ca.

It is possible to average several spectra in the computer, and to subtract a characteristic X-ray peak that overlaps another, such as the K K_β peak that overlaps the Ca K_α peak. Fig. 1 *A* shows an averaged raw spectrum of frog outer segments, and Fig. 1 *B* and *C* the same spectrum at higher gain, after the K K_α and K_β peaks were subtracted by the computer to display the Ca K_α peak. For comparison, a similarly processed spectrum of inner segments is superimposed. The difference between the Ca concentrations in the two regions, as well as the ability of the fitting routine to measure Ca accurately even in the presence of K, are thus graphically demonstrated.

Mitochondrial Ca was virtually zero in dark-adapted rods and increased very slightly upon illumination ($0{\cdot}4 \pm 0{\cdot}2$ mmol/kg dry wt.). These mitochondrial Ca concentrations are at the detectable limits (2–3 $\times$ S.E. of mean = 0·4–0·6 mmol/kg dry wt.) with the parameters of analysis used in this study, and the difference is just at

TABLE 1. Elemental composition of frog retinal rods in 0·18 mM-Ca Ringer solution (mean ± S.E. of mean, mmol/kg dry wt.)

	*n**	Na	Mg	P	S	Cl	K	Ca	Continuum†
Dark outer segments	81	70 ± 3	14 ± 0·5	597 ± 11	408 ± 8	55 ± 2	127 ± 2	0·40 ± 0·1	20968
Light outer segments	44	29 ± 2	12 ± 0·7	598 ± 13	418 ± 8	45 ± 2	174 ± 5	0·4 ± 0·2	18472
Dark inner segments	38	127 ± 5	66 ± 4	634 ± 22	368 ± 11	120 ± 5	326 ± 10	3·8 ± 0·4	13292
Light inner segments	27	46 ± 5	50 ± 2	595 ± 17	374 ± 12	104 ± 5	414 ± 14	3·6 ± 0·6	11220
Dark mitochondria	44	61 ± 2	43 ± 1	550 ± 12	413 ± 12	44 ± 2	253 ± 8	0·0 ± 0·2	23463
Light mitochondria	40	21 ± 2	36 ± 1	501 ± 10	397 ± 7	35 ± 2	257 ± 8	0·4 ± 0·2	19321

* Number of cells analysed (nine frogs). † Continuum counts are given as the number of counts.

TABLE 2. Na, K, Cl and Mg concentrations* in frog retinal rods in 0·18 mM-Ca Ringer solution (mean ± S.E. of mean, mmol/l cell H_2O)† and equilibrium potentials for Na, K and Cl (mV)

	n	H_2O† (%)	Na	K	Cl	Mg	E_{Na}	E_K	E_{Cl}
Frog Ringer solution	—	—	105	3·5	122·1	2·0	—	—	—
Dark outer segments	81	57	53 ± 2	96 ± 2	41 ± 1	11 ± 0·3	+17	−83	−27
Light outer segments	44	57	22 ± 1	131 ± 3	34 ± 2	9 ± 1·0	+39	−91	−32
Dark inner segments	38	73	47 ± 2	121 ± 4	44 ± 2	24 ± 1	+20	−89	−26
Light inner segments	27	74	16 ± 2	145 ± 5	37 ± 2	16 ± 1	+47	−94	−30

* Computed under the assumption that all Na, K and Cl are in solution. This assumption is probably not valid for Mg.

† See Methods section: the average concentrations and continuum counts from Table 1 were used.

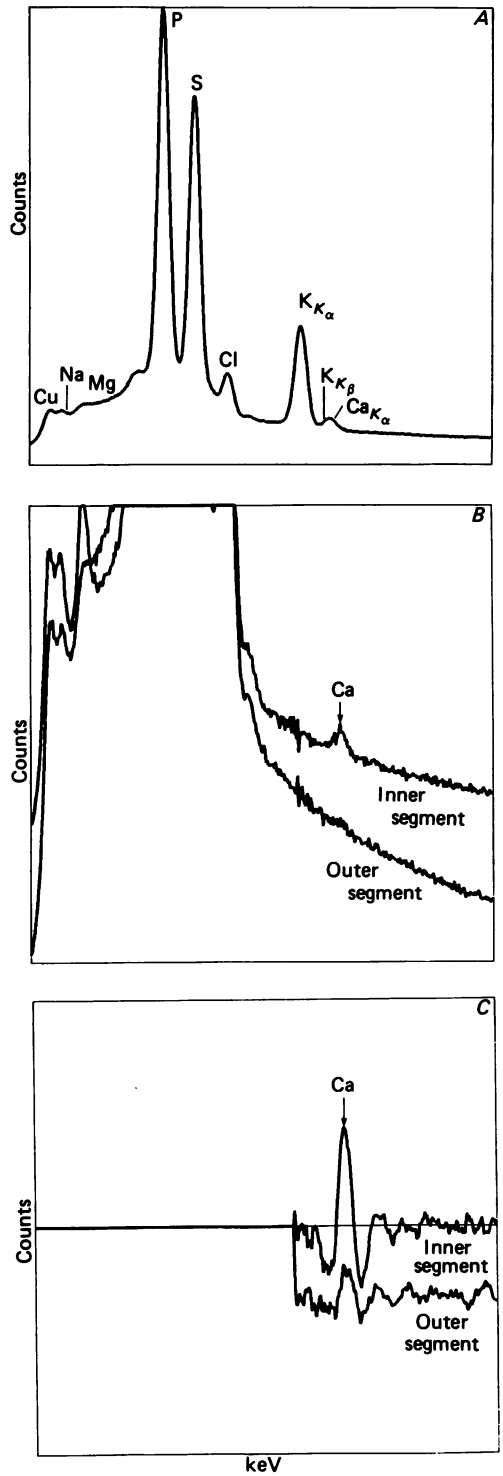

Fig. 1. For legend see opposite.

the borderline of significance ($P \approx 0.05$). The difference is just statistically significant ($P < 0.05$) if we include the additional twelve mitochondrial analyses of rods exposed to Na-free solutions, and may be indicative of Na-induced Ca release (Carafoli & Crompton, 1978).

The reduction of the Mg content (by 14 % in outer segment, 16 % in mitochondria and 24 % in inner segment) of the rods upon illumination was a rather unexpected finding. These changes were all statistically significant ($P < 0.05$ for outer segment; $P < 0.01$ for inner segment; $P < 0.001$ for mitochondria).

Dissecting and incubating the retina in a frog Ringer solution containing 1·8 instead of 0·18 mM-Ca did not result in a higher Ca content in the dark-adapted outer segments: the Ca concentration in the outer segment was 0.2 ± 1.5 mmol/kg dry wt. (mean $\pm$ S.D., $n = 4$). The concentrations (mmol/kg dry wt. $\pm$ S.D.) of monovalent ions were: Na, 53 ± 4.4; K, 120 ± 13.9; Cl, 48 ± 7.4. It is possible that the somewhat lower Na values in 1·8 mM-Ca Ringer solution than in 0·18 mM-Ca Ringer solution (Table 1) are due to the higher level of dark current in the solutions containing less Ca, but the possible significance of the difference in this small sample was not further explored.

In dark-adapted *toad* retinae incubated in *1·8 mM-Ca* Ringer solution, the Ca concentration in the outer segment was 0.3 ± 0.1 mmol/kg dry wt. (mean $\pm$ S.E. of mean, $n = 45$), not different from the Ca content of frog outer segments in 0·18 mM-Ca Ringer solution. The Ca concentration in the inner segment ($n = 20$) was 3.3 ± 0.4 mmol/kg dry wt. The monovalent ion concentrations (mmol/kg dry wt. $\pm$ S.E. of mean) were, in the outer segment: Na, 38 ± 3.8; K, 157 ± 3.8; Cl, 40 ± 3.4; in the inner segment: Na, 79 ± 9.5; K, 408 ± 12.5; Cl, 100 ± 4.6.

Na, K and Cl concentrations and the effects of Na-free solution

The concentration (in water) of Na was slightly higher and that of K slightly lower in the outer than in the inner segment of both dark-adapted and illuminated rods. This results in slightly different equilibrium potentials for these ions in the outer and inner segment, respectively. There was a very significant reduction in cell Na (Tables 1 and 2) and increase in K after prolonged illumination. These changes were accompanied by a small reduction ($P < 0.01$ for outer segment; $P < 0.05$ for inner segment) in cell Cl.

There was a marked reduction in mitochondrial Na content (by approximately $\frac{2}{3}$ of the content in the dark) upon illumination, paralleling the changes in cytoplasmic Na concentration.

Fig. 1. The Ca X-ray peaks after subtraction of the adjacent K X-ray peaks. *A*, average 'raw' spectrum of forty-seven rod outer segments: full scale = 5093 counts. *B*, same spectrum (outer segment) as *A*, displayed at higher gain (full scale = 512 counts), after subtraction of K peaks by the computer fitting routine. The upper trace (inner segment) is of a similarly processed averaged spectrum of twenty-six segments. The two spectra are displaced relative to each other for display. The Ca contents in these averaged spectra were (in mmol/kg dry wt. $\pm$ S.E. of mean) 0.6 ± 0.13 (outer segment) and 4.0 ± 0.26 (inner segment, $n = 26$). Difference in base-line slopes is due to variable contributions of the Cu grid to the background that were not subtracted. *C*, same spectra (3200–5120 eV) as *B*, after digital filtering (Shuman, Somlyo & Somlyo, 1976) to remove background. Ca peaks at 3·69 keV are evident, above noise level, in both spectra.

The Na content of outer and inner segments and of mitochondria was markedly reduced after 30–60 min in Na-free solution. The Na content of illuminated (2 or 10 min before freezing) or dark-adapted retinae was not significantly different and, therefore, Tables 3 and 4 contain the combined results from all these experiments. There was also a highly significant ($P < 0.001$) reduction in the Cl content of the rods

TABLE 3. Elemental composition of frog retinal rods (mean ± S.E. of mean, mmol/kg dry wt.) incubated in Na-free, 0·18 mM-Ca Ringer solution

	n*	Na	Mg	P	S	Cl	K	Ca	Continuum†
Outer segment	34	3±1	13±1	543±1	406±8	22±1	135±3	0·4±0·2	19847
Inner segment	18	17±2	75±4	671±30	428±15	55±3	363±17	2·8±0·5	10958
Mitochondria	9	7±2	31±2	485±12	430±12	22±4	222±12	0·6±0·4	19795

* Number of cells analysed (three frogs). † Number of X-ray continuum counts.

TABLE 4. Na, K, Cl concentrations in mmol/l cell H_2O* (mean ± S.E. of mean) and equilibrium potentials (mV) from frog retinal rods after incubation in Na-free, 0·18 mM-Ca Ringer solution

	n	H_2O (%)	Na	K	Cl	E_K	E_{Cl}
Outer segments	34	57	2±1	102±2	17±1	−85	−50
Inner segments	18	76	5±1	115±5	17±1	−88	−50

* See Methods section: calculated under the assumption that these ionic species are all in solution.

in Na-free solution. The somewhat higher Mg content of the inner segment was not statistically significantly different in this small sample, compared to the values in normal solution.

The Ca content of the outer segments was not affected by the Na-free solutions (Table 3). However, as electron probe analysis measures total, rather than free, Ca, our results shed no light on the effects of Na/Ca exchange on free rod Ca.

DISCUSSION

Rod Ca and Mg

The Ca content of the frog rod outer segments (0·4 mmol/kg dry wt.) is equivalent to 0·3 mmol/l cell H_2O and to 0·1 Ca/rhodopsin (rhodopsin concentration abut 3 mM; Liebman, 1975), and comparable to the lowest chemically measured values (Liebman, 1974; Szuts, 1980, 1981; Szuts & Cone, 1977). It is about tenfold less than the value (3·8 mM) reported by Hagins & Yoshikami (1975) for *Rana pipiens* on the basis of electron microprobe measurements of frozen dried and cleaved bulk retinae, but these authors noted Ca contamination of their samples by laboratory dust (Hagins, Robinson & Yoshikami, 1975). If all the Ca detected by us in the outer segment of dark-adapted retinae were to be concentrated in the approximately 2 nm (Chabre & Cavaggioni, 1975) wide intradiskal space, this would contain about 2·6 mmol Ca/l or $1·2 \times 10^5$ Ca atoms/disk.

We did not find a decrease in the Ca content of the outer segment following prolonged, intense illumination. The amount of Ca (about 10^7 Ca/outer segment) released by a saturating flash (Gold & Korenbrot, 1981) is only about 10 % of the total measured by us, and below the sensitivity of the measurements. The light-induced

release of Ca from rods (Gold & Korenbrot, 1980, 1981; Yoshikami, George & Hagins, 1980; George & Hagins, 1983) may also be due to the release of Ca from the endoplasmic reticulum (see below) in the inner segment. Gold & Korenbrot (1980) have rejected this possibility on the basis of diffusion times of Ca estimated with the Ca-selective electrode, but it is consistent with the faster current response when toad rods are locally illuminated at the basal than at the distal end (Baylor, Lamb & Yau, 1979), and with the abolition of the light response on breaking off the outer segment (Baylor *et al.* 1979). It is also possible that the endoplasmic reticulum merely functions as a source replenishing Ca released from the outer segment by illumination.

The highest concentrations of Ca in frog rods were in the inner segment. In this region, there were localized sites (*not* mitochondria) having distinctly high concentrations of Ca. We consider these sites to be cisternae of the endoplasmic reticulum. This finding suggests that in retinal rods, as in other non-muscle cells (for review, Martonosi, 1983; Somlyo, 1984) including photoreceptors (Ungar, Piscopo & Holtzman, 1980; Walz, 1982*a*, *b*) a large proportion of cell Ca is localized within the endoplasmic reticulum. Active Ca accumulation by the endoplasmic reticulum has been demonstrated in saponin-treated frog rods (Ungar *et al.* 1980).

A laser micromass analysis study (Schroder & Fain, 1984) published after this manuscript had been submitted resulted in conclusions completely opposite to ours, concerning the distribution and concentration of Ca in toad retinal rods. These authors found a high concentration of Ca in the outer segment. The preparatory method used in their study was freeze-substitution, followed by plastic embedment and flotation of the embedded sections on water. We attribute the difference between their results and ours to the elemental losses and translocation entailed by their preparatory method and to the very serious problems of accurate quantitation with laser micromass analysis (for review, Hall & Gupta, 1983). The concentration of Ca measured in rod outer segments with electron probe micro-analysis of cryosections of toad retina incubated in 1·8 mM-Ca solution is similar to that found in frog rods, and an order of magnitude lower than reported by Schroder & Fain (1984). The Ca content of the outer segments was similarly low in retinae dissected and incubated in Na-free solutions (Table 3), excluding the (unlikely) possibility of Ca loss during dissection due to Na/Ca exchange. Since Schroder & Fain (1984) found no detectable Ca by laser micromass analysis in the inner segment containing endoplasmic reticulum, where we observed the highest concentration of Ca in rods, the discrepancy between their results and ours is clearly not due to loss of Ca from our preparations or to insufficient sensitivity of electron probe micro-analysis.

The high Mg concentration (11 mM, if normalized to outer segment H_2O) is considerably in excess of the ATP concentration (3 mM, Hagins, 1972) and, unless Mg is bound to phospholipids, suggests a relatively high free Mg in rods.

Rather surprisingly, the rod Mg content was significantly reduced after prolonged illumination. This finding may be related to the observation of Hodgkin, McNaughton, Nunn & Yau (1984), who found, in rods in Na-free, Ca-free (EGTA) solutions, a dark current carried by Mg ions. The reduction in rod Mg by light (present study) could be explained if part of the dark current were normally carried by Mg ions. This interpretation also implies the presence of a mechanism that can extrude cellular Mg ions.

The reduction in mitochondrial Mg accompanying the cytoplasmic changes caused

by illumination is equivalent to a mitochondrial transport rate of approximately 0·02–0·03 nmol/mg protein . s. This is abut 100-fold lower than the maximal active Mg transport found in mitochondria isolated from smooth muscle (Sloane, Scarpa & Somlyo, 1978).

Cytoplasmic Na, K and Cl in dark and light and the effect of Na-free solutions

The concentration of Na was slightly higher, and that of K slightly lower, in the outer than in the inner segment of both dark-adapted and illuminated rods. This is consistent with the dark current representing the flow of Na ions through the plasma membrane of the rod outer segment to the inner segment and extrusion from the latter by the Na–K-ATPase-mediated Na pump (for review, see Owen & Torre, 1981). The resulting small (3–6 mV) differences between the K equilibrium potentials (Table 2) are presumably distributed over the length of the rod outer–inner segment distance and unlikely to cause a measurable difference in membrane potential.

The Na content of dark-adapted (frog) outer segments (70 mmol/kg dry wt.) in 0·18 mM-Ca Ringer was significantly higher than the Na content (38 mmol/kg dry wt.) of (toad) outer segments in 1·8 mM-Ca Ringer solution. This presumably reflects the increased dark current in low-Ca solution. The Na equilibrium potential was significantly more positive (+17 mV) than the dark-current reversal potential (about 0 mV; Owen & Torre, 1981) even in the (frog) rods bathed in the 0·18 mM-Ca Ringer solution, and it was +33 mV in the toad rod outer segments bathed in 1·8 mM-Ca Ringer. Therefore, *in vivo* the Na reversal potential probably significantly exceeds the dark-current reversal potential, and this finding is compatible with the limited selectivity of the dark-current channels (Owen & Torre, 1981).

The marked reduction in Na concentration (~ 58 % in the outer segment; 66 % in the inner segment), accompanied by an increase in K concentration, following illumination was presumably due to the continued operation of the Na pump in the inner segment, while the inward Na current was shut off by light. The magnitude of these changes during 5 min illumination is in agreement with calculations suggesting that the dark current (0·2 mM/s; Torre, 1982) is large enough to turn over all the cations in frog rods in about 8 min (Hagins *et al.* 1970).

The Cl equilibrium potential in the dark (−27 mV in frog in 0·18 mM-Ca, −35 mV in toad in 1·8 mM-Ca) is more positive than the probable resting potential (−46 mV in toad rods in 1·8 mM-Ca Ringer solution; Bastian & Fain, 1981), and suggests that Cl may not be completely passively distributed in rods. The reduction in cytoplasmic Cl both after illumination (Table 2) and in Na-free Ringer solution (Table 4) suggests the possibility of an inward Cl–Na co-transport mechanism.

The Na content of rods was markedly reduced, in some cells below detectable (2–3 mM) levels, after 30–60 min exposure to Na-free (choline Ringer) solution (Tables 3 and 4). This suggests that the intradiskal space cannot have an extracellular composition, if the disk membrane is truly impermeable to NaCl, as indicated by osmotic responses of rods (Chabre & Cavaggioni, 1975; Uhl, Kuras, Anderson & Abrahamson, 1980). An intradiskal space constituting 6 % of outer segment volume and containing 105 mM-Na would give rise to an outer segment Na content of approximately 15 mmol/kg dry wt., very much greater than the measured value (3 mmol/kg dry wt., Table 3). The loss of cytoplasmic Na into the Na-free solutions

might also account for failures to observe reversal of the receptor potential in (Mg-containing) Na-free solutions (Brown & Pinto, 1974).

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REFERENCES

Bastian, B. L. & Fain, G. L. (1982). The effects of low calcium and background light on the sensitivity of toad rods. *Journal of Physiology* **330**, 307–329.

Baylor, D. A., Lamb, T. D. & Yau, K.-W. (1979). The membrane current of single rod outer segments. *Journal of Physiology* **288**, 589–611.

Blaurock, A. E. & Wilkins, M. H. F. (1969). Structure of frog photoreceptor membranes. *Nature* **223**, 906–909.

Brown, J. E. & Pinto, L. H. (1974). Ionic mechanism for the photoreceptor potential of the retina of *Bufo marinus*. *Journal of Physiology* **236**, 575–591.

Carafoli, E. & Crompton, M. (1978). The regulation of intracellular calcium by mitochondria. In *Annals of the New York Academy of Sciences*, vol. 307, *Calcium Transport and Cell Function*, ed. Scarpa, A. & Carafoli, E., pp. 269–284. New York: New York Academy of Sciences.

Chabre, M. & Cavaggioni, A. (1975). X-ray diffraction studies of retinal rods. II. Light effect on the osmotic properties. *Biochimica et biophysica acta* **382**, 336–343.

George, J. S. & Hagins, W. A. (1983). Control of Ca^{2+} in rod outer segment disks by light and cyclic GMP. *Nature* **303**, 344–348.

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

Gold, G. H. & Korenbrot, I. J. (1981). The regulation of calcium in the intact retinal rod: A study of light-induced calcium release by the outer segment. In *Current Topics in Membranes and Transport*, vol. 15, *Molecular Mechanisms of Photoreceptor Transduction*, ed. Miller, W. H., pp. 307–330. New York: Academic Press, Inc.

Hagins, W. A. (1972). The visual process: Excitatory mechanisms in the primary receptor cells. *Annual Review of Biophysics and Bioengineering* **1**, 131–158.

Hagins, W. A., Penn, R. D. & Yoshikami, S. (1970). Dark current and photocurrent in retinal rods. *Biophysical Journal* **10**, 380–412.

Hagins, W. A., Robinson, W. A. & Yoshikami, S. (1975). Ionic aspects of excitation in rod outer segments. In *Energy Transformation in Biological Systems, CIBA Foundation Symposium*, **31**, 169–189.

Hagins, W. A. & Yoshikami, S. (1975). Ionic mechanisms in excitation of photoreceptors. *Annals of the New York Academy of Sciences* **264**, 314–325.

Hall, T. A. (1971). The microprobe assay of chemical elements. In *Physical Techniques in Biological Research*, vol. 1A, ed. Oster, G., pp. 157–275. New York and London: Academic Press.

Hall, T. A. & Gupta, B. L. (1983). The localization and assay of chemical elements by microprobe methods. *Quarterly Review of Biophysics* **16**, 279–339.

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.

Karp, R. D., Silcox, J. & Somlyo, A. V. (1982). Cryoultramicrotomy: Evidence against melting and the use of a low temperature cement for specimen orientation. *Journal of Microscopy* **125**, 157–165.

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

Kitazawa, T., Somlyo, A. P. & Somlyo, A. V. (1984). The effects of valinomycin on ion movements across the sarcoplasmic reticulum in frog muscle. *Journal of Physiology* **350**, 253–268.

Liebman, P. A. (1974). Light-dependent Ca^{++} content of rod outer segment disk membranes. *Investigations in Ophthalmology* **13**, 700–701.

LIEBMAN, P. A. (1975). Birefringence, dichroism and rod outer segment structure. In *Photoreceptor Optics*, ed. SNYDER, A. W. & MENZEL, R., pp. 199–214. Berlin: Springer.

MARTONOSI, A. N. (1983). The regulation of cytoplasmic Ca^{2+} concentration in muscle and nonmuscle cells. In *Muscle and Nonmuscle Motility*, vol. 1, ed. STRACHER, A., pp. 233–357. New York: Academic Press.

OWEN, W. G. & TORRE, V. (1981). Ionic studies of vertebrate rods. In *Current Topics in Membranes and Transport*, vol. 15, *Molecular Mechanisms of Photoreceptor Transduction*, ed. MILLER, W. H., pp. 33–57. New York: Academic Press.

PENN, R. D. & HAGINS, W. A. (1969). Signal transmission along retinal rods and the origin of the electroretinographic *a*-wave. *Nature* **223**, 201–205.

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

SHUMAN, H., SOMLYO, A. V. & SOMLYO, A. P. (1976). Quantitative electron probe microanalysis of biological thin sections: Methods and validity. *Ultramicroscopy* **1**, 317–339.

SIDMAN, R. L. (1957). The structure and concentration of solids in photoreceptor cells studied by refractometry and interference microscopy. *Journal of Biophysics, Biochemistry and Cytology* **3**, 15–33.

SLOANE, B. F., SCARPA, A. & SOMLYO, A. P. (1978). Vascular smooth muscle mitochondria: Magnesium content and transport. *Archives of Biochemistry and Biophysics* **189**, 409–416.

SOMLYO, A. P. (1984). Cellular site of calcium regulation. *Nature* **309**, 516–517.

SOMLYO, A. P. & SHUMAN, H. (1982). Electron probe and electron energy loss analysis in biology. *Ultramicroscopy* **8**, 219–234.

SOMLYO, A. V., SHUMAN, H. & SOMLYO, A. P. (1977). Elemental distribution in striated muscle and effects of hypertonicity: Electron probe analysis of cryo sections. *Journal of Cell Biology* **74**, 828–857.

SOMLYO, A. V. & SILCOX, J. (1979). Cryoultramicrotomy for electron probe analysis in biology. In *Microbeam Analysis in Biology*, ed. LECHENE, C. & WARNER, R., pp. 535–555. New York: Academic Press.

SOMLYO, A. P., SOMLYO, A. V. & SHUMAN, H. (1979). Electron probe analysis of vascular smooth muscle: Composition of mitochondria, nuclei and cytoplasm. *Journal of Cell Biology* **81**, 316–335.

SZUTS, E. Z. (1980). Calcium flux across disk membranes. *Journal of General Physiology* **76**, 253–286.

SZUTS, E. Z. (1981). Calcium tracer exchange in the rods of excised retinas. In *Current Topics in Membranes and Transport*, vol. 15, *Molecular Mechanisms of Photoreceptor Transduction*, ed. MILLER, W. H., pp. 291–305. New York: Academic Press, Inc.

SZUTS, E. Z. & CONE, R. A. (1977). Calcium content of frog rod outer segments and discs. *Biochimica et biophysica acta* **468**, 194–208.

TORRE, V. (1982). The contribution of the electrogenic sodium–potassium pump to the electrical activity of toad rods. *Journal of Physiology* **333**, 315–341.

UNGAR, F., PISCOPO, I. & HOLTZMAN, E. (1980). Calcium accumulation in intracellular compartments of frog retinal rod photoreceptors. *Brain Research* **205**, 200–206.

UHL, R., KURAS, P. V., ANDERSON, K. & ABRAHAMSON, E. W. (1980). A light scattering study on the ion permeabilities of dark-adapted bovine rod outer segment disc membranes. *Biochimica et biophysica acta* **601**, 462–477.

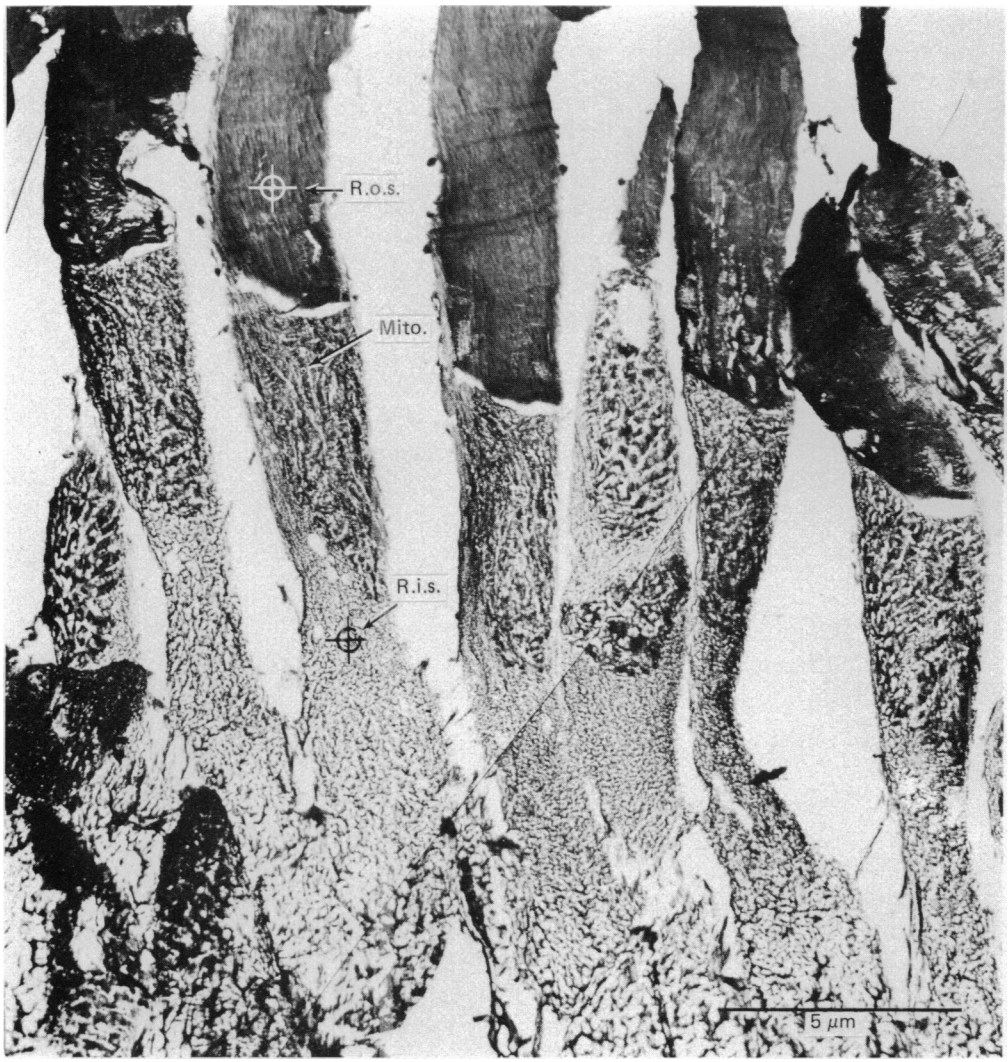
WALZ, B. (1982*a*). Ca^{2+}-sequestering smooth endoplasmic reticulum in an invertebrate photoreceptor. II. Its properties as revealed by microphotometric measurements. *Journal of Cell Biology* **93**, 849–859.

WALZ, B. (1982*b*). Calcium-sequestering smooth endoplasmic reticulum in retinula cells of the blowfly. *Journal of Ultrastructure Research* **81**, 240–248.

WALZ, B. & SOMLYO, A. P. (1984). Quantitative electron probe analysis of frog retinal rods. *Proceedings of the 8th International Congress of Biophysics*, p. 226.

YOSHIKAMI, S., FOSTER, M. C. & HAGINS, W. A. (1983). Ca^{++} regulation, dark current control, and Na^{+} gradient across the plasma membrane of retinal rods. *Biophysical Journal* **41**, 342a.

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


R.o.s.
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EXPLANATION OF PLATE

Electron micrograph of an unstained 100–150 nm thick cryosection of a frog retina used for electron probe analysis. The section plane is almost parallel to the longitudinal axis of the rods, but does not cut them at their meridian. The micrograph shows the base of rod outer segments (r.o.s.) as well as the ellipsoid (mitochondrial-containing region) and myoid region of the inner segments (r.i.s.). The circles labelled r.o.s. and r.i.s. illustrate the sites where analyses were typically performed with probe diameters of 500 nm. For mitochondrial analyses (mito.), the electron beam was focused to $\sim$ 75 nm and usually astigmated to cover one individual mitochondrion.