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Interaction of Bovine Rhodopsin with Calcium Ions

II: Calcium Release in Bovine Rod outer Segments Upon Bleaching*

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Abstract. The calcium content of bovine rod outer segment (ROS) suspensions was determined by flame spectrophotometry to be about 0.2 Ca^{2+} per molecule rhodopsin. After bleaching of rhodopsin, a release of 0.01–0.1 Ca^{2+} per molecule rhodopsin from ROS into the solution was observed. These figures agree with some data in the literature (Appendix).

A measured absorption increase of the Ca^{2+}-indicator phthalein purple (10 °C, 562 nm, pH 9.3) occurs apparently simultaneously with the formation of metarhodopsin II in ROS. This indicates that a light induced Ca^{2+}-release of 12 calcium ions per photoactivated rhodopsin is coupled in time with the formation of metarhodopsin II.

Key words: Rhodopsin – Calcium release – Flash spectrophotometry.

1. Introduction

According to Yoshikami and Hagins (Yoshikami, 1973; Yoshikami and Hagins, 1971, 1976) calcium is situated in the discs of the rod outer segments (ROS) (ca. 2 mM) and in the surrounding cytoplasma (10^{-8} M). During bleaching, the calcium concentration in the cytoplasma would increase to about 10^{-7} M. The calcium would block the sodium channels of the plasma membrane, reduce the sodium ion conductivity and thus transmit a light stimulus to the outer membrane. One important feature of this calcium hypothesis is the prediction of the release of a large number of calcium ions per bleached rhodopsin (at low light intensities about 100–1000 calcium ions; Hagins and Yoshikami, 1974; Cone, 1973). The table in the appendix is an attempt to list many of the experiments, which were undertaken in order to determine the calcium content in ROS and, if possible, to measure a calcium release upon bleaching. Ratios of one calcium ion released per one Rh molecule bleached are mentioned in several reports, the published results are not uniform,

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however. Two series of experiments were designed by us to see whether a change in the calcium concentration occurs upon bleaching of Rh and, if so, to which reaction step it corresponds.

2. Material and Methods

Bovine ROS were prepared in calcium-free physiological saline (137 mM NaCl, 5.36 mM KCl, 1.23 mM $MgCl_2$, 3.75 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, pH 7) as described in Part I of this publication.

The calcium content of ROS was measured using a flame spectrophotometer (Jarell-Ash-82-500 Atom-Absorption Spectrometer). A suspension of freshly prepared ROS was divided into two parts. One part was kept in the dark, the other was fully bleached in the bright light of a lamp at room temperature for 5 min and then in daylight for a further 1.7 h. Both samples were spun down (ca. 50,000 *g*) in the dark and the calcium concentration of the two sediments and of the two supernatants then measured.

Possibly occurring fast changes of the calcium concentration in ROS suspensions following illumination were measured in a flash photometer (see Part I, 2.1) with the aid of a calcium indicator. A metallochromic indicator, phthalein purple, was chosen as a specific indicator for calcium because of the following properties: It shows an acceptable absorption change dependent on the calcium concentration in a pH range below 11.4. [According to our measurements (Nöll, 1974), the transition from metarhodopsin I (MI) to MII can reproducibly be measured only in the pH range of 4—11.4. Other metallochromic indicators are sensitive to changes in ionic concentration especially in alkaline solution]. Furthermore, phthalein purple is stable for at least a few days, i.e., it does not precipitate from solution, and is soluble in water, whereas other indicators have to be dissolved in alcohol.

Metallochromic indicators are pH indicators as well. Due to the existence of a proton uptake in the reaction step MI to MII, Rh solutions show small pH changes upon illumination. To prevent responses of the calcium indicator to these pH changes, the ROS suspensions (2.5×10^{-6} M Rh, 137 mM NaCl, 5.36 mM KCl, 1.23 mM $MgCl_2$) were buffered at pH 9.3 with 0.1 M glycine-NaCl/NaOH buffer. Upon titration with 1 normal NaOH or 1 normal HCl no absorption change of the indicator (without Rh) was observed in the spectrophotometer. Metalphthalein on the contrary began to show a measurable almost linear absorption increase due to titration with calcium at this pH. The addition of ten times 1 µl 0.5 M calcium standard solution to 3.1 ml of the above described physiological saline, containing 0.1 ml of 2.8 mM metalphthalein, resulted in an absorption change of 0.15 per 1 µM of calcium.

The absorption maximum of the indicator (3×10^{-7} M, pH 9.3, 10 °C) lies at 562 nm. Rh also absorbs strongly at this wavelength. Normally, one has to compare the absorption decrease of Rh at 562 nm during the transition MI to MII (Rh alone) to the absorption changes of Rh plus indicator (Rh + I) at 562 nm. But the absorption band of the indicator shields the absorption band of Rh so that in the second case (Rh + I) less Rh is bleached than in the first (Rh alone). To avoid this difficulty, Rh and phthalein purple were put into one cuvette, and into the other (reference)

cuvette 0.25, 1, or 4 mM EGTA was added to buffer the calcium ions. (The higher the concentration of EGTA, the smaller the absorption peak of the indicator at 562 nm. Therefore at high concentrations of EGTA this absorption maximum was always adjusted to the value of Rh plus indicator alone by adding phthalein purple, making sure that the combined absorption spectra of Rh + I and Rh + I + EGTA resulted in the same shape.) Under these conditions titration with 1 μl volumes of 1 mM calcium did not change the absorption maximum of the indicator.

We bleached about 3% rhodopsin in the presence of metalphthalein.

3. Measurements and Results

3.1 Determination of the Calcium Content by Flame Photometry

The calcium content of ROS suspensions prepared in Ca^{2+}-free saline was determined before and after bleaching. The ratio Ca^{2+}/Rh was found to be 0.1—0.2. There was less calcium in the sediment of the centrifuged bleached ROS sample than in the sediment of the centrifuged unbleached sample. The supernatant of the bleached sample contained more calcium than that of the unbleached sample (three experiments). The ratio of calcium ions released to molecules of Rh bleached ranged from 2.5×10^{-3} to 1.3×10^{-1}. This ratio corresponds to a release of 3—65% of the endogenous calcium content.

Preliminary measurements on Rh-digitonin solutions, similar to those on ROS suspensions also indicated a small release upon bleaching (endogenous Ca^{2+}/Rh ratio ≈ 3, calcium change upon bleaching $\Delta Ca^{2+}/Rh \approx 0.1$; calcium change corresponds to 3% of the endogenous calcium content).

Our pilot experiments to detect calcium changes by use of neutron activation analysis [$^{48}Ca\ (n, \gamma)\ ^{49}Ca$] were promising. An optimal technique should be developed.

3.2 Measurements of Calcium Concentration Changes by Means of the Calcium Indicator Phthalein Purple

In order to measure the time dependence of changes in the calcium concentration, experiments were undertaken with the calcium indicator phthalein purple using the flash photometric technique. Figure 1 (curve 1) shows the extinction decrease of Rh at 562 nm, the wavelength of maximal absorption of the indicator, during the formation of MII in ROS suspension. The extinction decrease of an ROS suspension which contains the indicator (curve 2, Rh plus indicator) is to be compared to the larger extinction decrease of Rh in the presence of indicator and EGTA (curve 3), which buffers the calcium. The difference between curve 2 and curve 3 (curve 4) represents an absorption increase of the indicator, which suggests that phthalein purple has bound calcium. Figure 1, in which each curve is an average of twenty single signals from three samples respectively, represents one of several experiments performed with a 1 mM EGTA concentration. The following table lists the measurements at 0.25 mM and 4 mM EGTA concentration as well.

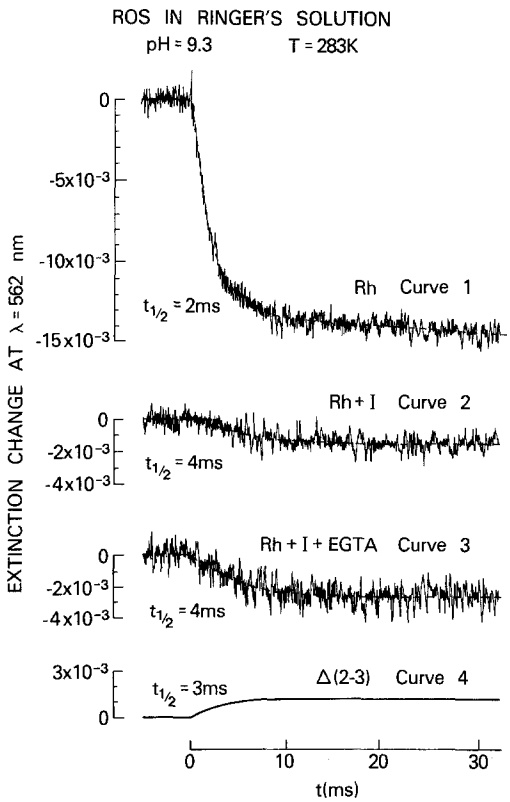

Fig. 1. Extinction decrease of rhodopsin (curve 1), rhodopsin plus indicator (curve 2), rhodopsin, the indicator additionally buffered with EGTA (curve 3) for rod outer segment suspension at pH 9.3, 10 °C, 562 nm. Curve 4 is the calculated difference curve (2–3) representing an absorption increase of the indicator

Table 1. Extinction changes of Rh, Rh plus indicator, Rh plus indicator plus EGTA during the transition Rh to MII in ROS suspensions at 562 nm, pH = 9.3, 283K

	Rh	Rh + I	Rh + I + EGTA		
			0.25	1.0	4 mM [EGTA]
$\Delta E \cdot 10^3$	12.7	1.5	1.9	2.2	1.9
n	3	4	5	3	3
SD · 10^3	0.9	0.3	0.4	0.1	0.5

n = Number of measurements
SD = Standard deviation

The time course of the absorption change of the indicator is not as well resolved as the MI to MII reaction (curve 1). However, the absorbance change of the calcium indicator as illustrated in curve 4 approximately parallels the extinction change of the MII-formation in time.

Preliminary measurements on Rh-digitonin solutions did not show a significant absorption change of metalphthalein upon bleaching Rh.

4. Discussion

The relatively wide scattered data in the literature concerning the calcium content and the calcium translocation in ROS reflect the considerable experimental difficulties involved in these measurements. The influence of extracellular media and the isolation procedure must not be neglected. An unknown fraction of the endogenous calcium content of the ROS can be lost by breakage of the ROS during the isolation and by the washing procedure. In addition, our preparations might differ in calcium content due to variable contamination by other cellular organelles such as mitochondria. Bownds et al. (1971) and Heller (1973) mention the possible effect of contamination by mitochondria. Heller found that his ROS preparations contained 1–5% mitochondria which possessed 1000 times more calcium than ROS and then suggested that the light induced calcium release could be due to contamination by mitochondria. Our ROS preparations contained less than 1% mitochondria (Krebs), an amount which cannot be neglected. However, it is unlikely that a light induced calcium release which occurs simultaneously with the formation of MII is due to such a mitochondria contamination. In addition, according to Winterhager (1975), the mitochondria in our preparations may have been inactivated. Szuts and Cone (1977) determined in a careful study the maximum possible contamination by other components but did not find any significant increase of the apparent calcium concentration in ROS by these organelles. Our finding of a calcium content of 0.1–0.2/Rh molecule in the ROS is in good agreement with the reports of Szuts and Cone (0.1–0.2 calcium/Rh in frog ROS; 1977) and Liebman (4 μM calcium/g protein corresponding to ca. 0.2 calcium/Rh; 1974), and fairly close to measurements by Hagins and Yoshikami (2–3.8 mM calcium in frog ROS, equivalent to about 0.7 calcium/Rh; 1975, 1976); the latter having the advantage of being performed with quick frozen samples which better resemble the physiological conditions. Hendriks et al. (1974) found a higher calcium content of up to 11 Ca/Rh for frog ROS.

Several studies suggest the existence of a light induced calcium release from ROS or disc membranes. The highest ratio is reported by Hendriks et al. (1974) with ca. 6 calcium/Rh for lysed frog ROS at a high bleaching percentage of Rh (50–80%). Liebman (1973, 1974) gave a calcium release of 0.2–0.8 calcium/Rh and mentioned that a calcium release might be concealed by a calcium pumping mechanism in the absence of EGTA. Mason et al. (1974) reported a light induced calcium release in frog and cattle ROS of about 1 calcium/Rh molecule bleached. Hemminki (1975a, b) found that illumination of outer segments before incubation decreased binding of Ca. Additionally, more Ca was retained by intact than by lysed outer segments after illumination (indicating that the plasma membrane acts as a barrier to the release of Ca). Weller et al. showed that if the disc membranes were exposed to light before loading with ^{45}Ca, the subsequent efflux of radioactivity on suspension in Ca-free medium was larger than in the material kept in the dark. Smith et al. (1977) found a light activated calcium release (0.75 calcium/Rh) from disc membrane vesicles, marked by the use of imidazole buffers. On the other side, Winterhager (1975) could not detect any significant Ca^{2+}-release from isolated discs or ROS on illumination before or after incubation in ^{45}Ca containing salines, neither did Sorbi and Cavaggioni (1975).

The calcium release we found, namely 0.01–0.1 calcium/Rh bleached has the

high light level for bleaching in common with the above mentioned findings. Our results led to a second series of experiments, the use of the calcium indicator metalphthalein in a flash spectrophotometer. Curves 1–3 in Figure 1 show extinction changes of Rh, Rh plus indicator, and Rh plus indicator with EGTA added. Hofmann et al. (1976) revealed that extinction changes of rhodopsin in ROS consist, depending on the measured wave length, of the absorption decrease of rhodopsin (formation of metarhodopsin II) and of changes in light scatter. They found and interpreted three different light scattering signals, which decrease in amplitude with increased bleaching of rhodopsin or disappear with a 1% bleach, as indication of structural changes within the disc membrane, as outer membrane effects, and as an influence of a light-induced transmitter release on the shape or mass of the discs. Since we average in our experiments twenty single flashes and bleach about 3% rhodopsin in the presence of metalphthalein, we can rule out the influence of at least one scattering signal on our results. It is not known how these signals behave at pH 9.3. But even if they contribute to our extinction changes, their influence should be cancelled in curve 4, since we compare our signals under the same conditions except the addition of EGTA (curve 3), which does not show any influence on the transition of Rh to MII (cf. Part I). Therefore we believe that the difference of curve 2 and curve 3 resembles an absorption increase of the indicator due to the release of calcium.

Using the calibration described in Material and Methods and a calculation of the data given in Figure 1 and in Measurements and Results (0.25 mM EGTA concentration) leads to a light induced calcium release of 12.4 calcium ions/Rh at a 3% bleaching level.

According to the hypothesis of Yoshikami and Hagins (Yoshikami, 1973; Yoshikami and Hagins, 1971) calcium ions released from the discs should be bound to the inner side of the outer membrane of the ROS and not penetrate into the surrounding medium. Our measurements suggest that calcium is partly released into the medium rather than exclusively bound by the ROS after the bleaching of Rh. This can be explained by damage to the ROS during the isolation procedure, resulting in leakage of the outer membrane (see above). According to Cone (1973) and Hendriks et al. (1974) the amount of light induced calcium release can be increased by lysis of the ROS.

Because of the small size of the absorbance changes caused by the calcium release, the time resolution of the flash photometric measurements was not as precise as hoped for. Still they allow the conjecture that the calcium release parallels the formation of MII in time, which would be in accordance with the calcium transmitter hypothesis.

Emrich and Reich (1974, 1977) suppose that the absorbance change of the indicator Eriochromschwarz T in Rh-digitonin solutions is a direct indication of the transition from the MI-conformation (with associated calcium) to the MII-conformation (with associated proton). Our experiments, however, are not directly comparable to theirs, since we used both a different indicator and a different rhodopsin preparation (in our case ROS suspensions, in Emrich and Reich's case Rh-digitonin solution which might provide a better accessibility of the Rh molecules for the indicator).

There are several possible locations for calcium ions before they are released into

the interdiscal space. They could be located either as free calcium ions in the disc-lumen or they could be bound to the disc membrane. If a light induced calcium release from Rh-digitonin-*solution* can be confirmed (we have two kinds of preliminary measurements so far) and is sufficiently high, this would favour the possibility that calcium is initially bound to Rh or lipids in the disc membrane (cf. Emrich and Reich, 1977). The observations that calcium is released (supposedly in the same time range as the MI to MII transition) and that the kinetics of the MI to MII transition and Rh regeneration are independent of the calcium concentration (see Part I) favour the assumption of a mechanism in which calcium is released from a site more remote from the Rh molecule (e.g. a neighbouring lipid molecule) and in which the calcium permeability of the disc membrane is increased.

Our results, performed with flame photometry and a calcium indicator, are in agreement with some reports in the literature and with the calcium hypothesis, but they do not quite prove it. Besides all the experimental implications, and only considering the figures, the results would mean, that we never could have gotten a higher ratio of calcium release/photoactivated rhodopsin, since we liberated all the free calcium in the disc, if the calcium content of 0.2–0.7 Ca/Rh is assumed to be the right order of magnitude. In order to show, that one bleached Rh can release 100–1000 calcium ions, which is predicted by the hypothesis, one has to go to lower light intensities.

ROS are evidently very delicate structures and it is possible, that they are sufficiently damaged by the experimental procedure, so that the calcium yields are much lower than in nature.

Appendix. *Calcium content and calcium release upon bleaching ROS of different animals and under different conditions. For exact details the original literature is cited*

Ca-content	Light-induced Ca-release	Material	Methods	Authors
10^{-3} M in discs 10^{-8} M in cytoplasma	yes (10^{-7} M in cytoplasma)	Rat ROS-discs	Estimation	Yoshikami and Hagins, 1971
10,000 free Ca/ROS $\triangleq 10^{-6}$ M	–	Rat ROS	Estimation	Yoshikami, 1973
1 Ca/Rh	Yes	Frog ROS	Atomic absorption photometry	Liebman, 1973
Ca/Rh = 1 : 10 in discs	1000 Ca/Rh ($\triangleq$50% of total Ca in discs)	Frog ROS	Osmotic lysis atomic absorbance	Cone (Szuts), 1973
1–2 Ca/Rh $\triangleq$ 0.1 M Ca/discs	0.2–0.8 Ca/Rh	Frog, bovine ROS	Atomic absorption spectrometer	Liebman, 1974
11 M Ca/M Rh $\triangleq$ 28 mM Ca/ROS	6 Ca/Rh	Frog ROS	Osmotic lysis atomic absorbance	Hendriks et al., 1974
0.1–0.2 Ca/Rh	$10^{-1} - 3 \cdot 10^{-3}$	Bovine ROS	Flame spectrophotometry	Nöll and Stieve, 1974
	12 Ca/Rh	Bovine ROS	Ca-indicator phthalein purple	Nöll and Stieve, 1974, this paper
27 M Ca/M Rh per rat photoreceptor (incl. inner segment)	No measurements	rat and mouse photoreceptor	Atomic absorbance	Farber and Lolley, 1976
19 M Ca/M Rh per mouse photoreceptor	No measurements			
0.25 M Ca/M Rh	No measurements	Frog ROS	Absorption spectro-photometry	Hess, 1975
3.8 mM in ROS	No measurements	Frog ROS	Electron microprobe	Hagins and Yoshikami, 1975
2 mM in ROS	No measurements	Frog, rat retinas	Electron microprobe	Yoshikami and Hagins, 1976
0.1–0.2 Ca/Rh	No	Frog ROS	Absorption spectro-photometry	Szuts and Cone, 1977
	1 Ca/30 Rh	Sonicated bovine ROS	Calcium indicator Arsenazo III	Kaupp and Junge, 1977

Ca-determinations with ^{45}Ca, ^{47}Ca

Ca-concentration of the medium	Ca-uptake (at saturation)	Light-induced Ca-release	Material	Method	Authors
10^{-5} M	Yes	$\leq$ 10% of total ROS Ca	Frog ROS	^{45}Ca, estimation	Bownds et al., 1971
Estimation : accumulation of 0.025 M Ca/M Rh $\triangleq 10^{-3}$ M Ca/disclumen					
$5 \cdot 10^{-6}$ M	0.3 nM Ca/mg protein (– ATP)	No	Sonicated bovine ROS	^{45}Ca	Neufeld et al., 1972
	0.7 nM Ca/mg protein (+ ATP)				
10^{-5} M	1.2 nM Ca/mg protein (+ ATP)				
10^{-5} M	Yes	1 Ca/Rh	Frog disc membrane vesicles	^{45}Ca	Mason et al., 1974
	Light induced Ca uptake	Yes	Sonicated frog ROS	^{45}Ca, ^{47}Ca	Baker and Mason, 1974
$1.3 \cdot 10^{-5}$ M	0.5–0.6 nM Ca/mg protein	Illuminated ROS retain less Ca	Bovine ROS	^{45}Ca	Hemminki, 1975a
10^{-3} M	20–30 nM Ca/mg protein				
High affinity binding site: 25 nM Ca/mg protein (dark) 22 nM Ca/mg protein (light)					
Low affinity binding site: 210 nM Ca/mg protein (dark) 160 nM Ca/mg protein (light)					
10^{-3} M	5 nM Ca/mg protein (maximal Ca-binding capacity)	Illumination of lysed ROS decreases Ca-binding	Bovine ROS	^{45}Ca, osmotic lysis	Hemminki, 1975b
10^{-5} M	ca. 1.2 nM Ca/mg protein	Yes	Calf ROS-discs	^{45}Ca	Weller et al., 1975
$2 \cdot 10^{-5}$ M	2 nM Ca/mg protein	No	Bovine ROS or discs	^{45}Ca	Winterhager, 1975
		No	Frog discs	^{45}Ca	Sorbi and Cavaggioni, 1975
		0.7 Ca/Rh	Sonicated bovine discs	^{45}Ca	Smith et al., 1977

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