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Interaction of Bovine Rhodopsin with Calcium Ions

I: The Metarhodopsin I–II Reaction and the Regeneration of Rhodopsin*

G. Nöll** and H. Stieve

Institut für Neurobiologie, Kernforschungsanlage Jülich,
D-5170 Jülich, Federal Republic of Germany

Abstract. The formation of metarhodopsin II in various bovine rhodopsin preparations (rod outer segment (ROS) suspensions and rhodopsin-detergent solutions) was measured by means of flash spectrophotometry. The half-lifetime and formation of metarhodopsin II in ROS did not depend on the calcium concentration in the range of less than 10^{-9} M (using EGTA or EDTA) to 15×10^{-3} M calcium at pH values of 5.0, 7.1, and 9.0 (Table 1).

The regeneration of rhodopsin from opsin by adding 11-cis retinal to ROS-suspensions and rhodopsin digitonin solutions was measured spectrophotometrically. It was not substantially different in either saline, one containing less than 10^{-7} M calcium (by adding EGTA), the other containing 10^{-3} M calcium (Table 2).

Key words: Rhodopsin – Calcium – Reaction kinetics – Regeneration.

1. Introduction

In 1971 Yoshikami and Hagins first published the calcium transmitter hypothesis, inspired by an experiment in which raising the extracellular calcium concentration reduced the dark current of rat photoreceptor cells ("calcium mimics the effect of light"). The conversion process of rhodopsin (Rh)[a] to metarhodopsin II (MII) is regarded to bring about a conformational change in the disc membrane of the rod outer segment (ROS) (cf. e.g. Hofmann et al., 1976; Emrich and Reich, 1977). In this way, according to the hypothesis, calcium ions are released from the discs into

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[a] *Abbreviations:* A = absorption, ΔA = absorption change; CTAB = N-Cetyl-N,N,N-trimethylammoniumbromide; E_{700} = extinction at $\lambda = 700$ nm; EDTA = ethylenediamine-NNN'N'-tetraacetic acid; EGTA = 2,2'-ethylenedioxybis [ethyliminodi (acetic acid)]; MI = metarhodopsin I, MII = metarhodopsin II; Rh = rhodopsin; ROS = rod outer segment

** Present address: Zentrum für Physiologie im Klinikum der Justus-Liebig Universität, Aulweg 129, D-6300 Gießen, Federal Republic of Germany

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the interdiscal space of the ROS. These ions then act on the outer membrane of the ROS reducing the sodium ion conductivity, thus transmitting the information of the light stimulus from the disc to the outer membrane.

The transition from MI to MII is considered to be of special physiological importance. The wide spectral shift from MI to MII and the large enthalpy and entropy of activation could support the idea of a conformational change in the chromoprotein (Abrahamson et al., 1960). The transition from MI to MII is the first in the intermediate sequence to involve water (Kühne, 1878), i.e., there is a proton uptake in this reaction step. It occurs at physiological temperatures in times of less than 1 ms. Therefore, it is suggested that this process plays a key role in triggering the receptor potential in vertebrates.

The calcium hypothesis has stimulated a number of important experimental investigations, but up to now the role of calcium ions in the transduction mechanism of the vertebrate photoreceptor excitation is controversial.

To clarify the role of calcium in vertebrate rods we have undertaken experiments concerned with the effect of calcium on the MI to MII reaction, and the effect of calcium on the regeneration of rhodopsin. The effect of illumination on the binding or seclusion of calcium ions in isolated ROS will be described in a subsequent paper (Part II).

2. Material and Methods

All measurements were carried out with bovine eyes obtained freshly from the slaughterhouse. The ROS were isolated according to the method of von Sengbusch (von Sengbusch, 1970; Nöll, 1974) by means of a stepwise sucrose density gradient and washed three times in a physiological saline (137 mM NaCl, 5.36 mM KCl, 1 mM $CaCl_2$, 1.23 mM $MgCl_2$, 3.75 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, pH 7). Also a calcium-free physiological saline, otherwise identical to the solution described above was used. The ROS were dropped into liquid nitrogen and stored at −196 °C until use.

Occasionally, ROS that had not been frozen, were used immediately after preparation. In some experiments, frozen ROS were dissolved in detergent solution containing either digitonin or CTAB (2% w/v) and the above mentioned salts. The extinction spectra of the ROS preparations indicated no contamination by blood cells. An electron microscopic examination showed that our ROS preparations contained less than 1% mitochondria. It also indicated that the ROS usually break off above the cilium (Krebs, pers. communication).

2.1 Ca^{2+}-Dependence of the MI to MII Transition

In these experiments, the Ca^{2+}-concentration in the physiological saline was varied. It contained 50, 5, 1, or 0.5 mM calcium, or no $CaCl_2$ was added. In order to further reduce the calcium content, 5 mM chelating agent EGTA or EDTA was added to the salines containing "no" or 1 mM $CaCl_2$. The molarity of free calcium ions was estimated to be less than 10^{-9} (EGTA) or 10^{-6} (EDTA) in the saline solutions to which no $CaCl_2$ was added, and less than 10^{-7} (EGTA) and $5 \cdot 10^{-5}$ (EDTA) in the

Table 1. Relative absorption increase ΔA and half-lifetime $t_{1/2}$ for the formation of MII measured at 389 ± 5 nm, 15 ± 1 °C, measuring time 160–640 ms, in physiological salines differing in calcium concentration and pH. (The measurements listet first under pH 5 were done with a different ROS preparation at a later time)

Calcium content of physiol. saline in mol/l		(1) $< 10^{-9}$	(2) 10^{-6}	(3) $5 \cdot 10^{-6}$	(4) $5 \cdot 10^{-4}$	(5) 10^{-3}	(6) $5 \cdot 10^{-3}$	(7) $5 \cdot 10^{-2}$
ROS suspensions								
pH 5[a]	rel. ΔA	13.9	14.0	14.1	14.2	–	13.9	13.6
	SD	0.3	0.2	0.3	0.3	–	0.7	0.4
	$t_{1/2}$ (ms)	4.3	3.5	4.5	4.5	–	4.4	2.6
	SD	0.2	0.6	0.5	0.7	–	0.5	0.2
pH 5[a]	rel. ΔA	136	131	125	–	129	–	–
	SD	13	9	5	–	8	–	–
	$t_{1/2}$ (ms) (± 20%)	4.1	4.1	4.1	–	4.1	–	–
Ph 7.1[b]	rel. ΔA	80	90	84	–	83	–	–
	SD	4	5	5	–	5	–	–
	$t_{1/2}$ (ms) (± 20%)	37	40	40	–	36	–	–
pH 9[a]	rel. ΔA	48	48	53	–	48	–	–
	SD	4	5	2	–	5	–	–
	$t_{1/2}$ (ms) (± 20%)	9.3	12	9.3	–	11	–	–
Rh-digitonin solution								
pH 7[a]	rel. ΔA	101	111	96	–	98	–	–
	SD	4	3	4	–	6	–	–
	$t_{1/2}$ (ms) (± 20%)	28	26	31	–	32	–	–

The composition of the physiological salines is as follows:
(1) 5 mM EGTA + (3) [Ca-concentration estimated (Portzehl et al., 1964)]
(2) 5 mM EDTA + (3)
(3) 137 mM NaCl, 5.36 mM KCl, 1.23 mM $MgCl_2$, 3.75 mM Na_2HPO_4, 1.25 mM NaH_2PO_4 at pH 7.1 (± 0.1); buffer at pH 5: 1.6 mM citric acid, 34 mM Na_2HPO_4; at pH 9: 4,46 mM Tris, 0.54 mM HCl
(4) 0.5 mM $CaCl_2$ + (3)
(5) 1 mM $CaCl_2$ + (3)
(6) 5 mM $CaCl_2$ + (3)
(7) 50 mM $CaCl_2$ + (3)
ΔA is a mean of minimal five experiments, SD the standard deviation of ΔA
[a] Retina stored in liquid nitrogen
[b] ROS neither frozen nor stored, used immediately after obtaining them from the slaughterhouse

case of saline solutions initially containing 1 mM $CaCl_2$ (Portzehl et al., 1964), when other sources for calcium (e.g. membrane bound calcium) are neglected (see legend of Table 1).

The extinction spectra of the ROS-suspensions and Rh-detergent solutions were recorded with a Cary 17 Spectrophotometer. Kinetic measurements were made in a

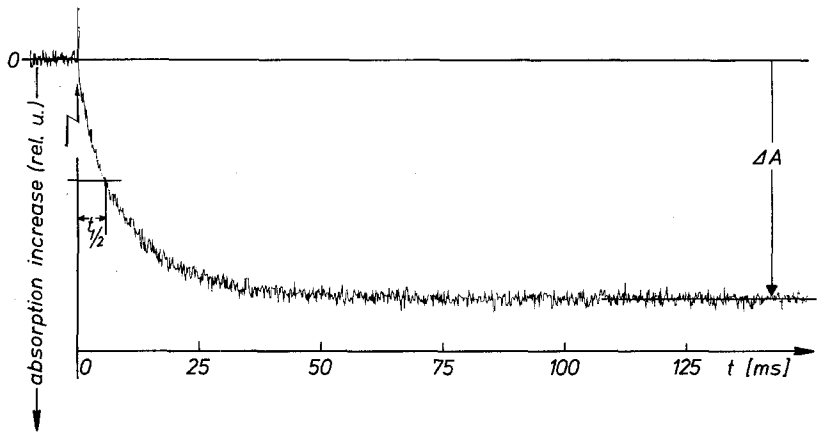

Fig. 1. Absorption increase for the formation of MII in ROS suspension (physiological saline containing 1 mmol $CaCl_2$/l, pH 7) at 389 nm, 22 °C. Excitation light at $t = 0$

flash photometer previously described (von Sengbusch, 1970; Nöll, 1974). The excitation light of the flash photometer (argon flash, half-lifetime ca. 20 μs) was filtered through cut-off filters (GG 475 and GG 495, Schott & Gen.). The time resolution was shorter than 200 μs. To improve the signal- to-noise ratio, twenty single signals from each sample, obtained at intervals of 1 s, were averaged (Biomac 1000, Datalab).

The absorption changes were monitored mostly at 389 nm for intervals of either 80, 320, or 640 ms. At 389 nm, only the increase of absorption due to the formation of MII can be seen. The signals (a typical one is reproduced in Figure 1) are characterized by the increase or decrease in relative absorption and by the half-lifetime.

2.2 Ca^{2+}-Dependence of the Regeneration of Rhodopsin

In the regeneration experiments, a suspension of ROS in physiological saline ($6.6 \cdot 10^{-6}$ M Rh, $E_{700} \approx 2.1$) was bleached at 2 °C through a GG 475 filter (Schott & Gen.) for 5 min. At this temperature, MII is rather stable (Matthews et al., 1963). Then 11-cis retinal (Sperling, 1973) was added to the sample in the spectrophotometer (the 11-cis retinal solutions were prepared by dissolving a small crystal in 1 ml methanol). The extinction spectra were then measured at different times and temperatures. From these curves, difference spectra were drawn using an unbleached sample as reference.

3. Measurements and Results

3.1 Calcium Dependence of the MI to MII Transition in Different Extracellular Media

Table 1 shows the half-lifetime and the relative absorption increase of the MI to MII transition following flash illumination in ROS-suspensions in media which differ in calcium concentration and in pH (cf. 2.1). Normally, previously frozen ROS were

used; for the measurements at pH 7.1, ROS were used immediately after preparation. At pH 5 a second series of measurements, which covered a larger range of calcium concentrations, is added.

In *ROS suspensions* the values for both the amount and the half-lifetime of the MII-formation are not significantly different (95% confidence interval) for the various calcium concentrations of the medium at the three different pH-levels. A decrease in the half-lifetime of the MI to MII transition is observed only at very high calcium concentrations (50 mM). The formation of MII, however, is pH dependent, in accordance with several reports (von Sengbusch, 1970; Emrich, 1971; Stieve et al., 1973; Nöll, 1974).

In Rh-digitonin solutions at pH 7, a somewhat higher variation in the relative absorption changes without any trend occurs during the formation of MII, which would be noteworthy with a 95% confidence interval, insignificant with a 99.9% confidence interval. The transition MI to MII accelerates with increasing calcium concentrations.

3.2 Calcium Dependence of the Regeneration of Rhodopsin

The experiments described above show that the calcium concentration does not considerably effect the bleaching reaction MI to MII. Nevertheless the question needs to be considered whether it is possible, that calcium mediates the reverse reaction, for instance the embedding of retinal into the opsin molecule. Therefore, rhodopsin regeneration experiments initiated by adding 11-cis retinal to MII were undertaken.

A *suspension of ROS* (cf. 2.2) was extensively bleached at 2 °C. Curve 1 of Figure 2 shows the formation of MII at 390 nm. When 11-cis retinal is added, the extinction increases at 390 nm (curve 2). The sample remained for 0.5 h at 2 °C. With time, the extinction decreases slightly at 390 nm and increases slightly at 520 nm (curve 3). On warming the ROS suspension to 20 °C, these extinction changes become larger (curves 4–6). These properties, and the presence of a distinct isosbestic point at 440 nm, are characteristic of Rh regeneration. The time course of regeneration at 520 nm is shown in Figure 3 and parameters are given in Table 2.

Table 2. Rhodopsin regeneration. Table 2 shows the time in which 30% extinction increase is reached at 520 nm after addition of 11-cis retinal to MII at 2 °C for ROS suspension and Rh-digitonin solution, both in normal and low calcium (5 mM EGTA) physiological saline (pH 7). 30 min (90 min respectively) after adding 11-cis retinal the ROS suspension (Rh-digitonin solution) was warmed up to 20 °C

	Ca^{2+} (M/l)	$t_{\Delta E = 30\%}$ (h)
ROS	10^{-3}	7.3
	$< 10^{-7}$	6.4
Rh-digitonin solution	10^{-3}	1
	$< 10^{-7}$	0.5

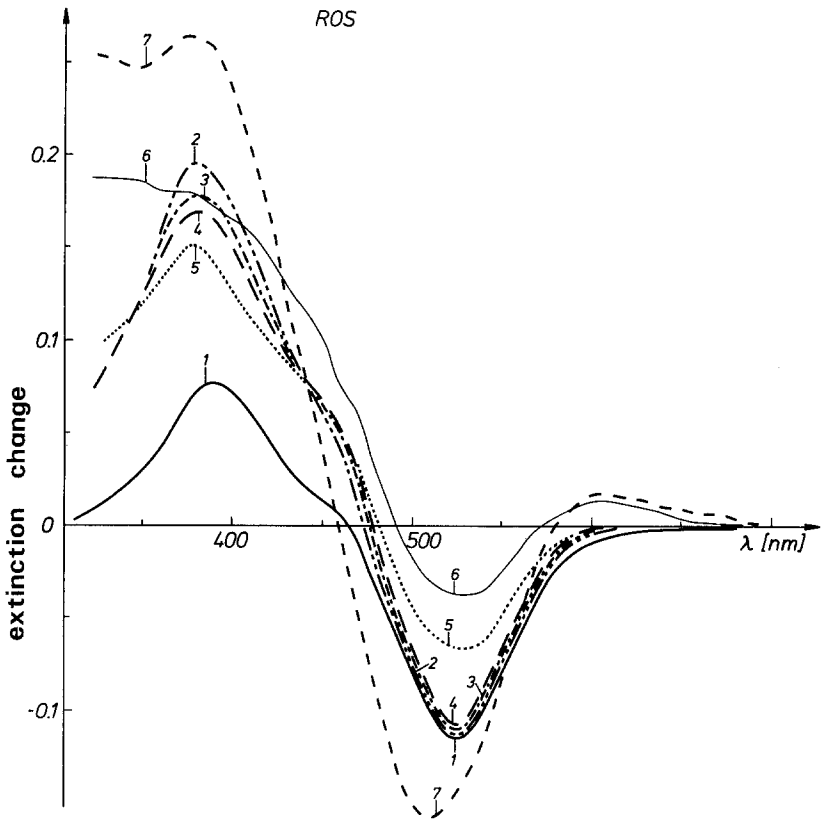

Fig. 2. Regeneration of Rh in ROS suspension (physiological saline containing 1 mM $CaCl_2$/l), bleached at 2 °C, with 11-cis retinal. Difference spectra were drawn using an unbleached sample as reference. Curve 1: bleached probe; Curve 2: immediately (2 °C); Curve 3: 30 min (2 °C); Curve 4: 1 h (20 °C); Curve 5: 10.5 h (20 °C); Curve 6: 30 h (20 °C) after addition of 11-cis retinal; Curve 7: final bleaching

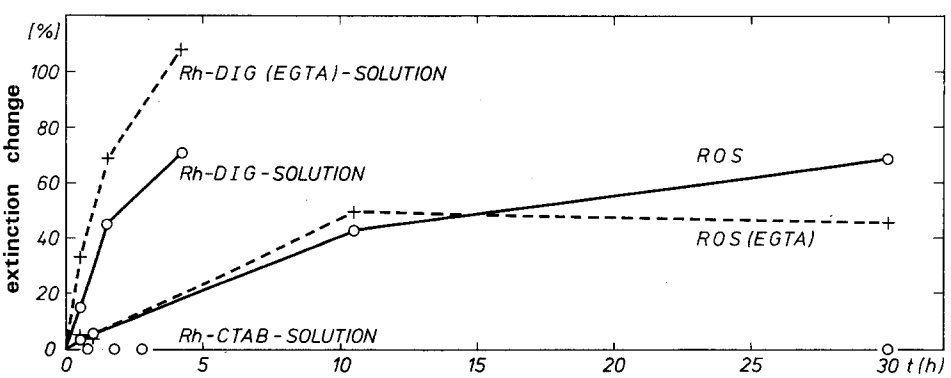

Fig. 3. Regeneration of Rh in ROS suspension (physiological saline containing 1 mM $CaCl_2$/l with and without 5 mM EGTA/l, pH 7), Rh-digitonin solution (1 mM $CaCl_2$/l physiological saline with and without 5 mM EGTA), Rh-CTAB solution (1 mM $CaCl_2$/l physiological saline) measured at 520 nm after addition of 11-cis retinal to MII [compare to Fig. 2: the extinction difference at 520 nm between the bleached (curve 1) and the unbleached (abscissa) sample is set to be 100%]

The time in which a 30% extinction increase at 520 nm is reached is used as parameter to measure the rate of regeneration.

To examine the calcium dependence of the regeneration, a parallel experiment was performed, in which 5 mM EGTA was added to the ROS suspension in order to bind the free calcium ions. In this case, regeneration also takes place (see Fig. 3 and Table 2).

The results obtained for ROS suspensions with and without EGTA cannot be directly compared because of the difficulty in bleaching the same amount of Rh and thus adding an equimolar quantity of 11-cis retinal corresponding to the amount of MII produced. A further experimental difficulty was the presence of small changes in extinction due to changes in light scatter over the course of the experiment, accentuated by the addition of EGTA.

Rh-digitonin solutions showed a faster time course of regeneration, compared to ROS suspensions (Fig. 3 and Table 2). 1.5 h after addition of 11-cis retinal the solution was warmed up to 20 °C. Again the difference in the time dependent extinction changes observed in the Rh-digitonin solutions with and without calcium is partly caused by the greater light scatter in the presence of EGTA.

In *Rh-CTAB solutions* regeneration does not take place, as already shown by Shichi (1971).

Regeneration of Rh in ROS suspension and digitonin solutions occurs at normal (10^{-3} M) as well as at very low ($< 10^{-7}$ M) calcium concentrations.

Taking into account the various possibilities of interference, we doubt that calcium has any influence on the Rh regeneration.

4. Discussion

Our measurements confirmed the finding of von Sengbusch (1970), that the properties of the MI to MII reaction in ROS suspensions at pH 6 are independent of the calcium concentration in the range between 10^{-5} and 10^{-3} M calcium. Hofmann et al. (1976) showed, that the absorption change during the transition MI to MII is accompanied by light-scattering signals which may indicate structural changes within the disc and plasma membrane of the ROS. These scattering signals should be negligible in our results, since we bleached about 30% rhodopsin and averaged 20 single measurements on one sample.

Taking into consideration the precipitation of calcium by phosphate and citric acid (used in our buffers) and hence a reduction of a possible calcium effect, as Emrich and Reich suggest (Emrich and Reich, 1976), our measurements on ROS still covered a larger range of free calcium concentrations and pH and did not indicate a distinct calcium dependence. Only a very high calcium concentration (about 15 mM) caused an acceleration of the MII-formation. On the other hand, Emrich and Reich (1976) suggest that calcium does influence this reaction at pH 5 in rhodopsin digitonin solutions; at calcium concentrations higher than about 3 mM the equilibrium between MI and MII should be shifted to MI. Our discrepancy with the data of Emrich and Reich may be explained by the employment of different systems. Emrich and Reich used rhodopsin digitonin solutions, whereas our experiments preferentially were performed in ROS-suspensions. Rhodopsin digitonin solutions were not measured in our experiments at pH 5.0.

Neither did our experiments show a noteworthy qualitative calcium dependence of the Rh regeneration. The comparison of the regeneration curves with and without calcium is impaired by the long-time changes in light scatter especially in the presence of EGTA. EGTA possibly increases the precipitation of digitonin and this in turn the extinction. Curve 6 in Figure 2 indicates by its parallel shift to curve 5, that after 10 h extinction changes are primarily caused by changes in light scatter. The fact that regeneration occurs faster in solution than in ROS-suspension can be expected, since the ROS-plasma membrane hinders the accessibility of retinal to opsin.

Hubbard and Wald (1952) have shown that the synthesis of Rh follows second order reaction kinetics under conditions in which 11-cis retinal and opsin are present in about the same ratio. The reaction is completed approximately 1 h after adding 11-cis retinal to opsin in the dark (23 °C, pH 6.4, reaction 50% complete after 7 min). Because of the longer regeneration time than that quoted by other authors (Hubbard and Wald, 1952; Weale, 1967; Baumann, 1972; Henselmann and Cusanovich, 1976), our results indicate that a regeneration under the conditions of our experiments did not start from MII when 11-cis retinal was added.

Rather, all-trans retinal must first be split off from the opsin before regeneration can start. This supports the assumption that 11-cis does not compete with all-trans retinal for the binding site of opsin in the state of MII.

Calcium plays an important role in many processes of excitable membranes. The significance of calcium for the reactions involved in the transduction process of vertebrate photoreceptors is still not satisfactorily understood. The results of the measurements reported in this paper allow to exclude a controlling role of calcium on some reactions involved in the visual excitation, namely the MII-formation and Rh-regeneration, whereas they do not permit a decision for or against the calcium transmitter hypothesis as formulated by Yoshikami and Hagins. One aspect of this hypothesis (Ca-release) will be dealt with in a following report.

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