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MEASUREMENT OF FAST LIGHT-INDUCED DISC SHRINKAGE WITHIN BOVINE ROD OUTER SEGMENTS BY MEANS OF A LIGHT-SCATTERING TRANSIENT

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Summary

A fast light-induced light-scattering transient, previously found in rod outer segment suspension, the so-called P-signal (Hofmann, K.P., Uhl, R., Hoffmann, W. and Kreutz, W. (1976) Biophys. Struct. Mechanism 2, 61—77), is described in more detail.

The effect has the same action spectrum as rhodopsin bleaching. It is not regenerated with 11-cis retinal.

The response is not linear with light-intensity for flashes which bleach more than 2.0% of rhodopsin; it saturates at an intensity corresponding to 15% rhodopsin bleaching.

The wavelength- and scattering angle dependence lead to the conclusion that the change in light-scattering reflects a shrinkage of an osmotic compartment of the rod outer segment.

The only compartment which we found to be intact in our rod outer segment preparations was the disc or rod sac; therefore, the effect must be attributed to a light-induced shrinkage of the rhodopsin-containing disc organelles.

The overall effect (15% of rhodopsin is bleached) is in the range of 0.5—1.5% of the original volume.

A light-induced passive cation-efflux from the disc, e.g. of Ca^{2+}, can be ruled out as a possible molecular origin of the disc-shrinkage in our preparations.

Introduction

Assuming that signal transduction within the receptor cell is connected with structural modifications, some researchers studied light-induced structural

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changes by means of X-ray diffraction and electron microscopy [2—8]. These methods allow an exact definition of the changing units but cannot resolve structure changes which are fast enough to be involved in the excitation of the receptor.

In addition to the study of birefringence [9], and of the structure-sensitive part of the electrical properties [10], the light-scattering measurement also may achieve higher time resolution. In a previous paper, we have shown that three different light-scattering signals in the millisecond range are obtained from bovine rod outer segments. These signals were assigned to three distinct structural regions [1].

One of the signals was originally detected as a step-like increase in the absorbance of a fresh bovine rod outer segment suspension, observed at the red end of the visible spectrum. We named it "P" (that means positive, i.e. by definition an absorbance increase) for distinction from the other signals. Generally defined, the P-signal is a light-scattering transient in the 10-ms range, which can be evoked by green flashes on rod outer segment suspension. The effect disappears with illumination 15—20 times more rapid than the content of unbleached rhodopsin. It appeared to be caused by some change in the structure of the whole disc body.

This paper presents some further data about the P-effect with special emphasis on its structural nature.

Material and Methods

Preparation of rod outer segment fragments

Bovine eyes were dissected in the slaughterhouse about 3 min after slaughtering and stored in an ice-cooled dark container. 50 eyes were collected for one preparation. 0.25 h after dissection of the last eye the retinae were dissected from the bulbi and stored in a preparation solution containing 280 mosmol KCl and 10 mM piperazine-1,4 diethenesulfonic acid (PIPES) [11], pH 7.0.

The retinae were then shaken and lightly pressed through a nylon net with 0.1 mm pores.

This rough suspension yielded approximately 25 ml. Rod outer segments were separated from mitochondria and other contaminating particles on a discrete sucrose density gradient with a specific density of 1.14. Rod outer segments were removed from the interface with a micrometer-adjusted injection needle, size 1 mm, bent at a right angle to the interface to avoid sucrose contamination and related osmotic damage. This suspension was twice washed in the above solution. The final rhodopsin concentration was approximately $5 \cdot 10^{-5}$ M. The plasma membrane was destroyed by two methods: (1) by pressing the suspension twice through an Eppendorf pipet tip filled with glass wool, and (2) a slow freezing to liquid N_2-temperature [12]. In the electron microscopic picture this preparation appears to consist of packets of discs partially enveloped by a membrane. The light-scattering control for the intact or resealed plasma membrane [1] was negative.

Measuring conditions

The reaction medium was the same as the preparation solution. Lower pH

values were adjusted with morpholino-ethanesulfonic acid (MES). The rhodopsin concentration in the reaction medium was $5 \cdot 10^{-7}$ M when measuring at a scattering angle $\theta = 0 \pm 2°$ and it was $1.5 \cdot 10^{-7}$ for measurements as a function of scattering angle.

Ionophores were stored as highly concentrated ethanolic solutions at −40° C. They were added to the reaction medium just prior to the measurements (up to an ethanol content of 0.5% the results are not influenced).

The measuring device

The measuring device is the same as previously described in detail [1].

Reproducibility

Amplitudes and kinetics of the signals are reproducible within the errors of concentration, noise and temperature ($\Delta T = \pm 0.1°C$), with thawed samples from one and the same preparation for about 30 min at room temperature. Then the amplitude decreases and the kinetics become erratic.

Measurements from different preparations run parallel to each other without quantitative conformity.

Results

A typical P-signal is shown in Fig. 1. The signal amplitude, A_p, is defined as the normalized total intensity change at the detector:

$$A_p = \frac{\Delta I}{I}$$

This paper only deals with the amplitude of the light-scattering signal. If the signal is measured with a scattering angle $\theta = 0 \pm 2°$ the term "change in apparent absorbance" will be used.

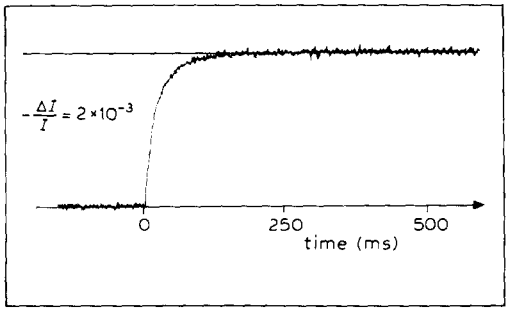
1. Excitation and regeneration

a. Wavelength of the exciting flash. A series of flashes was applied to a rod outer segment suspension and the change in apparent absorbance at 750 nm was measured with the first and second flash. With flashes numbers 20—36 the absorbance change at 380 nm (A_{380}) was measured. This experiment was done with six different samples from the same preparation at six excitation wavelengths: λ exc = 468 nm, 482 nm, 512 nm, 546 nm and 574 nm. Flash intensity was adjusted to a bleaching of 2% rhodopsin for each flash.

It turned out that:

$$\frac{A_p}{A_{380}} = \text{const. for all values of } \lambda_{exc.}$$

When more than 15% of the initially present rhodopsin within the rod outer segments is bleached, the flash-induced absorbance change observed at 380 nm, A_{380}, is proportional to the amount of rhodopsin bleached with this flash, A_R [1].



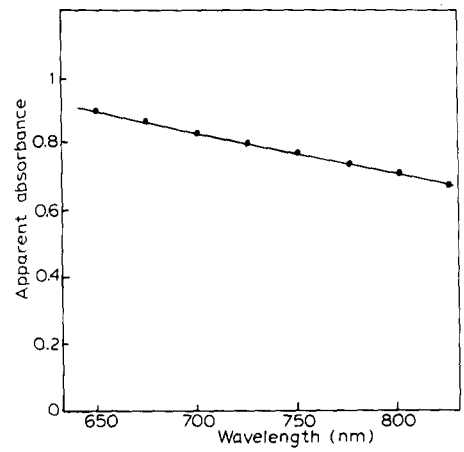


Fig. 1. Light-induced light-scattering transient of a bovine rod outer segment suspension. Time course of a typical "P-signal" (corresponding to a flash intensity bleaching 1.5% of the unbleached rhodopsin). The flash was applied at the zero-point of the time scale. Experimental conditions are as follows: $T = 22°C$, pH = 7. Flash wavelength: $530 \leqslant \lambda_{exc} \leqslant 550$ nm. Flash duration: 20 μs. Scattering angle: $\theta = 0 \pm 2°$.

Fig. 2. Apparent absorbance of a suspension of bovine rod outer segments as a function of the wavelength (pH = 7, $T = 22°C$, $\theta = 0 \pm 2°$).

Thus it follows that:

$$\frac{A_{380}}{A_R} = \text{const. and therefore } \frac{A_p}{A_R} = \text{const.}$$

for all λ_{exc}.

That means that the P-effect has the same action spectrum as rhodopsin bleaching.

b. Intensity of the exciting flash. The intensity of the exciting flash, I_b, is measured and defined by its bleaching strength. We applied flashes of different intensities ($530 \leqslant \lambda_{exc.} \leqslant 550$ nm) on three different samples from the same preparation. The flashes bleached 0.5, 1.0 and 1.5% rhodopsin, respectively. For these three intensities was found:

$$A_p \propto I_b$$

This proportionality holds not at all for flashes of larger intensity. While the amplitude for a 1.5% bleaching flash can achieve $\Delta I/I = 2 \cdot 10^{-3}$ (c.f. Fig. 1), the highest possible amplitude for the light-scattering change is about $\Delta I/I = 10^{-2}$.

This can also be observed in series of flashes as discussed in more detail in ref. 1.

The exact behaviour of A_p in series of flashes depends on the time between the flashes.

c. Addition of 11-cis retinal. We bleached a rod outer segment suspension with a series of flashes and observed the decrease of A_p. At the end of the flash series, 15% of the rhodopsin was bleached and the P-signal was no longer detected. Adding now 11-cis retinal in ethanolic solution, approximately 70% of the bleached rhodopsin was regenerated. The amplitude of the 380 nm

signal and of the light-scattering signal N [1] were enhanced correspondingly. The P-signal, however, was not regenerated.

2. Dependence on measuring parameters

a. Measuring wavelength. The apparent absorbance of a rod outer segment suspension as a function of the wavelength is shown in Fig. 2. This measurement was done with the same device as that used for the measurement of light-scattering.

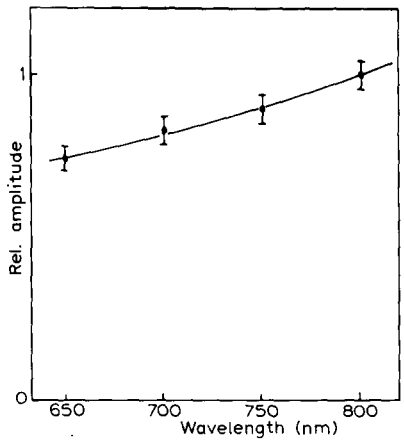
Fig. 3 shows the light-induced change in apparent absorbance as a function of the measuring wavelength. It is seen that A_p, as a transition of an unknown part of total light-scattering, behaves inversely to the apparent absorbance and therefore to the integral light-scattering of the rod outer segments within the range of scattering angle $2° \leqslant \theta \leqslant 180°$.

b. Scattering angle. Fig. 4 shows the amplitude of the light-scattering signal as a function of the scattering angle.

3. Non-light-induced light scattering changes

a. We added a small amount of a highly concentrated NaCl solution to an isotonic KCl suspension of rod outer segments. The angular dependence of the amplitude of the light-scattering change, thus induced, is shown in Fig. 5. For comparison the θ-dependence of the light-scattering signal amplitude is also drawn in.

b. Suspending rod outer segment fragments in an isotonic calcium acetate solution in the presence of $2 \cdot 10^{-6}$ M A 23187 (an ionophore which increases the membrane permeability both for Ca^{2+} and H^+, thus allowing an electron neutral exchange of the two cations [13]), a fast decrease in total light-scattering is detected. The extent of this light-scattering change has the same angular dependence as shown for the P-signal above.



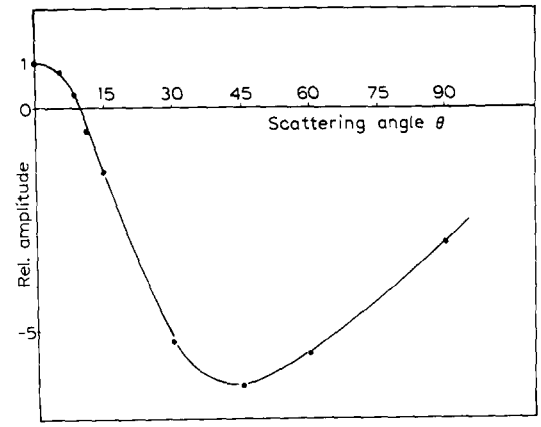


Fig. 3. Relative amplitude of the light-induced change in apparent absorbance (light scattering signal at a scattering angle $\theta = 0 \pm 2°$) as a function of wavelength (pH = 7, $T = 22°C$). Error bars correspond to variations of flash intensity and sample concentration.

Fig. 4. Relative amplitude of the light-induced change in light scattering as a function of the scattering angle (pH = 7, $T = 22°C$, $\Delta\theta = \pm 1°$). An identical course of the θ-dependence was reproduced three times with different rod outer segment preparations.

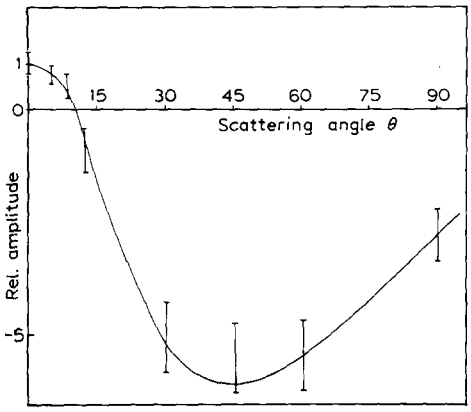


Fig. 5. Relative amplitude of an osmotically induced light scattering change as a function of the observation angle (pH = 7, $T = 22^{\circ}C$, $\Delta\theta = \pm 1^{\circ}$). Rod outer segment shrinking was induced by adding 0.5 ml NaCl solution, hypertonic by a factor of 30, to 50 ml rod outer segment suspension. This 30% increase in the osmolarity of the reaction medium produced a light-scattering change which was measured 5 min after the addition of NaCl. (30% osmolarity increase produced an extinction increase of 1%. Therefore the maximum possible amplitude of P would correspond to a change of osmolarity of about 30%.) Errors bars correspond to the deviations of the measured intensity over about 10 min. For comparison, the course of the θ-dependence of the light-induced light-scattering change is drawn in.

4. Dependence on ion concentrations

a. Fig. 6 shows the light-induced change in apparent absorbance as a function of Ca^{2+} or Mg^{2+} added.

b. The light-induced change in apparent absorbance remains the same in isotonic solutions of NaCl, Na_2SO_4, KCl, choline chloride and sucrose (in the latter case small amounts of Ca^{2+} must be added to stabilize the membrane [1]).

c. Addition of A 23187 (2 μM) making membranes permeable for Ca^{2+} and H^+ [13]

2,4-dinitrophenol (220 μM) + Valinomycin (1 μM) making membranes permeable for K^+ and H^+ [14—16]

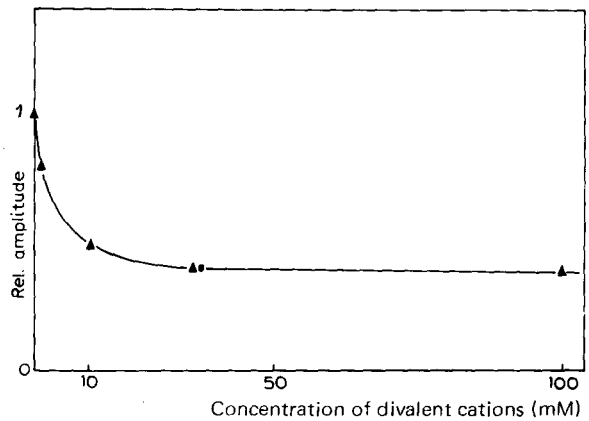


Fig. 6. Relative signal amplitude of the light-induced change in apparent absorbance (light-scattering signal at a scattering angle $\theta = 0 \pm 2^{\circ}$) as a function of the amount of divalent cations added. ▲, Ca^{2+} · Mg^{2+} (pH = 7, $T = 22^{\circ}C$).

Gramicidin D (50 nM) making membranes permeable for H^+ and all monovalent cations [17]

does not influence either amplitude or kinetics of the light-induced change in apparent absorbance.

Discussion

In discussing the P-effect, one has to distinguish between: (1) the observation, i.e. the light-scattering change, (2) the underlying event to the observation, i.e. the structural change, (3) the molecular origin; examples are: a flux of ions through the disc membrane thus changing the disc shape, or a rearrangement of membrane lipids.

1. Initiator

From the action spectrum (Results, 1a) it can be deduced, that the underlying event and its molecular origin are initiated by the light-induced rhodopsin reaction cycle. Thus, the triggering reaction is located within this cycle. The saturation of the response (Results, 1b) indicates, however, that the response per bleached rhodopsin decreases with the percentage of bleached rhodopsin within the rod outer segments: the transduction from rhodopsin to the events underlying our observation is dependent on the quantity of light previously applied. This is essentially an adaptation behaviour directly observed at the level of the isolated rod outer segment. It could play a role in the adaptation of the rod in vivo.

There are several models which could explain the observed behaviour [1].

Preliminary results on the dependence on flash-time interval, pH and ionic milieu favour the model of a rapidly changing interaction of rhodopsin. This interaction can be imagined via membrane-inherent processes or an agent released in the internal disc space.

2. Changing structural parameter

Since the calculations of Rayleigh [18] and of Gans [19] progress has been achieved in the understanding of light-scattering phenomena [20,21]. Information about the structural units which produce a certain observed light-scattering was obtained for symmetric and well defined systems [22]. With such a complicated system as the rod outer segment, however, we have to be satisfied with an empirical approach.

Two parameters possibly changing with the change in light-scattering must be considered: 1. The refractive index of the scattering particles (n). 2. The form of the scattering particles, i.e. their shape and their size (volume).

Taking into consideration that, with increasing "n", light-scattering as well as apparent absorbance increase, one would expect an amplitude for the light-induced change in apparent absorbance which decreases with increasing wavelength, if the changing parameter was n. On the other hand, if the light-scattering change was due to a form-change, the change in apparent absorbance could also increase with increasing wavelength, as Latimer et al. have calculated for particles having a size within the range of the wavelength [23,24].

This latter behaviour is observed, as shown in Fig. 2 (Results, 2a). It favours

a form change as the underlying structural event to our observation.

From the measurement of the light-induced light-scattering change as a function of the scattering angle a further decision can be made (Results, 2b). For particles smaller than the wavelength, a change of n would result in a light-scattering change independent of the scattering angle (Rayleigh-Gans approximation). For particles in the range of the wavelength there should be a deviation from that θ-independence only at small scattering angles. A form-change, however, shows itself in a marked angular dependence of the corresponding light-scattering signal. Several examples can be derived from the work of Latimer et al. [23,24], who calculated the light-scattering of different biological particles as a function of their volume and of the scattering angle.

A quite similar angular dependence as we measured for our signal amplitude (Fig. 4) can be derived from the results of these authors for a shrinkage of mitochondria with a volume of 0.5 μm^3. The agreement confirms the assumption of a form change as the structural origin of our observation, suggesting at the same time a shrinkage of the rod outer segments.

This hypothesis can be proved, if a shrinkage of the rod outer segments is osmotically induced from the outside (Results, 3), and the concomitant light-scattering change is observed. Related experiments were done by Heller et al. [25] and McConnell et al. [26,27].

The agreement of the two plots in Fig 5 shows: The structural change underlying to our observation is the shrinkage of an osmotic compartment of the rod outer segments. That does not necessarily mean that the underlying event is osmotically induced, it only means that the compartment shrinking after illumination is an osmotic compartment.

3. Producing osmotic compartment

Cohen has pointed out that in the rods of intact retinae there are three possible loci for osmotic control: the disc membrane, the plasma membrane and the extra-disc matrix which probably exhibits osmotic properties, due to its gel-like nature [28]. In the case of bovine rod outer segment preparations, McConnell [27] obtained evidence for more than one osmotic compartment and he proposed that they were the discs and the plasma membrane. Heller's results on frog rod outer segments, however, suggest that only one compartment is still intact namely the disc [25]. Corless' X-ray data on frog rod outer segments also suggest the only effect of increasing osmolarity to be a shrinkage of the discs [6].

We now have to prove that the osmotic compartment producing our light-scattering signal is also the disc.

Concerning the plasma membrane, we assume that it is no longer intact in our preparation (Materials and Methods). A gel-like matrix as proposed by Cohen [28] also seems to exist in our preparation; this matrix could in turn stabilize the disc packets. Such a matrix, however, cannot be made to swell by adding ionophores, and such a swelling can be deduced from the light-scattering observed with our preparations (Results, 3b).

Thus, the only intact osmotic compartment is the disc. From this finding and from the comparison of the angular dependences measured with the light-scattering change and with the swelling induced by the ionophore A 23187 we

deduce that the underlying event to our observation is a fast light-induced shrinkage of the disc vesicle.

4. Molecular origin

Since the light-induced change in light scattering can be mimicked by osmotic effects, it is quite obvious to assume that its molecular origin is an osmotic one as well, for instance a light-induced ion-flux across the disc-membrane. The most probable candidate for such an ionic flux would be a light-induced Ca^{2+}-efflux as postulated in Hagins' hypothesis on the mechanism of signal-transduction in vision [29].

The action of the Ca^{2+} concentration on the amplitude of the light-induced change in apparent absorbance is shown in Fig. 6 (Results, 4a). If Ca^{2+} reduced the extent of shrinkage by decreasing an existent Ca gradient, one could expect a quite different course, lowering the amplitude to zero or even inverting its sign. The observed saturation effect at about 20 mM Ca^{2+} or Mg^{2+} favours another explanation. It is known that divalent cations are bound to biological membranes giving them an increased rigidity. This increased rigidity could reduce the sensivity of the discs to light-induced forces making the discs shrink. A concentration of about 20 mM therefore would correspond to the saturation point where the disc membrane is completely loaded with Ca^{2+} or Mg^{2+}.

Moreover, the action of other ions (Results, 4b) makes it improbable that there is any ion gradient across the disc membrane in our preparations which is reduced by light. The experiments with ionophores (Results, 4c) support this suggestion: The light-induced light-scattering change should not be observed without any change if an ion gradient were a necessary condition for its underlying event. Therefore it is quite likely that the effect does not reflect any passive cation-efflux from the discs due to a light-induced permeability change.

5. Disc volume change

According to Koch [30] the apparent absorbance of scattering particles varies with their volume obeying the equation: $A \alpha V^{-2/3}$. As Bryant pointed out, there are some cases where the exponent lies between $-2/3$ and -2 [31]. Therefore the overall amplitude of the light-scattering change ($-\Delta I/I = 10^{-2}$) corresponds to a volume change between 0.5 and 1.5% only.

Thus, the observed nonlinearity of the response with flash intensity (Results, 1b) should not be caused by the nonlinearity of the shrinkage but must have its basis in the molecular origin itself (see also Discussion 1).

6. Comparison with X-ray data

Chabre and Cavaggioni, on the base of X-ray data, found a light-induced shrinkage of the disc lattice distance in the range of approximately 1% when studying isolated frog rod outer segments [3]. This shrinkage is markedly reduced when the Ca^{2+} content of the reaction medium is increased. With our effect an analogous behaviour is observed: its amplitude is reduced to 40% of its original value when 20 mM Ca^{2+} is added and it is enhanced with EDTA. As the shrinkage described by Chabre occurs to its full extent before full bleaching is achieved, just like the response we observe saturates, we consider the shrink-

age which is derived from X-ray data to be the same phenomenon as that visualized by our observation. That means that the diminution of the lattice distance is due to a shrinkage of the single discs.

Our results on the molecular origin of the disc shrinkage (it is not due to a passive light-stimulated ion-efflux from the discs) do not disprove Hagins' Ca hypothesis nor any other ionic transmitter model. They only mean that there is no evidence at all for such an ionic flux in our preparations. It is possible that a permeability change as postulated by Hagins still takes place, but it cannot be visualized since there are no longer ion-gradients which can be removed by light. Another possibility is that the Ca^{2+} acting as transmitter is not released from inside the discs but from the disc-membrane itself, or that Ca^{2+} is ejected in an active step. Even if the disc shrinkage itself does not prove to be of direct importance for signal-transduction in vision it might be a sensitive indicator for studying the role of the disc membrane in signal transduction.

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