Volume 235, number 1,2, 103–108 FEB 06113 August 1988

Rapid transducin deactivation in intact stacks of bovine rod outer segment disks as studied by light scattering techniques

Arrestin requires additional soluble proteins for rapid quenching of rhodopsin catalytic activity

Rafael Wagner, Nicholas Ryba* and Rainer Uhl

Max-Planck-Institut für Biophysikalische Chemie, Am Faßberg, D-3400 Göttingen, FRG

Received 17 May 1988

In photoreceptors of the living retina both activation and deactivation of transducin must occur in less than 1 s. In ROS preparations used for in vitro studies, however, deactivation takes minutes. This is due to the fact that activated transducin is released into the free aqueous space, whereby GTPase activity and consequent deactivation of the protein are slowed down, and due to the dilution of soluble ROS proteins involved in the quenching of rhodopsin activity. In this paper, using a convenient, non-invasive light scattering assay, we demonstrate that in an intact stack of disks, where active transducin stays membrane associated and is rapidly deactivated, the activity of rhodopsin can also be quenched in the time range of seconds when soluble ROS proteins are supplemented. Arrestin, the 48 kDa protein of the photoreceptor, is one of the proteins required for rapid recovery, however, it requires the synergistic action of other soluble proteins (besides rhodopsin kinase) in order to exert its effect: When arrestin is included in the reaction mixture without the 'helper protein(s)', it cannot speed recovery, and when a mixture of soluble proteins is added which lacks arrestin, there is also no effect. The nature and identity of this (these) helper protein(s) are still unclear.

Photoreception; Transducin deactivation; Rhodopsin phosphorylation; 48 kDa protein; Arrestin; Light scattering

1. INTRODUCTION

In a previous article [1], we have demonstrated that a particular, light-induced light scattering transient from intact stacks of bovine photoreceptor disks, P_A, reflects the transient activation of transducin, and later [2] we have provided evidence for a GTPase turnover number of more than 1 s^{-1} in this particular preparation. It was the first direct in vitro demonstration of rapid transducin deactivation, close to the speed of the in vivo process. Previously reported values were more than one order of magnitude lower (reviews [3,4]). We have attributed the considerably faster GTPase rates that we measured to the fact that in our system transducin never leaves the disk surface, during either activation or deactivation.

The rate of recovery of the P_A signal and hence the rate of transducin deactivation is slow, much slower than that of GTPase activity, and it depends on the light intensity of the test flash [2]. This indicates that in our in vitro system rhodopsin deactivation is slower than in vivo. Here, we have attempted to bring our in vitro system a step closer towards the in vivo state by complementing it with soluble photoreceptor proteins. One of them, arrestin (48 kDa protein), has been reported to bind to phosphorylated rhodopsin, thus quenching its ability to activate (and reactivate) transducin [5]. Arrestin, like other soluble ROS proteins, is con-

Correspondence address: R. Uhl, Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, FRG

* *Present address:* Dept of Biochemistry, University of Leeds, Leeds LS2 9JT, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

siderably diluted in our assay, since we have to rupture the photoreceptor plasma membrane in order to establish physiological levels of nucleotides in the rod interior.

We found that additions of purified 48 kDa protein had no effect on the rate of transducin deactivation. However, in the presence of small amounts of other soluble ROS proteins a dramatic effect of the 48 kDa protein was observed: transducin was deactivated within a few seconds, even at flash intensities more than 100-times above those needed for saturation of the light response of dark-adapted photoreceptors. This means that our intact disk stack, when complemented with a cocktail of soluble ROS proteins, constitutes a convenient in vitro system in which activation and deactivation processes can be studied in the physiological time range.

2. MATERIALS AND METHODS

The procedure for preparation of structurally intact disk stacks with perforated plasma membrane has been detailed elsewhere [6]. Briefly, retinas of fresh bovine eyes were isolated in Hepes-Ringer, passed through a nylon mesh after vortex-mixing and spun at 30000–60000 × *g* for 20 min on a discontinuous sucrose gradient (31%, w/v). ROS at the interface were harvested and washed once in Ringers. Finally, pellets were resuspended in a sucrose/Ringers medium where 50 mM NaCl was replaced by 100 mM sucrose, at a rhodopsin concentration of about 0.1 mM. Small aliquots were frozen quickly in liquid N_2 and thawed immediately prior to an experiment. In our hands, this yields ROS with mostly intact disk stacks but perforated plasma membranes.

Soluble ROS proteins were obtained using a modification of the procedure in [5] (purified 48 kDa protein) or by simple low ionic salt washes.

Light extract: ROS were isolated [6] under room light from previously light-adapted eyes. The ROS were washed twice for 30 min in 15 mM NaCl, 5 mM Hepes (pH 7.4), and 0.5 mM $MgCl_2$. The supernatants were combined and concentrated by ultrafiltration.

Dark extracts were prepared analogously, except that dark-adapted eyes were used and that light exposure (15 min under room light) preceded the washing step.

Purified 48 kDa protein was obtained by washing the pellet (left over from the preparation of the light extract) with 5 mM Hepes, pH 7.4, 0.5 mM $MgCl_2$, and 0.1 mM GTP-γ-S to remove transducin and PDE, and then twice without GTP-γ-S. The membranes were resuspended and incubated for 12 h in 500 mM NaCl and 5 mM Hepes (pH 7.4) to dissociate the 48 kDa protein. The resulting supernatant was diluted with 5 mM Hepes (pH 7.4) to 100 mM NaCl and concentrated by ultrafiltration. Identical results were obtained when purified 48 kDa protein was used that was given to us by Hermann Kühn.

All buffers were at 4°C, contained 1 mM DTT and were thoroughly saturated with argon. Supernatants were clarified by centrifugation for 30 min at 100000 × *g* before use.

Light scattering measurements were performed in a multi-angle flash photolysis apparatus (MAFPA), the design of which has been described in [7].

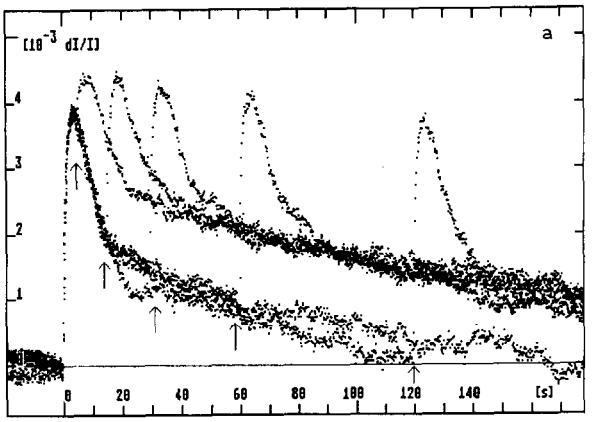
Before each experiment ROS samples were thermally equilibrated in the monitoring (IR) beam of the MAFPA and subsequently flashed once (10^{-3} bleach) in the presence of GTP. This way all damaged ROS fragments lost their transducin molecules irreversibly, as judged from gel electrophoresis (not shown) and from the occurrence of a small 'loss signal' [8]. Subsequent flashes, which gave rise to the signals displayed in this paper, stimulated no further transducin loss (no loss signal, no transducin on the gel).

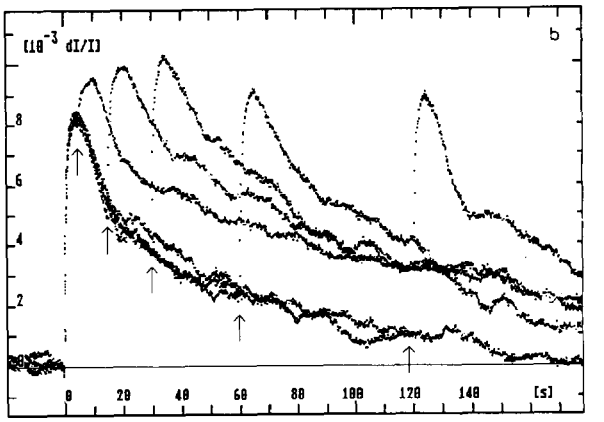
All chemicals were of the highest grade available from Sigma.

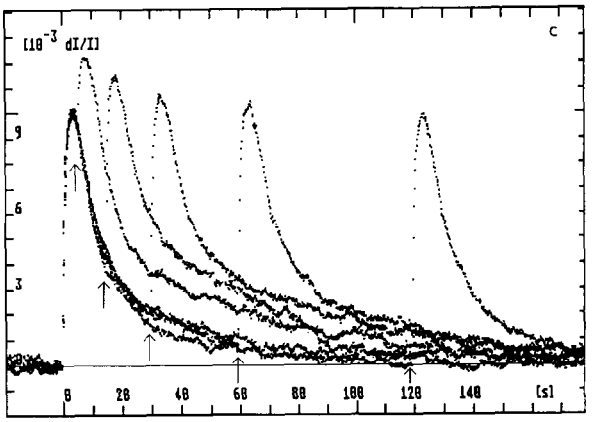
3. RESULTS AND DISCUSSION

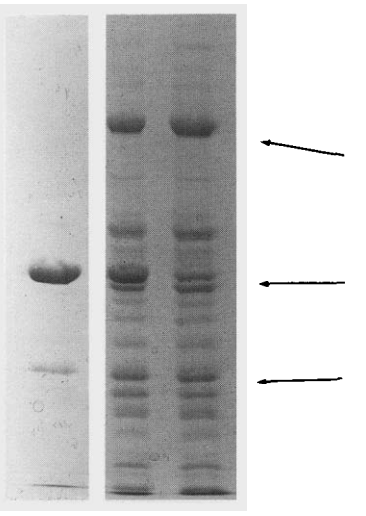
A flash, bleaching a fraction of 2.7×10^{-4} of the total rhodopsin, caused activation of 90% of the total transducin pool under our assay conditions, i.e. in structurally preserved disk stacks [2]. Deactivation occurred in two phases, a fast one, being completed in ~10 s and a slow one of 120 s [2]. The time course of the light scattering signal P_A indicated that both components contributed to equal extents to the overall deactivation process (fig.1a). We have previously shown [2] that, under these conditions, GTPase activity is much faster than the observed overall deactivation and that rhodopsin deactivation must therefore be rate-limiting. Rhodopsin deactivation requires rhodopsin to be (multiply) phosphorylated [9] and, subsequently, 48 kDa protein (also known as 'arrestin'), to be bound [5]. Rhodopsin kinase, responsible for rhodopsin deactivation by ATP-dependent phosphorylation, is not easily washed out from ROS membranes [10]. Arrestin, on the other hand, is a soluble protein [5], which is 4000-fold diluted in our experiment. Gel electrophoresis data (figs 1e,2b) show vanishingly small amounts of arrestin in our assay mixture, and increasing or decreasing the ROS concentration in the assay had no influence on the kinetics of P_A [2]. The recovery observed in fig.1a must therefore reflect rhodopsin deactivation solely due to phosphorylation.

Fig.1b shows P_A signals in the presence of 3×10^{-6} M exogenous, purified arrestin, which, in separate experiments, was shown to bind to bleached rhodopsin (fig.1e). No acceleration of the recovery was observed. Instead, the signal-to-noise ratio of the light scattering transients was reduced









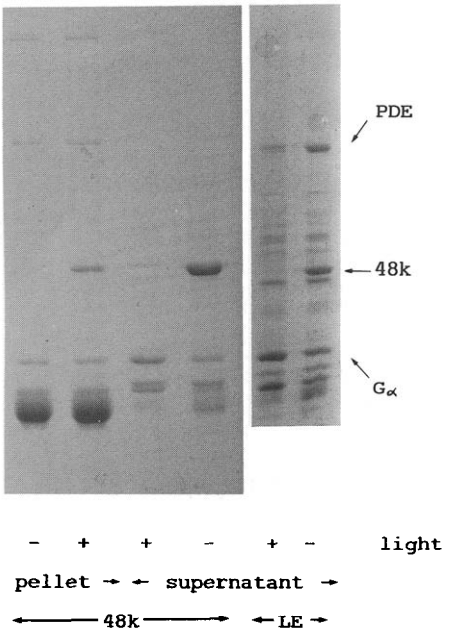


Fig.1. Double-flash experiments in the absence and presence of soluble ROS proteins. (a) Control, no proteins added; (b) addition of 3×10^{-6} M purified 48 kDa protein; (c) addition of a light extract of soluble ROS proteins, including 3×10^{-6} M 48 kDa protein. Freshly thawed ROS were suspended (at a rhodopsin concentration of 10^{-6} M) in 100 mM NaCl, 2 mM $MgCl_2$, 0.2 mM BAPTA, 10 mM Hepes (pH 7.4) and incubated for 3 min in the infrared light beam of the MAFPA. After a first flash, removing transducin from damaged ROS, doublets of flashes were applied, each bleaching a fraction of 2.7×10^{-4} of the total rhodopsin. Flashes were separated by 5, 15, 30, 60 and 120 s. All five curves, recorded sequentially from one sample, were superimposed on a control curve representing a single-flash experiment. The scattering angle was 24° and the temperature 21°C. (d) Characterization of soluble ROS proteins by SDS-PAGE. 48k, 48 kDa protein; LE, light extract; DE, dark extract. (e) Control of the binding properties of 48 kDa protein in two 48 kDa protein-containing fractions: 48k, purified 48 kDa protein; LE, light extract.

(this was observed consistently in a variety of experiments) and the fast component of the recovery was even slowed down. However, when a 'light extract' of soluble ROS proteins, containing similar amounts of arrestin (fig.1d), was added, a clear effect could be observed (fig.1c): the signal quality improved, the fast component of the recovery became slightly faster, and the slow component was greatly accelerated. Due to the increased rate of recovery the light sensitivity was reduced, i.e. the light titration curve was shifted towards higher light intensities, signifying that more light was required to activate all transducin. Because of this the test flash then activated only 80% of the total transducin.

There are three possible explanations for our observation:

(i) The 48 kDa protein in the light extract differs from the purified form.

(ii) It is not arrestin, but some other soluble ROS protein, which speeds the recovery of the P_A signal.

(iii) Arrestin requires one or more additional proteins to exert its effect.

The results depicted in fig.2 allow one to decide between the three possibilities. We have measured the recovery, i.e. the decrease in response amplitude to saturating (double intensity compared to fig.1) flashes which were applied every 2 min. Such a series of light pulses leads to a decrease in signal amplitude with time, both in the absence of soluble proteins and in the presence of purified arrestin or of the dark extract, which contained little arrestin. On the other hand, upon the addition of light extract or a mixture of dark extract and purified 48 kDa protein, the gradual decrease in response amplitude was prevented. In both cases the time course of the signals was identical (not shown), indicating that the fast recovery required both 48 kDa protein and one or more protein component(s) of the ROS extract. This rules out possibilities (i) and (ii).

P_A signals under optimal conditions, i.e. in the presence of a light extract and of additional, purified 48 kDa protein, are shown in fig.3. The total 48 kDa protein concentration was 7×10^{-6} M and the temperature 37°C. Under these conditions, the signal-to-noise ratio improved tremendously and the rate of recovery was further increased, approaching physiological values. Note that in the double-flash experiment in fig.3b the

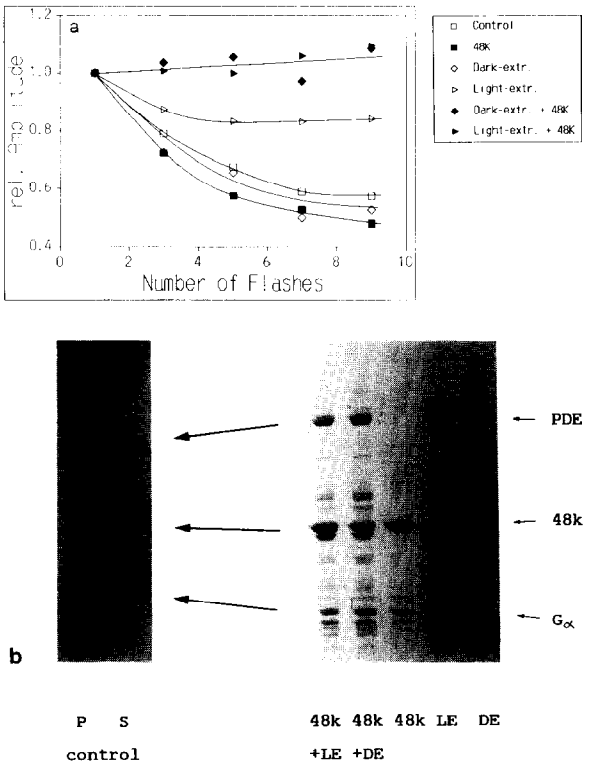


Fig.2. Decline of the P_A amplitude upon the addition of different mixtures of soluble ROS proteins. (a) ROS were exposed to a sequence of flashes (0.5 min^{-1}), each bleaching 5.4×10^{-4} of the total rhodopsin, and amplitudes were taken at the relative maximum. Depending on the presence or absence of certain soluble ROS proteins the amplitude decreased rapidly, slowly, or not at all. (b) Polyacrylamide gels, taken from the supernatant of the six light scattering experiments. The control contained hardly any 48 kDa protein, the dark extract less than 1.3×10^{-6} M, the light extract 3×10^{-6} M, the purified 48 kDa also 3×10^{-6} M, the mixture of dark extract and purified 48 kDa 3.7×10^{-6} M, and the mixture of light extract and purified 48 kDa approx. 7×10^{-6} M. Relative concentrations were judged by gel scans. Absolute concentrations were roughly estimated from the absorbance at 280 nm (A_{288}), assuming that 1 mg/ml protein corresponds to a value of 2 absorbance units. Abbreviations as in fig.1d,e.

flash intensity was increased 2-fold compared to fig.2 and 4-fold compared to fig.1. It bleached 10^{-3} of the total rhodopsin, a value more than 200-times higher than the bleaching required for saturation of the light response of a dark-adapted photoreceptor cell (5×10^{-6}).

In our assay the arrestin concentration appeared to be the limiting factor for the speed of recovery. The concentration of the 'helper proteins' could be

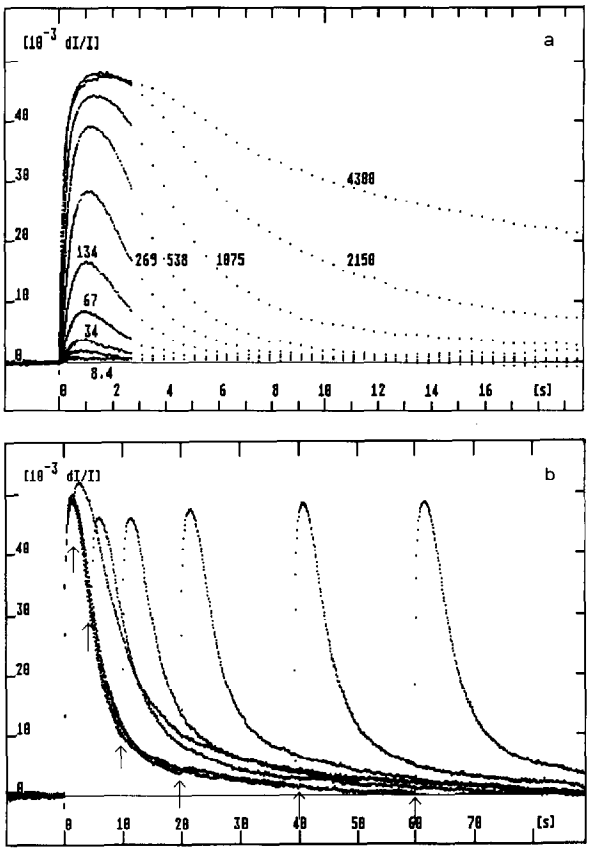


Fig.3. P_A signals at physiological temperature, in the presence of arrestin (7×10^{-6} M) and other soluble ROS proteins. (a) Light titration. The flash energy was incremented by a factor of two, starting at a bleaching of 8.4×10^{-6}. (b) Double-flash experiment as in fig.1a–c. The flash intensity was increased 4-fold, i.e. one flash bleached 0.1% of the total rhodopsin.

reduced by a factor of two without slowing the response, and an increase in their concentration, such as an increased concentration of rhodopsin kinase, had no further accelerating effect (not shown). The arrestin concentration, on the other hand, could not be reduced without a loss in the speed of recovery (not shown). So far we have not tested arrestin concentrations above 7×10^{-6} M, since arrestin polymerises to varying extents at higher levels. We are confident, however, that the rate of recovery that we measure does not constitute an upper limit for our in vitro system and that our assay will prove very helpful for further tests of other factors governing response speed and adaptation.

4. CONCLUDING REMARKS

In this and a previous paper we have shown, using light scattering as a convenient probe, that an in vitro assay of the photoreceptor can be constructed, which allows one to study transducin activation and deactivation in the physiological time domain. While rapid transducin activation is easily achieved, the inactivation process in the in vitro system has always been very slow. When intact stacks of disks were used, however, prepared as we have previously described [6], one factor governing the deactivation, the GTPase rate, was greatly accelerated [2], and when soluble ROS proteins were added back to the disk stacks, the rhodopsin deactivation was also speeded up.

Our results clearly demonstrate that arrestin, the 48 kDa protein of the photoreceptor, plays an important role in this deactivation process, as previously suggested [5]. However, they also indicate that the binding of arrestin alone is not sufficient for rapid quenching, since under conditions where 48 kDa protein binding to bleached rhodopsin occurs, no effect on the recovery of the P_A signal is seen unless other soluble protein components are supplemented. This is in contrast to previous results from others [5,10], who have reported an effect of purified arrestin on the shut-off time of the rod phosphodiesterase (PDE). While this would seem to point to a direct interaction between arrestin and PDE, as suggested in [11], we favour another explanation for the observed discrepancies: in our intact system transducin deactivation occurs within seconds, even in the absence of arrestin, whereas in the reconstituted systems used in [5,10] it takes minutes. We assume that the fast phase of recovery, which we measure in the absence of arrestin, reflects the transfer of one phosphate group to the activated rhodopsin, reducing its activity, and that the slow phase reflects multiple phosphorylation, causing complete quenching. This occurs in our system before an interaction with purified arrestin can take place, whereas in the reconstituted system [5,10] phosphorylation is so slow that arrestin has the chance to interact before phosphorylation is completed. The rapid, physiological effect of arrestin, however, which sets in long before complete phosphorylation has been achieved and which can only be observed in

a well-preserved system, requires the presence of (an)other soluble ROS protein(s). While the exact mechanism of this synergistic effect is still unclear, the present data clearly demonstrate that our light scattering assay is a powerful tool for the answering of this and related questions in the future.

Acknowledgements: Some of the results of this work have been presented at the 1987 FASEB meeting on visual transduction, held at Copper Mountain, Colorado. The authors gratefully acknowledge the financial support from the DFG (research grant to R.U. UH30/7). R.U. was a Heisenberg fellow and N.R. was supported by a European Science Exchange Fellowship. The authors are grateful to H. Kühn for the generous gift of 10 mg of purified 48 kDa protein.

REFERENCES

[1] Wagner, R., Ryba, N.J.P. and Uhl, R. (1987) FEBS Lett. 221, 253–259.
[2] Wagner, R., Ryba, N.J.P. and Uhl, R. (1988) FEBS Lett. 234, 44–48.
[3] Dratz, E.A., Lewis, J.W., Schaechter, L.E., Parker, K.R. and Kliger, D.S. (1987) Biochem. Biophys. Res. Commun. 146, 379–386.
[4] Arshavsky, V.Yu., Antoch, M.P. and Philippov, P.P. (1987) FEBS Lett. 224, 19–22.
[5] Wilden, U., Hall, S.W. and Kühn, H. (1986) Proc. Natl. Acad. Sci. USA 83, 1174–1178.
[6] Uhl, R., Desel, H., Ryba, N. and Wagner, R. (1987) J. Biochem. Biophys. Methods 14, 127–138.
[7] Uhl, R., Desel, H. and Wagner, R. (1985) J. Biochem. Biophys. Methods 11, 31–43.
[8] Vuong, T.M., Chabre, M. and Stryer, L. (1984) Nature 311, 659–661.
[9] Liebman, P.A. and Ari, S. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17, 215–225.
[10] Kühn, H. (1981) in: Current Topics in Membranes and Transport, pp.171–201, Academic Press, New York.
[11] Zuckerman, R. and Cheasty, J.E. (1986) FEBS Lett. 207, 35–40.