Volume 221, number 2, 253–259 FEB 05084 September 1987

The amplified P-signal, an extremely photosensitive light scattering signal from rod outer segments, which is not affected by pre-activation of phosphodiesterase with Gα-GTP-γ-S

Rafael Wagner, Nicholas J.P. Ryba and Rainer Uhl

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

Received 19 June 1987

A light scattering signal from bovine rod outer segments in the presence of GTP is described. It has the same angular dependence as the P-signal but is 3 orders of magnitude more sensitive to light and therefore we have called in the amplified P-signal. Adding Gα-GTP-γ-S has no significant effect on the light scattering signal despite the activation of PDE. cGMP affects the amplified P-signal, but subsequent addition of Gα-GTP-γ-S restores the normal signal character. All these facts strongly support the view that the amplified P-signal reflects G-protein activation rather than that of PDE. This is in striking contrast to an interpretation of a very similar light scattering signal previously described by other groups.

Rod outer segment; Light scattering; G-protein; Phosphodiesterase

1. INTRODUCTION

Recently, it has become clear that a light-induced enzymatic cascade plays a key role in visual transduction. The nature of this cascade has been established mainly using classical biochemical methods (review [1]). However, light scattering experiments have also provided important information, particularly with respect to the kinetics of the various reactions [2,3]. Light scattering provides a very sensitive, noninvasive method of study. Unfortunately, the interpretation of changes in light scattering suffers from a lack of specificity of the technique and requires great care. Despite this problem, light scattering studies of ROS have helped significant advances to be made in understanding transport processes, across the disk and plasma membranes [4], and also light-induced changes in protein interactions. Here, we focus on the light scattering changes associated with these protein interactions.

There are several distinct transients reflecting the different stages of the enzymatic cascade. The signals can be distinguished by differences in their sensitivity to light activation, and by the angular dependence and time course of the light scattering transients. The process which converts the rhodopsin from its inactive to its active form, is accompanied by the so-called N-signal (N representing its negative sign). The N-signal is strictly proportional to the amount of meta-rhodopsin II (MII) formed and its kinetics and relative amplitude parallel the meta-rhodopsin I–meta-rhodopsin II reaction [5].

In the absence of GTP the next step of the en-

Correspondence address: R. Uhl, Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, Nikolausberg, FRG

Abbreviations: GTP-γ-S, guanosine, 5′-*O*-(3-thiotriphosphate); ROS, rod outer segments; G-protein, GTP-binding protein or transducin; PDE, cyclic nucleotide phosphodiesterase; Gα-GTP-γ-S, GTP-γ-S bound to the α-subunit of G-protein; DTT, dithiothreitol; NMG, *N*-methylglucamine; BAPTA, 1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

zymatic cascade in ROS, the rhodopsin–G-protein interaction is reflected by the P-signal (P, positive sign). The P-signal saturates at approx. 10% bleaching, which corresponds to the molar ratio of G-protein to rhodopsin. It has been proposed that this signal arises from the light-induced MII–G-protein binding and is therefore often called the binding signal [2].

Under more physiological conditions (i.e. in the presence of GTP), one activated rhodopsin can interact with many molecules of G-protein thus resulting in a signal with enhanced light sensitivity. This signal has a negative sign and saturates at approx. 0.1% bleaching. Since this change in light scattering was ascribed to the reversal of the binding of G-protein to MII it was referred to as the dissociation signal [2].

The next step in the amplification cascade, the activation of PDE, has been reported to be reflected by another light scattering signal, the AT signal, which exhibits an even higher sensitivity to light. One rhodopsin bleached per disk surface is sufficient for evoking the full amplitude of this change in light scattering [7].

We present evidence that a light scattering transient that we have measured, which appears very similar to the AT signal, reflects the activation of the G-protein rather than that of the PDE.

2. MATERIALS AND METHODS

Our preparation of ROS has been extensively described [8]. Briefly, retinas of fresh bovine eyes were dissected into Hepes-Ringer, passed through a nylon mesh after vortex-mixing and spun at 30–60000 × g for 20 min on a single-step sucrose gradient (31%, w/v). ROS were harvested from the buffer/sucrose interface and washed once in Ringers. ROS pellets were resuspended in 200 mM sucrose, 2 mM $MgCl_2$, 5 mM NMG-Hepes (pH 7.4) to a final concentration of about 200 μM rhodopsin. The absorption ratio A_{280}/A_{500} was typically between 2.2 and 2.4. Aliquots of 50 μl of this ROS suspension were frozen quickly in liquid nitrogen. Under these conditions we generally obtain ROS as an intact disk stack with a perforated plasma membrane [8].

Light scattering measurements were performed in a multi-angle flash-photolysis apparatus (MAFPA), the design of which has been described previously [6]. Using MAFPA we can record light scattering changes at 8 different scattering angles from 0 to 28° in steps of 4°. Flash photolysis was initiated with a xenon flash, calibrated by evoking a series of N-signals. A full flash bleached 11.2% of the residual rhodopsin, lower bleaching being obtained using high-precision neutral density filters (Schott).

PDE activity was determined by the measurement of the concomitant proton release, using a calomel pH electrode [9]. The measuring buffer contained 100 mM NaCl, 2 mM $MgCl_2$, 5 mM Na-Hepes, 0.2 mM Ca-buffer BAPTA, 0.1 mM $CaCl_2$, 2 mM DTT, pH 7.5. The assay buffer has an unusually flat calibration curve (δpH/δH^+ vs pH) with a buffer capacity of 2.5 δpH·(mol H^+ added)$^{-1}$ over the pH range 7.5–6.5. Freshly thawed samples of frozen ROS were added to a final concentration of 2 μM rhodopsin. Nucleotides were added from concentrated stock solutions: 20 mM Mg-GTP, 100 mM Mg-ATP, 200 mM cGMP, each adjusted to pH 7.5 with NaOH. All measurements were performed at room temperature (22°C).

G-α-GTP-γ-S was purified from isotonic washed ROS. ROS from 15 eyes were exposed to bright red light at 4°C, lysed by rapid freezing in 30 ml Ringer diluted to an osmolarity of 40 mosM and contained 50 μM GTP-γ-S. ROS were pelleted and the supernatant was recentrifuged (80000 × g, 40 min) to remove traces of membrane. The crude Gα-GTP-γ-S was loaded directly onto a DE-52 cellulose column (1.5 ml bed volume) washed with 100 mM NaCl, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.4) and eluted with 0.5 M salt. The concentrated fraction was applied to a Sephadex G-75 column (120 ml bed volume). Protein-containing fractions were checked for purity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [10] and Gα-containing fractions were pooled, diluted to 100 mM salt and concentrated using the DE-52 column. After concentration Gα appeared to be at least 95% pure (SDS-PAGE), and the free GTP-γ-S was <1 μM as judged by absorption spectroscopy.

3. RESULTS

Light scattering signals in the presence and absence of GTP were characterised with respect to

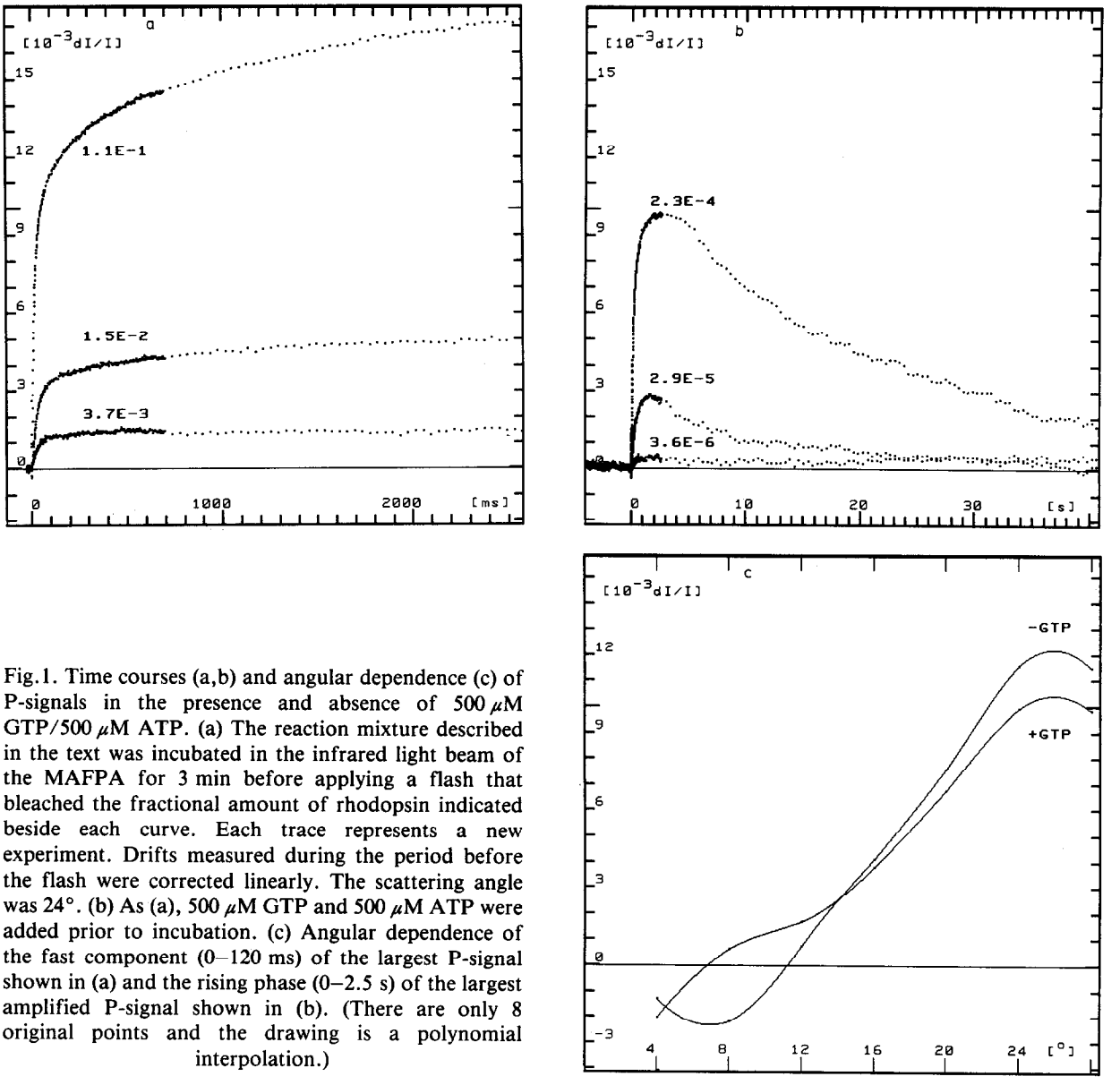

Fig.1. Time courses (a,b) and angular dependence (c) of P-signals in the presence and absence of 500 μM GTP/500 μM ATP. (a) The reaction mixture described in the text was incubated in the infrared light beam of the MAFPA for 3 min before applying a flash that bleached the fractional amount of rhodopsin indicated beside each curve. Each trace represents a new experiment. Drifts measured during the period before the flash were corrected linearly. The scattering angle was 24°. (b) As (a), 500 μM GTP and 500 μM ATP were added prior to incubation. (c) Angular dependence of the fast component (0–120 ms) of the largest P-signal shown in (a) and the rising phase (0–2.5 s) of the largest amplified P-signal shown in (b). (There are only 8 original points and the drawing is a polynomial interpolation.)

their light sensitivity (fig.1a,b) and angular dependence (fig.1c). In the absence of GTP the well known P-signal was obtained. This signal is easily identified by the typical angular dependence of its fast component. In the presence of GTP there was a dramatic increase in light sensitivity of the P-signal. Comparison of the fast component of the signal in the absence of GTP and the rising phase of the signal in the presence of GTP demonstrated that both the angular dependence and the total amplitude of the transient remained essentially unchanged on addition of GTP. Because of the highly amplified response to light and the unchanged nature of the light scattering we have called this signal the amplified P-signal. The kinetics and light sensitivity of the amplified P-signal very closely resemble the AT-signal described by Kamps et al. [7,11].

PDE activity was measured as a function of Gα-GTP-γ-S addition (fig.2). Increasing activity of

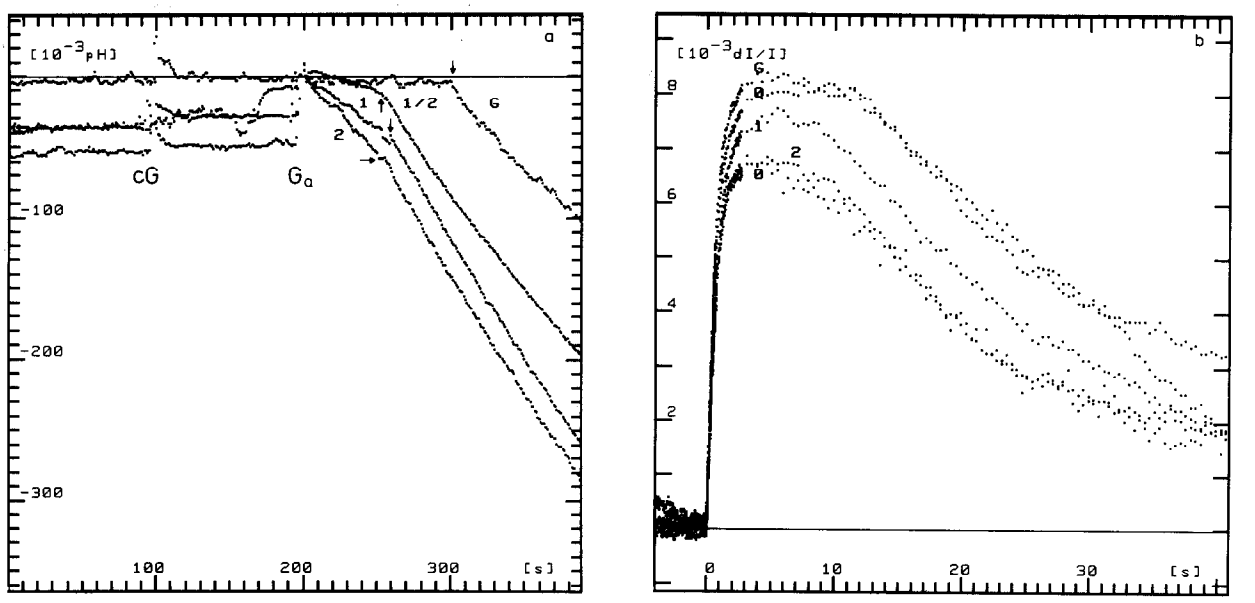

Fig.2. Effect of Gα-GTP-γ-S on PDE activity and amplified P-signal. (a) pH traces showing PDE activity. The reaction mixture containing GTP and ATP described in fig.1 was incubated for 3 min in complete darkness. Then 5 mM cGMP was added (at cG on the trace) and at Ga, Gα-GTP-γ-S was added, a saturating flash (several percent rhodopsin) was delivered at the time indicated by the arrow. The Gα-GTP-γ-S concentration is given in units of 1/10 of the rhodopsin concentration, i.e. units of the natural G-protein content; G, 5 μM GTP-S was added at Ga instead of Gα-GTP-γ-S. The pH jumps recorded at Ga are due to different pH values of the Gα-GTP-γ-S stock and the reaction mixture. Fluctuation of one of the curves before Ga results from repositioning of the pH electrode. (b) Amplified P-signals. Preparations as above without addition of cGMP. Scattering angle was 24°. The flash at $t = 0$ bleached a fraction of 2.3×10^{-4} of the rhodopsin. (The two controls without Gα-GTP-γ-S give an upper limit of the variation of amplitude between experiments.)

PDE was evoked by increasing amounts of Gα-GTP-γ-S without light activation of rhodopsin. The dark activity measured before addition of Gα-GTP-γ-S was essentially constant and the maximum activity obtained after a saturating flash was independent of Gα-GTP-γ-S. In striking contrast to the activation of PDE, the amplified P-signal remains almost unchanged on addition of Gα-GTP-γ-S (fig.2b). Experimental conditions were the same for measurement of PDE activity and light scattering except that there was no cGMP in the light scattering buffer.

We found that cGMP at concentrations which are needed to record PDE activity (several mM), but not at levels which are reported to be physiological (<70 μM [12]) affect the angular dependence of the amplified P-signal. The changes in the rising phase of the signal diminished as the amount of Gα-GTP-γ-S present was increased, i.e. with increasing PDE activity prior to the flash used to produce the light scattering signals. The kinetics and angular dependence of these effects are shown in fig.3. The angular dependence of the light scattering in the presence of cGMP did not change at the end of the fast component (300 ms) but was uniform throughout the period of measurement (2.7 s).

4. DISCUSSION

The P-signal clearly reflects the interaction of activated rhodopsin with G-protein, which in the absence of GTP constitutes the binding of G-protein by meta-rhodopsin II [2]. In the presence of GTP one activated rhodopsin can interact with many G-proteins. Photolysed rhodopsin catalyses the exchange of GDP for GTP at a binding site on Gα, and this allows dissociation of the MII–G-protein complex. The Gα-GTP activates PDE [13]. Under these conditions the G- or dissociation

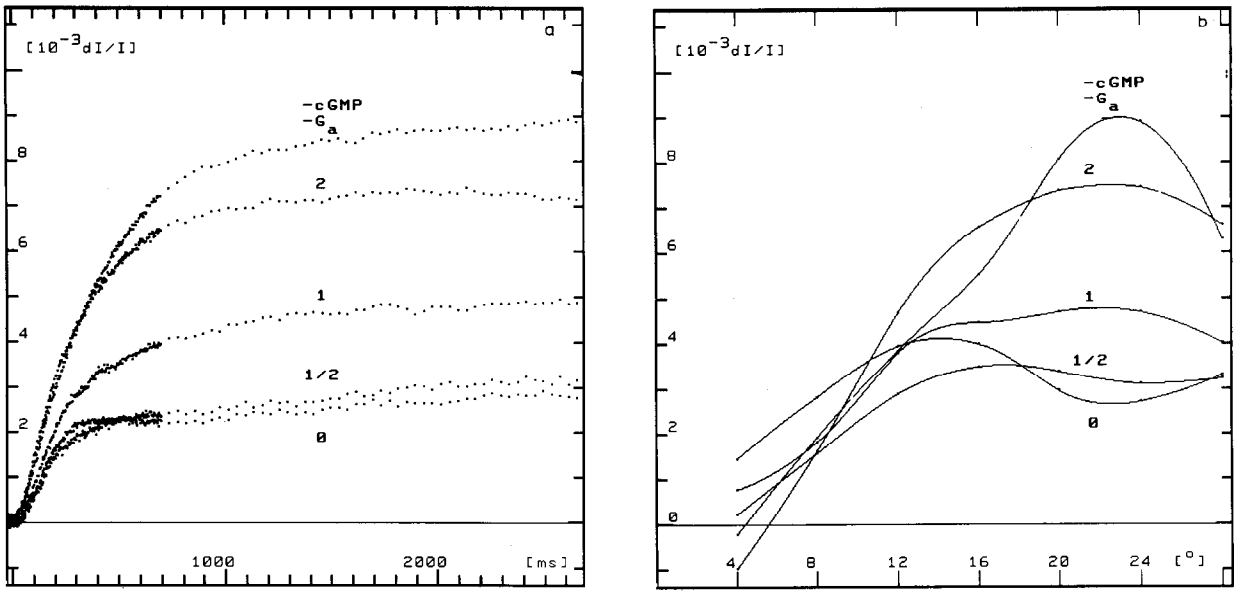

Fig.3. (a) Time course (scattering angle 24°) and (b) angular dependence (0–2.5 s) of amplified P-signals in the presence of cGMP and Gα-GTP-γ-S. Numbers indicate the amount of Gα-GTP-γ-S in units of 1/10 of the rhodopsin concentration as in fig.2a. Trace – Ga – cGMP is the control without cGMP and Gα-GTP-γ-S but with GTP and ATP present as before.

signal has been previously reported [2]. It was assumed that this signal represented the activation of G-protein in the presence of GTP and that it was the reversal process of the P- or binding signal [2]. We also find dissociation signal light scattering transients but only in preparations where the stacked disk integrity has been damaged. For instance, sonication of the ROS or treatment of the preparation with glasswool increases the amplitude of this signal at the expense of the amplified P-signal [6]. The lower light sensitivity resulting from such treatments might be due partially to smaller vesicle size with a significant reduction in the amount of rhodopsin per vesicle. However, as we have demonstrated before [6], the binding and dissociation signals are not merely the reversal of one another, but result from completely distinct structural changes. Thus, no matter exactly what processes these signals reflect, the terminology of binding and dissociation signal is misleading. We therefore prefer to use a more phenomenological nomenclature, and refer to these transients as the P-signal and G-signal respectively.

In undamaged preparations of stacked disks, we measure a light scattering signal which has the same angular dependence as the P-signal but is 3 orders of magnitude more sensitive to light. The amplified P-signal closely resembles the previously described AT-signal [7,11] both in its light sensitivity and in its time course, and we conclude that the two signals are identical. The question arises as to whether this signal reflects G-protein activation, as suggested by the similar angular dependence and the total amplitude of the P- and amplified P-signals, or if it is due to PDE activation, as reported previously [7,11]. To test these possibilities we looked for an alternative but non-perturbing way of activating the PDE without affecting the G-protein. Other groups [7] have used aluminium fluoride, which together with GDP and G-protein activates PDE [14,15] and is therefore not a suitable method of discrimination of the activation of PDE and G-protein. Protamine was also reported to affect specifically PDE activity [7], although, in our hands, no consistent light scattering signals were obtained after treatment of ROS preparations with this peptide. Gα-GTP-γ-S, on the other hand, should have few perturbing properties, because the only modification of the natural activator of PDE is that it is permanently

active due to the non-hydrolysable nature of the protein-bound GTP analogue.

The activation of PDE after addition of Gα-GTP-γ-S is extremely fast, there being no lag within the time resolution of our measurement. We have therefore checked the possibility that free GTP-γ-S in combination with internal G-protein activated PDE, because <1 μM free GTP-γ-S has been reported to cause activation of PDE [16]. We found this to be true only in the absence of GTP and ATP, and conclude that this activity is due to trace amounts of rhodopsin that are bleached under dim red light and activate the photocycle. We found that this PDE activity was shut off within 3 min of incubation of ROS with GTP and ATP in complete darkness. With ROS pre-treated in this fashion, we found no measurable activation of PDE on addition of GTP-γ-S to concentrations of up to 5 μM (fig.2). The presence of such a concentration of GTP-γ-S as a contamination in our Gα-GTP-γ-S preparation was ruled out by absorption spectroscopy. Moreover, if such a concentration of GTP-γ-S were present the amplified P-signal would be affected no matter whether it reflects activation of PDE or of G-protein.

Therefore, we conclude that the amplified P-signal monitors the activation of G-protein rather than that of PDE. The light sensitivity of G-protein activation determined from measurements of the amplified P-signal equals that of PDE [17], and it therefore follows that activation of the total pool either of G-protein or of PDE requires the bleaching of only one rhodopsin per disk surface. This implies that even at very low light intensity a substantial excess of Gα-GTP over PDE may be present.

We have demonstrated that cGMP does affect the amplitude and angular dependence of the amplified P-signal. However, pre-activation of PDE with Gα-GTP-γ-S suppresses these effects to a degree which is correlated with the fractional amount of PDE activated before light exposure. Thus the effects of addition of cGMP on the amplified P-signal seem to result from its action on inactive, light-activatable PDE. The concentration of cGMP required to produce half-maximal effect is approx. 1 mM (not shown), comparable with the K_m of light-activated PDE for cGMP [18,19]. PDE-specific, cGMP-dependent light scattering signals were reported previously [20,21]. However, the cGMP effects on the amplified P-signal when taken together with the fact that Gα-GTP-γ-S restores the normal characteristics of this transient add more weight to our conclusion that the amplified P-signal is not PDE specific, but related to the activation of G-protein.

PDE and cGMP light scattering effects have been extensively investigated in systems with disrupted disk stack and reconstituted protein content [20–22]. Although these experiments are useful in the investigation of the enzymatic properties of ROS proteins direct comparison with our data is dangerous. The intact disk stacks which we have used in our measurements appear to show much higher levels of biochemical coupling than is normally measured, and any disruptive handling leads to large differences in the light scattering properties of the preparation. Finally, as pointed out earlier, light scattering signals result from a wide variety of physical changes and alterations in preparations that may lead to very different light scattering transients without requiring fundamental differences in the biochemistry of the preparations.

ACKNOWLEDGEMENTS

N.R. acknowledges the Royal Society, London, for a European Exchange Programme award and R.U. the DFG for grant Uh 30/7 and the Heisenberg Foundation for a long-term fellowship.

REFERENCES

[1] Stryer, L. (1986) Annu. Rev. Neurosci. 9, 87–119.
[2] Kühn, H., Bennett, N., Michel-Villaz, M. and Chabre, M. (1981) Proc. Natl. Acad. Sci. USA 78, 6873–6877.
[3] Vuong, T.M., Chabre, M. and Stryer, L. (1984) Nature 311, 659–661.
[4] Uhl, R., Kuras, P.V., Anderson, K. and Abrahamson, E.W. (1980) Biochim. Biophys. Acta 601, 462–477.
[5] Uhl, R. (1976) PhD Thesis, Universität Freiburg.
[6] Uhl, R., Desel, H. and Wagner, R. (1985) J. Biochem. Biophys. Methods 11, 31–43.
[7] Kamps, K.M.P., Reichert, J. and Hofmann, K.P. (1985) FEBS Lett. 188, 15–20.
[8] Uhl, R., Desel, H., Ryba, N.J.P. and Wagner, R. (1987) J. Biochem. Biophys. Methods 13, in press.

[9] Liebmann, P.A. and Evanczuk, A.T. (1982) Methods Enzymol. 81, 532–542.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Kamps, K.M.P. and Hofmann, K.P. (1986) FEBS Lett. 208, 241–247.
[12] Goridis, C., Virmaux, N., Cailla, H.L. and Delaage, M.A. (1974) FEBS Lett. 49, 167–169.
[13] Sitaramayya, A., Harkness, J., Parks, J.H., Gonzales-Olivia, C. and Liebman, P.A. (1986) Biochemistry 25, 651–656.
[14] Bigay, J., Deterre, P.D., Pfister, C. and Chabre, M. (1985) FEBS Lett. 191, 181–185.
[15] Stein, P.J., Halliday, K.R. and Rasenick, M.M. (1985) J. Biol. Chem. 260, 9081–9084.
[16] Yamananka, G., Eckstein, F. and Stryer, L. (1986) Biochemistry 25, 8094–8181.
[17] Liebman, P.A. and Pugh, E.N. (1979) Vision Res. 19, 375–380.
[18] Robinson, P.R., Kawamura, S., Abramson, B. and Bownds, M.D. (1980) J. Gen. Physiol. 76, 631–645.
[19] Kawamura, S. and Murakami, M. (1986) Biochim. Biophys. Acta 870, 256–266.
[20] Caretta, A. and Stein, P.J. (1985) Biochemistry 24, 5685–5692.
[21] Caretta, A. and Stein, P.J. (1986) Biochemistry 25, 2335–2341.
[22] Lewis, L.W., Miller, J.L., Mendel-Hartvig, J., Schaechter, L.E., Kliger, D.S. and Dratz, E.A. (1984) Proc. Natl. Acad. Sci. USA 81, 743–747.