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Sub-second turnover of transducin GTPase in bovine rod outer segments

A light scattering study

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A fast, regenerative light scattering signal from bovine ROS, the P_A-signal, reflects the light-induced, transient activation of transducin. Its rate of recovery depends on the number of photolysed rhodopsin molecules, indicating that rhodopsin deactivation and not GTPase activity is rate limiting in our in vitro system. When rhodopsin deactivation is accelerated (in the presence of NH_2OH), P_A-signal recovery is also accelerated. A GTPase turnover number of more than 2 s^{-1} (at 37°C) can be derived from these experiments. This is more than one order of magnitude faster than the GTPase rates so far described in the literature and is rapid enough for a physiological shut-off mechanism. The fast GTPase is attributed to a highly intact disk stack, which never releases transducin into the free aqueous space.

Photoreception; Transducin deactivation; GTPase turnover; Light scattering

1. INTRODUCTION

G-proteins have been shown to play a key role in the stimulus-response coupling of various biological systems [1]. In the photoreceptor cell they mediate communication between photoexcited rhodopsin and a cGMP-specific phosphodiesterase [2,3]. The cGMP concentration, in turn, modulates the conducting properties of the plasma membrane and hence the current entering the photoreceptor [4,5]. Because of its central role in visual transduction the photoreceptor G-protein has been termed transducin [3]. In vivo several hundred G-proteins are activated by a single bleached rhodopsin via GDP-GTP exchange, and all G-proteins can be activated in less than 1 s. Similar values have been successfully demonstrated in in vitro studies [6,7]. To deactivate transducin the bound GTP must be hydrolysed and to prevent its reactivation the active form of rhodopsin has to be quenched, presumably by phosphorylation and subsequent binding of an 'arrestin' termed 48 kDa protein [8,9]. While in vivo deactivation takes place in ~1 s (in light-adapted photoreceptors it is even faster), similarly high rates have never been observed in in vitro studies. The GTPase turnover numbers, in particular, which have been reported in the literature, are more than one order of magnitude too slow, indicating that crucial factors involved in deactivation are lost or diluted in the in vitro system (review [10]). A recent study [10], which demonstrates a dependence of the GTPase rate on the concentration of ROS material, supports this idea. Extrapolating the data given in this report to physiological concentrations of ROS proteins yields a turnover number for the rod GTPase of 1–2 s^{-1}, close to the in vivo value. It would be desirable, however, to have an in vitro system which is so close to the in vivo state that it can be

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used without extrapolations, and in this communication we will demonstrate that we have succeeded in achieving this goal.

In a previous paper [11] we have demonstrated that a highly light-sensitive light scattering signal P_A, which can be obtained from intact stacks of photoreceptor disks, reflects rapid activation of transducin. Here we extend our observation in showing that this signal also reflects rapid transducin deactivation and that this deactivation process can be accelerated such that a total recovery occurs within 2–3 s, indicating a GTPase rate of 1–2 s^{-1}. This is the first experimental demonstration of a 'physiological' GTPase turnover number.

2. MATERIALS AND METHODS

The preparation procedure for structurally intact disk stacks with perforated plasma membrane has been detailed elsewhere [12]. 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/Ringer medium where 50 mM NaCl was replaced by 100 mM sucrose, at about 100 μM rhodopsin. The purity-indicating ratio A_{280}/A_{500} was between 2.2 and 2.4. Portions of 50 μl each were frozen quickly in liquid nitrogen. In our hands this yields ROS with mostly intact disk stacks but perforated plasma membranes.

Light scattering measurements were performed in a multi-angle flash photolysis apparatus, the design of which is described in [13]. It records 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 with a series of N-signals [14]. A full flash bleached 12.9% of the remaining rhodopsin, lower bleaches being obtained with the help of high-precision neutral density filters from Schott.

All chemicals were of the highest grade available from Sigma.

The measuring protocol was the same as in [11]. In all experiments, except that of fig.3b, the temperature was 21°C.

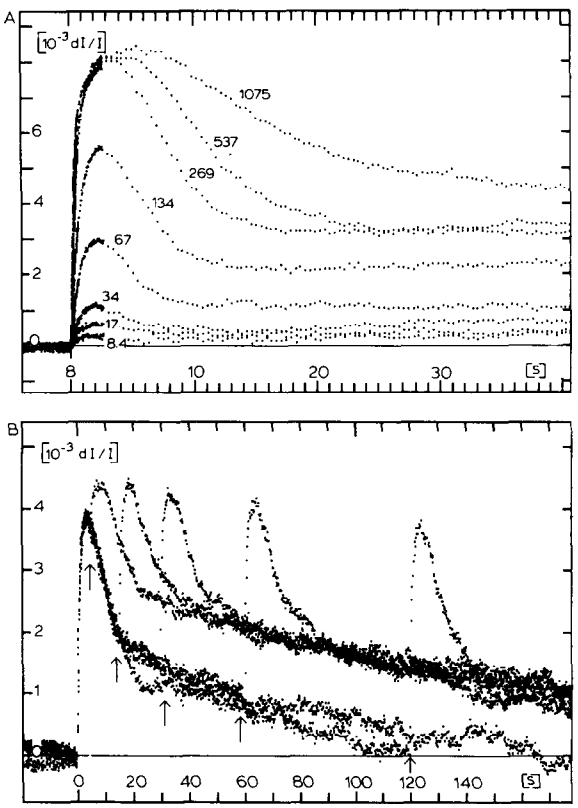

Fig.1. (a) Time course and flash intensity dependence of P_A-signals in the presence of 100 μM GTP and 500 μM ATP. Freshly thawed ROS were suspended (to a rhodopsin concentration of 1.5 μM) in 100 mM NaCl, 10 mM Hepes (pH 7.4), 2 mM $MgCl_2$, 0.2 mM BAPTA, and incubated for 3 min in the infrared light beam of the MAFPA. Then a first flash, bleaching a fraction of 8.4 × 10^{-6} of the total rhodopsin was applied. 2 min later a second flash followed, bleaching twice as much, and so on. The signals were fully reproducible, i.e. when the light titration was repeated, going from saturating to small flashes, identical signals were obtained. The scattering angle was 24° and temperature 21°C. (b) Double-flash experiments. Saturating flashes were repeated 3, 10, 20 and 40 s after a first saturating flash. All four curves were superimposed on a curve representing a single-flash experiment. All signals were recorded from one sample. Experimental conditions as in a.

3. RESULTS

A highly light-sensitive, regenerative light scattering signal, the P_A-signal, was obtained from our ROS preparation in the presence of GTP and ATP (fig.1a). It has been shown to be due to transient activation of G-protein [11]. Its dose-response curve was Poisson-shaped, which is expected if transducin activation due to one bleached rhodopsin is restricted to a particular disk surface. The recovery of P_A consisted of a fast component (3–20 s, depending on the bleaching level), and a slow component, which was completed in less than 3 min. The slow component was sometimes obscured by small drifts. When barely saturating flashes were applied at frequencies lower than 0.3 min^{-1}, identical P_A-signals were obtained over periods of several hours. The first signal was indistinguishable from the hundredth, indicating that the process which is monitored is fully reversi-

ble. At higher flash frequencies (fig.1b), signals resulting from subsequent flashes exhibited smaller amplitudes, their particular size reflecting the degree of recovery that had occurred by a given time. It is interesting to note that a particular total amplitude could not be exceeded. This points to a close link between the maximal amplitude of P_A and a maximally activated transducin pool.

In the presence of increasing amounts of GTP-γ-S, the recovery of P_A was progressively inhibited and the amplitudes of P_A responses to subsequent flashes were diminished accordingly (fig.2). This behaviour is expected if the rising and falling phase of the P_A-signal reflect transducin activation and deactivation, respectively.

Transducin deactivation requires that both

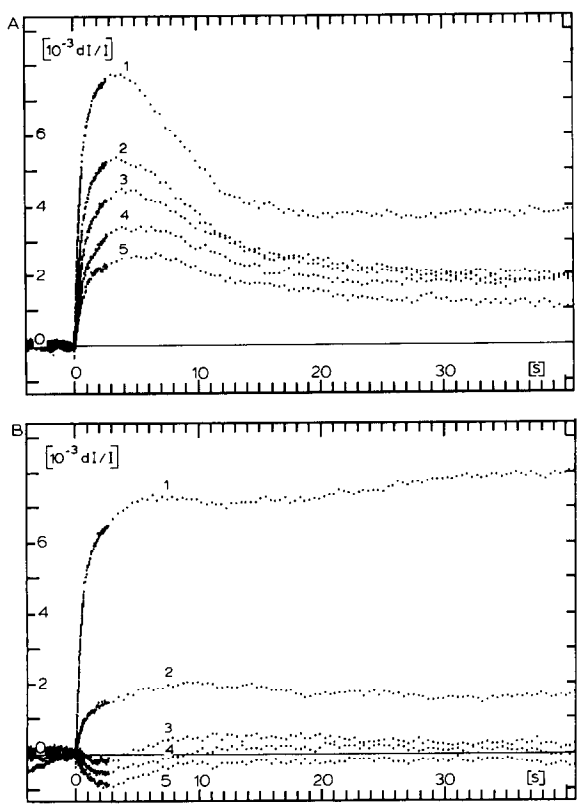

Fig.2. Effect of an increasing amount of GTP-γ-S in the reaction mixture on amplitude and kinetics of P_A. (a) Five saturating flashes in the presence of 5 μM GTP-γ-S and 100 μM GTP. (b) Same experiment in the presence of 100 μM GTP and 20 μM GTP-γ-S. GTP-γ-S was added after 3 min of dark incubation in the presence of GTP and ATP. With increasing concentration of GTP-γ-S the recovery diminishes and the amplitude evoked by subsequent flashes is decreased.

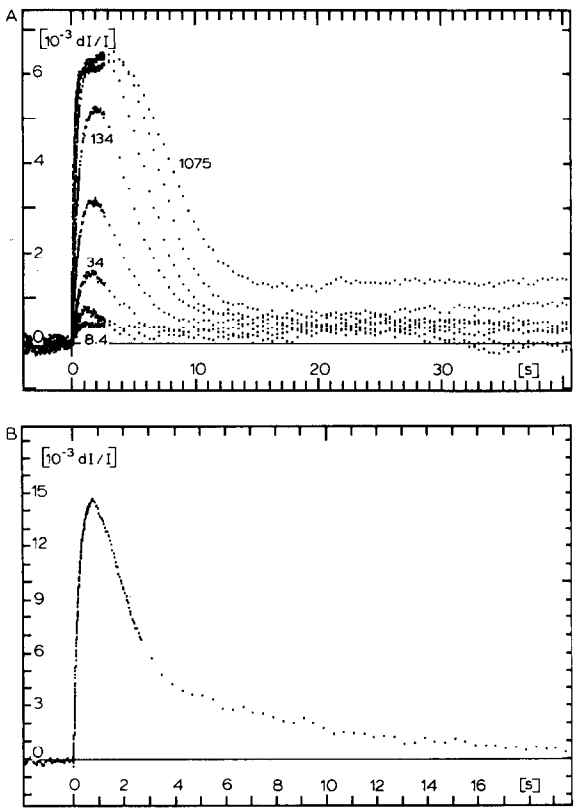

Fig.3. (a) Time course and flash intensity dependence of P_A-signals in the presence of 100 μM GTP, 500 μM ATP and 100 mM hydroxylamine. Experimental conditions as in fig.1, except that 50 mM NaCl was replaced by 100 mM NH_2OH. (b) Time course of P_A-signal at 37°C in the presence of 100 mM NH_2OH.

rhodopsin and G-protein have been deactivated. The observation that the (fast component) of the P_A-signal recovery depended on the level of bleaching suggested that the rate-limiting step in our system was rhodopsin deactivation and not the GTPase turnover. We have tested this by removing the active form of rhodopsin artificially, using hydroxylamine. Hydroxylamine has been shown to quench the ability of bleached rhodopsin to activate transducin by converting metarhodopsin II into retinal oxime and opsin [15]. Fig.3a shows a series of light responses in the presence of 100 mM NH_2OH. The fast phase of recovery was accelerated and the slow phase vanished almost completely. Moreover, the dependence of the recovery rate on the bleaching level was much less pronounced, though still visible. It was demonstrated that at physiological temperature the half-time of

recovery can be as fast as 0.5 s in the presence of NH_2OH (fig.3b). From this it can be concluded that the GTPase turnover rate must be at least 2 s^{-1} under these conditions.

Finally, we have investigated whether an increased concentration of ROS material has an effect on transducin activation and deactivation rates, as suggested by the work of others [10]. P_A-signals were recorded at three different ROS concentrations, in the absence (fig.4a) and presence (fig.4b) of hydroxylamine. The identical nature of the recorded signals clearly demonstrated that there was no dependence of the ROS concentration on P_A. This was observed under conditions where rhodopsin kinase (fig.4a) and GTPase (fig.4b) were (predominantly) rate-limiting, indicating that in our in vitro system, in contrast to [10], soluble cofactors played no major role. Increasing the ATP and GTP concentration also did not yield faster kinetics (not shown).

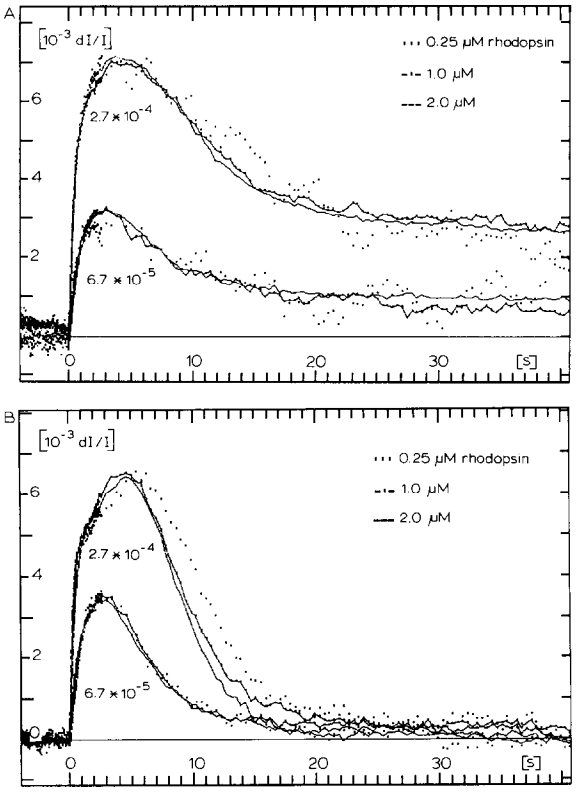

Fig.4. P_A-signals at two different flash intensities and three different ROS concentrations. (a) In the absence of hydroxylamine. (b) In the presence of 100 mM hydroxylamine.

4. DISCUSSION

Activation and deactivation of transducin occur, in vivo, at the disk membrane surface. Both reactions are fast. In disrupted ROS or in reconstituted systems only the activation step is fast, while deactivation is considerably slowed down. We believe that this reflects the fact that in commonly used in vitro systems activation still occurs at the membrane surface – transducin in its inactive, GDP-binding form being membrane associated – while deactivation takes place, at least partially, in the free aqueous space, since the active form of transducin is released into the reaction medium [16]. In our structurally well preserved system, however, the active form of transducin does not leave the disk stack. Proof for this is provided by the fact that P_A-signals show complete recovery and a constant, fast rise time, even after 100 saturating flashes. Moreover, their kinetics is independent of the ROS concentration (fig.4) and SDS gel electrophoresis reveals no loss of transducin due to isotonic washes, even in the presence of GTP and after physiological bleaches (to be published). We have, therefore, an in vitro system that has retained most, or even all, factors governing rapid transducin deactivation via GTPase activity.

The recovery rates of P_A in the presence of 100 mM NH_2OH set lower limits for the rate of GTPase. A first-order time constant of 2 s^{-1}, as observed at 37°C, must correspond to a GTPase turnover number close to this value. The observation that this fast recovery is still dependent on the flash intensity (fig.3a) suggests that even under these conditions rhodopsin deactivation is still (at least partially) rate-limiting and hence the actual GTPase rate even faster. This is the first direct demonstration of a GTPase turnover high enough to play its key role in visual transduction.

5. CONCLUDING REMARKS

This paper clearly demonstrates the advantages of using an in vitro system which is as close as possible to the in vivo state. Our ROS preparation appears to be such a relative physiological system and light scattering – in this particular case the light scattering transient P_A – appears to be a very powerful, non-invasive technique for its examination.

The major difference between our in vitro system and the intact photoreceptor lies in the intactness of the plasma membrane. While we can prepare ROS with an intact plasma membrane, we cannot administer nucleotides necessary for visual transduction without destroying the plasma membrane. We found that quick freezing in liquid nitrogen, in the presence of 100 mM sucrose as cryoprotectant, is the most gentle way of perforating the plasma membrane without disrupting the disk stack. While this leaves, as shown here, the deactivation of transducin itself relatively unaffected, that of rhodopsin is clearly slower than in vivo (fig.2). This points to the involvement of soluble protein factors in rhodopsin deactivation. It can be tested using the light scattering assay described here in combination with preparative biochemistry, i.e. by adding selected, isolated protein components to the system. Such experiments have been successfully carried out in our laboratory.

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