ATP can promote activation and deactivation of the rod cGMP-phosphodiesterase

Kinetic light scattering on intact rod outer segments

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Received 20 June 1986

The AT (amplified transient) signal is a flash-induced increase of the near-infrared light scattering from isolated bovine rod outer segments and is interpreted as a monitor of cGMP-phosphodiesterase activation [(1985) FEBS Lett. 188, 15–20]. We have investigated the effects of ATP and cyclic GMP on this signal. It has been found that ATP enhances the AT signal, the relative effect being the largest for low photoexcitation (~1 rhodopsin per disc membrane). At a high rhodopsin turnover, which saturates the AT amplitude, the effect of ATP is to accelerate the rise of the signal. ATP can also accelerate the falling phase of the signal. This deactivating effect depends on the simultaneous presence of cyclic GMP. The results indicate that ATP acts on the phosphodiesterase activation cycle, promoting activation as well as deactivation, dependent on cGMP as a cofactor.

G-protein *Rhodopsin* *Cyclic-GMP phosphodiesterase* *Time-resolved light scattering*

1. INTRODUCTION

For the light-dependent activation of the cGMP-phosphodiesterase in rods, four steps are described in the literature (reviews [1,2]): (i) relaxation of photoexcited rhodopsin (R*) into the interactive state (R_M); (ii) interaction of R_M with the G-protein in its GDP-binding state (G_{GDP}); (iii) release of the G_α-subunit from $R_M \cdot G$ after GTP/GDP exchange; (iv) interaction of G_{GTP} with the PDE inhibitor (I). Deactivation requires the switch-off of the activators R_M and G_{GTP} and the return of I to the PDE. Because the deactivating reactions are relatively slow, one R_M molecule can interact with many G_{GDP} molecules. This leads to a fast (100 ms) accumulation of the activator G_{GTP} and a high transient level of activated PDE over a period of seconds. Obviously, the lifetime of the activated forms of R, G and PDE provides a potentially regulating factor in this particle amplifier and kinetic studies can help to come closer to their understanding.

The role of cGMP and ATP as cofactors in the enzyme cascade of the rod has been the subject of various investigations: cGMP is a messenger of the amplified light signal from the R/G/PDE cascade to the light-dependent plasma membrane conductances [3]; in addition, cGMP has been shown to control a disc membrane conductance [4,5]. In

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Abbreviations: R*, photoexcited rhodopsin; [R*], mol fraction of R*; R_M, interactive state of rhodopsin; G, G-protein (or transducin); G_{GTP}, G-protein in its GTP-binding form; NIR, near infrared; AT signal, amplified transient near-infrared light-scattering signal; ROS, rod outer segment; PDE, cyclic-GMP phosphodiesterase; 8-Br cGMP, 8-bromo cyclic GMP; AMP-PNP, adenosine 5′-[β,γ-imido]triphosphate

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00145793/86/$3.50

frog ROS, cGMP-dependent phosphorylation has been described for two small peptides, components I and II [6] and an effect of cGMP on the binding of GTP and GDP to the G-protein has been demonstrated recently [7].

The most obvious effect of ATP shown so far, is the rapid reversal of PDE activation after a flash [8]. Phosphorylation of photoexcited rhodopsin [9] has been discussed as a possible mechanism for this accelerated ATP-dependent deactivation [8,10]. A completely different effect of ATP on PDE activity might originate from a light-sensitive, Mg-dependent ATPase of the disc membrane [11,12] which is cGMP-sensitive and can be measured by light scattering [12,13].

Real-time monitoring of the $R^* \cdot G$ interaction (steps i–iii) is possible in situ by accompanying changes of light scattering [14,15]. Recently, a new light-scattering signal, the so-called AT (amplified transient) signal, has been found in structurally intact ROS which has been proposed to reflect a structural change linked to step iv [16]. This interpretation is now supported by the observation of a similar signal in a reconstituted R/G/PDE system [17] which has been correlated to a transition of PDE to and from the membrane.

In this study, the effect of ATP and cGMP on the PDE activation-deactivation cycle, reflected by the AT signal, has been investigated. We provide evidence for an acceleration by ATP of the whole PDE-activation cycle rather than only of its deactivation, as previously described (cf. [8]).

2. MATERIALS AND METHODS

The measurements were done on structurally intact but permeabilized ROS in order to supply the regulating protein inventory with various mixtures of nucleotides at comparable concentration. This measuring system provides sufficient kinetic resolution to measure the high speed of the scattering signal in the presence of ATP. The preparation of the isolated ROS was similar to that in our previous study [16]. Fresh bovine retinae were dissected under very dim red light in 5 mM Tris-acetate (pH 7.6), 10 mM glucose, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA and 660 mM sucrose. After vortex-mixing for 15 s and filtration of the crude suspension through a nylon mesh, the outer segments were isolated by density centrifugation on a continuous sucrose gradient (23–36%, w/w) containing 1 mM $Mg(OAc)_2$ buffered with 2 mM Tris-acetate (pH 7.6) at 15000 × g, for 10 min at 10°C.

Light-scattering measurements were carried out in 70 mM KCl, 40 mM $NaHCO_3$, 5 mM $Mg(OAc)_2$, 0.5 mM KH_2PO_4, 1 mM DTT (pH 7.6). This medium was adjusted to pH 7.6 with 2 mM Tris-Hepes. Free Ca in the micromolar range up to 10^{-5} M had no significant effect on the scattering signals.

The nucleotides and 0.008–0.01% Triton X-100 were added immediately prior to the measurements. Samples of 800 μl (10 mm path length) containing 2 μM rhodopsin each were used. The geometry for measuring the flash-induced changes of near-infrared light scattering was the same as in our previous study [16] (scattering angle, 25–30°; detection normal to the plane of ROS axis and optical axis).

GTP, ATP and 8-Br cGMP were supplied by Serva, Heidelberg.

3. RESULTS

Orientation of the ROS in the magnetic field takes ~30 s until equilibration and can be readily observed by the accompanying increase of the radial light scattering [16]. After full orientation, an additional slow ATP-sensitive scattering increase is observed which indicates most probably an increase of the anisotropic order in the ROS. It normally lasts ~3 min, enhancing the radial scattering of the oriented sample by 3–5%, and seems to correlate to an ATP-dependent component of the light-induced AT signal, as described below. Some preparations do not show the regular dark effect but rather display large fluctuations of the scattering intensity which can even cover any flash-induced signal. However, if signals can be evoked, they are reproducible from one sample to another and can be repeated many times on the same sample.

A typical AT signal is shown in the first trace in fig.1. Under the conditions applied (1 mM GTP, 2 flash-excited rhodopsin molecules per disc membrane), the AT signal is saturated to 80% of its amplitude. A higher GTP level does not further increase the amplitude, nor the rate of the signal. However, when 1 mM ATP is given in addition to

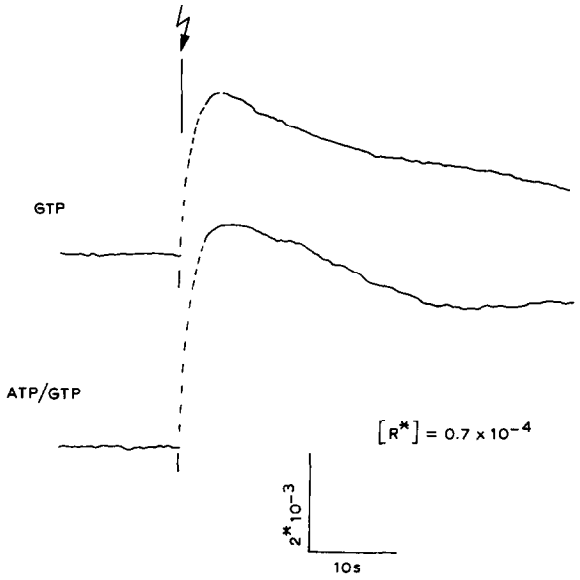

Fig.1. Amplified NIR scattering transient (AT signal) from isolated Triton-permeabilized bovine ROS, in the presence of 1 mM GTP (first trace) and of 1 mM ATP plus 1 mM GTP (second trace). The flash produced a relative rhodopsin turnover [R*] of 0.7×10^{-4}; this corresponds to an average photoexcitation of 2.3 rhodopsin molecules per disc membrane.

GTP, a larger signal is measured (second trace in fig.1). This signal is even larger than the one measured with a saturating flash in the presence of GTP alone. The relative enhancement by ATP of the scattering change is generally the larger, the slower and smaller the signal in the absence of ATP. This applies especially when less than one rhodopsin molecule per disc membrane is photoexcited and both the amplitude and rate of the AT signal decrease in proportion to R* [16].

The rising phase of some signals is shown and analysed in more detail in fig.2. Consider first the signals in the lower part of the figure (same as fig.1, with an expanded time scale). Both the GTP and the ATP/GTP signals are well fitted by a consecution of two first order reactions (solid lines). This means that the rise of the signals, apart from the delay, obeys first order kinetics. The delay is equally long in both signals which means that ATP does not involve an additional rate limiting step. In the upper part of the figure, the same comparison is made for a ten-fold more intense flash. With GTP alone (second trace), the rise with this higher

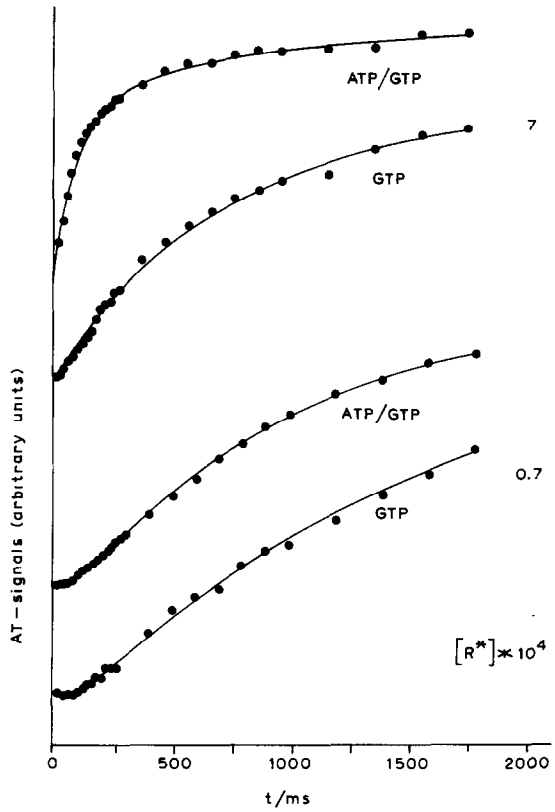

Fig.2. Measured (points) and calculated (solid lines) waveforms of the AT signal for two different flash-induced photoexcitations ([R*] = 0.7 and 7×10^{-4}). The signal amplitudes are all normalized to the same value. The signals for [R*] = 0.7×10^{-4} are the same as in fig.1, with an extended time scale. Note the acceleration of the rise for [R*] = 7×10^{-4} which is exclusively observed with ATP (uppermost trace, 1 mM ATP plus 1 mM GTP). The rise of this signal is biphasic; the solid line represents a sum of two positive exponentials, according to

$$A_o - A_t = A_1 \cdot e^{-k_1 t} + A_2 \cdot e^{-k_2 t}.$$

The signals at lower flash intensity rise with single first order kinetics (rate constant k). Their delay can be approximated by a first order precursor reaction (rate constant k_i, according to

$$A_o - A_t = (1/(k - k_i)) \cdot (k \cdot e^{-k_i t} - k_i \cdot e^{-kt}).$$

For the ATP/GTP signal at [R*] = 0.7 (third trace), the fit yields $k = 1.06\ s^{-1}$ and $k_i = 8.9\ s^{-1}$.

[R*] is only slightly faster than the one at the lower [R*] (fourth trace). This type of rate saturation has already been observed in our previous study [16].

However, when 1 mM ATP is added in the presence of 1 mM GTP (first trace), the signal is strongly accelerated. The rise of this signal deviates systematically from first order kinetics. Apart from the very short delay, the signal is well fitted by a sum of two positive first order components with rate constants $k_1 = 9.5\ s^{-1}$ and $k_2 = 1.3\ s^{-1}$ in a relative weight of $A_1/A_2 = 7/3$. The biphasic fit was obtained for several ATP/GTP signals but never for signals with GTP alone (see the legend of fig.2 for an example) or with AMP-PNP/GTP. Thus hydrolysable ATP appears to be an essential cofactor for a faster species of reacting units, presumably a faster fraction of activatable PDE.

In fig.3, the computed rates k_1 and k_2 are plotted as a function of [R*]. It is to be seen that at low [R*] a slow rate is found under all conditions. At higher intensities, the GTP records and the ATP/GTP records separate significantly from each other: both the rate k of the GTP signals and the slow rate k_2 of the ATP/GTP signals saturate while the fast ATP/GTP component increases and can be even approximately proportional to [R*].

A possible effect of a light-induced vanadate-sensitive ATPase activity [11–13] has been tested by incubating ROS for 5 min with 250 μM vanadate. It reduces or even prevents the ATP-dependent signal component. However, this result should be considered with care since vanadate can affect the activation of G-protein, measured via 'extra-metarhodopsin II' (cf. [18]; Schleicher and Hofmann, unpublished).

The effect of cyclic GMP on the AT signal is reported below. Addition of exogenous hydrolysable cGMP to permeabilized ROS leads to complicated and poorly reproducible waveforms of the scattering signal. Clearer results have been obtained with the non-hydrolysable analog 8-Br cGMP. It was generally observed that this agent enhances the scattering change of the amplified transient in the presence of GTP. However, in contrast to ATP, addition of 8-Br cGMP does not pro-

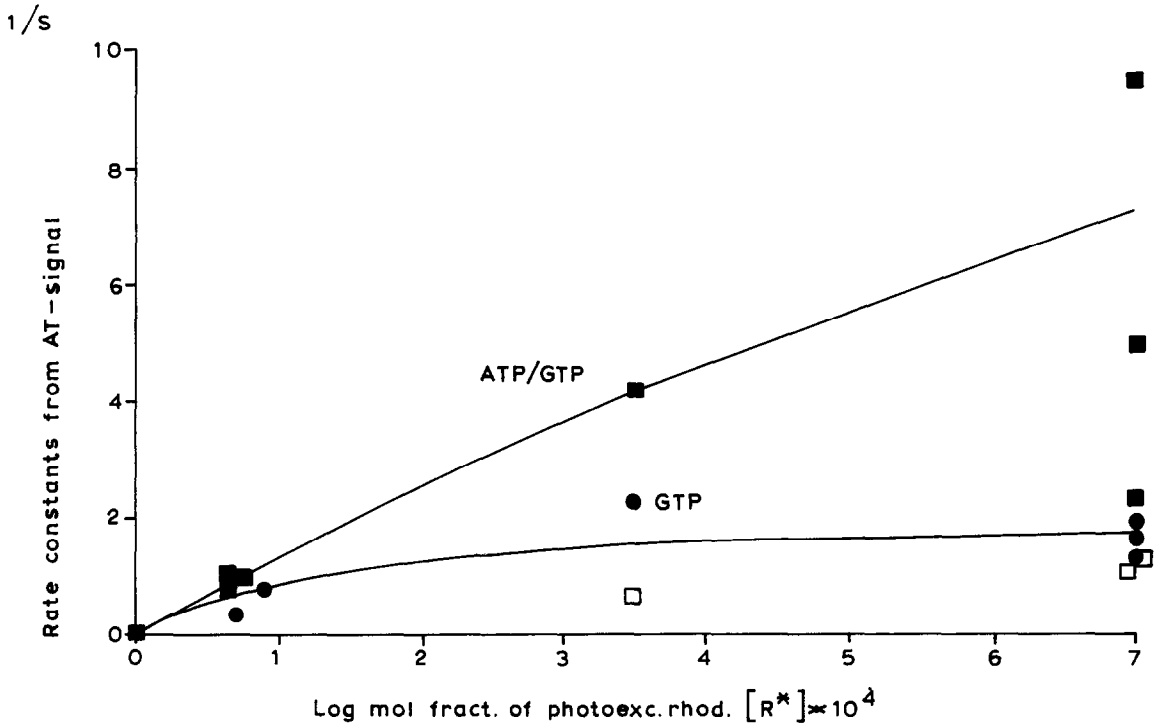

Fig.3. Calculated rate constants from AT signals (as shown in fig.2) as a function of [R*]. The signals in fig.2 and other records were fitted, as described in fig.2, by first order kinetics. For biphasic ATP/GTP signals, the rate constants k_1 and k_2 are plotted as filled squares (k_1) and empty squares (k_2). The rate k of the monophasic GTP signals is plotted as filled circles. Note the parallel behavior of k and k_2.

duce an additional faster component but the signals are simply scaled up by a factor between 1.0 and ~1.5 with respect to signals with GTP alone. The largest relative enhancement by 8-Br cGMP is found when poorly hydrolysable GTP analogs are applied instead of GTP and PDE deactivation is inhibited resulting in a persistent level [16] of the AT signal (not shown).

The effects of ATP and 8-Br cGMP depend on each other. A typical record with 8-Br cGMP is shown in fig.4. The first trace is from the third flash on the sample; the signal from this flash – as the one from the previous flash – does not run back to the baseline but to a positive level indicating a more persistently activated fraction of PDE. 8-Br cGMP promotes this behavior which is sometimes also observed without exogenous cGMP and is not suppressed by ATP alone (fig.1). However, when ATP is added to the 8-Br cGMP sample after the third flash, the signal from the 4th flash – and those from the subsequent flashes – shows no persistent component any longer. It returns exactly to the baseline indicating that PDE deactivation is faster under these conditions. Thus ATP can also promote PDE deactivation and cGMP favors or is even necessary to achieve this effect.

4. DISCUSSION

The data will be discussed on the basis of our previous interpretation of the AT signal as a structural change in the ROS which is directly linked to the transient removal of the inhibitory subunit from the phosphodiesterase [16]. The idea of such a structural change in the intact ROS is now supported by the recent work of Caretta and Stein [17] on isolated rod disc membrane vesicles. They demonstrate a correlation between a light scattering change caused by vesicle aggregation and the light- and nucleotide-dependent binding of purified PDE to the membranes. The physical forces causing vesicle aggregation might also explain the AT signal via a contraction of the ROS or parts thereof.

Regardless of its physical interpretation, the AT signal can serve as a kinetic monitor of PDE activation in situ and give heuristic information which cannot be obtained otherwise. However, the question arises whether all the components of the scattering change correspond to PDE activation. In addition to the PDE, a number of other proteins depend on light in their membrane binding [1]. It is therefore not a priori excluded that the transition to the membrane of proteins other than PDE could

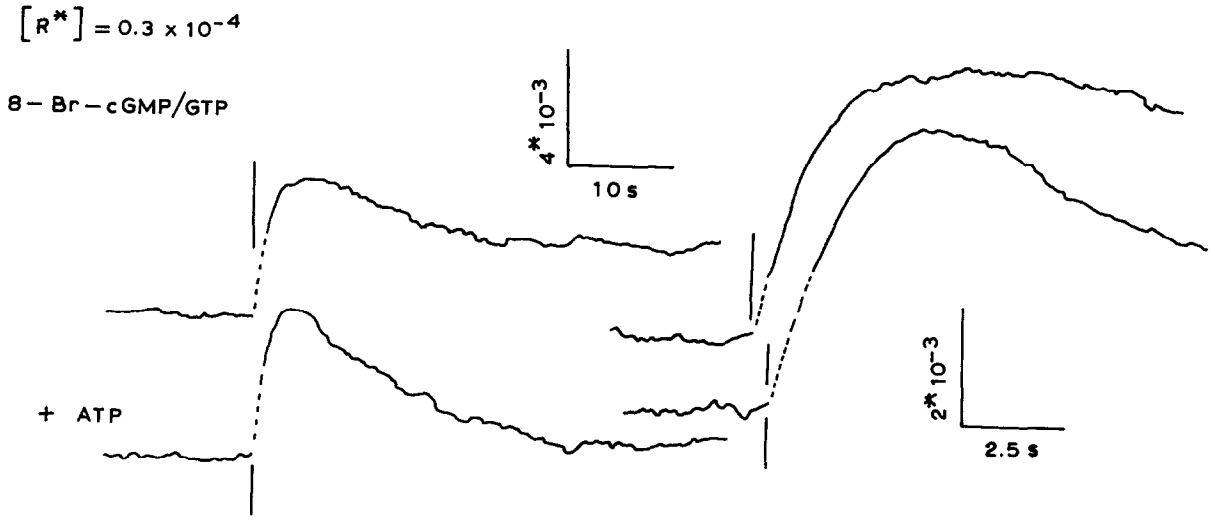

Fig.4. AT signals from a ROS suspension containing 1 mM GTP and 0.2 mM 8-Br cGMP. Traces on the right are the same signals in higher vertical and horizontal resolution. The upper signal is from the third flash on the sample. After this record, 1 mM ATP was added; the lower signal was recorded 3 min after the addition of ATP. The fast phase of the signals is shown on the right side (expanded scales as indicated).

be directly reflected in the ATP-dependent part of the scattering increase (fig.1). However, such a mechanism would have to be even faster and more light-sensitive than PDE activation (which would then appear in the slower and smaller ATP-independent part of the AT signal).

Recent experiments on isolated bovine retinae (Kahlert and Hofmann, unpublished) rather favor an interpretation of the AT signal as a homogeneous 'PDE-signal': in this system, a positive scattering signal is observed (together with the electroretinogram) which fits within a computer model of PDE activation. This signal from the retina is kinetically very similar to the ATP-dependent part of the AT signal from ROS (fig.2).

Thus we propose that both the ATP-dependent and ATP-independent components of the scattering signal in figs 1–3 reflect PDE activation. They can be understood as two fractions of the PDE which are activated with different speed and performance. Only one of them (varying in its extent from one preparation to the other) approximates the speed of PDE activation in the retina.

Given this interpretation, it follows that ATP can promote PDE activation (figs 1–3) as well as cGMP-dependent deactivation (fig.4).

When cGMP is present as substrate (as it is in the cGMP-hydrolysis assay for the PDE; cf. [8]) it must act at the same time as a cofactor for deactivation. It is therefore not excluded that the ATP-mediated reversal of PDE activation found by Liebman and Pugh [8] might originate from a cGMP- (and vanadate-)sensitive mechanism, as suggested by our results, rather than from a quench of R_M by phosphorylation [10]. Since the rhodopsin kinase seems to be cGMP-insensitive [19,20], both these mechanisms have to be distinguished. On the other hand, deactivation of R_M is certainly required to deactivate the R/G/PDE cascade. It has been shown recently that PDE activation is effectively quenched when phosphorylated rhodopsin binds the 48 kDa protein [21]. The cGMP-dependent deactivation might be additional and proceed at the G- or PDE-level (or even involve transmitter proteins other than G_α).

It is interesting to compare fig.4 in this study with the PDE-activity transient derived from a record of cGMP hydrolysis (cf. [22], fig.3). The falling phase is similarly accelerated by ATP (plus cGMP) in both measurements. What is different is that the AT signal is ATP-stimulated also in its rise which leads to an even higher peak with ATP in spite of the accelerated decay. This might reflect a property of the intact ROS which is lost in the membrane preparation used by Liebman et al. [22].

The AT signal shares its dependence on ATP and cGMP and its sensitivity to vanadate with another light-scattering signal (so-called A signal [12,13]) which is slow and driven in the dark by ATP. The A signal might reflect the dark reaction ('energetization', cf. [13]) which holds the PDE cascade in a highly light-activatable form. At the present state of our knowledge, the effects of cGMP and ATP on the PDE-activation cycle can also be discussed in terms of a cGMP-controlled, light-induced phosphorylation of proteins which interact with the cascade. In frog ROS, the binding of GDP and GTP to the G-protein depends on cGMP [7] and the cGMP-dependent phosphorylation of two small peptides, components I and II [6] can be considered as a possible mechanism for this control (Hamm, H., personal communication).

Further analysis will require purified components to be introduced and to test their influence on the AT effect. It would certainly be of interest to maintain the kinetic resolution and uniformity of the scattering signal in the intact ROS which seems to provide a real time measure of the PDE-activation cycle. However, intact structure, closed compartments and undisturbed protein inventory might contribute to the fast structural response in this measuring system, and the relative influence of these factors is hard to estimate.

ACKNOWLEDGEMENTS

We thank H. Hamm and A. Schleicher for discussions. The financial support of the Deutsche Forschungsgemeinschaft (SFB 60) is gratefully acknowledged.

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