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Effect of GTP on the rhodopsin-G-protein complex by transient formation of extra metarhodopsin II

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The light-induced transient interaction between rhodopsin and G-protein in the presence of GTP has been measured by the formation of extra metarhodopsin II. Disc membranes were recombined with the hypotonic extract containing the G-protein. Without GTP, a flash induces stable rhodopsin-G-protein complexes which dissociate upon addition of GTP. In low GTP (less than 10 μM) transient rhodopsin · G-protein interaction is observed. Rhodopsin · G-protein dissociates the faster, the more GTP is present (rate of dissociation, 0.3/s at 5 μM GTP; $T = 3.5$°C). The results corroborate that the uptake of GTP terminates the rhodopsin-G-protein complex and allow an estimation of the rhodopsin · G-protein lifetime.

Light absorption in rods is coupled to the activation of a cGMP phosphodiesterase via membrane-associated interaction of the receptor protein (rhodopsin) with a transducer protein (G-protein, transducin; Ref. 1). Two optical monitors are available for measuring this interaction in situ. Kinetic light scattering in the near infrared range was the first method which provided information on the kinetics and stoichiometry of the rhodopsin · G-protein interaction [2]. The dependence of the scattering data on the structural preservation of the disc membrane stack complicates the interpretation in structurally heterogeneous preparations [3,4]. These limitations do not occur with a spectroscopic monitor of the rhodopsin · G-protein interaction provided by the 380 nm photoproduct metarhodopsin II (so-called extra MII, Ref. 5): photoexcited rhodopsin is forced into the MII state when G-protein is bound [3,6] and this is measured by a strong shift of the equilibrium between MII and the preceding 480 nm product metarhodopsin I (MI). We have proposed the following reaction scheme [3], in which G stands for G-protein and R for rhodopsin:

$$\overset{1}{\underset{}{}}\qquad\overset{2}{\underset{}{}}$$

$$\underset{}{\overset{}{}}\qquad\overset{}{G}$$

$$R \rightsquigarrow \rightarrow MI \rightleftharpoons MII \rightleftharpoons MII.G$$

Scheme I

Under appropriate conditions (e.g., $T = 5$°C, pH 7.5) the normal MI/II equilibrium (step 1 in Scheme I) is strongly shifted to the left and the G-induced shift (step 2 in Scheme I) is, therefore, quite pronounced. Quantitative measurements can be made and evaluated from Scheme I [7,8].

So far, kinetic work on extra MII has used conditions where the MII-G complex is artificially stabilized (absence of GTP). The present study is the first to investigate the application of this moni-

Abbreviations: MII, metarhodopsin II; MI, metarhodopsin I; G_{GTP}, G-protein in its GTP-binding form; G_{GDP}, G-protein in its GDP-binding form.

tor in the presence of GTP. Transient formation of the rhodopsin-G-protein complex should reflect itself in a transient formation of extra MII. Under conditions where enough extra MII accumulates (high rhodopsin turnover, low (GTP), low temperature), one should be able to observe the actual lifetime of the complex.

Bovine rod outer segments were prepared according to a standard procedure [3,5] and stored in liquid N_2. For the extraction of the peripherally bound protein (see for example, Refs. 2 and 3) a washing step at moderate ionic strength was applied first. Then, the rod-outer segments were osmotically shocked in low-ionic strength and sedimented. The supernatant (containing the peripheral proteins) was centrifuged again and yielded the extracted proteins. The pellet was washed once more at low-ionic strength resulting in the washed membranes fraction. Prior to the measurements, the thawed washed membranes were diluted and recombined with protein extract to a final concentration of $3 \cdot 10^{-6}$ M rhodopsin. The final ratio of rhodopsin to G-protein was 6 : 1. The measurements were carried out in isotonic saline containing 130 mM KCl/0.5 mM $MgCl_2$/1 mM $CaCl_2$/0.5 mM EDTA/1 mM dithiothreitol/10 mM 1,4-piperazine diethanesulfonic acid (Pipes) (pH 7.5).

Aliquots from one suspension were used for the measurements in Fig. 1. The sample was stored in darkness for 2 h on ice prior to the measurements to allow the decay of MII; GTP was added immediately before measuring. The shutter for the measuring light was open only during the records.

The absorption changes were measured in a Shimadzu UV300 two-wavelength spectrophotometer (2 nm slit, $\lambda_1 = 380$ nm, $\lambda_2 = 417$ nm, 1 cm path). This instrument provides sufficient time and amplitude resolution at low levels of measuring light. The flash (delivered by a light guide) photoexcites 4% (mol fraction) of rhodopsin; the sample is cooled to 3.5°C and mounted as close as possible to the end-on photomultiplier.

Fig. 1 shows the 380 nm absorbance changes (signals) which were corrected for light-scattering contributions by subtracting the 417 nm absorbance change. At the latter wavelength MII is isosbestic to MI.

The initial (not time-resolved) deflection seen in

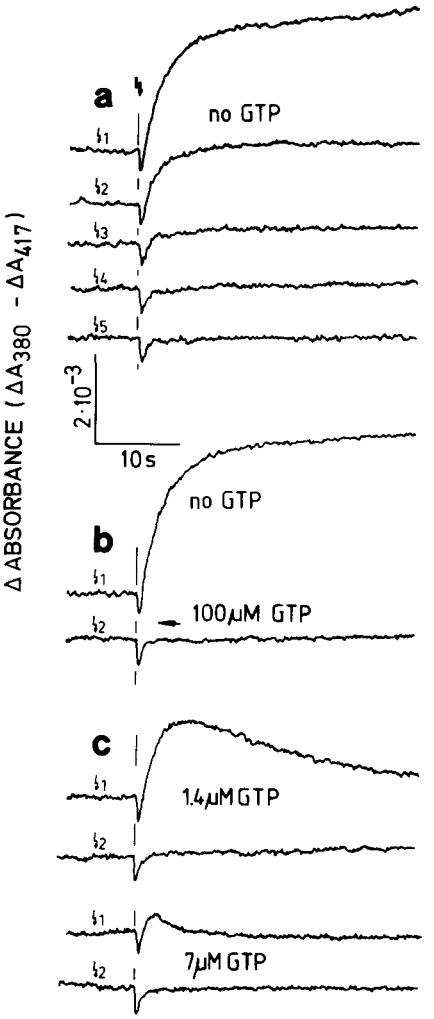


Fig. 1. Flash-induced absorption signals in a suspension of bovine photoreceptor membranes (original records). Membranes were isolated and recombined with peripheral proteins as described. Signals are the absorbance change at 380 nm (A_{380}) minus the absorbance change at 417 nm (A_{417}); this correction by the absorbance change at the MI/MII isosbestic point eliminates the scattering contributions almost totally (cf. Ref. 5). The amplitude of the MI/II transition can be taken starting from the deepest point of the initial jump and is a direct measure of MII formed. Temperature and pH ($T = 3.5$°C, pH 7.5) are adjusted in such a way that the normal equilibrium amount of MII (not influenced by binding of G-protein to MII, see text) is equal to the negative initial jump. (a) MII production in the absence of GTP. A succession of five flashes was applied to the sample at 2 min intervals; each flash bleached 4% of the rhodopsin present, the time interval between the flashes was 2 min. (b) MII production in the absence (flash no. 1) and presence (flash no. 2) of GTP (100 μM). GTP was added 2 min after flash no. 1 and 30 s prior to flash no. 2. (c) MII production measured in two different samples in the presence of low GTP.

all signals represents the 380/417 nm absorbance change by all earlier photoproducts including MI (if no MII were produced, the track would be negative steplike and would remain at this negative level). Because of the production of MII on the time scale of seconds, the absorbance increases after this negative deflection.

Consider first the case in which no free G-protein is present. This applies, for example, to flashes no. 4 and 5 in Fig. 1a. In this case the total of G-protein has already complexed with MII from preceding flashes and the same MII production as on washed membranes without G is observed [3]. The small MII absorption increase upon flashes no. 4 and 5 is the formation of the 'normal' equilibrium amount (step 1 in Scheme I, step 2 virtually absent). Under the conditions ($T = 3.5°C$, pH 7.5) this absorption increase is just as large as the initial negative deflection. Extra MII is observed as a positive absorbance difference as is seen, for example, in flashes no. 1–3 in Fig. 1a. Stable binding of the G-protein to MII is indicated by the persistent level of the extra MII absorption changes and by the exhaustion of free complexable G-protein in the succession of flashes.

In fig. 1b the first flash again produces stable MII · G-protein (same extra MII as in Fig. 1a, flash no. 1). Addition of 100 μM GTP (2 min after flash no. 1, 30 s prior to flash no. 2) prevents any extra MII formation upon the second flash; all G-protein is now present in a non-complexable form. Following Ref. 1, this form is identified as G_{GTP}, produced by multiple sequential interaction of MII (formed by the first flash) with G_{GDP} and GDP/GTP exchange. Apparently, the G-protein remained in this form during the 30 s interval between the application of GTP and the second flash (slow GTPase activity).

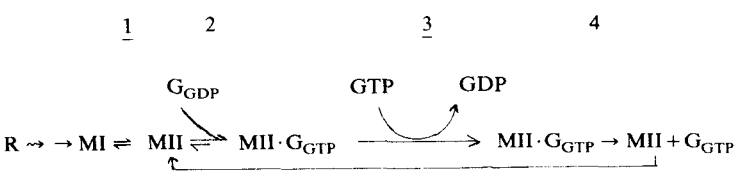
The question arises whether or not the exchange of GTP for GDP at the G-unit terminates the MII · G interaction [9,10]. The idea that the GTP/GDP exchange is causal for R · G dissociation relies so far on the acceleration of the so-called dissociation signal with increasing (GTP) [2,9]. Fig. 1c shows two typical records of extra MII at low GTP concentrations. A transient formation of extra MII is observed. The following is seen in more detail: (1) The rise of the transient is the same for both GTP concentrations applied; the formation of MII is rate limiting (compare Fig. 1a). This verifies that GTP does not influence the formation of MII · G. (2) The decay of MII · G is the faster the more GTP is present; the rates of the records shown in the figure (0.4/s at 7 μM and 0.07/s at 1.4 μM) are approximately proportional to the concentrations.

Also, in other records (not shown) the transients became smaller and shorter with increasing (GTP). No formation of extra MII was detectable for [GTP] > 20 μM. The acceleration by GTP of the MII · G dissociation indicates that, under the conditions, the uptake of GTP is the rate-limiting step for breaking up the MII · G binding interaction.

After this reaction step and the (putative) complex MII · G_{GTP} have been introduced the Scheme I can be extended up to the recycling of MII [9].

Our version of the scheme (see Scheme II), in which R stands for rhodopsin and G for G-protein, gives in addition the rate-limiting steps (underlined). The rate constant of step 3 is the same as the measured k_D of MII · G dissociation.

Extrapolation to 1 mM GTP (the normal level in rods) should allow to estimate the degree to which the GTP uptake limites the speed of the rhodopsin · G-protein sequential interaction. The time required for one complete rhodopsin-recycling step (i.e., rhodopsin · G-protein binding, GTP/GDP exchange, rhodopsin · G-protein dissociation and diffusion to the next successful rhodopsin · G-protein encounter) is 0.5 to 1.5 ms at room temperature (reviewed in Ref. 10). A



Scheme II

measure for the temperature dependence of rhodopsin · G-protein recycling is that of the scattering signal linked to phosphodiesterase activation (so-called AT-signal; Ref. 11 and unpublished observations). This signal is at 22°C 2.3 times faster than at 13°C; extrapolation to 3.5°C (where this signal is undetectable) with an assumed high coefficient of six yields a maximal rhodopsin · G-protein recycling time of $1.5 \times 2.3 \times 6 = 21$ ms.

On the other hand, extrapolation of the rhodopsin · G-protein dissociation rates at μM GTP (this study) to 1 mM GTP gives rates between 50/s and 60/s (e.g., $k_D(1\ \text{mM}) = (1000/7) \times k_D(7\ \mu\text{M}) = 145 \times 0.4 = 60/\text{s}$). This yields a lower limit of 17–20 ms for the rhodopsin · G-protein lifetime which is comparable to the time required for a whole rhodopsin · G-protein recycling step (as estimated above). Thus, the uptake of GTP appears to limit the rate of the rhodopsin · G-protein sequential interaction even at 1 mM GTP.

A problem of this comparison is that the MII · G dissociation is best measured on isolated membranes, while the recycling data [10] are from fresh preparations. The GTP uptake capacity might be selectively injured and slowed down by the isolation procedure. On the other hand, rate limitation by GTP uptake fits also to theoretical considerations [10] which have already suggested that 1 mM GTP is indeed required to meet the observed speed of the rhodopsin · G-protein amplifier.

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