Complex formation between metarhodopsin II and GTP-binding protein in bovine protoreceptor membranes leads to a shift of the photoproduct equilibrium

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Complex formation between metarhodopsin II and GTP-binding protein in bovine photoreceptor membranes leads to a shift of the photoproduct equilibrium

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1. INTRODUCTION

The transition of metarhodopsin I (MI) to metarhodopsin II (MII) is the last step in the decay reaction chain of vertebrate rhodopsin photoproducts which occurs on a ms time scale and which is therefore sufficiently rapid to be involved in triggering visual transduction [1]. The two photoproducts MI and MII are in a temperature- and pH-dependent equilibrium while MII slowly decays further. This equilibrium has been extensively studied (reviewed in [2]). We have shown that a first flash bleaching 2% rhodopsin in dark-adapted rod outer segment (ROS) membranes does not lead to the 'normally' observed equilibrium of MI and MII; in contrast, MII is virtually the only photoproduct formed [2]. With increasing photolysis by further flashes delivered on the same sample, more and more MI per flash is formed. Only at bleaching levels $\gtrsim$ 10%, further flashes produce the 'normal' mixture of MI and MII described by the classical MI/MII equilibrium [2].

The bleaching range (0–10%) in which this 'anomalously' high ratio of MII *vs* MI is formed ('extra MII'-formation) is the same range in which also light-evoked, rapid changes of the scattered light intensity, the so-called 'P-signals' [3,4] or 'binding signal' [5], are observed, suggesting that these effects may be closely related to one another. The light-scattering 'binding signal' is related to the stoichiometric association of GTP-binding protein (G-protein) to photoexcited rhodopsin [5]. The formation of 'extra MII' is determined by some peripheral protein factor of the disc membrane [2]. This study provides evidence that the formation of 'extra MII' is caused by a shift of the MI/II equilibrium due to rapid binding of the G-protein to MII.

Abbreviations: ROS, rod outer segments; G-protein, GTP-binding protein; MI, metarhodopsin I; MII, metarhodopsin II; GTP-γS, guanosine 5′-*O*-(3-thiotriphosphate); NIR, near-infrared

2. MATERIALS AND METHODS

All spectroscopic measurements were done in isotonic saline containing 130 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM ethylene-diamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 mM piperazine-1,4-diethane sulfonic acid (PIPES) (pH 7.5).

Bovine ROS were prepared according to a standard procedure [2] and were stored frozen in liquid N_2. Experiments with ROS were performed under dim red light unless otherwise stated. Washed membranes devoid of G-protein [6] were prepared by 2-fold centrifugation of ROS (at 100 μm rhodopsin) in 2 mM PIPES (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT. G-protein was purified as in [7] and was separated from excess GTP by Sephadex G-25 column chromatography. It was pure ($> 99\%$), as judged by gel electrophoresis [8], and contained the 3 polypeptides of $\sim$ 37 000, 35 000 and 6000 M_r [9]. Supernatants from bleached and unbleached ROS (see fig.1) were prepared as in [6,7].

The apparatus for the simultaneous measurement of absorption and near-infrared (NIR) scat-

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tering signals was as in [2,10]. All scattering signals were measured on randomly oriented ROS and were corrected for the small so-called N-signal as in [10]. Each flash bleached 3.1% of the rhodopsin present. All measurements were performed at pH 7.5, I = 5.5°C. Under this condition, the 'classical' MI/MII equilibrium is 3.0/1 (see fig.4); i.e., sufficient MI is present to allow for convenient observation of 'extra MII'. Furthermore, the kinetic analysis is considerably simplified, since at 5.5°C, the 3 kinetic components which are contained in the NIR scattering signal of randomly oriented ROS [3,4] degenerate to one single rate-limiting process which is the MI/MII transition (in preparation).

3. RESULTS

Two experimental systems have been used to study the influence of G-protein on the MI/MII equilibrium:

(i) A reconstituted system of hypoosmotically washed disc membranes with various amounts of purified G-protein added;
(ii) ROS in which the stack of disc membranes is still relatively intact, with and without reactive, membrane-associated G-protein.

For (ii) a method was developed to solubilize the G-protein (mainly its α-subunit) from the membranes without hypoosmotic shock, simply by adding GTP-γS in the dark to ROS suspended in the experimental saline. At moderate (isotonic) ionic strength, most of the G-protein is normally membrane-associated, i.e., it is absent from the supernatant (fig.1a). In the presence of 20 μM GTP-γS, the α-subunit of the G-protein becomes preferentially solubilized, in the dark (fig.1c) as well as in light (fig.1b).

The reaction is virtually completed within 4 min at 0°C (fig.1c); similar results were also obtained at higher temperature (2–60 min at 20°C, not shown). Quantitative evaluation of gel densitograms shows that 80–85% of the total extractable α-subunit is contained in the first supernatant from bleached as well as from unbleached ROS (fig.1b,c). About 3 times as much α-subunit as compared to the β-subunit is extracted by GTP-γS, most of the β-subunit remaining membrane-bound at this ionic strength.

The influence of purified G-protein on the 387/417 nm absorption signal (MII signal) is shown in fig.2. Addition of G-protein to washed disc membranes obviously increases the amplitude of the signal, i.e., the amount of MII formed by a flash. It is further seen that the signal in absence of G-protein (small signal in fig.2) is considerably faster than that in presence of G-protein (large signal). To facilitate comparison of the kinetics, all amplitudes have been normalized to the same value in fig.3. This figure compares NIR scattering signals and MII-formation (387/417 nm) signals for ROS and for washed disc membranes.

Consider first the cases where G-protein is either totally absent (washed membranes without added G-protein, bottom line in fig.3), or where it is present but saturated with GTP-γS and therefore dissociated from the membranes (ROS + GTP-γS, second line in fig.3). No scattering signal is observed in either case, confirming that the scattering signal depends on the presence of G-protein in its unreacted (GTP-free) form [5]. The MII signals from the first flash and from flashes no 7–14 are kinetically indistinguishable in these cases; the rate constant of MII formation is $2.4 \leq k \leq 3.1\ s^{-1}$ both in washed membranes and in ROS + GTP-γS, regardless of the flash number.

When G-protein is present in the preparation (ROS without GTP-γS, first line, and membranes with added G-protein, third line in fig.3), the MII-signal from the first flash is slower, its rate constant being $0.8 \leq k \leq 1.05\ s^{-1}$ both in discs and in ROS. Parallel to this MII signal, the NIR scattering signal [3,5] is observed in ROS; it is kinetically identical to the MII-signal. Recombined membranes (third line in fig.3) yield only the much slower scattering signal P_D [3]. Apparently, the fast scattering signal requires not only the presence of G-protein but also a relatively intact structure of the disc stack. Apart from this complication, there are exactly two types of signals: the slower one, observed in absorption and scattering upon the first flash; and the faster one, which is only found in absorption. The slower reaction mode needs a dark-adapted membrane and the presence of G-protein. The faster one takes place in the absence of reactive G-protein, i.e., in washed membranes as well as in ROS + GTP-γS as well as in ROS at bleaching levels above that needed for saturation of the G-protein effect.

The relative amounts of MII formed in response to a succession of flashes are plotted in fig.4 for various preparations. In cases where the formation

Fig.1. Solubilization of G-protein with GTP-γS. An ROS suspension (80 μM rhodopsin) in 120 mM KCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM DTT (pH 7.2) was divided into 3 equal portions that were subjected to different treatments before extraction of soluble proteins: (a) untreated dark control; (b) ROS fully bleached for 1 min at 20°C in the presence of 20 μM GTP-γS; (c) ROS kept dark for 4 min at 0°C in the presence of 20 μM GTP-γS. The suspensions were centrifuged, and 50 μl of the corresponding clear supernatants were applied to SDS–polyacrylamide gels [7]. Note the light-induced binding of the 48 000 M_r protein, i.e., its absence from the supernatant (b), as described in [5,8]. The G-protein (α and β subunit) is normally membrane-associated under these ionic conditions (gel a). It becomes solubilized (particularly its α-subunit) by the treatment with GTP-γS in darkness (D) as well as in light (L). The solubilization is related to the exchange of GTP-γS for bound GDP on the G-protein (see [5,6,11]).

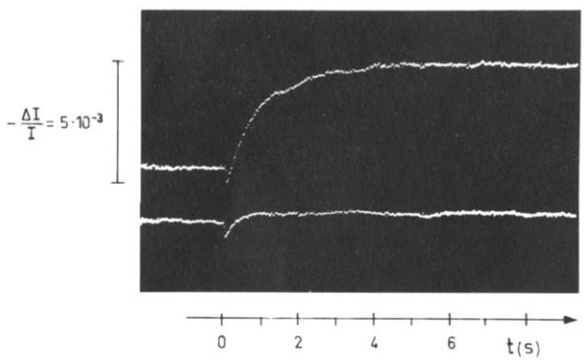

Fig.2. Flash-induced absorption signals in a suspension of washed disc membranes (3 μM rhodopsin). Flash applied at $t = 0$; original recordings. Signals are the negative difference of the relative absorption changes at 387 nm and at 417 nm [10]. The dual wavelength measurement eliminates the scattering contributions as in [2,10]. The negative initial jump is the absorption change due to early photoproducts including MI (not time-resolved); the production of MII follows on the *s*-time scale under these conditions (5.5°C, pH 7.5). The amplitude of the MI/MII transition is taken starting from the deepest point of the initial jump; it serves as a direct measure of the amount of MII formed. Both signals shown are from a first flash bleaching 3.1% rhodopsin. The small signal is obtained in the absence of G-protein, and the large signal after addition of purified G-protein (1 mol G-protein/7 mol rhodopsin) to another aliquot of the same disc suspension.

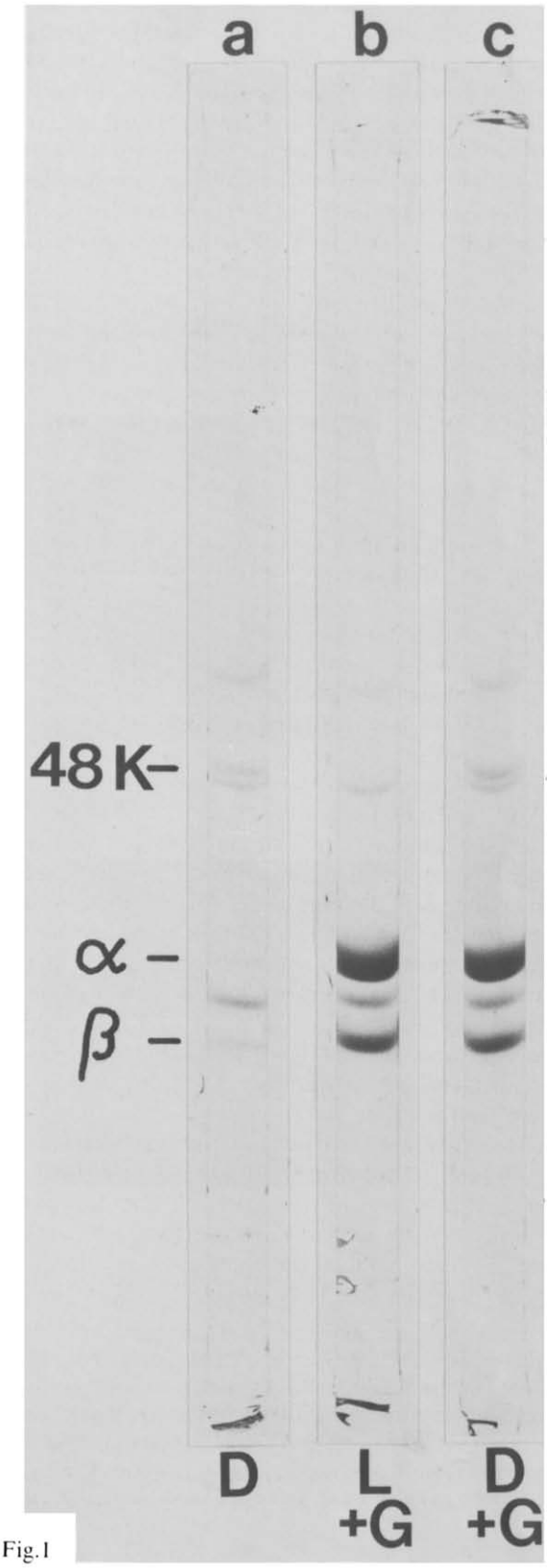

Fig.1

of MII obeys the 'classical' MI/MII equilibrium, the resulting 'exhaustion curve' is a straight line in this semilogarithmic plot (see [2]). This is approximately the case for ROS plus GTP-γS, and for washed membranes in absence of G-protein. Positive deviations from the straight line indicate the formation of 'extra MII', as observed in the case of ROS without added GTP-γS (A) and in washed membranes with added purified G-protein (B). The more G-protein is added, the more 'extra MII' is formed. The straight line is reached when an amount of rhodopsin approximately equimolar to the amount of G-protein present has been bleached, i.e., when the binding of G-protein to photoexcited rhodopsin is saturated [5,6].

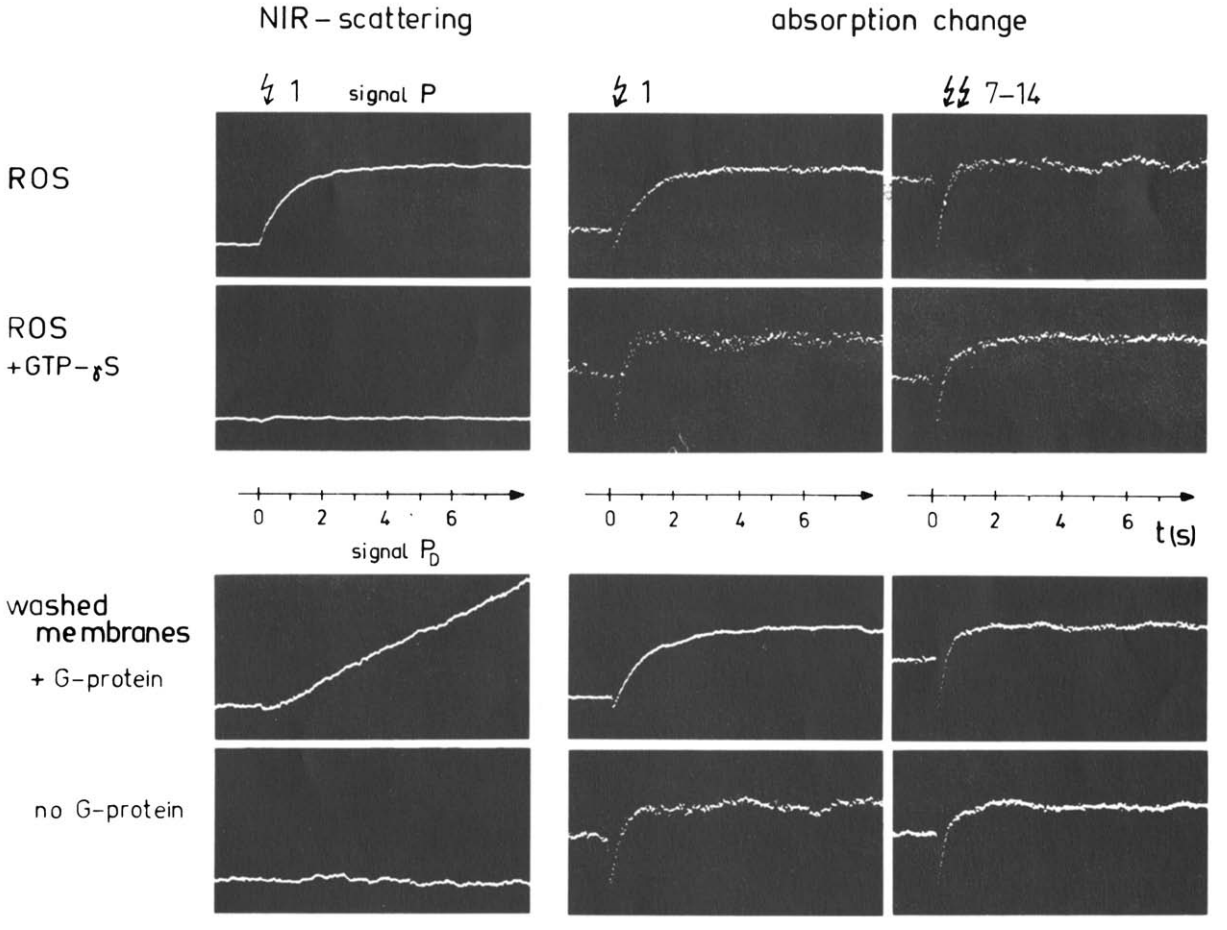

Fig.3. Kinetics of the flash-induced near-infrared scattering and absorption signals in 4 different preparations. The absorption signals (right side) measure the formation of MII as described in the legend to fig.2; their amplitudes are normalized to the same value. Scattering signals (left side) are the relative intensity changes at 800 nm measured in an angular range of $6.5° < \vartheta < 23°$. The 3 signals in one line belong to one and the same sample the composition of which is defined on the left side. The scattering signal P (left side) and the first absorption signal (middle) are simultaneous measurements from the first flash; the absorption signal at the right is an average of flashes no. 7–14. It is seen that the kinetics of the slow, G-protein dependent mode of MII formation (middle signals of first and third line) are identical to the kinetics of the NIR scattering signal in ROS. GTP-γS abolishes the NIR scattering signal and accelerates the MII formation in ROS, i.e., it leads to a situation similar to that found in washed membranes in the absence of G-protein.

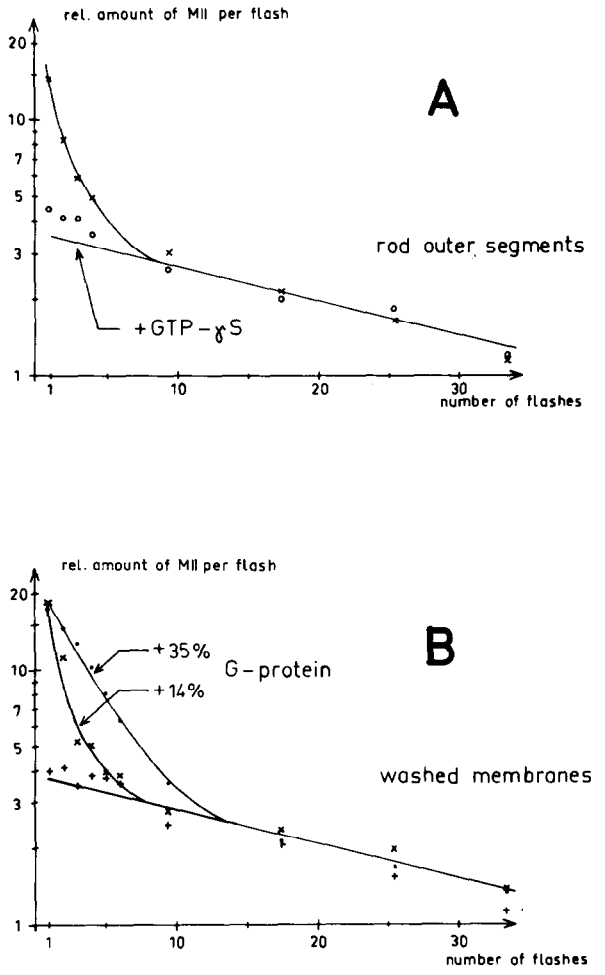

Fig.4. Formation of MII upon successive photolysis of rod outer segments, (A), and of washed membranes, (B), in series of flashes (exhaustion curves). Every flash bleaches 3.1% of the rhodopsin still present. The amplitudes of the flash-induced 387/417 nm signals (see fig.2) are plotted as single-flash recordings for flashes no. 1–6 and as the average of groups of 8 flashes at higher bleaching extents: (A) rod outer segments; (x) 5.5°C; (○) 5.5°C and presence of 50 μM GTP-γS; The MI/MII equilibrium at 5.5°C is K = MII/MI = 3.6/(14.5 – 3.6) = 1/3.0 as seen by comparison of the amplitude for flash no. 1 (virtually pure MII) and of the ordinate intercept (mixture of MI and MII) [2]. 'Extra MII' is observed for untreated ROS as in [2]; GTP-γS almost quantitatively abolishes the formation of extra MII. (B) Washed membranes without and with purified G-protein added. The more G-protein was added (molar ratio of G-protein *vs* rhodopsin indicated in percent), the more pronounced is the 'extra MII' effect.

4. DISCUSSION

The results show that G-protein is the determining factor for the MII formed in excess of the normal equilibrium ('extra MII'), as well as for the NIR scattering signal. The underlying process appears to be the formation of a complex between G-protein and photoexcited rhodopsin, as suggested by the approximate stoichiometry between the 'extra MII' formed and the amount of G-protein added (fig.4), as well as in [5,6]. The exact kinetic congruence between the scattering signal and the MI/MII signal at the first flash indicates that both signals reflect the same rate-limiting reaction step. The simplest reaction scheme which explains the data is the following:

$$\mathrm{MI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MII} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{MII\text{-}G} \qquad (1)$$

The formation of the complex MII-G, indicated by the scattering signal, draws MII from its equilibrium with MI, and this MII is observed as the 'extra MII'. The results show that, for flash no. 1, the equilibrium is fully shifted to MII, whereas the flashes no. 7–14 are already completely unaffected (fig.4). The saturation effect at higher bleaching levels is easily explained as follows: the G-protein already complexed with MII from the first flashes cannot bind to MII from later flashes, leading to the 'normal' MI/MII equilibrium upon later flashes.

The reaction scheme (1) assumes that both the 'normal' and the 'extra' MII are formed via the same pathway (same rate constant k_1) and that the G-protein binds only to MII which has already been formed. This assumption can be tested using the following kinetics: Since MII is virtually the only photoproduct found upon the first flash, and since the formation of the complex MII-G must be much faster than the MI/MII transition (indicated by the kinetic congruence between scattering signal and MII signal), the first part of the scheme (1) degenerates to:

$$\mathrm{MI} \overset{k_1}{\rightarrow} \mathrm{MII}, \text{ solution, } \mathrm{MII}(\infty) - \mathrm{MII}(t) \backsim e^{-k_1 \cdot t}$$

at the first flash;

For flashes no. 7–14, it results:

$$\mathrm{MI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{MII}, \text{ solution, } \mathrm{MII}(\infty) - \mathrm{MII}(t) \backsim e^{-(k_1 + k_{-1}) \cdot t}$$

Under the conditions used (5.5°C, pH 7.5), the equilibrium is K = MII/MI = 1/3.0 (see legend to fig.4). With $K = k_1/k_{-1}$, one obtains $k_{-1} = 3.0\, k_1$. Thus, if scheme (1) is valid, the MI/MII signal from flashes no. 7–14 must be 1 + 3.0 = 4.0-times faster than that from no. 1. The average experimental relation is 2.75/0.92 = 3.0, in reasonable agreement with the assumption.

It is not totally excluded that the G-protein binds already to MI or to an earlier photoproduct. However, one has then to assume that MI in such a complex decays to MII at the same rate k_1 as uncomplexed MI does. Scheme (1) avoids this somewhat improbable assumption.

Scheme (1) replaces our former scheme where an irreversible trigger step for the P-signal and a postponed equilibrium was assumed [12].

The 'extra MII' is formed with identical kinetics in both experimental systems used, i.e., similarly in ROS and in the reconstituted system of extensively washed membranes and purified G-protein. This indicates that the formation of 'extra MII' depends only on the presence of G-protein but not on an intact ROS structure. The fast NIR-scattering signal, however, requires in addition structural integrity of the ROS (see fig.3, signals P and P_D. (More gentle washing with 10 mM buffer yields faster scattering kinetics also in the reconstituted system, see [5].) The physical event indicated in light scattering may, in the case of extensively washed membranes (signal P_D), be much slower than its biochemical trigger reaction, the binding of G-protein to MII.

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