

G PROTEINS AND PHOTOTRANSDUCTION

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■ **Abstract** Phototransduction is the process by which a photon of light captured by a molecule of visual pigment generates an electrical response in a photoreceptor cell. Vertebrate rod phototransduction is one of the best-studied G protein signaling pathways. In this pathway the photoreceptor-specific G protein, transducin, mediates between the visual pigment, rhodopsin, and the effector enzyme, cGMP phosphodiesterase. This review focuses on two quantitative features of G protein signaling in phototransduction: signal amplification and response timing. We examine how the interplay between the mechanisms that contribute to amplification and those that govern termination of G protein activity determine the speed and the sensitivity of the cellular response to light.

INTRODUCTION

G Proteins Regulate the Amplification and Timing of Cell Signaling

Heterotrimeric G proteins are the molecular mediators that link G protein-coupled receptors (GPCRs) to their effector proteins — enzymes or ion channels; thus, they form the second stage in a three-step signaling cascade. The first step is the activation of the GPCR by the binding of an agonist molecule. In the second step the activated GPCR activates multiple molecules of G protein by catalyzing the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the G protein α subunit ($G\alpha$). The G proteins activated in this way constitute an amplified representation of the activated GPCR. In the third step of the cascade the activated G protein binds to its effector and thereby switches it either on or off in different systems. The timing of the effector's activity is controlled by the G protein, because hydrolysis of the GTP bound to $G\alpha$ terminates the activity of

both the G protein and the effector. The duration of this signal can be set by the intrinsic GTPase time constant of the G protein or it can be regulated by interaction with a GTPase-activating protein (GAP), either alone or in combination with the G protein effector.

The Vertebrate Rod: A Model System for Investigating G Protein Signaling

Phototransduction is the process by which a photon of light captured by a molecule of visual pigment generates an electrical response in a photoreceptor cell. Visual pigments are members of the superfamily of GPCRs whose natural ligand, the chromophore 11-*cis* retinal, is already covalently attached (1, 2). The 11-*cis* isomer acts as a powerful antagonist, and it is only when a photon of light isomerizes the chromophore to its all-*trans* form, which acts as a powerful agonist, that the visual pigment GPCR becomes active. In all vertebrate and invertebrate photoreceptor cells investigated thus far, phototransduction has been found to be based on a heterotrimeric G protein signaling cascade. The cascade of vertebrate photoreceptors has proven to be an especially productive model system for elucidating general principles of G protein signaling, thanks to several advantages it offers to investigators.

The first advantage arises from the fact that phototransduction takes place in a highly specialized organelle, the outer segment (Figure 1) of the photoreceptor cell. The outer segment contains high concentrations of the proteins involved in the transduction cascade—typically $\sim 500 \mu\text{M}$ of the G protein, transducin (see Table 3 in Ref. 3)—and, moreover, it lacks most of the proteins involved in other cellular functions. The outer segments of rods, in particular, can easily be detached from the retina and harvested in quantities sufficient for purification of most key protein components, and for conducting biochemical experiments to characterize their properties and interactions.

A second advantage is that the light-evoked electrical responses of vertebrate photoreceptors have been extensively investigated with an array of electrophysiological techniques, providing a rich, quantitative database that has to be explicable in terms of the interactions between the biochemical components of the transduction cascade. Because the electrophysiological recordings are obtained from intact photoreceptors (and even from cells *in vivo*), it follows that such recordings reflect transduction when the components of the cascade are present in their natural dispositions and concentrations, and are interacting with one another under conditions selected by evolution for their signaling function.

A third advantage of vertebrate photoreceptor preparations, one that applies equally well to *in vitro* biochemical assays and to electrophysiologically measured responses, is that they allow exquisite precision in quantification of the natural stimulus, measured as the number of photopigment molecules isomerized by light exposure. As a consequence, investigations of rod phototransduction have achieved

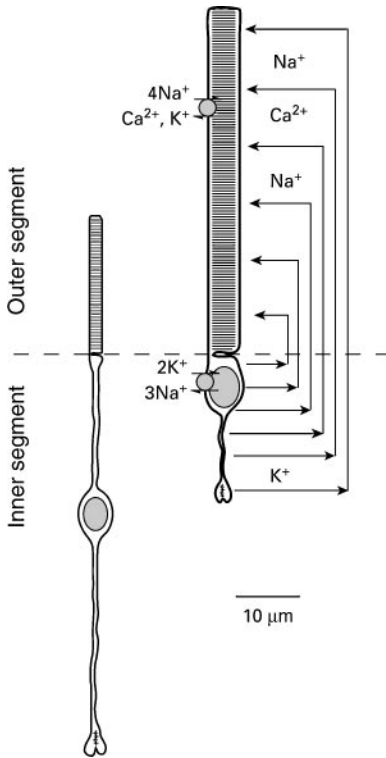


Figure 1 The structure of the mammalian rod (*left*) and a toad rod (*right*) (drawn roughly to scale). In the dark state the cells have a circulating current, characterized by the influx of Na^+ and Ca^{2+} cations into the outer segment through cGMP activated channels, and a balancing outward flux of K^+ through channels in the inner segment. A $\text{Na}^+/\text{Ca}^{2+}-\text{K}^+$ exchanger in the outer segment membrane, and a conventional Na^+/K^+ exchanger in the inner segment membrane maintain the overall ionic gradients that drive the two limbs of the circulating current.

a level of quantitative analysis that would be difficult to attain with any other second messenger system. Indeed, rod phototransduction provides the unique opportunity to analyze a cellular response evoked by the activation of a single GPCR, i.e., one rhodopsin molecule (4–7).

A number of excellent reviews published over the past decade examine various aspects of vertebrate phototransduction and are recommended to the reader (3, 8–26). This review focuses on recent developments in the understanding of two quantitative features of G protein signaling in phototransduction: signal amplification and response timing. It also focuses entirely on rods, which are the more abundant photoreceptors in the retinas of most vertebrates, with the consequence that the quantitative aspects of G protein signaling have been studied in much greater detail in rods than in cones.

The Major Steps in the Activation Phase of Vertebrate Phototransduction

The major steps of the G protein cascade in vertebrate rods are illustrated schematically in Figure 2.

PHOTOISOMERIZATION Vision begins when the chromophore, the preattached ligand of a single molecule of visual pigment, is isomerized by a captured photon. Within a millisecond rhodopsin undergoes a series of intramolecular transitions leading to a conformational state called metarhodopsin II (or R^*), which is capable of activating the photoreceptor-specific G protein, transducin.

TRANSDUCIN ACTIVATION R^* interacts with the GDP-bound form of the transducin $\alpha\beta\gamma$ trimer; i.e., with $G\alpha_t\text{-GDP-G}\beta\gamma_t$. The R^* activates the transducin by triggering rapid exchange of bound GDP for GTP on the $G\alpha_t$; this is followed very rapidly by dissociation of the transducin from the R^* , as well as by dissociation of the active $G\alpha_t\text{-GTP}$ (or G^*) from $G\beta\gamma_t$.

PHOSPHODIESTERASE ACTIVATION At the next step of the cascade $G\alpha_t\text{-GTP}$ stimulates the activity of its effector enzyme, the cGMP phosphodiesterase (PDE), also known as PDE6 (27). The PDE is a heterotetramer consisting of two identical or nearly identical catalytic subunits ($\alpha\beta$ in rods, $\alpha\alpha$ in cones) and two identical regulatory γ subunits (PDE γ), which serve as protein inhibitors of PDE activity and which are responsible for maintaining the activity in the nonactivated state at its very low basal level. Activation of the PDE results from the binding of $G\alpha_t$ to the γ subunit, thereby removing the inhibitory constraint that the PDE γ had imposed on the catalytic site of the PDE α or β subunit.

CYCLIC GMP HYDROLYSIS AND cGMP-CHANNEL CLOSURE Activation of the PDE causes a reduction in cytoplasmic concentration of cGMP, the second messenger in phototransduction, and this in turn causes closure of the cation selective cGMP-gated channels located in the plasma membrane. Closure of these channels reduces the steady inward current that is normally carried by Na^+ and Ca^{2+} ions in the dark, resulting in membrane hyperpolarization and decreased release of the synaptic transmitter glutamate at the photoreceptor terminal.

Timely Termination of Phototransduction

As in all G protein signaling pathways, timely termination of the photoreceptor signal requires that all the activated intermediates be inactivated rapidly, restoring the system to its dark, basal state, ready for signaling again. Thus, the three protein intermediates, R^* , $G\alpha_t\text{-GTP}$, and activated PDE, must all be inactivated, and the concentration of cytoplasmic messenger cGMP must be restored to its dark level by guanylyl cyclase. Because the focus of this review is on transducin, we do not consider the inactivation of R^* or the restoration of cGMP levels by guanylyl cyclase; these topics are covered in several of the reviews cited above.

In phototransduction the active state of the effector enzyme (PDE) persists until the GTP bound to its activator ($G\alpha_t$) is hydrolyzed to GDP and P_i , permitting the

dissociation of $G\alpha_i$ from PDE and returning the effector to its inactive state. The two functional features of phototransduction reviewed here, signal amplification and termination of activity, involve distinct molecular and cellular mechanisms, yet are tightly intertwined in determining the response of the photoreceptor. Thus, the activation phase of the signal needs to be very highly amplified to elicit a reliable response to each photon. In addition, the cascade needs to be inactivated rapidly enough to allow the cell to signal reductions in light intensity and repetitive stimulation. A goal of this review is to examine the mechanisms that contribute to signal amplification and those that lead to termination of cascade activity, whose interplay determines the sensitivity of the cell to light.

AMPLIFICATION IN ROD PHOTOTRANSDUCTION IS ACHIEVED IN THREE GAIN STEPS

A Framework for Quantifying Amplification in Phototransduction

The remarkable ability of rods to reliably signal individual photoisomerizations has presented a long-standing challenge to investigators of vertebrate phototransduction (4, 5). The capture of a single photon by a rod results in suppression of 2–5% of the total cGMP-activated current at the peak of the photoresponse (4–7) and a consequent membrane hyperpolarization of about 1 mV. This exquisite signaling capacity is achieved through three steps of amplification, or gain. First, a single R^* activates many transducin molecules in the course of its lifetime. Second, each PDE activated by a transducin hydrolyzes many cGMP molecules during its lifetime. Third, the cGMP-gated channels contribute a gain factor corresponding to the cooperativity of their opening (their Hill coefficient).

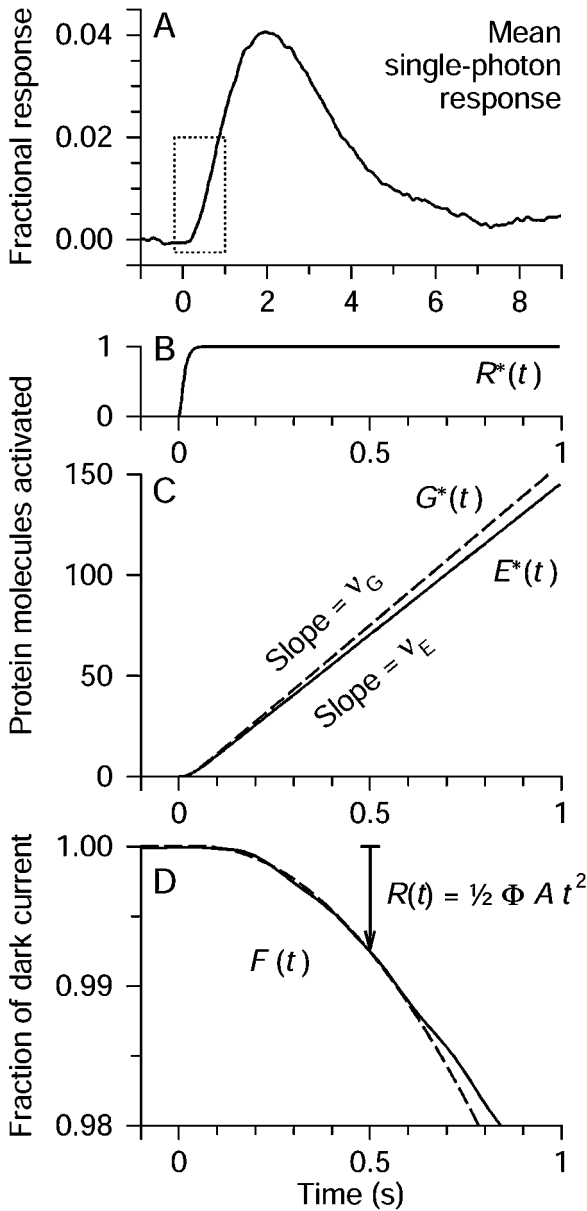
The contribution of the individual molecular components of the cascade to the overall gain of phototransduction has been quantified in a theoretical framework developed by Lamb & Pugh (28). Details of that LP analysis can be found in (3, 14, 28); here we simply present a summary as a background for key aspects of this review.

The LP analysis predicts, and many studies have confirmed, that the rod's response to a brief flash initially rises as a parabolic function of time (Figure 3):

$$R(t) \approx \frac{1}{2}\Phi A t^2. \quad 1.$$

Here $R(t)$ is the normalized response, Φ is the number of photoisomerizations (R^*) per rod generated by the flash, and A is a constant characterizing the rate of parabolic rise, and thus the overall signal amplification: The greater the magnitude of A , the more rapidly the response rises per R^* . The normalized response $R(t)$ is the fractional suppression of the preexisting circulating current, expressed as the observed response divided by the steady level present before the flash. The initial

suppression of current follows a parabolic function of time because the quantity of cGMP hydrolyzed at time t after the stepwise activation of R^* is determined by two cascaded integrating processes: the activation of multiple transducins (and as a consequence, multiple PDEs) by each R^* , followed by the hydrolysis of multiple cGMPs by each activated PDE.



The LP analysis further shows that the parameter A , called the “amplification constant,” can be expressed as the product of four gain factors arising at different steps of the cascade:

$$A = \nu_G c_{GE} \beta_{\text{sub}} n_{cG}. \quad 2.$$

Here ν_G is the rate of activation of transducin molecules per R^* and c_{GE} is the coupling efficiency from G^* activation to PDE activation, so that $\nu_G c_{GE} = \nu_E$ is the rate of activation of PDE catalytic subunits per R^* ; we use the letter E to denote “effector enzyme,” which in this cascade is the PDE. β_{sub} is the rate constant of cGMP hydrolysis per activated PDE catalytic subunit, and n_{cG} is the Hill coefficient describing the cooperativity of channel opening by cGMP. The value of n_{cG} is 2–3 (29–31). The parameters ν_G and ν_E , characterizing the rates of activation of transducin and PDE, per R^* , respectively, are examined below.

The parameter β_{sub} captures the gain contributed by the PDE and is defined as the rate of change in cytoplasmic concentration of cGMP elicited by a single activated PDE catalytic subunit. This rate constant can be expressed in terms of the Michaelis parameters of the PDE as

$$\beta_{\text{sub}} = \frac{k_{\text{sub}}/K_m}{N_{\text{Av}} V_{\text{cyto}} BP_{cG}}, \quad 3.$$

where k_{sub} represents the average cGMP turnover rate of an active PDE catalytic subunit (PDE*), given by $1/2 k_{\text{cat}}$, with k_{cat} denoting the catalytic rate of a fully activated PDE holomer (PDE**), and where K_m is the Michaelis constant of the PDE for cGMP. In addition, N_{Av} is Avogadro’s number, V_{cyto} is the cytoplasmic volume of the outer segment, and BP_{cG} is the cell’s cytoplasmic buffering power for cGMP.

Figure 3 Kinetics of the rod’s response to a single photon of light, as a function of time t after delivery of the photon. (A) The mean electrical response of a toad rod to a single photon is plotted on a slow time-base (from 32). (B, C) Kinetics of activation of the disc-based proteins, rhodopsin, G protein, and phosphodiesterase (PDE) to their excited forms (R^* , G^* , and E^* , respectively), when inactivation reactions are ignored; note the faster time-base. A single photoisomerization activates a single R^* , which triggers activation of the G protein at a constant rate (of $\nu_G \approx 150 \text{ G}^* \text{ s}^{-1}$ at room temperature), and which in turn couples to E^* activation at almost as high a rate, ν_E . (D) The steadily increasing quantity of activated PDE, $E^*(t)$ leads to hydrolysis of cGMP at an accelerating rate, which in turn causes the fraction $F(t)$ of cGMP-gated channels remaining open to decline according to a Gaussian function of time. The fractional response, $R(t)$ plotted in A, is given by the reduction in $F(t)$ from unity, which can be shown to follow parabolic kinetics (arrow) at early times. The noisy trace in D is redrawn from the part of panel A highlighted by the dotted box on the faster time-base and in terms of fractional circulating current.

The LP analysis provides a framework for quantitative comparison of estimates of the gain factors obtained in biochemical experiments, with the overall signal amplification derived from electrophysiological recordings. In other words, the amplification constant of an intact rod provides a benchmark that must be accounted for by the combined effect of the biochemical parameters, as described by Equations 2 and 3. We examine this issue in detail below, after first discussing an example that illustrates the important role of the volume of the cytoplasm, V_{cyto} , in amplification.

Comparative Analysis of the Amplification of Amphibian and Mammalian Rods

Whereas both amphibian and mammalian rods reliably respond to single photons, those of mammals reach a reliable signal about 10 times faster than those of amphibia, a factor that cannot be explained solely by the temperature dependence of chemical reactions, which would have a Q_{10} no higher than 3. Perhaps even more puzzling, a body of electrophysiological evidence has established that the amplification constant is up to 100 times higher in mammalian rods ($A \approx 5\text{--}10\text{ s}^{-2}$) than in amphibian rods ($A \approx 0.1\text{--}0.2\text{ s}^{-2}$) (reviewed in 3 and 14). In the LP analysis this large difference is accounted for principally by the smaller volume of mammalian rods: Thus, for amphibian rod outer segments $V_{\text{cyto}} \approx 1\text{ pl}$, whereas for mammalian rods $V_{\text{cyto}} \approx 20\text{--}40\text{ fl}$, a factor of 25- to 50-fold smaller. Because V_{cyto} appears in the denominator, Equation 3 predicts that the mammalian rod will have a 25- to 50-fold higher value of β_{sub} , owing to its smaller cytoplasmic volume alone; thus, a given number of activated PDE catalytic subunits can much more rapidly alter the cGMP concentration in the smaller volume. The higher temperature of the mammalian rod is also expected to increase ν_E and k_{sub} , providing, together with the cytoplasmic volume ratio, a full accounting of the 50- to 100-fold higher value of A in two cells that use the same molecular machinery. One can readily see that the higher value of A in the mammalian rod endows it with greater response speed: In Equation 1 an n -fold increase in A causes R to reach a criterion level (e.g., 5%) at a speed \sqrt{n} -fold faster, so that a 100-fold higher value of A in the small mammalian rod translates into a 10-fold faster attainment of a criterion response amplitude. Thus, improved temporal resolution is an evolutionary advantage of the small packaging of the mammalian rod photoreceptor.

THE RATES OF ACTIVATION OF TRANSDUCIN AND PHOSPHODIESTERASE BY R^* : BIOCHEMICAL MEASUREMENTS

Biochemical assays with radiolabeled nucleotides (usually with the nonhydrolyzable GTP analog, $\text{GTP}\gamma\text{S}$) provide a straightforward method of measuring the rate of transducin activation per R^* (ν_G). Another, less direct, way to estimate

ν_G is to derive it from the measured rate of PDE activation per R^* (ν_E) based on the assumption that activation of each catalytic subunit of PDE results from the activation of one $G\alpha_t$. The legitimacy of this assumption is discussed below.

There is great variation among published estimates of ν_G and ν_E derived from biochemical assays, with values ranging from 10 to 200 $G^* s^{-1}$ per R^* (reviewed in 14; their Tables 3, 4). Furthermore, concerns have been expressed regarding the legitimacy of using biochemical assays to determine ν_G and ν_E (reviewed in 14; see 32, 33 for recent updates). Among the concerns are the low time resolution of such assays relative to the speed of normal photoresponses and the relatively dilute suspensions of disrupted photo-receptor membranes that are typically used. Dilution often results in the loss of a substantial fraction of transducin into the aqueous phase, and severe disruption could potentially alter the natural kinetics, for example by altering the lateral diffusion of proteins in or at the membrane surface.

We recently undertook an investigation aimed at addressing a number of issues associated with the estimation of ν_G and ν_E using biochemical assays (32). The experiments first determined, for fresh frog rod outer segment suspensions, the conditions that maximized the activation rates; the factors that were studied included the means of permeabilization of the outer segments, as well as the concentration of membranes and the concentrations of GTP and divalent cations. This investigation found that at 22°C and under optimized conditions ν_E and ν_G were on average 120 $G\alpha_t$ (or subunits of activated PDE) s^{-1} per R^* , with values as high as 150 in individual experiments.

THE RATE OF TRANSDUCIN ACTIVATION BY R^* : MEASUREMENTS WITH INFRARED LIGHT SCATTERING

Mechanisms of Light Scattering by Rod Disc Membranes

Monitoring the light-evoked changes in the scattering of infrared light by suspensions of rod outer segment fragments has played an important role in the investigation of photo-transduction. In this section we review some of the findings obtained by this technique and compare them with related findings obtained using other methods.

To understand the basic idea of light scattering, imagine a red laser beam passing through a dilute suspension of milk in a fish tank: Looking down on the tank, one can see the path of the beam through the tank, because of the light that is scattered by the suspended milk droplets in the direction of the observer's eye. Scattering occurs in all directions (though not equally), and an off-axis detector, such as the eye, will monitor the component of light scattered in that direction; in contrast, a detector on the axis of the laser beam will monitor mainly the light that has not been scattered, and this intensity is usually much higher. Typically one measures the fractional change in intensity, $\Delta I/I$, either off-axis, or axially, or both, using a near-infrared source, and in response to stimulation of the rod particle preparation with a brief flash of visible light that isomerizes a known fraction of the rhodopsin.

The intensity of the scattering in any particular direction depends on a number of factors, including the direction of the detector with respect to the illumination beam, the wavelength of the light, the size distribution and material properties of the suspended particles, and of course, the concentration of particles in the solution. Theoretical analysis shows that, for a dilute suspension of particles whose major dimension is not much greater than the wavelength of the measuring light, the principal factor contributing to a change in the scattering intensity in a particular direction is a change in the mass density of the particles relative to the density of the bulk solution (34, 35). Thus, in a suspension of rod discs, any gain of protein mass onto the membranes from the solution will cause the scattering of light to increase, whereas any loss of mass from the membranes to the solution will cause the scattering to decrease.

Scattering by larger and more organized structures, such as entire rod outer segments, is more complicated to analyze theoretically than that by small fragments, as a number of factors other than the electron density of the particles come into play. In the next section we focus on two signals that are obtained from suspensions of discs or small fragments of outer segment.

The Discovery of the Binding and Dissociation Signals for Near Infrared Light Scattering from Rod Disc Suspensions

Near infrared light scattering by rod outer segments was first reported by Hofmann and colleagues (36) and has subsequently been the subject of numerous investigations by many researchers. Kühn et al. (37) presented the first experiments demonstrating effects of transducin activation on light scattering changes. Using a suspension of outer segment fragments and measuring the intensity of light in the forward direction, they found and analyzed two distinct transducin-dependent infrared scattering signals, which they named the “binding” and “dissociation” signals. The binding signal, which they observed in the absence of GTP, was seen as a negative-going deflection in $\Delta I/I$ in the axial direction. When the membranes were stripped of all proteins but rhodopsin and then reconstituted with G_t , the amplitude of the binding signal was found to saturate when the concentration of R^* was very nearly equal to the total concentration of G_t in the reaction volume. Other investigations had shown that, in the absence of GTP, G_t binds very tightly to R^* (38), and therefore Kühn et al. (37) concluded that the binding signal was likely to represent the formation of an R^*-G_t complex with 1:1 stoichiometry. The second transducin-dependent signal reported by Kühn et al. (37) required the presence of GTP and was seen as an increase in $\Delta I/I$ measured axially; i.e., it corresponded to a reduction in scattering and hence presumably a loss of mass from the membrane. Based on prior work showing that G_{α_t} dissociates from the membrane when rhodopsin is activated (38), it was concluded that this signal most likely reflects the rapid dissociation of G_{α_t} -GTP from the membranes into solution, and so it was called the “dissociation” signal.

The Role of Transducin Redistribution Between Soluble and Membrane-Bound Pools in the Binding and Dissociation Signals

Subsequent research has substantiated the explanations put forward by Kühn et al. (37) for the molecular nature of the two scattering signals they observed but has refined it by focusing attention on the critical role of the soluble and membrane-bound fractions of G_t . Arshavsky and coworkers (39) hypothesized that the binding and dissociation signals both reflected the redistribution of transducin between the soluble and membrane fractions. Because it had been established that G_t dissociates from membranes at low ionic strength (38), they manipulated the ionic strength of the medium to vary the amount of G_t bound to membranes previously stripped of proteins and reconstituted with G_t in the dark. They found that the amplitudes of the binding and dissociation signals varied with the quantities of G_t expected to be in the corresponding fractions: Thus, in the low ionic strength medium (in which most G_t is dissociated) the binding signal was large and the dissociation signal small, whereas the converse was true in normal ionic strength solution. Moreover, the absolute difference between the maximal amplitudes of both signals remained unchanged with the manipulation, consistent with the idea that the two signals reflect the light-dependent redistribution of a fixed pool of transducin.

Comprehensive evidence that both the binding and dissociation signals are generated by a redistribution of the mass of G_t between membrane-bound and soluble phases has recently been presented by Heck & Hofmann (33). Under standard experimental conditions (3 μM rhodopsin-containing membranes reconstituted with 0.5 μM G_t), the saturated amplitudes of the binding and dissociation signals were proportional to the quantities of G_t recovered from the membrane and soluble phases and quantified with SDS-PAGE, at an approximately 50%:50% ratio. In addition, a linear relationship was observed between the maximal amplitude of the dissociation signal and the total added G_t over the concentration range of 0.1–1 μM G_t . These results provided the authors with a basis for using the saturating amplitude of the dissociation signal to estimate the fraction of G_t that was initially membrane bound under the experimental conditions.

Further evidence for the hypothesis that G_t mass redistribution underlies the dissociation signal can be obtained from a calculation of the saturating amplitude of the signal. Thus, the first-order prediction for off-axis scattering by a dilute solution of small rod fragments is that $\Delta I/I = 2\Delta M/M$, where M is the total mass of the fragment; the factor 2 arises because the intensity of light scattered by small particles depends on the square of the number of scattering electrons in the particle (35). Thus, given that (a) rhodopsin comprises 31% of the dry mass of rod discs (41); (b) that the concentration of rhodopsin was 3 μM , and that of the membrane-bound fraction of transducin ($G_{t,\text{mem}}$) was 0.25 μM in the experiments of Heck & Hofmann (33); and that (c) the molecular masses of rhodopsin and $G\alpha_t$ are both approximately 38 kDa, the saturated amplitude of the dissociation signal

is predicted to be

$$\frac{\Delta I}{I} \approx \frac{2\Delta M}{M} \approx 2 \frac{0.25 \mu\text{M} \times 38 \text{ kDa}}{(3 \mu\text{M} \times 38 \text{ kDa})/0.31} \approx 0.05, \quad 4.$$

whereas the observed magnitude was 0.03. (Possible reasons for the modest discrepancy between prediction and observation are that the rod particles may be too large to undergo coherent electronic oscillation, that the mass of water inside the membrane vesicles is not taken into account in this calculation, and that in addition to $G\alpha_t$ some $G\beta\gamma_t$ may dissociate from the membranes.) Experiments by other investigators (e.g., 37) with similar preparations give saturating amplitudes of comparable magnitude, though the fractions of soluble and membrane-bound G_t were not specified.

The kinetics of the binding signal in dilute suspensions of rod fragments reveal that the rate at which R^* reacts with soluble G_t must be far slower than the rate at which it reacts with membrane-bound G_t . It is therefore important to estimate the fraction of G_t that might be membrane bound in intact rods. The dissociation constant for the binding of holo- G_t to rod outer segment membranes is $K_D \approx 1\text{--}3 \mu\text{M}$ (33), in the case of reconstituted membranes, and has been estimated to be an order of magnitude lower for native membranes (42); here K_D is expressed in terms of the bulk concentration of rhodopsin in solution, because rhodopsin is present in a fixed ratio to the membrane lipids (43). Hence, in an intact rod, where the rhodopsin concentration is $R = 6000 \mu\text{M}$ relative to the cytoplasmic volume and where the concentration ratio G_t to rhodopsin is 1:12 (44, 45), it is expected that the membrane-bound fraction of G_t will be at least $R/(R + K_D) = 6000/(6000 + 1) = 99.98\%$; i.e., essentially unity.

Estimation of ν_G with the Dissociation Signal

The unequivocal assignment of the molecular mechanisms underlying the binding and dissociation signals, together with the finding that the fraction of membrane-bound G_t can be determined from the ratio of the maximal amplitudes of the two signals, provided Heck & Hofmann (33) with a rigorous basis for determining ν_G , the rate of activation of G_t per R^* . Using preparations of bovine rod disc membranes reconstituted with purified transducin, they recorded the kinetics of the dissociation signal over a wide range of GTP and GDP concentrations, at a range of temperatures. They then fitted an analytical model of the interactions and determined the single set of parameters that provided the best global fit to the entire ensemble of results, at each temperature. At 22°C the extrapolated maximal rate of G_t activation was $\nu_{G,\text{max}} \approx 590 G\alpha_t \text{ s}^{-1}$ per R^* , and the concentration of membrane-bound G_t required to reach the half-maximal rate (i.e., the K_m for G_t) was 3100–3800 molecules per μm^2 of membrane surface (see Reference 33, tables 1 and 2). At 34°C the corresponding parameters were 1300 s^{-1} and $3000 \mu\text{m}^{-2}$ (Reference 33, table 2).

From Heck & Hofmann's results and analysis one can derive an estimate of the rate ν_G that would apply *in vivo*. Given that G_t in intact frog and mammalian rods

is present at a molar ratio to rhodopsin of 1:12 (44, 45), and taking the rhodopsin density to be 25,000 molecules μm^{-2} (43), the membrane density of transducin in amphibian rods is calculated to be $G_{t,\text{mem}} \approx 2100 \mu\text{m}^{-2}$. Hence, in the presence of a saturating concentration of GTP and in the absence of GDP, the rate ν_G in rods at 22°C is predicted from the analysis of Heck & Hofmann to be

$$\nu_G = \nu_{G,\text{max}} \frac{G_{t,\text{mem}}}{G_{t,\text{mem}} + K_m} = 220 G\alpha_t \text{ s}^{-1} \text{ per R}^*, \quad 5.$$

where K_m has been set to the average of the two values measured by Heck & Hofmann, $3500 \mu\text{m}^{-2}$. The estimate of ν_G derived with Equation 5 for amphibian rods depends on several assumptions; namely, that frog and bovine membranes at 22°C are functionally equivalent, that the GDP concentration is negligible, and that peripheral proteins (which are not present in the reconstituted system) have negligible effect on ν_G . Despite these uncertainties, the value of $220 G\alpha_t \text{ s}^{-1} \text{ per R}^*$ derived from the light-scattering measurements using bovine rod membranes (33) is remarkably close to the value of 120–150 $G\alpha_t \text{ s}^{-1} \text{ per R}^*$ derived from biochemical assays using frog rod membranes (32).

The Release or Amplified Transient Signal Likely Reflects G_t -Phosphodiesterase Interaction

Another distinct light-scattering signal that has been used to estimate ν_G is the “release” signal, first described by Vuong et al. (46). They used a magnetic field to orient fragments of frog rod outer segments, with normally spaced disc stacks but permeabilized plasma membranes. With the rods oriented at 45° to the incident beam and with two detectors positioned at right angles to the incident beam, they measured infrared scattering simultaneously at the two detectors. Physical analysis showed that changes in scattering recorded by the detector at -90° should reflect mass displacement along the rod’s axis, whereas the detector at $+90^\circ$ should reflect mass displacement in a radial direction.

Upon flash illumination of the rod suspension, Vuong et al. (46) measured a small but rapid transient increase in scattering in the “axial” signal, together with a larger and slower decrease in scattering in both directions. They attributed the difference between the two signals to release of $G\alpha_t$ from the membrane into the cytoplasm and termed this the release signal, whereas they attributed the common reduction in scattering to the subsequent leakage of $G\alpha_t$ out of the outer segments, and they termed this the “loss” signal. In analyzing the release signal, they measured a fractional rate of change of scattering of $10^4 \text{ s}^{-1} \text{ per R}^*$. Then, by assuming that the maximal level of this signal corresponded to the release of the total complement of $G\alpha_t$ from the membrane, they multiplied this value by an assumed ratio of G_t to rhodopsin, 1:10, to obtain a transducin activation rate of $\nu_G = 1000 G\alpha_t \text{ s}^{-1} \text{ per R}^*$. In our view, the assumption by Vuong et al. (46), that the maximal release signal corresponds to release of the entire pool of G_t , is questionable. For the reasons discussed below, we instead take the view that this signal is more likely to arise from the interaction between G_t and PDE.

Subsequent to the investigation by Vuong et al., a number of other studies of light scattering from rod preparations with highly organized structure have been carried out, including further experiments with magnetically oriented rods (47, 48) as well as experiments with isolated retinas (49, 50). In those preparations that most nearly retain their natural structure (despite having been permeabilized), the measured off-axis scattering, $\Delta I/I$, exhibits a simple positive-going form; when ATP is present in addition to GTP, the signal recovers to baseline and can be elicited repeatedly, indicating that the cascade can recover completely (reviewed in 51; see 50). Pepperberg et al. (49) called this simplified, positive-going signal the amplified transient or "AT signal," and it has generally been assumed to have the same molecular origin as the release signal described by Vuong et al. (46). Other investigators using the AT signal have also concluded that $\nu_G \approx 1000 \text{ s}^{-1} \text{ per R}^*$ (51).

Space limitations do not permit a thorough discussion of the substantial literature on the AT signal. However, a number of observations suggest that the AT signal might originate in an interaction between $G\alpha_t$ and PDE. First, to our knowledge, the AT signal has only been recorded in preparations in which both G_t and PDE were present. Second, using a preparation of magnetically oriented rod outer segments, Kamps et al. (52) tested the hypothesis that the presence of PDE is required for the signal, by preactivating the PDE with protamine, prior to delivery of the light flash. The AT signal was not observed in protamine-treated rods, although a normal dissociation signal was observed. Third, Heck & Hofmann (53), investigating rod outer segment membranes stripped of peripheral proteins and reconstituted with purified G_t and PDE, found (a) that an AT-like signal was only seen when native PDE was present in the sample prior to flash activation and (b) that preactivation of the PDE with $G\alpha_t$ -GTP γ S removed this signal stoichiometrically, although a normal dissociation signal was still observed (indicating the competence of the G_t that was not preactivated).

The apparent major conflict between the value of ν_G estimated from the AT signal ($\sim 1000 \text{ s}^{-1} G\alpha_t \text{ s}^{-1} \text{ per R}^*$) and the estimates obtained from the dissociation signal and the GTP γ S binding assay (220 and $150 \text{ s}^{-1} G\alpha_t \text{ s}^{-1} \text{ per R}^*$, respectively) can be resolved if the AT signal actually represents a binding interaction between G_t and PDE. Because the amount of PDE in rod outer segments is about an order of magnitude smaller than the total amount of G_t (54, 55), the binding interaction of these two proteins will saturate when $\sim 1/10$ of the G_t pool is activated. Thus, if we assume that the AT signal and the axial release signal from magnetically oriented rods are essentially the same, and originate from a $G\alpha_t$ -PDE interaction, then the estimate of ν_G derived from these experiments should be scaled by $\sim 1/10$ relative to the estimates derived with the assumption that the maximum signal represents total activation of G_t . This rescaling yields $\nu_G \approx 100 \text{ s}^{-1} \text{ per R}^*$ for the AT signal and release signals, bringing the estimates from the three types of experiments into reasonable agreement. In summary, we think that the most plausible reconciliation of the estimates of ν_G obtained from the AT/release signal with the estimates from other methods is that the AT/release signal represents the activation of only that

fraction of the G_i pool that has interacted with PDE. At a mechanistic level, we think that the rapid (though transient) increase in apparent mass at the membrane might result from the binding of $G\alpha_t$ to the PDE.

THE STOICHIOMETRY BETWEEN ACTIVATED TRANSDUCIN AND PHOSPHODIESTERASE SUBUNITS

Another issue impacting the molecular nature of signal amplification in the phototransduction cascade is the stoichiometry of activation of PDE by $G\alpha_t$ under in vivo conditions. Two factors should be considered in this regard: the fraction of the total PDE activity evoked by the binding of a single $G\alpha_t$ -GTP molecule, and a proportionality factor arising from the finite time required for a newly produced $G\alpha_t$ -GTP to find, bind to, and activate a PDE.

Given that the PDE consists of two nearly identical functional units, each containing one catalytic α or β subunit and one γ subunit (serving both as the inhibitor of nonactivated PDE and the binding site for transducin), a starting hypothesis would be that each PDE unit is activated independently by a $G\alpha_t$ (cf. 56). However, a variety of other hypotheses have been proposed, ranging from the idea that one $G\alpha_t$ activates both catalytic subunits (57) to the idea that the binding of transducin to the first PDE γ results in only 5% of maximal PDE activity (58).

Determination of the coupling ratio from $G\alpha_t$ to PDE, with assays that measure $G\alpha_t$ production and PDE activity, requires a precise determination of the total PDE amount in the reaction mixture. The most convenient preparation for estimating the PDE content of outer segment membranes is frog rods, because frog PDE has two exchangeable, high affinity noncatalytic bindings sites for cGMP (59, 60). Thus, the total amount of the PDE holoenzyme in a frog rod outer segment preparation can be determined as half the maximal radiolabeled cGMP bound to the membrane fraction. Using this approach to quantify PDE, Leskov et al. (32) found that during the initial phase of activation (up to about one third of the total PDE activity), the ratio of PDE catalytic subunits activated per $G_i\alpha$ was unity. Thus, there is neither gain nor loss of signal amplification in the coupling between $G\alpha_t$ and PDE.

Another finding (61) indicates that in frog rod outer segments $G\alpha_t$ interacts preferentially with the γ subunit associated with one of the PDE catalytic units. Although this would suggest that, under well-stirred conditions, PDE activity would only be driven beyond 50% maximal by a large excess of $G\alpha_t$, it is important to note that the situation in the rod is far from well stirred and that $G\alpha_t$ is produced at high concentration at the location of a single R^* . Thus, even though only 100 or so molecules of $G\alpha_t$ may be produced by the time-to-peak of the single-photon response (100–200 ms in a mammalian rod), they will have been at such a locally high concentration that they may well have bound doubly to molecules of PDE in the vicinity of the isomerization.

The delay in interaction between a newly formed $G\alpha_t$ and a PDE can be quantified in terms of the proportion of those $G\alpha_t$ s that have been activated and have

bound to PDE at any given time relative to the total $G\alpha_s$ s that have been activated. This proportion has been expressed as the coupling efficiency c_{GE} in Equation 2, and factors affecting its magnitude have been analyzed by Lamb & Pugh (28) and Lamb (62). Theoretical analysis indicates that this proportionality factor should be quite close to unity, for the measured protein densities and diffusion coefficients, in combination with the measured rate ν_G of transducin activation.

THE KINETIC PARAMETERS OF TRANSDUCIN-ACTIVATED PHOSPHODIESTERASE

Phosphodiesterase is a Nearly Perfect Effector Enzyme

From the measured values of the amplification constant and the rate of transducin activation in amphibian rods, it is possible to use Equations 2 and 3 to obtain a lower limit for the catalytic efficacy of PDE. In Equation 2 we can substitute the values discussed previously, $A \approx 0.1\text{--}0.2 \text{ s}^{-2}$ and $\nu_E (= \nu_G c_{GE}) \approx 120 \text{ s}^{-1}$, together with the accepted cooperativity of channel activation of $n_{cG} \approx 2\text{--}3$, to obtain a value of $\beta_{\text{sub}} \approx 4 \times 10^{-4} \text{ s}^{-1}$. This parameter, β_{sub} , represents the rate constant at which cGMP is hydrolyzed in the intact outer segment by a single activated hydrolytic subunit of PDE and is related to the underlying physical parameters according to Equation 3. Substituting into Equation 3, with a cytoplasmic volume of $V_{\text{cyto}} \approx 1 \text{ pl}$ for a frog rod, we obtain a lower bound for the catalytic efficacy of the PDE of $k_{\text{sub}}/K_m \geq 2 \times 10^8 \text{ M s}^{-1}$. For this “ \geq ” relation, the equality applies in the case that there is no buffering of cGMP in the outer segment ($BP_{cG} = 1$), whereas the required value of k_{sub}/K_m must be even higher than this if buffering of cGMP does occur. This value places PDE among the handful of most efficient enzymes known, for which k_{cat}/K_m exceeds $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (63), and qualifies PDE as a nearly perfect effector in fulfilling its function of maximally amplifying the signal during the photoreceptor response to light.

Turnover Rate and Michaelis Constant of Phosphodiesterase

It has been established for many years that the turnover rate of the fully activated PDE** holomer (k_{cat}) exceeds 4000 s^{-1} (3), corresponding to a subunit turnover number (for PDE*) of $k_{\text{sub}} \geq 2000 \text{ s}^{-1}$. On the other hand, the estimated value for the enzyme’s Michaelis constant, K_m , has recently been substantially revised. Over the years, the estimates of K_m reported by different investigators for light-activated PDE have varied by almost two orders of magnitude, with many values well into the millimolar range and none below $80 \mu\text{M}$ (Table V in 14). In 1994 Dumke et al. (55) proposed that this wide variation might result from preparations exhibiting widely different degrees of preservation of the original disc-stacking structure, in conjunction with the phenomenon of cGMP “diffusion with hydrolysis.” The idea was that when the stacking of discs resembled that in intact outer segments, and at the same time a great deal of PDE activity was stimulated, then substantial

gradients of cGMP concentration would arise along the interdisc diffusional paths. As a result, activated PDE molecules located near the center of the disc stacks would be exposed to much lower concentrations of cGMP than the level set in the bulk solution by the experimenter, and therefore the K_m would be greatly overestimated. Consistent with this analysis, it was found that the K_m measured in suspensions of large rod outer segment fragments was about six times higher than the value observed with severely disrupted membrane fragments. Dumke et al. proposed that the lowest observed value, $K_m \approx 100 \mu\text{M}$, measured with the most severely disrupted preparations represented the true Michaelis constant of the transducin-activated enzyme.

Subsequently it was discovered that even this value represented a substantial over-estimate (32), because diffusion with hydrolysis was found to occur even in the most severely disrupted preparations of frog rod outer segment membranes. To reduce the effects of this phenomenon, Leskov et al. (32) chose to activate only a small proportion of the PDE, which they accomplished by activating only small amounts of $G\alpha_t$ by using very low concentrations of $\text{GTP}\gamma\text{S}$. At very low levels of PDE activation, engaging only 1–2% of the total PDE, the measured K_m stabilized at $10 \mu\text{M}$ cGMP, which they concluded to be the true K_m of transducin-activated PDE. Importantly, the measurements of the PDE activation rate of $\sim 120\text{--}150 \text{ s}^{-1}$ obtained in the same study indicate that only 1–2% of the total PDE is activated within an individual interdiscal space during a single-photon response. This means that during the single-photon response the PDE will indeed operate at its true K_m of $\sim 10 \mu\text{M}$.

This result reconciled the estimates of the K_m for PDE activated by trypsin proteolysis of the inhibitory γ subunits that is also accompanied by the solubilization of activated PDE from the surface of rod disc membranes to solution (see Table V in 14; see 64 for a more recent update). The previous discrepancy resulted not from any fundamental difference in catalytic properties of PDE when activated by different means, but from what might be described as a geometrical factor in the membrane stack that led to an artificially high value of apparent K_m .

Reconciliation of Biochemical and Electrophysiological Measurements

With the results and insights described above, it is possible to present a unified set of parameters that accounts both for the biochemical and the electrophysiological measurements in the literature. Thus, if we take $k_{\text{sub}} = 2200 \text{ s}^{-1}$ and $K_m = 10 \mu\text{M}$ for PDE, and a cytoplasmic volume of $V_{\text{cyto}} = 0.85 \text{ pl}$ (based on frog or toad rod outer segments dimensions of $6 \times 60 \mu\text{m}$ and the cytoplasm occupying 50% of the envelope volume), and if we assume that $BP_{\text{cG}} = 1$ (implying the absence of cGMP buffering), substitution in Equation 3 yields $\beta_{\text{sub}} = 4.3 \times 10^{-4} \text{ s}^{-1}$. Then, with the mean value of ν_E obtained in biochemical experiments of $120 \text{ E}^* \text{ s}^{-1} \text{ per R}^*$ and assuming the most conservative value of the channel Hill coefficient, $n_{\text{cG}} = 2$, the overall amplification constant is predicted by Equation 1 to be $A = 0.10 \text{ s}^{-2}$.

Furthermore, substitution of the best numbers obtained in the biochemical assays (32) or the even higher value of $\nu_G \approx 220 \text{ s}^{-1}$ derived above from analysis of the light-scattering signals (33), together with a channel cooperativity of $n_{cG} = 3$, yields values for A that exceed $A = 0.3 \text{ s}^{-2}$ and therefore allows for the possibility that $BP_{cG} > 1$ (cf. 60).

POSSIBLE CONTRIBUTIONS OF THE G PROTEIN CASCADE TO ADAPTATIONAL CHANGES

The Amplification of Transduction is Unaltered in the Short Term by Exposure to Backgrounds of Moderate Intensity

It has long been known that the sensitivity of the visual system decreases in the presence of steady background illumination, a phenomenon known as light adaptation. In the rod and cone photoreceptors light adaptation is characterized both by desensitization, measured as a reduction in the peak of the incremental response to a dim flash, and by acceleration of the response, manifested by a shortening of the time-to-peak and a more rapid final recovery to the baseline level (e.g., 65).

Although it has been clearly established that photoreceptor light adaptation is mediated to a substantial degree by a light-induced reduction in calcium concentration (66–68), it is important to emphasize that the reduction in sensitivity is not actually caused by the lowered calcium concentration (69, 70). Indeed, the reduced calcium concentration does just the opposite: It rescues the photoreceptor from the massive reduction in sensitivity that would otherwise occur. Such saturation would inevitably accompany the complete closure of channels that would be induced by even quite dim backgrounds, were it not for the occurrence of adaptational changes. The lowered calcium concentration prevents saturation and thereby raises the sensitivity from the very low level that occurs when calcium concentration is “clamped.”

As described above, the amplification of transduction can be quantified by measuring the early parabolic rise of the response to a dim flash. Three studies on isolated amphibian rods have reported that during background illumination the amplification constant A is unaltered (70–72), though two other studies have reported considerable reduction (73, 74). The most extensive of these studies (70) found that the reactions that mediate recovery cause deviation of the response from its initial trajectory at much earlier times than previously thought (less than 100 ms for moderate backgrounds), making it essential to restrict analysis to the earliest region of the rising phase. Analysis of the fractional response at these very early times showed the initial rise to be invariant with adaptational state, for backgrounds suppressing up to 75% of the circulating current and applied for periods of several minutes; thus, there was no evidence of any reduction in the amplification constant. Accordingly, the simplest interpretation is that neither the rate of G protein activation, nor any of the other factors that combine to form the

amplification constant (Equations 2, 3), are altered during short-term adaptation at moderate intensities.

However, there are no grounds for extending this conclusion beyond the adaptational conditions tested. In particular, it seems possible that exposure to saturating intensities, or to extended durations of illumination (as in the diurnal cycle), might elicit changes in amplification.

Long-Term Changes in Amplification Might be Mediated by Changes in Protein Concentration

Consideration of Equations 2 and 3 indicates that changes in a multitude of different parameters could in principle modulate the gain of phototransduction. One class of possibility that we now consider is whether the rate ν_G of G protein activation might be modulated by alteration of the level of G_t in the disc membranes, either by changes in bulk concentration or by regulation of the competence of the G_t (e.g., by phosducin binding).

An initial question is whether alterations in the concentration of G_t would be expected to alter the rate ν_G of G_t activation or whether transduction operates under conditions in which this rate is saturated. Three lines of evidence have recently emerged in support of the first of these possibilities, that ν_G does depend on the level of G_t .

First, Heck & Hofmann (33) systematically varied the concentration of G_t in their preparation and observed a strong dependence of the rate of activation upon the level of G_t over the physiological range, with a K_m of 3100–3800 molecules of the membrane-bound G_t per μm^2 of membrane. Because the actual level of G_t in amphibian and rodent rods is considerably less than this [~ 2000 – $2500 \mu\text{m}^{-2}$, calculated from a G_t :rhodopsin ratio of 1/12 (44, 45)], the rate ν_G would be expected to depend strongly on the actual amount of G_t present. Second, it has been found that elevation of the level of G_t in suspension of frog rod outer segments above its normal content, elicited by the addition of purified frog transducin, leads to an increase in the activation rate ν_G assayed by GTP γ S binding (V. A. Klenchin & M. D. Bownds, unpublished data). Third, it has recently been found in mouse rods that a genetic manipulation that is presumed to increase the frequency of interaction between R^* and G_t leads to an increase in amplification constant, A (75). In these rods, which were hemizygous for rhodopsin, the quantity of rhodopsin in the membrane was approximately halved from normal, whereas the level of transducin was unaltered. The observed doubling of A was interpreted to be caused by an increased rate of lateral diffusion of proteins at the disc membrane surface. This result suggests that the rate ν_G in vivo is dependent on the rate at which R^* contacts molecules of G_t , and it is expected that this rate will depend on the concentration of G_t in the membrane.

Given that ν_G is affected by the concentration of G_t , two scenarios need to be investigated: whether the effective concentration of G_t is modulated by the binding of other proteins and whether changes in bulk concentration of G_t in the outer segment occur.

PHOSDUCIN It has been suggested that the effective concentration of G_t might be modulated by interaction with phosducin—a soluble phosphoprotein that complexes tightly with $G_t\beta\gamma$ (76–79). The presumption is that in this bound state G_t would not be competent to be activated by R^* . It has been shown that the unphosphorylated form of phosducin binds to $G_t\beta\gamma$ with much greater affinity than does the phosphorylated form (80–81), and it has therefore been hypothesized that light-induced dephosphorylation of phosducin could mediate a reduction in the gain of transduction, by reducing the concentration of competent G_t . However, a result obtained in three recent studies argues against this possibility. Measurements of the distribution of phosducin throughout the subcellular compartments of rod photoreceptors indicate that the great majority of phosducin is present in the inner segment and that the amount of phosducin in the rod outer segments is less than 10% of the total amount of transducin (82–84). On this basis it would seem that the amount of phosducin in the outer segment would be insufficient to influence the effective level of G_t .

REDISTRIBUTION OF TRANSDUCIN Another possible mechanism for gain modulation would be a redistribution of transducin between the inner and outer segments. Although such a redistribution of G_t was originally reported in the late 1980s, the method was questioned at the time, but new results confirm the original findings. A redistribution of G_t was reported by four groups (85–87a) when immunohistochemical techniques were applied to rodent retinas prepared under different adaptational conditions, though Roof & Heth (88) argued that these results might have been compromised by an artifact: light-dependent masking of the antibody recognition epitopes. However, a novel technique combining tangential microdissection of the flat-mounted retina with Western blot analysis of protein in the sections has recently confirmed that major movements of transducin do indeed occur in rat rods (89), with a large proportion of G_t translocated into the inner segment during extended light exposure and translocated back to the outer segment during dark adaptation. In those experiments the intensity of light exposure that was used caused closure of all the cGMP-gated channels (i.e., response saturation), so it could not be determined whether the amplification constant had changed during the light exposure. However, experiments in progress (M. Sokolov, A. L. Lyubarsky, K. J. Strissel, A. Savchenko, V. I. Govardovskii, E. N. Pugh, Jr. and V. Y. Arshavsky, submitted) have shown that during dark adaptation there is a window of time during which the rods have recovered sufficiently from saturation for meaningful electrophysiological measurements to be made. The reduction in the rod outer segment levels of G_t observed in those experiments was accompanied by a reduction in the amplification constant measured in the same animals.

GARP Yet another possible mechanism for regulation of the gain of transduction involves proteins called GARPs (glutamic acid-rich proteins), discovered by Sugimoto et al. (90). At least one of these proteins, GARP2, binds to PDE, thereby preventing its activation, under strongly light-adapted conditions (91). However,

electrophysiological measurements to test for alteration of gain have not been conducted yet, so this role of GARP2 is entirely hypothetical.

In closing this section, we draw attention to two points. The first is that the hypothesized mechanisms for the modulation of gain in the cascade may conceivably serve roles not in light adaptation per se, but in protection of the cell's metabolism. In the continued presence of illumination that leads to closure of all the cGMP-activated channels and hence the continued absence of signaling, the rod has little to gain (and perhaps much to lose) by maintaining elevated turnover of substrate in each of the contributing steps. The second point is that the topic of light adaptation is in fact a very complex one, and here we have examined only those phenomena relating to activation of the light response. Most of the mechanisms thought to influence light adaptation under physiological conditions (i.e., when some cGMP channels remain open) instead contribute to the shut-off (or recovery) of the response and are not addressed here. For recent reviews of light adaptation, see (25, 26, 69).

TRANSDUCIN INACTIVATION IN PHOTORECEPTORS IS TIGHTLY CONTROLLED BY SEVERAL REGULATORY PROTEINS

The shape of the rod's response to a single photon embodies an evolutionary compromise between the mutually conflicting factors of speed and sensitivity. Assuming the amplifying mechanisms underlying the activation phase of the response are optimized, the sensitivity, measured by the peak amplitude of the single-photon response, is determined primarily by the lifetimes of the three primary intermediates of the cascade: the lifetime of R^* , the lifetime of E^* ($= G\alpha_t$ -PDE), and the lifetime of cGMP. (The lifetime of cGMP is the reciprocal of the rate constant of steady cGMP hydrolysis.) Because the inactivation of E^* occurs when the terminal phosphate of $G\alpha_t$ -GTP is hydrolyzed, the GTPase rate of transducin is a determinant of both the time course and the sensitivity of the light response. The lower the GTPase rate, the longer the duration of PDE activity, the larger the response amplitude, and the greater the sensitivity. But such sensitivity comes at the price of a slower response and poorer time resolution.

Rods and cones, the two classes of photoreceptor in the vertebrate retina, represent different compromises between speed and sensitivity: In a given species the rods are more sensitive than the cones, in part because they inactivate more slowly. For example, in an amphibian retina the activation stages of transduction appear to have comparable amplification (as defined by Equations 1-3), and the 10- to 20-fold differences in sensitivity arise in large part from the differences in time-to-peak of the response to a dim flash, which may be around 100 ms for a cone but around 1 s for a rod (at 22°C) (3). Differences in rod and cone response timing reflect differences in evolutionary pressures for speed vs. sensitivity under nighttime and daylight conditions.

Not surprisingly for its critical role in response timing, the rate of transducin GTPase in photoreceptors is tightly regulated by several phototransduction proteins. Working together, a complex of proteins optimize the lifetime of activated transducin to fit the needs of photoreceptors from different species and individual rod and cone photoreceptors of the same retina. In this section we first introduce the proteins known to be involved in regulating the GTPase activity of transducin. We then discuss the roles of these proteins and the contributions of their major structural domains.

Discovery of Transducin GTPase–Regulating Proteins

The early studies of transducin GTPase reaction revealed a paradox: The rate of GTP hydrolysis measured with purified transducin was ~ 100 times slower than the apparent time constant with which rods recover from flashes (compare, e.g., 92 and 93 with 4 and 5). The resolution of this paradox began when experimental measurements of transducin's GTPase activity were made under the more physiological conditions of either concentrated suspensions of rod outer segments (94, 95) or of rod outer segments with highly preserved disc stack structure (96). These studies found a GTPase rate much higher than had been observed in cell-free systems reconstituted with purified proteins. Subsequent studies revealed that photoreceptors contain a molecular mechanism capable of activating the relatively slow intrinsic GTPase activity of transducin by at least two orders of magnitude. Because many aspects of the regulation of transducin's GTPase activity have been described in detail in two excellent recent reviews (97, 98), we provide only a brief historical outlook and then consider a few of the most recent developments in the field.

The first photoreceptor protein shown to act as a GTPase activating protein (GAP) for transducin was the immediate target of $G\alpha_t$ -GTP, the inhibitory γ subunit of PDE (PDE γ). Arshavsky & Bownds (99) found that addition of either purified PDE or recombinant PDE γ to photoreceptor membranes (containing G_t but lacking most other soluble and peripherally bound membrane proteins, including endogenous PDE) caused a several-fold stimulation of transducin's GTPase activity. In the same year Berstein and colleagues (100) showed another G protein effector, the $\beta 1$ isoform of phospholipase C (PLC $\beta 1$), to be capable of stimulating the GTPase activity of the corresponding G proteins, $G_{q/11}$. Together, these observations raised the possibility that regulation of the lifetime of activated G proteins by their effectors might represent a general principle of operation (101). However, PDE γ is now known not to be the primary GAP for transducin, and the only two conventional effectors known to confer GAP activity on their G proteins are PLC $\beta 1$ and type 5 adenylate cyclase (102).

A much more ubiquitous mechanism of regulating the GTPase activity of G proteins became evident with the discovery of a family of GAPs called regulators of G protein signaling, or RGS proteins (see 97, 103–105 for recent reviews of RGS protein classification and properties). All members of the RGS family examined so far have been shown to serve as GAPs for a broad range of G protein α subunits,

acting allosterically by stabilizing the transition conformation of the $G\alpha$ subunit that is most favorable for hydrolysis of GTP.

In the field of phototransduction, evidence that photoreceptors contain a GAP distinct from PDE γ came from experiments in which PDE γ failed to activate transducin's GTPase activity in the absence of photoreceptor membranes (106), experiments in which photoreceptor membranes with PDE removed retained their ability to activate GTPase (107) and experiments showing the GAP effect of PDE γ to be higher with larger concentrations of photoreceptor membranes in the reaction mixture (108–110). Taken together, these results indicated the role of PDE γ in this regard to be one of potentiating the GAP activity of another membrane-associated protein, rather than being the sole GTPase regulator itself.

Wensel and colleagues made a major breakthrough in identifying this membrane-associated GAP, with their discovery that the short splice variant of the ninth member of the RGS family (RGS9) is the photoreceptor-specific GAP responsible for stimulating transducin's GTPase activity (111, 112). Shortly afterwards it was shown that RGS9 exists in photoreceptors as a constitutive complex with the long splice variant type 5 G protein β subunit ($G\beta 5L$) (113). The expression of RGS9 and $G\beta 5L$ in photoreceptors occurs under strict reciprocal control. Mice lacking the RGS9 gene do not possess functional $G\beta 5L$ in their rods, in spite of the presence of the corresponding mRNA at normal level (114). Likewise, successful expression of RGS9 in both Sf9 cells and in vivo, in the rod and cone photoreceptors of transgenic *Xenopus laevis* tadpoles and in murine cones, requires coexpression of $G\beta 5$ (115, 116).

Recent studies indicate that RGS9- $G\beta 5L$ belongs to a subfamily of RGS proteins, including mammalian RGS6, RGS7 and RGS11, whose members share a common structural domain composition, are found predominantly in the nervous system, and appear to exist as complexes with $G\beta 5$ in vivo (117–121). RGS9 from photoreceptors is the only member found to complex with $G\beta 5L$; other members complex with a short splice variant of $G\beta 5$, $G\beta 5S$ (117–122). As illustrated in Figure 4, the binding of $G\beta 5$ occurs via a G protein γ subunit-like domain, or GGL, located next to the RGS homology domain in the sequence of all RGS9 subfamily members (123). For further information on the structural and functional aspects of this subfamily we refer the reader to two recent reviews (98, 122).

RGS9 is Necessary for Normal Inactivation of the Phototransduction Cascade in Rods and Cones

Experiments with mice that have the RGS9 gene knocked out have established that this protein is essential for normal inactivation of the cascade in vivo in both rods and cones (114, 116). In single mouse rods the dominant or rate-limiting time constant of recovery of the flash response increases from 0.2 s to about 9 s, a 45-fold increase in the absence of RGS9 (114). Cone-driven responses to strong flashes measured with electroretinographic methods show an ~60-fold increase in their half-time of recovery (116). Combined with histochemical results that show

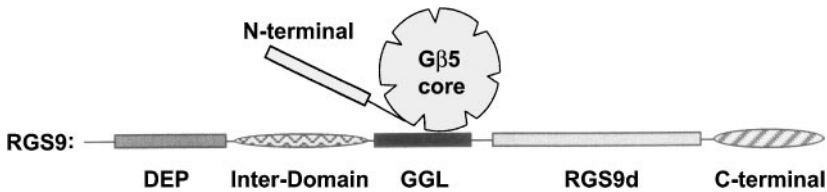


Figure 4 Domain composition of the RGS9-G β 5L complex. RGS9 contains five distinct structural regions: the \sim 110–amino acid N-terminal domain called DEP because it is also present in Disheveled, EGL-10 and Pleckstrin (136); the \sim 80–amino acid sequence (interdomain) with the lowest degree of homology among the members of RGS9 subfamily; the \sim 80–amino acid G protein γ subunit–like domain (GGL); the RGS homology domain (RGS9d) of \sim 120 residues; the C-terminal extension of \sim 55 amino acids, unique among other members of this subfamily. G β 5L is thought to have a core seven-bladed β -propeller structural domain characteristic of all G protein β subunits and mostly the α helical N-terminal region, including a photoreceptor-specific 42–amino acid extension that is absent in G β 5S and in other known G protein β subunits (137).

expression in both types of photoreceptors (112), these electrophysiological data unequivocally establish the necessity of RGS9 for normal cascade inactivation.

Cooperation Between RGS9-G β 5L and PDE γ is Required for Timely Transducin Inactivation In Vivo

The identification of three different proteins (RGS9, G β 5L, and PDE γ) involved in the regulation of transducin GTPase has raised the issue of whether these proteins perform their GAP function as a coordinated ensemble or simply provide redundancy in an important regulation. Consider first the mechanism of PDE γ action. In principle, there are two possibilities. First, PDE γ could directly contribute to GTP hydrolysis, for example, by further stabilizing the G α_t transition state beyond the action of the RGS domain. Second, PDE γ could act by increasing the affinity between activated G α_t and RGS9. Kinetic analysis of the \sim 15–30-fold PDE γ potentiation of the GAP activity of the native RGS9-G β 5L complex from bovine rod outer segment membranes has shown that this potentiation consists entirely of an equal magnitude increase in the affinity between RGS9-G β 5L and the G α_t -PDE γ complex as compared with activated G α_t alone (124). The same study revealed another remarkable feature of the native RGS9-G β 5L complex. At 22°C and saturating G α_t concentration, RGS9-G β 5L is able to stimulate transducin GTPase activity to a rate of \sim 100 turnovers per second, making RGS9-G β 5L the most efficient GAP known for a heterotrimeric G protein. This rate is sufficiently high to account for photoresponse turnoff in the most rapid photoreceptors, such as human cones, which are capable of resolving the oscillations of light flickering at frequencies of 60 Hz.

The observations that RGS9-G β 5L plays the central role in stimulating transducin GTPase activity and that PDE γ acts by increasing the affinity between G α_t and RGS9-G β 5L raise the hypothesis that PDE γ plays no essential role in regulating the rate of GTP hydrolysis in physiologically intact rods. Thus, in intact rods both G α_t and RGS9-G β 5L are present at concentrations higher than in most in vitro experiments, so that their affinity may be sufficient for timely transducin inactivation without any additional impact from PDE γ . A definitive rejection of this hypothesis was provided by the analysis of the responses of the rods of a transgenic mouse whose PDE γ was substituted with a mutant PDE γ containing a single amino acid substitution at position 70 (W70A) (45). The mutation of this residue had been previously shown to reduce the binding affinity between transducin PDE γ (125, 126) and to abolish the ability of PDE γ to activate transducin GTPase in the presence of the RGS9-containing photoreceptor membranes (126). The impaired G α_t -PDE interactions in the W70A mice resulted in a greatly lowered amplification of the rod photoresponses and slowed kinetics of the response recovery. The lowered amplification was expected from the greatly weakened binding affinity of G α_t for W70A PDE γ . The slowed recovery is particularly important for this discussion, because it establishes that the binding interaction between RGS9-G β 5L and wild type PDE γ is essential for a normal rate of inactivation of G α_t in vivo. The time course of the photoresponse recovery in W70A rods assessed from an exponential fitted to the final response decline ($\tau \approx 1$ s) was about sevenfold slower than that fitting the responses of rods of wild type mice ($\tau \approx 0.15$ s). The factor 7 provides a rough estimate of the degree to which PDE γ activates transducin GTPase in intact rods. Interestingly, rods from knockout mice completely lacking RGS9 recover from responses of similar amplitude only ~ 2.5 -fold slower ($\tau \approx 2.5$ s) than rods from W70A mice (114). Taken together, the data from these two studies argue that PDE γ is essential for the normal regulation of transducin GTPase in vivo and that the relative impact of PDE γ in activating GTP hydrolysis is on the same order of magnitude as the impact of RGS9.

Physiological Utility of the Dual Regulation of Transducin GTPase Activity by an RGS Protein and its Target Enzyme

The physiological utility of a dual regulation of transducin GTPase activity by an RGS protein and its target enzyme can be understood from the following consideration. When a molecule of transducin is activated by an R* it needs to accomplish two competing goals. First, it must transduce the signal from the R* to PDE with high efficiency, i.e., without losing any signal before PDE is activated. Second, it has to inactivate rapidly, within 200 ms in mammalian rods. If G α_t were to be inactivated by RGS9-G β 5L before it complexed with PDE, some G α_t molecules would never activate PDE, and signal amplification would accordingly be smaller. Thus, the dependence of GTPase activation on G α_t association with PDE γ ensures high efficiency of signal transmission between G α_t and PDE, because the hydrolysis of GTP does not occur without the binding of G α_t to PDE. At the same time,

the combination of the high affinity of RGS9-G β 5L for G α_t -PDE γ together with the high GTPase activity of the complex, leads to rapid termination of the signal. Achieving both efficient transmission and adequate time resolution is a general problem in all signal transduction pathways utilizing G protein α subunits as messenger molecules. In this sense, photoreceptors provide an instructive example of how this problem can be solved at the molecular level by a coordinated action of an RGS protein and a G protein effector.

Targeting Specificity of RGS9-G β 5L: An Interplay of Contributions from the Catalytic and Noncatalytic Domains of RGS9-G β 5L

Another way to view the preferential ability of RGS9-G β 5L to interact with the G α_t -PDE γ complex as compared with free activated G α_t is to consider it as an example of specificity in RGS protein action. The problem of RGS specificity has been among the most intensively studied topics in G protein signaling since RGS proteins have been discovered. It is now recognized that the catalytic domains of most RGS proteins accelerate GTPase activity of multiple G protein α subunits and usually show only limited selectivity toward individual G α subunits (reviewed in 97, 98). Consistent with these observations, the interacting surfaces between RGS and G proteins are among the most conserved regions present in proteins of both families (127, 128).

The promiscuity of the RGS catalytic domains suggests that the large degree of specificity in the interactions between RGS and G proteins is likely to be achieved through a variety of mechanisms including specific expression of individual RGS proteins in appropriate cell types, their precise targeting to specific subcellular compartments, posttranslational modifications, and the action of additional domains and subunits with which many RGS proteins are equipped (reviewed in 97, 98, 104, 105, 122). The ability of RGS9-G β 5L to distinguish between free activated G α_t , and G α_t bound to PDE γ is a particularly interesting example of RGS protein specificity because it cannot be achieved by simple compartmentalization, i.e., localization of a correct RGS-G protein pair in a particular cellular compartment. Free G α_t and G α_t -PDE γ must coexist during the light response, so RGS9-G β 5L needs to be able to discriminate the two species, interacting predominantly with the latter to ensure signal transmission efficiency.

RGS9-G β 5L is unique among all tested RGS proteins for its ability to cooperate with PDE γ upon stimulating transducin GTPase activity. The GAP activity of other RGS proteins and their recombinant catalytic domains is inhibited by PDE γ (115, 129–132). We now discuss how this property of RGS9-G β 5L results from contributions of both the catalytic domain of RGS9 (RGS9d) and other structures within the RGS9-G β 5L complex.

The fact that RGS9d itself is able to positively cooperate with PDE γ upon stimulating the GTPase activity of transducin was first reported by Wensel and colleagues (111) and confirmed later by other investigators (111, 133, 134). More

recently, Sowa et al. (132, 135) provided important structural insights to the effector regulation of RGS9d activity. They conducted site-specific mutagenesis of the RGS homology domain of RGS7, which is the closest relative of RGS9 but whose GAP activity toward transducin is inhibited by PDE γ , and found that as little as three amino acid residues, corresponding to L353, R360, and G367 of RGS9, determine whether PDE γ inhibits or activates the GAP activity of RGS7 (132). They further found that the substitution of the first two residues of RGS7 by the corresponding residues of RGS9 results in a complete reversal of the inhibitory effect of PDE γ into a stimulatory effect.

These results are consistent with an earlier report that the residues required for positive cooperation with PDE γ reside within the $\alpha 3$ – $\alpha 5$ region of RGS9d (133). They are also consistent with information obtained from the crystal structure of RGS9d in complex with $G\alpha_t \cdot \text{GDP} \cdot \text{AlF}_4^-$ and the PDE γ fragment responsible for regulating transducin GTPase activity (128). In this structure PDE γ and RGS9d are located very close to one another, make extensive interactions with the same region of transducin (switch II), and form one direct contact between V66 of PDE γ and W362 of RGS9. Yet neither one of the three RGS9 residues that determine the direction of the PDE γ effect interacts with PDE γ directly, indicating that the mechanism of their action must be allosteric (132) and remains to be elucidated.

Although RGS9d itself is able to positively cooperate with PDE γ upon stimulating transducin GTPase activity, the degree of this cooperation is small and has not been found to exceed threefold (111, 132–134). This suggests that most of the ~ 20 -fold stimulatory effect of PDE γ on the activity of native RGS9-G $\beta 5$ L (124) originates from the action of the structural domains within the RGS9-G $\beta 5$ L complex other than RGS9d (Figure 4). Indeed, He et al. (115) and Skiba et al. (139) reported that essentially all structures within RGS9-G $\beta 5$ L modulate its catalytic properties and/or enhance its cooperation with PDE γ . The latter study argues that the noncatalytic domains of RGS9-G $\beta 5$ L act by modulating its affinities for free $G\alpha_t$ and the $G\alpha_t$ -PDE γ complex. The structure, including the seven-bladed β -propeller core of G $\beta 5$ and the GGL domain of RGS9, reduces the affinity between RGS9 and $G\alpha_t$, whether $G\alpha_t$ is present in its free GTP-bound form or is complexed with PDE γ . Several other domains, including DEP and the C-terminus of RGS9 and the N-terminus of G $\beta 5$ L, increase the RGS9 affinity for $G\alpha_t$ -GTP-PDE γ but not for free $G\alpha_t$ -GTP, thus counteracting the affinity reduction (or inhibition) imposed by GGL-G $\beta 5$. The overall effect of these two affinity shifts of opposite direction is manifested in the physiologically large difference in the RGS9-G $\beta 5$ L affinities for free $G\alpha_t$ -GTP and $G\alpha_t$ -GTP-PDE γ . What remains to be determined is whether the noncatalytic domains act allosterically by modifying the binding properties of RGS9d or act directly by forming their own contacts with $G\alpha_t$ and/or PDE γ .

This mechanism by which the noncatalytic domains of RGS9-G $\beta 5$ L modulate the affinities of this complex for correct and incorrect targets suggests a general principle by which targeting specificity may be achieved by the members of the RGS9 subfamily. The G $\beta 5$ -GGL module may serve as an inhibitor of their GAP

activity by reducing the affinity of highly promiscuous RGS homology domains to G protein α subunits (cf. 118). Other domains, for instance DEP present in all RGS9 subfamily members, may restore the affinity specifically toward an appropriate target of each individual RGS protein.

CONCLUDING REMARKS

Phototransduction in vertebrate rods stands as a prototypical example of the operation of a G protein cascade of signal transduction. We understand the molecular steps contributing to activation in great detail and we can account quantitatively for the immense gain of the rising phase of the response to light. In addition, we now appreciate the molecular nature of many of the reactions involved in terminating the light response, though the quantitative details of the interactions are less clear than in the case of activation. Of particular importance to the photoreceptor in achieving both a high gain and a rapid recovery is transducin's ability to resist shut-off until after it has interacted with its effector enzyme, the PDE; central to this ability is the role played by RGS9-G β 5L. It appears likely that the subtleties of the molecular interactions contributing to the remarkable abilities of G proteins to produce both highly amplified and well-timed signals will be unraveled in the next few years.

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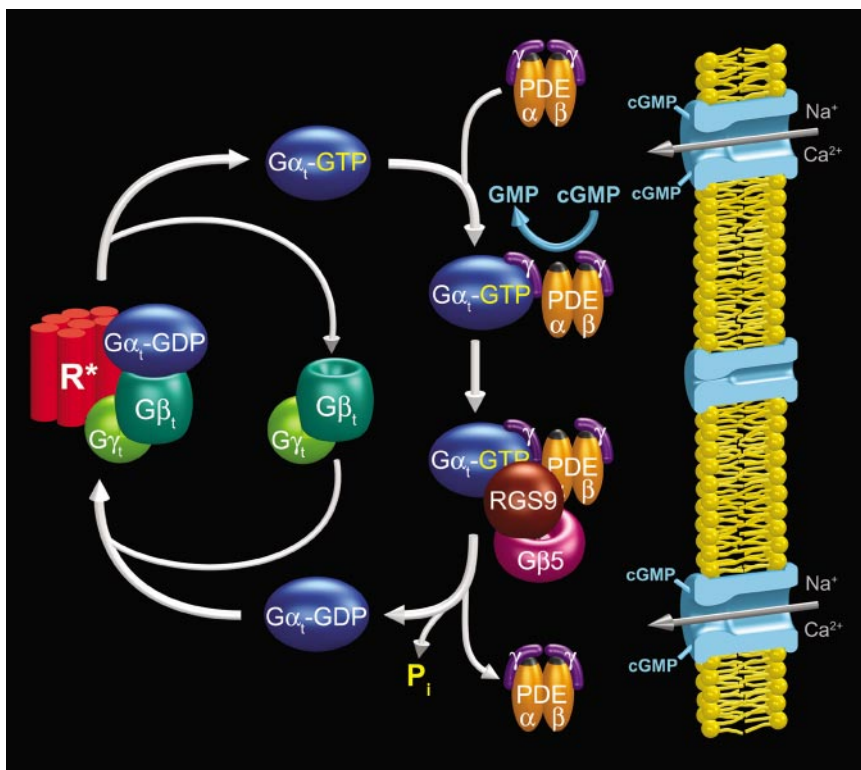


Figure 2 The cycle of G protein activation and inactivation in phototransduction. See text for details.



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