

Chapter 2

G Protein Deactivation Mechanisms in Vertebrate Phototransduction

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Abstract Heterotrimeric G proteins are widely used in nature to facilitate cellular responses to extracellular stimuli. In humans, these G proteins mediate vision and other senses, modulate neurotransmission, and are required for hormonal actions. The signaling system involves three groups of molecules, namely, the receptors with heptahelical transmembrane motifs, the trimeric G proteins themselves, and the effectors through which G proteins alter cellular homeostasis. Advances in genome sciences have revealed the full complement of this system in multiple species, and the current challenges are to elucidate which, when, where, and how each component is used. The field of phototransduction has historically provided unrivaled details in describing general principles of G-protein signaling. This chapter intends to cover the reactions that dominate the rate of phototransduction recovery in rod and cone photoreceptors.

Phototransduction Mechanisms

Vertebrate retinal photoreceptors are terminally differentiated cells with organized subcellular compartments tailored for distinct functions [5, 68]. Other than organelles common to eukaryotic cells, a unique compartment of photoreceptors that allows its identification is the outer segment. This structure contains tightly packed membrane discs enclosed in plasma membranes in rods and an invaginated, sometimes tapered, plasma membrane in cones. The outer segment links to the inner segment through a delicate connecting cilium, a microtubule-based structure broken off easily in solution by mechanical means. This allows the membrane-rich, and hence buoyant, outer segment to “float” during centrifugation for rapid isolation [75]. The

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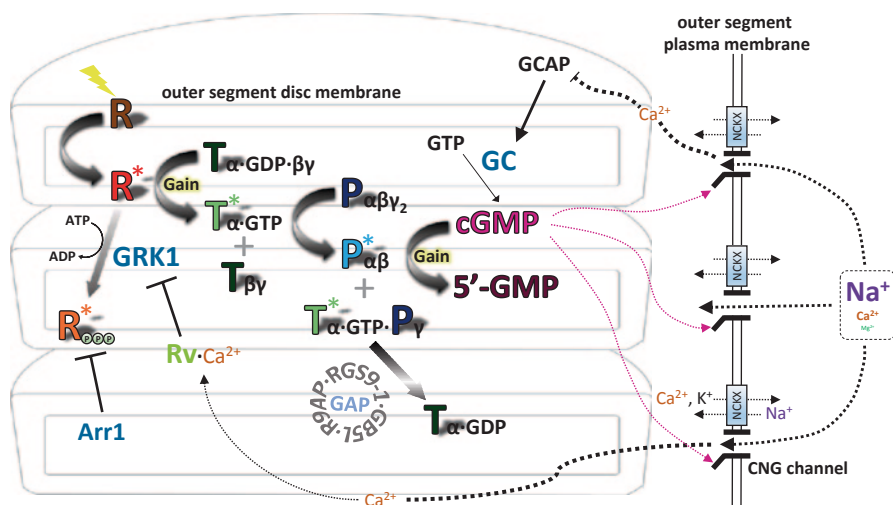


Fig. 2.1 Phototransduction activation and recovery in a vertebrate rod. Photon absorption by rhodopsin (R) leads to the formation of metarhodopsin II (R^*), which in turn catalyzes guanine nucleotide exchange on α subunit of the heterotrimeric transducin. Activated transducin dissociates into $T^*\alpha\text{-GTP}$ and $T\beta\gamma$ moieties, with the former ($T^*\alpha\text{-GTP}$) interacting with the inhibitory γ subunits of phosphodiesterase (PDE ; $P_{\alpha\beta\gamma 2}$) to disinhibit the catalytic subunits ($P^*_{\alpha\beta}$) and allow free cGMP to be rapidly hydrolyzed to 5'-GMP. The decline of free cGMP leads to the closure of cyclic-nucleotide-gated (CNG) channels and membrane hyperpolarization. While CNG channels are close and Ca^{2+} entry is blocked, the Na/Ca, K exchanger (NCKX) continues to extrude Ca^{2+} , leading to a decline in intracellular free Ca^{2+} concentration. This reduction in Ca^{2+} is sensed by two calcium-binding proteins, recoverin (Rv) and guanylate cyclase-activating proteins (GCAPs). Rv binds and inhibits G-protein-coupled receptor kinase 1 (GRK1). GCAPs activate de novo synthesis of cGMP by guanylate cyclases (GC). During the recovery phase, GRK1 phosphorylates R^* to enable its quenching by arrestin ($Arr1$). The intrinsic GTPase activity of transducin α subunit hydrolyzes GTP to GDP. The GTPase activity is accelerated by a GTPase activating protein (GAP) complex that consists of $RGS9-1$, $G\beta 5L$, and $RGS9$ anchoring protein ($R9AP$)

ease of obtaining sufficient outer segments enabled a detailed biochemical characterization of reactions involved in phototransduction activation and deactivation, with many enzymatic activities unambiguously assigned to homogeneously purified outer segment proteins [29, 31, 42, 43, 57, 103]. Back when recombinant DNA was at its fledgling stage, this strong foundation together with electrophysiological interrogations of the system jointly infused the G protein field with enviable real-time kinetic information that still remains unparalleled today [9]. By virtue of its simplicity and accessibility, rod phototransduction has been studied extensively and regarded as one of the most exquisite examples of G-protein signaling pathways. In the dark, a “dark current” circulates between the outer and inner segments of rods and sustains a depolarized membrane potential and continuous glutamate release at axonal terminals. As depicted in Fig. 2.1, illumination activates a G-protein-coupled visual pigment (rhodopsin) located on disc membranes when the attached chromophore 11-*cis*-retinal is photoisomerized into the all-*trans* configuration. The

protein then adopts a conformation capable of catalyzing the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α -subunit of the G protein heterotrimer named transducin. During its lifetime, a photoactivated rhodopsin molecule may activate ~ 20 transducin in a mouse rod [54]. The GTP-bound transducin α -subunit dissociates from the $\beta\gamma$ dimer to interact with the effector cGMP phosphodiesterase (PDE) by sequestering its inhibitory γ -subunit (PDE γ). The disinhibited PDE catalytic $\alpha\beta$ subunits are near perfect enzymes that hydrolyze cGMP at a rate near the diffusion limit [4, 59]. This leads to a hasty decline in free cGMP concentration and closure of cyclic-nucleotide-gated (CNG) channels, which otherwise allow extracellular Na^+ , Ca^{2+} , and Mg^{2+} [16, 109] to enter the outer segment to drive the dark current. The interruption of inward current causes membrane hyperpolarization and a reduction in tonic synaptic glutamate release. The activities of rhodopsin and PDE endow the pathway with the needed amplification and together with the remarkable thermal stability of rhodopsin in the dark [111], they account for the long-appreciated fact that a human rod can detect single photons [38]. In contrast to the light-induced decrease of Ca^{2+} influx through CNG channels, the light-insensitive and electrogenic $\text{Na}^+/\text{Ca}^{2+}$, K^+ exchanger (NCKX) located on the outer segment plasma membrane extrudes Ca^{2+} continuously during channel closure [110]. This leads to a drop in intracellular Ca^{2+} level, measured in both rods and cones of different species to change from 600 to 700 nM in the dark to < 50 nM in saturating light [33, 83, 84, 104]. The decline of cytoplasmic Ca^{2+} concentration regulates several reactions involved in phototransduction inactivation.

The overall duration of a rod photoresponse, and the temporal sensitivity of rod vision, is determined by how fast phototransduction is terminated. For deactivation to occur in a timely manner, all active intermediates need to be turned off and cGMP levels restored. At the receptor level, the activated rhodopsin is phosphorylated by rhodopsin kinases (aka G-protein-coupled receptor kinase 1 (GRK1)) and then bound by arrestin to cap its catalytic ability. Rhodopsin kinase activity is regulated by Ca^{2+} through its Ca^{2+} -dependent interaction with recoverin, the study of which exemplifies the power of parallel characterizations of phototransduction using biochemical and electrophysiological means. As a small photoreceptor-enriched calcium-binding protein, recoverin was initially thought to stimulate guanylate cyclase (GC) activity [23]; but see also [45] when intracellular calcium levels fall following light stimulation—this allows the rapid restoration of cGMP needed for response recovery [50]. However, when internally dialyzed into outer segments, exogenous recoverin did not alter recovery kinetics but instead prolonged the activation phase [26, 34], quite the opposite of what one might have expected for a protein involved in recovery. These results necessitated another search for new calcium-binding protein(s) for this particular activity, leading to the identification of guanylate cyclase-activating proteins (GCAPs) [24, 32]. The GCAP-activated and GC-dependent *de novo* cGMP synthesis replenishes free cGMP levels in the outer segment, facilitating the reopening of CNG channels and depolarization of rods. As for recoverin, it binds and inhibits rhodopsin kinase [10, 46, 49], thus participating in recovery in a counterintuitive way [19, 66]. Increasing recoverin levels in the outer segment prolongs phototransduction by holding back rhodopsin kinase to

prevent phosphorylation of activated rhodopsin, and hence delays its quenching mediated by subsequent arrestin binding. At the G protein level, the α -subunit itself has intrinsic GTPase activity, which can be accelerated by the GTPase-activating protein (GAP) complex comprising RGS9-1, G β 5-L, and R9AP. The GAP activity can be further enhanced by PDE γ , ensuring timely deactivation of transducin only after its interaction with PDE. Details regarding the discovery of the transducin GAP complex will be discussed in more detail below.

Akin to rods, cone phototransduction uses cGMP as the second messenger but signaling is carried out by a distinct set of proteins. For example, instead of rhodopsin, cones express visual pigments of distinctive spectral sensitivities, or opsins, to enable color vision. Furthermore, some proteins such as RGS9-1 and rhodopsin kinase are expressed by both rods and cones albeit to different levels, which may also contribute to the dissimilar response kinetics and light/dark adaptation properties between the two cell types.

The GAP for Transducin Is a Protein Complex

Another windfall from both biochemical and physiological characterizations of phototransduction is the insight that transducin required a GAP for its timely deactivation [2, 3]. Depending on species and recording conditions, a typical photoresponse of a vertebrate rod lasts for a few hundred milliseconds [9]. In order for response recovery to occur, all active G proteins must be inactivated, as must all other active intermediates generated during the phototransduction activation phase. In the case of transducin, the deactivation relies on its intrinsic GTPase activity [29, 40], which is assayable by measuring the release of radioactive γ -phosphate of bound GTP. The measured rate of γ -phosphate release from purified transducin was too slow in vitro, roughly once every 10–20 s [2, 3, 40] to commensurate with the short duration of recorded rod photoresponses. Therefore, factor(s) exists in the outer segment that can dramatically accelerate GTP hydrolysis by transducin.

Since the GAP activity is assayable, fractionating outer segment components seemed to be a straightforward way to identify it. However, the GAP activity was found to be tightly associated with membranes and labile in the presence of detergents. Direct purification of the GAP turned out to be a formidable task. However, these attempts were fruitful to establish the role of the inhibitory subunit of the cGMP-PDE [3, 96], which is not the GAP itself but it significantly augments the GAP activity [2, 14]. Around that time, a new group of proteins coined regulators of G-protein signaling (RGS) were discovered in the trimeric G protein field, first in yeast and worm then in mammals [25, 51]. These RGS proteins are quite different in sizes but all possess a highly conserved RGS domain of approximately 120 amino acids in length that is necessary and sufficient for their GAP activity toward G $_{i/o}$ proteins [101]. Several laboratories went on to test the possibility that the long sought-after transducin GAP might be an RGS protein. For several members that were tested, they indeed all helped transducin hydrolyzing bound GTP in vitro [12,

27, 37, 68, 69]. The investigation then turned to determine which RGS protein is the actual transducin GAP, or to accommodate a notion that multiple RGS proteins are redundantly present in the outer segment for this important task. To sort out these possibilities, additional criteria such as photoreceptor-specific expression and whether or not PDE γ could enhance their GAP activity were considered [37, 102]. RGS9-1 stood out as a promising candidate because it satisfied both of these requirements [37], while others did not [102]. The speculation was confirmed as recovery in mouse rod and cone was severely delayed when the RGS9 gene was knocked out [14, 62].

These loss-of-function approaches in genetically malleable species, such as mouse and zebra fish, have proved quite useful to establish the genes critical for phototransduction (for examples, see [13, 58, 63, 71, 80, 106]). In the case of identifying the transducin GAP and in addition to RGS9-1, this approach verified the presence of two more components, namely G β 5-L [98] and R9AP (RGS9 anchoring protein, [41]), in the ternary GAP complex in photoreceptors that helps transducin hydrolyze GTP. The delays in phototransduction recovery seen in RGS9-knockout rods are also found in rods lacking G β 5 or R9AP [48]. Furthermore, these studies also revealed a unique obligate partnership between RGS9-1 and G β 5-L [14, 17, 48], suggested during biochemical characterization of then elusive transducin GAP [65], wherein the loss of one leads to the instability of the other despite the presence of messenger RNA. Equally if not more interesting is that the expression level of this GAP complex is higher in cones than in rods [21]. Depending on the species, this difference can be as high as tenfold [113, 114] and hence fuels the speculation that the GAP level might be an important factor governing kinetic differences between rod and cone photoresponses.

Regulation of Rhodopsin Lifetime

The decay of purified activated rhodopsin *in vitro* is also too slow to account for the short duration of rod photoresponses. To quickly shut down activated rhodopsin, the C-terminal tail of rhodopsin which contains multiple serine and threonine sites is indispensable [11]. In fact, one of the early biochemical reactions readily characterized in purified outer segment preparations was light-dependent rhodopsin phosphorylation [60, 89]. The kinase responsible for this activity is rhodopsin kinase [44, 61], which belongs to the G protein receptor kinase (GRK) family and is expressed in both rods and cones in humans [15, 55, 115]. Phosphorylation of rhodopsin triggers the binding of arrestin [55], which quenches rhodopsin's catalytic activity and prevents it from activating additional transducin molecules. Human Oguchi disease patients with loss-of-function mutations in either arrestin [28] or rhodopsin kinase gene [108] are night blind because their rods remain saturated due to the prolonged catalytic activity of photoactivated rhodopsin. However, their daytime vision is minimally affected [20, 28, 108] because additional cone arrestin [22] and cone opsin kinase (aka GRK7) [15, 99, 100] are present to prevent excessive signaling from

cone visual pigments. When assayed in vitro on rhodopsin, recombinant GRK7 is a superior enzyme to rhodopsin kinase [72, 95]. In carp retina where pure rod and cone populations can be isolated in sufficient quantity for side-by-side comparison, efficiency of light-dependent visual pigment phosphorylation was reported to be much higher in cones than in rods [95]. It is unclear whether the presence of both GRK7 and rhodopsin kinase in cones of certain species, such as humans, contributes to the sensitivity and kinetic differences between the two photoreceptor types. Unlike Oguchi disease patients, however, mice lacking rhodopsin kinase have much delayed cone photoresponse recovery [13, 63]. The lack of GRK7 expression in mouse cones may account for this discord between human and mouse [99]. It is noteworthy that there exist other kinases in photoreceptors that can phosphorylate activated visual pigments. One such kinase that has been extensively characterized is protein kinase C (PKC) [35]. However, in the context of phototransduction recovery, PKC apparently plays little role [13, 63]. Finally, the N-terminal portion of rhodopsin kinase is phosphorylated by protein kinase A (PKA) at serine 21 in dark-adapted retina, which leads to a reduction in catalytic activity toward activated rhodopsin [73]. It was speculated that this regulation is involved in photoreceptor light adaptation, an interesting idea that awaits future investigation.

Rate-Limiting Step of Phototransduction Recovery

The relatively short duration of rod's photoresponses intrigued many laboratories. The activation reactions, initiated by photoisomerization of rhodopsin's chromophore, proceed sequentially in the phototransduction cascade (Fig. 2.1). This activation phase can be robustly recorded from photoreceptors of many species and can be modeled by a delayed Gaussian function put forth by Lamb and Pugh [56]. The LP model can account for the diversity of response parameters due to species difference in outer segment dimensions and different recording temperatures. Unlike the activation phase, however, reactions involved in the recovery phase occur in parallel and all active intermediates produced during activation must be turned off, and cGMP must be replenished to reopen CNG channels to resume the dark current [9]. Conceivably, the slowest reaction(s) among the many that must occur determines the overall rate of recovery. Mechanistic investigations along this line thus aim to identify the so-called rate-limiting step of recovery. Many elegant studies were conducted [47, 54, 67, 79, 81, 112] but thus far the identities of this rate-limiting step appeared mixed, which may reflect species difference or an intrinsic difference between rods and cones within the same species [62, 67, 112]. All experiments, in vitro and in vivo, compared recovery time constants in manipulated versus control conditions, procedurally or reverse-genetically. If the speed of a reaction when accelerated increased the overall rate of recovery, the rate-limiting step was then said to be identified or otherwise excluded. Several seminal findings along this line of thought are worth mentioning. First, it was shown that preventing outer segment calcium changes in salamander rods has no effect on the rate of rod recovery,

indicating that the rate-limiting step therein is a calcium-independent process [62]. However, this finding appears not applicable to cones of the same species as calcium appears to alter the rate of cone recovery when the light-induced drop in outer segment calcium level was delayed [67, 112]. While it is unclear where calcium exerts this effect on cone recovery in salamander, existing data suggest that it is at the pigment level. The identity of the rate-limiting step is a bit clearer in mouse. A loss-of-function approach by inactivating GCAPs has ruled out the involvement of the calcium/cGMP negative-feedback loop [7]. Knocking out the calcium-binding protein recoverin hastens the recovery of rod phototransduction [66]. As mentioned earlier, recoverin binds rhodopsin kinase in a calcium-dependent manner [10, 49], so this deductively implies that rhodopsin deactivation might be the long thought-after rate-limiting step. However, in another gain-of-function experiment, overexpressing rhodopsin kinase by three to four folds either has no significant effect [54] or accelerates rod recovery to a moderate extent [82].

A surprise emerged when elevation of the level of transducin GAP complex by overexpressing its membrane anchor R9AP leads to a dramatic speeding up of rod recovery [54]. The degree of acceleration correlates well with moderate level of overexpression but approaches an asymptote when expression is pushed higher. This is because rod recovery becomes more limited by the next slowest reaction(s) than by transducin deactivation. Taken together, these data indicate pretty clearly that transducin deactivation is the rate-limiting step in normal mouse rod recovery. However, this is a conclusion that needs reconciliation with the observed moderate acceleration of recovery in recoverin-less rods [66], the robust binding and inhibition of rhodopsin kinase by recoverin [10, 49], and moderately accelerated rod recovery when rhodopsin kinase is overexpressed [19, 82]. Given the precedence that calcium appears to differentially regulate amphibian rod and cone phototransduction recovery and that cones express a higher level of transducin GAP than rods [21, 113, 114] in multiple species, it will also be important to see whether transducin deactivation similarly rate-limits mouse cone recovery. Along this line, it is noteworthy that overexpressing GCAP1 was found to speed up the recovery of cone-derived electroretinogram (ERG) responses. However, it remains to be determined whether such acceleration occurs directly at the level of cone phototransduction [76]. Finally, from an evolutionary point of view, it is worthwhile to revisit amphibian phototransduction to determine whether species differences (vs. mouse) account for the disparity in reactions that limit the rate of recovery. For instance, while salamander rod recovery is not influenced by holding outer segment calcium fixed, it is unclear whether G protein deactivation is the rate-limiting step. Similarly, while calcium is important for timely recovery of salamander cones, the identity of the rate-limiting step therein has not been shown by a gain-of-function approach under normal and not under calcium-clamped conditions. The salamander genome has been sequenced and annotated [91] and novel genome-editing tools have emerged [86] that may allow comparative genetic studies in this species with those from laboratory mice. These experiments are necessary to determine the importance of G proteins and their timely deactivation in the recovery of photoreceptor light responses among different species.

The Role of Recoverin in Phototransduction

The binding and inhibition of rhodopsin kinase by recoverin is robust and occurs at physiological calcium levels [8, 10, 49]. The notion that transducin deactivation rate-limits mouse rod recovery needs to be reconciled with the moderately accelerated recovery observed in recoverin-knockout rods [66]. By breeding the recoverin-knockout mice into a background where transducin GAP is highly overexpressed [19, 53], the moderately accelerated rod recovery can be further hastened. This indicates that the rate of recovery in recoverin-knockout rods is rate-limited by transducin deactivation. The moderate acceleration of recovery can also be recorded in mice with greater than tenfold rhodopsin kinase overexpression, which can similarly be speeded further by GAP overexpression [19]. Thus, it appears that in addition to regulating rhodopsin lifetime [18, 26, 34, 44], recoverin and rhodopsin kinase also play an unintuitive role in normal rod recovery by speeding up PDE shutoff, as previously reported [66].

It is unclear how recoverin and rhodopsin kinase regulate PDE deactivation. However, the presence of the GAP complex in mouse rods is required to see this moderate acceleration effect on recovery [19], indicating that transducin, GAP complex, and/or PDE itself may be the target and that the N-terminal RGS homology (RH) domain of rhodopsin kinase [88] cannot substitute as a surrogate transducin GAP. Along these lines, it has been shown that removing phosphorylation sites of the inhibitory γ subunit of PDE alters rod recovery kinetics [97]. Although a direct link between rhodopsin kinase and PDE γ phosphorylation has not been established, it is an interesting target for future investigation. Furthermore, recoverin is abundantly expressed in photoreceptors and the distribution therein is regulated by light [94]. The physiological significance of this light-dependent recoverin redistribution in photoreceptors remains unclear, nor is the mechanism. Despite a lack of mechanistic understanding, the physiological importance of recoverin-mediated acceleration of rod recovery in light adaptation is now appreciated. It is well established that background light accelerates rod recovery [104]. The acceleration is in large part mediated by recoverin's action on rhodopsin kinase because knocking out recoverin hampers it [19]. Thus, with respect to recoverin's role in phototransduction, it appears to modulate the lifetime of activated rhodopsin and to speed up recovery during light adaptation. Finally, one enigmatic function of recoverin remains unsolved as it appears to enhance signal transmission between rod and downstream neurons [85].

Future Directions

Understanding how generalizable the knowledge we gain from phototransduction applies to other trimeric G protein pathways throughout the body is a worthwhile endeavor. Even within the visual system, we still are putting together the tit bits of information available to construct G-protein signaling cascades in retinal depolar-

izing bipolar cells [52, 76, 87] and in intrinsically photosensitive retinal ganglion cells [107]. Future research efforts may benefit from a focused approach in the retina, which is an approachable part of the central nervous system with abundant anatomical detail to assist mechanistic inquisition. While mechanisms of rod phototransduction are well described, cone phototransduction remains incompletely understood. A simultaneous comparative examination of both rods and cones will provide valuable insights into how these mechanisms produce light responses with distinct characteristics. Finally, the field of G-protein signaling in the retina may exert further impact by advancing the biophysical characterizations [78] of important molecules such as visual pigments [74, 93], G proteins [92], G protein GAPs [90], arrestins [39], PDEs [4], rhodopsin kinase [87], GCAPs [1], and GCs, to name just a few, to understand how mutations therein affect complex formation and/or biochemical activity, and how functional perturbations may cause blinding diseases in humans.

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