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cGMP SIGNALING IN VERTEBRATE RETINAL PHOTORECEPTOR CELLS

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1. ABSTRACT

The visual transduction pathway in vertebrate photoreceptors transforms a light stimulus entering the photoreceptor outer segments into an electrical response at the synapses of rod and cone photoreceptor cells. This process is mediated by complex biochemical pathways that precisely regulate cGMP levels, thereby controlling the extent, duration, and adaptation of the photoreceptor to the light stimulus. This review first summarizes the major mechanisms of regulating cytoplasmic cGMP levels (synthesis, degradation, buffering, and efflux) as well as the primary targets of action of cGMP (cyclic nucleotide-gated ion channels, cGMP-dependent protein kinase, and cGMP-regulated phosphodiesterases). This information is applied to our current understanding of how these processes operate in the signal-transducing outer segment of rod and cone photoreceptors to carry out visual excitation, recovery, and adaptation in response to light stimulation.

2. INTRODUCTION

All eukaryotic cells employ cyclic nucleotides—specifically, guanosine 3',5'-cyclic monophosphate (cGMP) and adenosine 3',5'-cyclic monophosphate (cAMP)—to serve as internal messengers to transduce extracellular signals into an appropriate cellular response. Many cells have multiple routes of synthesis and degradation of cyclic nucleotides, as well as numerous targets of action which are regulated by dynamic changes in the intracellular concentration of cAMP and/or cGMP. The prior discovery of cAMP and elucidation of its role as a second messenger led many signal transduction researchers to initially assume that the closely related cGMP molecule would be regulated and function in a similar manner. The past twenty five years of cyclic nucleotide research have proven otherwise. Despite many similarities in the structure and function of the enzymes and receptors involved in cAMP and cGMP action, cAMP and cGMP levels are regulated differently and each cyclic nucleotide plays distinct roles as second messengers.

cGMP is involved in regulating numerous physiological processes, including vascular smooth muscle relaxation, natriuresis, platelet function, neutrophil adhesion, sperm motility, neuronal signaling, and sensory transduction (1). The metabolism of cGMP is controlled by the synthetic enzymes, guanylate cyclases, and hydrolytic enzymes, cyclic nucleotide phosphodiesterases. Cytoplasmic levels of cGMP may also be modulated non-

enzymatically by sequestration by cGMP binding proteins (2), and/or by transport mechanisms that cause cGMP efflux from the cell (3). Changes in cytoplasmic cGMP concentration affect cGMP signaling pathways by changing the extent of binding to specific cGMP binding proteins (receptors). Targets of cGMP action include cGMP-dependent protein kinases (PKG), cyclic nucleotide-gated (CNG) ion channels, and cGMP-binding phosphodiesterases, all of which are allosterically regulated by cGMP binding to non-catalytic regulatory sites on these proteins.

3. METABOLIC AND NON-METABOLIC MECHANISMS TO REGULATE THE cGMP CONCENTRATION IN CELLS

3.1. Guanylate cyclase (E.C. 4.6.1.2)

Guanylate cyclases (GC) synthesize cGMP from GTP using a catalytic mechanism analogous to that used by adenylate cyclases to synthesize cAMP. Indeed, all vertebrate adenylate and guanylate cyclases share a common structural motif that comprises the catalytic domain (Pfam accession number: PF00211) of this enzyme superfamily (4, 5, 6). Within the vertebrate GC family, there exist two distinct types of GC, soluble and membrane-associated (historically referred to as "particulate"). In humans, there are four genes (two alpha subunits and two beta subunits) that code for subunits of the heterodimeric soluble GCs (GC-1), while seven distinct isoforms exist for the single transmembrane domain-containing membrane-associated GCs (GC-2A through GC-2G).

The soluble GCs are found as an alpha-beta heterodimer in the cytoplasm, and their subunit structure consists of an N-terminal regulatory domain, a dimerization region, and the C-terminal catalytic domain. Soluble GC is subject to regulation by binding of nitric oxide (NO) to a heme prosthetic group located in the regulatory domain; other ligands (e.g., carbon monoxide, protoporphyrin IX) can also activate soluble GC, but much less effectively.

Membrane-associated GCs have a domain organization typical of a single transmembrane receptor protein (e.g., growth hormone receptors), and exist in a homodimeric state in the membrane. Each subunit of this class of GC contains an extracellular ligand binding domain at its N-terminus, a short transmembrane segment, a kinase homology domain, and, finally, the C-terminal catalytic domain. In the case of GC-2A, GC-2B, and GC-2C, enzyme activation results from binding a specific peptide ligand (e.g., atrial natriuretic peptide for GC-2A) to the extracellular domain. For the other GC-2 family members, it is unclear whether extracellular ligand binding has physiological relevance. Indeed, for those GC-2 family members whose primary tissue localization is in sensory cells, intracellular signaling pathways are likely to allosterically regulate catalytic activity. The kinase-like domain in GC-2 lacks kinase activity [except in the case of the photoreceptor enzyme (7)] but may serve to bind ATP and allosterically stimulate catalysis for all family members. The physiological significance of the kinase homology domain is unclear. For the photoreceptor enzymes (GC-2E and GC-2F), three distinct GC-activating proteins (GCAP1, GCAP2, and GCAP3) serve to regulate catalytic activity in a calcium-dependent manner (discussed later). Reversible phosphorylation of GC-2 represents another potential mechanism for feedback regulation of this family of enzymes (5, 6).

3.2. Cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17 and E.C. 3.1.4.35)

Phosphodiesterase (PDE) catalyzes the hydrolysis of the cyclic phosphate bond of cGMP and cAMP to form the corresponding 5'-nucleoside monophosphate. At least three classes of eukaryotic PDEs have been discovered, each with its own distinctive catalytic domain structure. Class I PDEs contain the characteristic PDEase I catalytic domain (Pfam accession number: PF00233) and represent the largest class of cyclic nucleotide phosphodiesterases, including all known vertebrate PDEs. Several Class II PDEs have been identified in slime molds, fungi, and bacteria, consisting of a distinct catalytic domain structure (Pfam: PF02112) that is selective for cAMP as a substrate. Class III PDEs exist only in prokaryotes, and have a completely different catalytic mechanism that resembles that of the purple acid phosphatases (8).

Within the Class I PDE superfamily, there exist 11 distinct gene families of PDEs: PDE1 through PDE11. These families are readily distinguished by comparison of their primary amino acid sequences, but can also be classified based on their regulatory mechanisms, substrate preference, pharmacological inhibitor specificity, and expression patterns (9, 10, 11). Three PDE families (PDE5, PDE6, and PDE9) strongly prefer cGMP as the substrate, three are cAMP-specific (PDE4, PDE7, PDE8), and the rest do not discriminate between the two substrates. Almost half of the PDE families (PDE2, PDE5, PDE6, PDE10, PDE11) contain two tandem GAF domains (Pfam number: PF01590) in their N-terminal regulatory domain which in most, but not all, instances bind cGMP with high affinity (see below). In addition to cGMP-regulated PDEs, other PDE families are subject to regulation by calcium/calmodulin, phosphorylation, and extrinsic regulatory proteins. While much is still to be learned about PDE regulation, a common theme that is emerging is that the N-terminal regulatory domain responds to signals from upstream signaling pathways by undergoing a conformational change; this structural alteration is communicated to the C-terminal catalytic domain, resulting in a change in conformation at the enzyme active site that alters catalysis. Changes in PDE expression levels also play an important role in long-term regulation of PDE function.

3.3. Transport and buffering of cGMP

In addition to metabolic control of cGMP levels by GC and PDE, the free cytoplasmic cGMP concentration can be regulated by cGMP efflux across the plasma membrane and/or by cGMP sequestration (i.e., binding to specific binding sites). These non-metabolic mechanisms for controlling cGMP levels depend on cell-specific expression of plasma membrane transport proteins to pump cGMP out of the cell down its concentration gradient,

and/or high affinity cGMP binding proteins to serve as a cGMP buffer.

While many studies have documented efflux of cGMP from cells into the extracellular space, the physiological relevance of this process has not been conclusively demonstrated. Earlier studies (12, 13, 14) documented the appearance of cyclic nucleotides in the extracellular medium in response to activation of intracellular signal transduction pathways. More recently, organic anion transport systems have been identified that selectively pump cGMP out of the cell in an ATP-dependent process (15, 3). However, there is no unequivocal evidence that cGMP efflux can occur at sufficient rates and in a regulated manner to serve as a regulator of cytoplasmic cGMP levels. Instead, cGMP efflux is more likely to serve a signaling role in providing extracellular guanine nucleotides that participate in other autocrine and paracrine pathways.

Considering that cellular concentrations of cGMP are $< 10^{-7}$ M in most cell types (1), reversible sequestration of a portion of total cellular cGMP would require cGMP binding proteins of high affinity and of a concentration in the same range as the cellular cGMP concentration. Of the known cGMP binding proteins, the CNG ion channel family (see below) is unlikely to serve as cGMP buffers because of their relatively weak affinity (dissociation constant, Kd = 10^{-6} M) affinity and low abundance in most cells. The cGMP-dependent protein kinase (PKG; described below) has high affinity cGMP binding sites in its regulatory domain [Kd = 10^{-7} M; (16)], and may be present in some tissues in amounts sufficient to impact the cytoplasmic cGMP concentration (2). Thus, it represents one candidate for serving as an endogenous cGMP buffer. Of the five families of GAF-containing PDEs, PDE2, PDE10, and PDE11 are reported to have relatively low affinity for cGMP, and the enzyme concentration relative to the cellular cGMP concentration in specific cell types has not been determined (17). Both PDE5 and PDE6 are high-affinity cGMP binding proteins, and exist at cellular concentrations where they are likely to contribute substantially to reducing the free cGMP concentration (2).

4. TARGETS OF cGMP ACTION

4.1. cGMP-dependent protein kinase

The cGMP-dependent protein kinase (PKG) is a member of the serine/threonine protein kinase family and is less widely distributed than the closely related cAMP-dependent protein kinase (PKA). This enzyme is most abundant in lung and smooth muscle tissues, and consists of two families in mammals: Type I (gene name: PRKG1) and Type II (PRKG2). Both PKG families consist of two tandem cGMP binding sites in the N-terminal region, and a protein kinase catalytic domain whose substrate specificity differs for the two families. Type I PKG is found as two distinct isoforms (I-alpha and I-beta) which differ in cGMP binding properties but have identical catalytic properties (18). The cGMP binding sites in PKG are members of the same cyclic nucleotide binding domain (Pfam number: PF00027) as is found in PKA, in CNG ion channels, and in the CAP family of transcriptional regulators. The stoichiometry of binding to Type I PKG is 2 cGMP molecules per subunit, with each site having unique exchange kinetics but similar overall binding affinities [Kd = 100-200 nM; (16)].

Activation of PKG results from cGMP binding or by autophosphorylation. cGMP binding and autophosphorylation occur with positive cooperativity, both events promoting conformational changes that causes activation of protein kinase activity at the active site. Phosphorylation of PKG-specific substrate proteins then serves to propagate the signaling pathway that initially induced the elevation of cytoplasmic cGMP concentration. Furthermore, in those cells where the concentration of PKG is significant relative to the cellular cGMP concentration, cGMP sequestration by high-affinity cGMP binding sites on PKG may represent a negative feedback loop to accelerate the recovery of the pre-stimulated state of the cell (2). The high affinity and slow exchange rate of cGMP with PKG implies that transient increases in cGMP levels will have the effect of rapidly activating PKG, but that activation may persist for some time after the cGMP levels return to the basal state.

4.2. Cyclic nucleotide-gated ion channels

The CNG ion channel family belongs to a large super-family of ion channels that share a similar structure, including a cyclic nucleotide binding domain in the C-terminal region of the primary sequence. (19). Six genes (CNGA1-4, and CNGB1 and CNGB3) combine to form three different heterotetrameric channels. Along with the CNG family, the hyperpolarization-activated pacemaker ion channel (HCN) family (20) also appears to be subject to regulation of ion flow by cyclic nucleotide binding, whereas the EAG channel family is insensitive to cyclic nucleotide regulation (21).

CNG channels are abundant in sensory neurons (e.g., retinal photoreceptors, chemosensitive olfactory neurons) but also present in many other tissues. Activation (opening) of the CNG ion channel results from a highly cooperative binding of four cyclic nucleotide molecules to the channel. CNG channels in retinal photoreceptors are selective for cGMP over cAMP, but in other cases the CNG channels lack ligand selectivity. While modulation of CNG channels by calcium-calmodulin, phosphorylation, lipids and retinoids has been observed (22, 19), the fundamental feature of CNG channels is their function as a molecular switch that responds rapidly to changes in cytoplasmic cyclic nucleotide levels.

4.3. cGMP-binding phosphodiesterases

Three of the five GAF domain-containing PDEs (PDE2, PDE5, and PDE6) may serve as feedback regulators of cGMP signaling pathways by virtue of their high affinity, noncatalytic cGMP binding sites. [PDE10 and PDE11 bind cGMP poorly, if at all, when the recombinant enzyme is examined (23, 24).] Binding of cGMP to the regulatory GAF domains of PDE2 is responsible for a direct, allosteric stimulation (approximately10-fold) of catalytic activity. For PDE5, there is a cooperative effect of

phosphorylation of the catalytic subunit and cGMP binding that increases enzymatic catalysis of PDE5 (17). For both PDE2 or PDE5, this allosteric modulation of GAF-PDEs by cGMP has been shown to be physiologically relevant in controlling cyclic nucleotide metabolism (25, 26). (Regulation of PDE6 by cGMP binding is treated in a later section.)

PDE5 and PDE6 show a very strong preference for cGMP over cAMP (27, 28), indicating that cross-talk between cGMP and cAMP signaling pathways cannot occur via these two cGMP-binding PDEs. PDE2 discriminates cGMP over cAMP less well (29, 30), leaving open the possibility that elevated cAMP levels might allosterically regulate PDE2 in a negative feedback manner. Another important feature shared by PDE5 and PDE6 (but not by PDE2) is the high affinity (Kd approximately 100 nM) with which cGMP binds to the GAF domains. There are two important consequences of these PDEs having a high intrinsic cGMP binding affinity. First, dissociation of bound cGMP from these sites can be quite slow compared to dynamic changes in cGMP concentration. Thus, a rapid elevation of cGMP may cause immediate occupancy of the GAF domains, but the subsequent reduction of cGMP levels during response recovery may occur more quickly than release of cGMP bound to GAF-PDEs. Second, in cells where GAF-PDE concentrations are comparable to the total cellular cGMP concentration, high affinity cGMP-binding PDEs may buffer cGMP (Section 3.3).

4.4. Other potential targets of cGMP action

Depending on the concentrations of cAMP and cGMP in the cell, proteins containing cAMP binding motifs could potentially bind cGMP and thus contribute to cross-talk between the cGMP and cAMP signaling pathways. For example, PKA regulatory subunits also contain cyclic nucleotide binding sites (with the same domain structure as PKG) that can bind cGMP, although with 50-fold lower affinity than cAMP (18). There are 65 human proteins reported to contain the same cyclic nucleotide binding domain (Pfam number: PF00027) as is found in PKA, PKG and the CNG channels), many of whom serve unknown functions at present.

5. SENSORY TRANSDUCTION IN VERTEBRATE ROD AND CONE PHOTORECEPTORS

The remarkable ability of the human eye to capture photons, transduce this light stimulus into an electrical response, and then to generate a neural output to the brain that we call "vision," has evolved from a very ancient plan that is shared by all animals with eyes. Although there is diversity in the ways in which eyes collect and focus light, there have arisen only two basic types of photoreceptive cells, those with cilia and those with microvilli. Vertebrate retinas rely exclusively on ciliary photoreceptor cells, and most animals contain two distinct classes, namely rods and cones. Another fundamental difference between vertebrate and invertebrate vision is the fact that vertebrate rods and cones hyperpolarize their cell membranes upon light stimulation, whereas most invertebrates undergo depolarizing photoresponses (31, 32). We restrict this section to discussing the cell biology and physiology of vertebrate retinal photoreceptors.

5.1. Rod and cone photoreceptor cells

All vertebrate retinas contain two classes of photoreceptor cells (rods and cones) that are responsible for converting photons of light into a neural signal. Rods are best suited to operate at low light intensities, and are actually quite reliable single photon detectors. The rod photoresponse becomes saturated when the light stimulus is sufficiently bright (for example, during daylight illumination). Cones are less light sensitive than rods, but possess the important property that their photoresponses can adapt to the brightest illumination conditions on earth and still detect changes in light intensity. Most retinas contain more than one type of cone photoreceptor that differs in the absorption properties of its visual pigment. This confers the potential for color discrimination by comparing the neural outputs of the different cone classes (33).

Rods and cones are highly specialized neurons which consist of several functionally and structurally distinct cellular compartments. The phototransducing outer segment portion of the cell consists of a densely packed membranous system optimized for photon capture and signal transduction. In rods, the disk membranes constitute a physically separate stack of over a thousand flattened membranes that is distinct from the plasma membrane. In contrast, cone outer segments are formed as continuous invaginations of the plasma membrane. The outer segment is the compartment in which phototransduction occurs and where the initial membrane hyperpolarization is triggered. The closely packed disk membranes in the outer segment greatly reduce free diffusion in the cytoplasm, resulting in the restricted spread of the photoexcitation process.

The outer segment of rods and cones is connected to the inner segment by a narrow, non-motile "connecting cilium." By mechanisms not well understood, the connecting cilium actively regulates the transport of proteins and metabolites between the outer and inner segments. The metabolic machinery residing in the inner segment portion of the photoreceptor cell is also highly compartmentalized. Mitochondria are concentrated in the "ellipsoid" region nearest the connecting cilium, thereby facilitating delivery of metabolic energy to the highly metabolically active outer segment. Organelles dedicated to protein biosynthesis are located between the mitochondria-rich ellipsoid region and the cell nucleus. The synaptic terminals of rods and cones are where the receptor potential (originally generated in the outer segment) is communicated to second-order neurons. [For reviews, see (34, 35).]

5.2. Photoreceptor electrophysiology

In the dark-adapted, resting state, vertebrate photoreceptors generate a circulating current called the "dark current." This current results from entry of sodium (primarily) and calcium through open CNG ion channels in the outer segment plasma membrane, concurrently with the

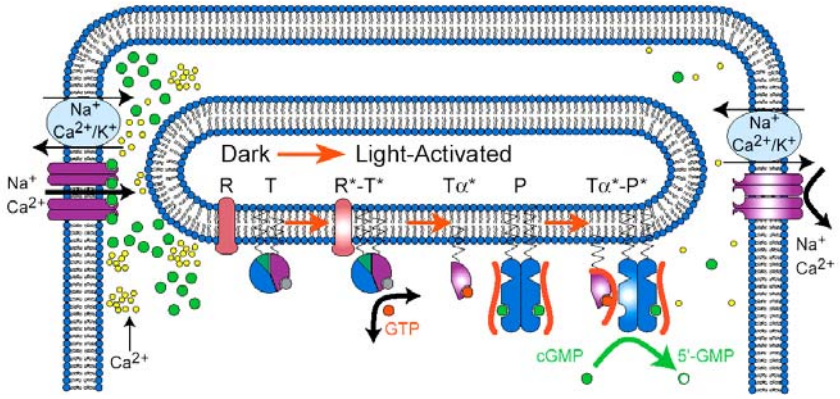

Figure 1. The visual excitation in vertebrate photoreceptors. The integral membrane protein, rhodopsin (R), absorbs a photon of light and isomerizes to its activated state (R*). The heterotrimeric G-protein, transducin (T), is attached to the disk membrane by both a farnesylated gamma subunit (green) and a fatty acylated alpha subunit (violet). T is activated upon forming a high-affinity complex with activated rhodopsin (R*-T*). Exchange of bound GDP for GTP induces dissociation of the activated alpha subunit of transducin (Talpha*) from the beta-gamma subunits. The activated Talpha* (with GTP bound) then binds to the inhibitory gamma subunit (red) of PDE6 (P) to form activated complex, Talpha*-P*. Hydrolysis of cGMP to 5'-GMP is accelerated when PDE6 (itself prenylated at each catalytic subunit) is activated. High levels of cGMP (green circles) in the dark-adapted state decline upon light activation, causing closure of cGMP-gated ion channels (purple). Blocking the inward flow of Na^+ and Ca^{2+}, in conjunction with continued operation of the Na^+/Ca^{2+}-K^+ exchanger (gray) leads to a drop in the free calcium concentration (yellow circles) in the outer segment.

extrusion of sodium by a Na^+/K^+-ATPase and the efflux of potassium by K+ channels (both localized to the inner segment). A Na^+/Ca^{2+}-K^+ exchanger (in the outer segment) and other ion channels in the inner segment further regulate ion conduction and transport in photoreceptors. Upon illumination, the circulating dark current is interrupted due to closure of the CNG ion channels in the outer segment. The hyperpolarization of the membrane potential which results from channel closure is passively propagated from the outer segment through the inner segment to the synaptic terminal, where glutamate transmitter release at the synapse is inhibited (36, 37, 38).

Remarkably, rod photoreceptors can reliably detect single photons, highly amplify the signal via the cGMP signaling pathway, and generate a discrete, stereotypical electrical response (39). As the light intensity is increased, the extent of dark current suppression also increases until photoresponse saturation is reached. The speed of the photoresponse is rapid: single photons responses attain peak amplitude in d 1 sec, while bright flashes can suppress the dark current within milliseconds. Cone photoreceptors are less light-sensitive than rods, and their photoresponses are smaller and faster than for rods. The rising phase of the photoresponse (termed "visual excitation") is dominated by the kinetics of activation of the cGMP signaling pathway [(37); discussed in detail below].

In order to resolve rapid changes in light stimuli, the initial process of visual excitation described in the preceding paragraph must be followed by a rapid recovery (termination) process. The mechanism of photoresponse recovery itself is tightly coordinated with, and results from, the excitation process. This is because all of the components activated during visual excitation must be deactivated in order to restore the dark-adapted state. In addition to components of the cGMP signaling pathway, regulation of the outer segment calcium concentration is of fundamental importance in determining the inetics of photoresponse recovery (40, 41, 42).

In addition to excitation and recovery/termination, photoreceptors are capable of adapting to constant illumination by the process of light adaptation. As the light intensity of background illumination is increased, the sensitivity of rods and cones to flash stimulation is decreased, resulting in smaller photoresponses that have faster recovery kinetics without much change in the excitatory, rising phase of the flash response. Light adaptation serves to increase the range of light intensities over which visual transduction can operate. Calcium plays a central role in the underlying mechanisms of light adaptation, acting through calcium regulatory proteins to modulate several steps in the cGMP signaling pathway (43, 42).

The unique cellular anatomy of the photoreceptor cell (with its outer segment serving as a self-contained phototransducing organelle), combined with the wealth of electrophysiological data on the light-evoked electrical responses of intact photoreceptors, has fueled rapid advances in our understanding of the signal transduction pathways involved in excitation, recovery, and adaptation of photoreceptor cells. The level of quantitative analysis that correlates the electrical responses to photic stimuli with the underlying cascade of biochemical reactions that are triggered by light is unmatched in any other field of signal transduction. This is most evident in the mathematical modeling of the molecular mechanisms responsible for the amplification, kinetics, and adaptational properties of vertebrate photoreceptor cells to light stimulation (37, 41, 42).

5.3. Molecular components of visual excitation

The first step in vertebrate vision is the photoisomerization of the retinal chromophore (11-cis retinal) of the visual pigment on the outer segment membrane (Figure 1). Remarkably, many prokaryotes,

along with all animals, use a retinal chromophore-based visual pigment which is covalently attached to the opsin apoprotein. Rhodopsin is a member of the G-protein coupled receptor superfamily. Photoactivation in most vertebrates (including humans) results from isomerization of 11-cis retinal to all-trans retinal. This causes movement of the transmembrane alpha-helices that surround the chromophore, producing metarhodopsin II, the activated form of the receptor (44).

Conformational changes in the cytoplasmic loops of metarhodopsin II allow rhodopsin to bind with high affinity to the heterotrimeric G-protein, transducin. This interaction catalyzes the exchange of bound GDP for GTP on the transducin alpha-subunit, causing dissociation of the alpha-subunit (with bound GTP) from the beta-gamma dimer. Activated transducin then binds to its effector in this signaling cascade, PDE6, displacing the inhibitory PDE6 gammasubunit and accelerating the catalysis of cGMP. Because of the relatively long lifetime of metarhodopsin II, more than one hundred transducin molecules (and hence PDE6 molecules) can be activated per photoisomerization event. This first step in signal amplification is followed by a second stage, in which each activated PDE6 catalyzes the destruction of up to thousands of cGMP molecules. The overall gain of this amplified excitation pathway is $>10^5$ cGMP molecules hydrolyzed per activated rhodopsin. The rapid drop in cytoplasmic cGMP levels then causes bound cGMP to dissociate from binding sites on the CNG ion channel in the plasma membrane. Channel closure in the plasma membrane induces hyperpolarization of the membrane potential. This sequence leading from photoisomerization of visual pigment to membrane hyperpolarization constitutes the set of reactions defined as visual excitation.

As the central effector of visual excitation, photoreceptor PDE6 is under very stringent regulatory controls. In rod photoreceptors, PDE6 is present at a concentration of 20 micromolar (45), and is localized to the disk membranes in the outer segments. Post-translational modification of the C-terminus of the catalytic subunits by prenylation and carboxymethylation confers high affinity association with the disk membranes (46, 47). In its nonactivated (darkadapted) state, the catalytic dimer of PDE6 binds two gamma subunits with very high (Kd = 1 pM) affinity (48, 49, 50). This ensures that the basal hydrolytic activity of PDE6 is very low. Upon stimulation of the visual excitation cascade, the transducin alpha subunit binds to PDE6, relieving the inhibitory constraint of the gamma subunit. This accelerates cGMP hydrolytic rates approximately 300-fold. Interestingly, transducin-activated PDE6 achieves only about one-half of the catalytic potential of which it is capable when both gamma subunits are removed *in vitro,* consistent with transducin activating only one of the two PDE6 catalytic subunits (51, 52). This extraordinary catalytic power of activated PDE6 drives down cytoplasmic cGMP levels on the millisecond time scale, as well as consuming cGMP as quickly as it dissociates from the CNG channels.

5.4. Molecular components of photoresponse termination and adaptation

In order to maximize both the temporal resolution of visual stimuli as well as the ability of photoreceptors to adapt over the 10^{12} range of photic stimuli on earth, the visual excitation pathway must be feedback-regulated for rapid termination and response modulation. Every individual step in the excitation pathway is subject to regulation during the recovery to the dark-adapted state. Many of the individual reactions are subject to regulation by calcium.

(1) Metarhodopsin II inactivation involves phosphorylation by a specific G-protein receptor kinase (GRK1 in rods, GRK7 in cones) whose activity is regulated by the calcium binding protein recoverin/S-modulin (53, 54, 55). This is followed by binding of arrestin to phosphorylated opsin to complete the inactivation process (56). Finally, pigment regeneration of the photobleached chromophore requires enzymatic and transport reactions, termed the “retinoid cycle” (57).

(2) The inactivation of the alpha-subunit of transducin requires hydrolysis of bound GTP. The GTPase rate is itself a highly regulated process that involves a protein complex of the regulator of G-protein signaling-9 (RGS-9), the type 5 G-protein beta-subunit, the RGS9 anchor protein (R9AP), and the PDE6 gamma subunit (58).

(3) PDE6 inactivation occurs when the PDE6 gamma subunit is released from its binding site on the alphasubunit of transducin and re-inhibits the enzyme’s active site. The strength of the interaction of the gamma subunit for transducin versus PDE6 is modulated by the state of occupancy of cGMP at the PDE6 regulatory GAF domains. Reciprocal positive cooperativity between cGMP binding and PDE6 gamma subunit binding to PDE6 may be responsible for determining which function of PDE6 gamma predominates: potentiation of GTPase acceleration of transducin or inhibition of PDE6 catalysis (59).

(4) Restoring cGMP levels following PDE6 activation is primarily accomplished by activation of photoreceptor guanylate cyclases (GC-2E and GC-2F; also abbreviated as ROS-GCs or Ret-GCs in the literature). GC activation is a consequence of the drop in cytoplasmic calcium concentration that results from channel closure (Figure 2). Calcium regulation of GC-2E and GC-2F is mediated by a group of GC-activating proteins (GCAPs) that release bound calcium when the cytoplasmic concentration drops below 500 nM (60, 61, 62). A non-enzymatic mechanism for restoring cGMP levels by utilizing cGMP bound to high-affinity sites on the PDE6 GAF domains (63) is theoretically possible based on the concentration of high affinity cGMP binding sites in the photoreceptor cell (40 micromolar; see below). However, unless PDE6 had already been inactivated, any released cGMP would also be subject to hydrolysis (2).

(5) Re-opening the CNG ion channel during photoresponse recovery directly results from restoration of the cGMP levels and the binding of cGMP to allosteric sites on the channel protein. However, this ligand gating mechanism can be modulated to control the sensitivity of the channel to cGMP levels. In rod photoreceptors,

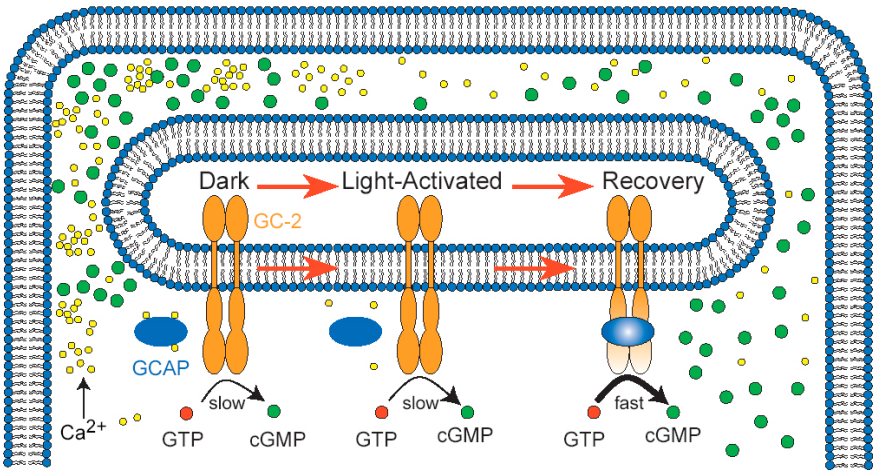

Figure 2. Recovery of cGMP levels following PDE6 activation is mediated by the calcium-regulated photoreceptor GC-2. In the dark-adapted state when the calcium concentration is high (left), calcium ions bind to GCAPs (blue). Calcium-bound GCAP is unable to bind to GC-2 (orange), and cGMP synthesis is slow. During the initial phase of light activation when the free calcium concentration drops, GCAP dissociates its bound calcium (middle). Calcium-free GCAP then binds with high affinity to GC-2, causing acceleration of cGMP synthesis from GTP (right). As PDE6 returns to its inactivated state, activated GC-2 is able to elevate cGMP levels. The subsequent rise in calcium concentration completes the recovery to the dark-adapted state.

calmodulin is the primary regulator of cGMP binding affinity, and calcium-calmodulin binding to the beta-subunit of the channel serves to decrease the cGMP sensitivity of the channel. In cone photoreceptors, modulation of cGMP sensitivity by calcium is greater than in rods. Retinoids, lipid metabolites, channel phosphorylation and interactions with other proteins may also contribute to modulation of the ligand sensitivity of CNG ion channels (64, 22, 65, 66).

Whereas the mechanism of visual excitation requires consideration only of one second messenger (i.e., cGMP), it is evident that calcium serves an integral role in several processes related to photoresponse turnoff and adaptation. The cytoplasmic concentration of calcium in dark-adapted photoreceptor outer segments (400-600 nM) rapidly decreases upon light exposure to 10-50 nM as a consequence of channel closure concomitant with continued extrusion of calcium by the Na+/Ca2+-K+ exchanger. Of the well-known calcium regulatory proteins and their effectors (i.e., recoverin/S-modulin and GRK1 and GRK7; GCAPs and GC-2E and GC-2F; calmodulin and rod CNG ion channel), the dominant effect of calcium on recovery and adaptation is mediated through its regulation of GC activity. The 10-fold increase in cGMP synthesis resulting from the decline in calcium concentration actually extends the operating range of the photoresponse during light adaptation. Furthermore, the simultaneous light-induced activation of PDE6 and GC accelerate cGMP metabolic flux and accelerate the kinetics of the photoresponse (43, 41, 42).

6. REGULATION OF cGMP LEVELS IN PHOTORECEPTORS

In this section, we apply the general understanding of how cellular cGMP concentrations are regulated (Section 3) to the specific case of the regulation of cytoplasmic cGMP concentration in photoreceptor cells prior to and following activation of the phototransduction cascade.

6.1. cGMP metabolic flux sets the dynamic steady state cGMP concentration

In darkness, a steady state condition exists for cGMP in the photoreceptor outer segment, in which the hydrolytic and synthetic rates are equal in magnitude and opposite in sign. The metabolically active concentration of cGMP is maintained in darkness at several micromolar (67). Both electrophysiological (68) and biochemical (69) measurements agree that cGMP metabolic flux is sufficient to cause all of the free cGMP in the outer segment to turn over every second (rate = 1-2 micromolar cGMP per sec). This metabolic flux reflects hydrolysis of cGMP by nonactivated PDE6 at a rate that is only 0.1% of its fully activated catalytic turnover number (70, 49, 58), consistent with essentially all PDE6 existing in its fully inhibited state. Steady state cGMP levels in dark-adapted rods require equal rates of cGMP synthesis by the calcium-regulated GC. At the resting, dark-adapted calcium concentration in the rod outer segment, the steady state GC activity is neither inhibited nor activated (71).

Upon light activation, the instantaneous rate of change in the concentration of metabolically active cGMP in the photoreceptor outer segment is defined by the difference in its rate of synthesis (by GC) and in its rate of degradation (by PDE6). During the response to a flash of light, activation of PDE6, the decline in cGMP levels, and the closure of channels (Section 5.3) precedes by tens of milliseconds the decline in calcium concentration and the activation of GC. During this initial period, PDE6 is fully activated by transducin but fails to achieve its full catalytic potential. This is because the enzyme is operating with a substrate concentration much less than its Michaelis constant, and so the hydrolytic rate is a zero-order reaction.

Regardless, activated PDE6 is predicted to rapidly reduce the metabolically active cGMP concentration to sub-micromolar levels.

Even when calcium declines and GC is activated, the 10-fold elevation in rate of cGMP synthesis cannot match the hydrolytic potential of activated PDE6. During this period in which both PDE6 and GC are activated, cGMP metabolic flux increases 10-fold (68, 69, 72, 73) without significant recovery of cGMP levels. Rather than a waste of metabolic energy, acceleration of cGMP metabolic flux following light exposure functions to decrease light sensitivity as well as extending the operating range of a light-adapted photoreceptor to brighter stimulus intensities (43).

Following cessation of the light stimulus, inactivation of PDE6 (resulting from the set of feedback reactions that limit the activated lifetime of rhodopsin and transducin; Section 5.4) is the primary factor that governs the ability of the photoreceptor to recover to its dark-adapted state. Activation of GC will persist until cGMP levels are increased sufficiently to cause opening of CNG channels and an elevation in calcium concentration. While other feedback mechanisms certainly play a role in shaping the recovery of the dark-adapted state, the dominant mechanism that shapes the kinetics of the recovery process is the calcium-regulated changes in GC activity that restore cGMP levels to their pre-illumination (41).

6.2. Buffering and sequestration of cGMP in rod outer segments

In the previous section, non-metabolic mechanisms that might modulate the cytoplasmic cGMP levels in the dark or upon light activation of the phototransduction pathway were not considered. Two lines of evidence unequivocally demonstrate that most of the total cGMP in dark-adapted rod outer segments is bound to specific sites and not in rapid equilibrium with cytoplasmic cGMP: (1) only 15% of the total cGMP pool (60 μM) is immediately susceptible to light-induced hydrolysis during the time frame in which the electrical response occurs (74, 75). Furthermore, even after prolonged exposure to light, the cGMP content of rod outer segments never declines close to zero, but routinely undergoes a maximal 50% decrease (74, 76, 75, 77, 78). [This observation cannot be accounted for by GC activity establishing a new steady state cGMP concentration, since activated PDE6 hydrolytic activity is >10-fold larger than fully activated GC synthetic capacity.] (2) Quantitative analysis of the fractional activation of CNG channels in dark-adapted rods (<5%) permits estimation of a cGMP concentration of 2-4 micromolar in equilibrium with the channel in intact rods (79, 80).

Direct measurements of cGMP binding sites in rod outer segments identified two classes of cGMP binding sites: one class has high affinity (Kd = 60 nM) and is capable of binding two-thirds (40 micromolar) of the total cellular cGMP, while a low affinity (Kd = 7 micromolar) class of sites was present in higher concentration and predicted to bind most of the remaining cGMP (81). Together, it is calculated that of the 60 micromolar total cellular cGMP in rod outer segments, only 2 micromolar represents unbound cGMP in the dark-adapted state (2). Barring changes in cGMP affinity upon light activation of the visual transduction pathway, the low affinity class of sites would be expected to lose their cGMP (which would then be subject to hydrolysis) while cGMP bound to high affinity sites would be protected from degradation. The estimates of free and bound pools of cGMP in dark-adapted and illuminated rods agree with the biochemical and electrophysiological observations cited above.

The question arises as to the identity of these cGMP binding sites. The CNG ion channel can be immediately ruled out as a buffer for sequestering cGMP, because its concentration in rod outer segments is too low (about 1 micromolar for the channel), its affinity for cGMP is too weak, and its function is to serve as a sensor of the instantaneous cGMP concentration in order to regulate ion flow across the plasma membrane (64). PKG, another cGMP binding proteins with high affinity for cGMP, is believed to be present in rod photoreceptors, but its concentration in rod outer segments is unknown. Photoreceptor PDE6 is the only protein known to bind cGMP and to be present in sufficient abundance in rod outer segments [20 micromolar enzyme concentration; (45, 82)] to sequester a significant fraction of the cellular cGMP. The high affinity with which nonactivated rod PDE6 binds cGMP (83, 84, 45) leads to the conclusion that the high affinity cGMP binding sites that are not metabolically active reside on the GAF domains of PDE6. With a binding stoichiometry of 2.0 cGMP binding sites per nonactivated PDE6 holoenzyme (84, 45), PDE6 can account for the 40 micromolar high affinity cGMP binding sites in the rod outer segment. The identity of the low affinity sites that bind most of the remaining cellular cGMP in rod outer segments is not currently known. The possibility that PDE6 binds cGMP to an additional class of binding sites is plausible, since each PDE6 holoenzyme consists of a catalytic dimer, each subunit of which contains a cGMP-binding GAFa domain (85, 86) and a second GAFb domain whose function is not known.

7. TARGETS OF cGMP ACTION IN PHOTORECEPTOR OUTER SEGMENTS

This section focuses on specific topics related to the targets of cGMP action in photoreceptors that were not already presented in Section 6.

7.1. Cyclic nucleotide-dependent protein kinases

Little is known about the presence of PKG in retinal photoreceptor cells, potential substrates for phosphorylation, or the significance for regulation of phototransduction. While some evidence suggests that PKA predominates over PKG in rod outer segments (87, 88), most results do not unequivocally distinguish PKG from PKA (89, 90). Because cGMP levels in photoreceptors are >10-fold higher than in most other cells, the potential for cGMP to “cross-talk” with the cAMP signaling pathway by activating PKA must be considered.

Cyclic nucleotide-dependent phosphorylation of

photoreceptor proteins has been studied, with identification of several PKA/PKG substrates. Light-dependent dephosphorylation of Components I and II (in amphibian photoreceptors) and phosducin (in mammalian and fish photoreceptors) is consistent with the idea that PDE6 activation—and the resulting drop in free cGMP levels—inactivates PKA/PKG and leads to dephosphorylation of these proteins (91, 92, 93, 94, 95, 96, 97). The abilities of these dephosphorylated proteins to preferentially bind to transducin beta-gamma dimers (98, 97, 99) and to assist in light-dependent protein translocation within the photoreceptor cell (100) are consistent with a role for cyclic nucleotide-dependent protein kinases in some aspects of long-term light adaptation.

7.2. Cyclic nucleotide-gated ion channel

The primary target of action of cGMP in photoreceptor cells is the cyclic nucleotide-gated ion channel present in the plasma membrane of rod and cone outer segments. The native CNG channel in rod photoreceptors is an oligomer of three alpha subunits and one beta subunit (101). The beta subunit confers unique properties on the functional channel, including sensitivity to calcium-calmodulin. The rod CNG channel is found in a complex with the Na^+/Ca^{2+}-K^+ exchanger (102). Cone photoreceptors have a distinct CNG channel composed of highly similar alpha and beta subunits. However, regulation of the cone CNG channel by calcium differs significantly than what is found for the rod CNG channel (66, 103).

Photoreceptor CNG channels are optimized to rapidly respond to changes in cGMP levels by opening or closing their pores that allow sodium and calcium to flow into the outer segment. Rapid responsiveness to fluctuations in cGMP levels is a result of the relatively low affinity of cGMP binding, with fast dissociation and association rates for the ligand binding sites. The cooperativity (Hill coefficient = 3) with which four cGMP molecules bind amplifies small changes in cGMP concentration. As mentioned above, calcium regulatory proteins (calmodulin in rods, an as-yet uncharacterized calcium binding protein in cones) can bind to the photoreceptor CNG channel and reduce the cGMP sensitivity of channel gating. Desensitization of CNG channels by reducing cGMP binding affinity is also reported to be affected by tyrosine or serine/threonine phosphorylation, diacylglycerol and/or related metabolites, and retinoids (22, 19, 104, 105).

The calcium permeability of the CNG channel generates a calcium feedback signal (in concert with the exchanger) when the channels close during visual excitation, thus allowing for calcium-dependent reactions involved in recovery and adaptation to occur (64, 104). Divalent cations also partially block current flow by transiently binding during passage through the pore, thereby serving to reduce background noise associated with random channel openings (106).

7.3. Photoreceptor PDE6

The central role of the activation and inactivation of PDE6 catalysis during the excitation and termination processes of phototransduction was described in Sections 5 and 6. Here we focus on the function of the cGMP-binding GAF domains in PDE6 regulation.

In its nonactivated state, the PDE6 holoenzyme (2 catalytic subunits, 2 inhibitory gamma subunits) binds two cGMP molecules with unequal affinity. While the binding affinity to each site is high, the values for the cGMP dissociation constant (Kd) vary considerably among species and between rod and cone isoforms. Unlike PDE2 and PDE5 where cGMP binding stimulates catalysis by a direct allosteric mechanism, no similar effects of cGMP occupancy on PDE6 catalysis have been detected for the catalytic dimer of PDE6 (107, 49). Instead, cGMP binding to the GAF domains of PDE6 enhances the affinity of the PDE6 gamma subunits for the catalytic dimer, thereby reducing the basal activity of the enzyme (108, 70). The effect is reciprocal, in that binding of the gamma subunit to the PDE6 catalytic dimer enhances cGMP binding affinity to one class of cGMP binding sites (45, 52).

Following transducin activation of PDE6, displacement of one gamma subunit on the PDE6 holoenzyme not only relieves inhibition of catalysis, but also causes rapid dissociation of cGMP from one of the GAF domains (45, 52). cGMP dissociation correlates with gamma subunit dissociation from PDE6 (73, 52). The positive cooperativity between cGMP and gamma subunit dissociation from transducin-activated PDE6 may allow the gamma subunit to interact with RGS-9 in order to accelerate the GTPase rate (109, 110, 73). This negative feedback mechanism to shut off activated PDE6 during prolonged light activation may be relevant to the mechanism of light adaptation (58). In this hypothetical model, whether or not cGMP is bound to the GAF domains of PDE6 determines whether the gamma subunit remains in a transducin-PDE6 complex (cGMP bound to PDE6) or dissociates with transducin to form a transducin-RGS-9 complex (cGMP dissociated from PDE6).

8. PERSPECTIVES

While visual transduction in vertebrate rod photoreceptors is the best studied cGMP signaling system, there remain many gaps in our knowledge. For example, most of the work reported in this review pertains to phototransduction in rod photoreceptors. Much less is known about the biochemical differences in cone photoreceptors that underlie the remarkably different physiological responsiveness of cones (Section 5.2). While a quantitative account of the major biochemical reactions responsible for visual excitation has been accomplished, the processes responsible for termination and modulation of the photoresponse are not fully known. Although cGMP and calcium signaling through known phototransduction proteins can account for many aspects of photoresponse recovery and adaptation, there remain unexplained features of recovery and adaptation that may involve novel regulatory mechanisms that act on cGMP signaling (78, 111).

In some instances, very little is understood about

certain aspects of cGMP signaling in photoreceptors when compared with other cells and tissues. For example, cyclic nucleotide transport across the plasma membrane—and its potential for regulating cytoplasmic cGMP concentration—has not been experimentally addressed in photoreceptor cells. While PKG is a primary target of cGMP action in many other cell types, virtually nothing is known about its subcellular localization and abundance in photoreceptors, its regulation, or its substrates.

Even when considering the well-studied rod PDE6 enzyme, we lack critical information in several areas. We currently are limited to low-resolution images of the structure of PDE6 (112), with no x-ray crystal structures published to date. Novel mechanisms regulating PDE6 may yet be revealed, particularly with regard to reactions involved in the termination and modulation of the photoresponse. Two high-affinity PDE6 binding proteins, glutamic acid-rich protein2 (GARP2) and a 17 kDa prenyl binding protein (PrBP/delta) have been reported (113, 114), both of which alter cGMP binding properties to the GAF domains of PDE6 *in vitro* (115); Pentia and Cote, unpublished data]. Signaling pathways in other cells that utilize the PDE6 gamma subunit in conjunction with MAP kinase, src tyrosine kinase, and other signaling components (116, 117, 118) need to be critically evaluated for their potential occurrence in photoreceptor cells as well. Finally, recent reports of photoreceptor PDE6 and transducin as elements of signaling pathways unrelated to phototransduction (119) suggest that our knowledge of the cGMP signaling pathway in photoreceptors may be applicable to understanding signal transduction pathways in other cell types.

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