Adaptation in Vertebrate Photoreceptors

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Fain, Gordon L., Hugh R. Matthews, M. Carter Cornwall, and Yiannis Koutalos. Adaptation in Vertebrate Photoreceptors. *Physiol Rev* 81: 117–151, 2001.—When light is absorbed within the outer segment of a vertebrate photoreceptor, the conformation of the photopigment rhodopsin is altered to produce an activated photoproduct called metarhodopsin II or Rh[*]. Rh[*] initiates a transduction cascade similar to that for metabotropic synaptic receptors and many hormones; the Rh[*] activates a heterotrimeric G protein, which in turn stimulates an effector enzyme, a cyclic nucleotide phosphodiesterase. The phosphodiesterase then hydrolyzes cGMP, and the decrease in the concentration of free cGMP reduces the probability of opening of channels in the outer segment plasma membrane, producing the electrical response of the cell. Photoreceptor transduction can be modulated by changes in the mean light level. This process, called light adaptation (or background adaptation), maintains the working range of the transduction cascade within a physiologically useful region of light intensities. There is increasing evidence that the second messenger responsible for the modulation of the transduction cascade during background adaptation is primarily, if not exclusively, Ca^{2+}, whose intracellular free concentration is decreased by illumination. The change in free Ca^{2+} is believed to have a variety of effects on the transduction mechanism, including modulation of the rate of the guanylyl cyclase and rhodopsin kinase, alteration of the gain of the transduction cascade, and regulation of the affinity of the outer segment channels for cGMP. The sensitivity of the photoreceptor is also reduced by previous exposure to light bright enough to bleach a substantial fraction of the photopigment in the outer segment. This form of desensitization, called bleaching adaptation (the recovery from which is known as dark adaptation), seems largely to be due to an activation of the transduction cascade by some form of bleached pigment. The bleached pigment appears to activate the G protein transducin directly, although with a gain less than Rh[*]. The resulting decrease in intracellular Ca^{2+} then modulates the transduction cascade, by a mechanism very similar to the one responsible for altering sensitivity during background adaptation.

I. INTRODUCTION

Sensory receptors respond to specific stimuli with an electrical response and are vital to our perception of the external world. Most sensory receptors give graded responses, whose amplitude increases proportionally with the intensity of the stimulus. The relationship between stimulus intensity and response is, however, not fixed for most sensory receptors but is altered as the mean level of stimulation is changed. This process, called adaptation, is a nearly universal feature of sensory transduction, responsible for example for the fading of intense odors and our accommodation to the sound of city traffic (see for example Ref. 65). It is also responsible for adjustments in the sensitivity of photoreceptors and the visual system, which make it possible for us to detect objects in our environment at nearly constant contrast despite large changes in the level of ambient illumination.

For rod photoreceptors, which are specialized for the detection of dim illumination and can respond to single photons of visible light (13), the linearity of signal detection and very high absolute sensitivity has the necessary consequence that response amplitude reaches a maximum value for flashes of relatively dim intensity (see for example Refs. 14, 60, 64, 132). The process of adaptation acts to decrease the sensitivity of the transduction cascade, reducing the amplitude of the single-photon response. In steady light bright enough initially to produce a response of maximum amplitude in a dark-adapted receptor, the sensitivity of transduction is rapidly reduced so that saturation does not occur and the receptor can respond to increments or decrements of illumination in the presence of the background illumination.

The electrical responses in Figure 1 illustrate the phenomenon of photoreceptor adaptation. These recordings were all made from the same rod, isolated from the retina of the salamander *Ambystoma tigrinum*. The responses in Figure 1*A* were recorded from the dark-adapted photoreceptor. In darkness there was a steady current of ~40 pA entering the outer segment of the cell through the cGMP-gated channels. This is usually referred to as the circulating current or dark current (see Ref. 220). Brief flashes of illumination reduce this current by closing the cGMP-gated channels, as we describe in detail in section II. This produces a change in circulating current whose peak amplitude, for dim flash intensities, varies approximately linearly with the intensity of the stimulus. Bright flashes close all of the cGMP channels and reduce the dark current to zero, effectively saturating the rod (see Ref. 184).

When the rod in Figure 1 was stimulated with steady light (Fig. 1, *B* and *C*), there was an initial relaxation in the response waveform to the steady illumination, indicating that the sensitivity of the rod was decreasing. The sensitivity to flashes also decreased so that a brighter flash

intensity was now required to produce a response of the same amplitude. This had the effect of shifting the entire operating range of the receptor to brighter intensities. Careful examination of the responses in Figure 1, *B* and *C*, shows that the sensitivity to flashes is approximately inversely proportional to the intensity of the background.

This relationship of inverse proportionality between sensitivity and background intensity is known as Weber's law and was first proposed not only for the visual system but for all sensory systems by Weber in the 1800s (278). Careful psychophysical investigation (summarized in Refs. 8, 23, 243) shows that Weber's law is valid for visual perception particularly for large visual fields and for flashes of relatively long duration. For single photoreceptors, Weber's law seems to be followed quite closely for background lights over at least two to three orders of magnitude. This is true for both rods (14, 64) and cones (12) in lower vertebrates, and also for both rods (139, 172, 196, 267, 268) and cones (254) in mammals.

For photoreceptors, Weber's law can be expressed in the following way (see Refs. 12, 14, 64). We define the flash sensitivity (S) as the amplitude in millivolts or picoamps of the response to a flash (evoking only a small response) divided by the intensity of the flash, in units of photons per square centimeters or rhodopsin molecules bleached per photoreceptor. If S_F is the flash sensitivity of the receptor in the presence of a background and S_F^D the flash sensitivity in darkness, then

$$\frac{S_F}{S_F^D} = \frac{I_0}{I_0 + I_B} \qquad (1)$$

where I_B is the intensity of the backgound. The constant I_0, sometimes called the dark light (8), is equal to the background intensity required to reduce sensitivity by one-half. This equation says that for background lights sufficiently greater than I_0 (i.e., for $I_B \gg I_0$), S_F is nearly equal to $(S_F^D \cdot I_0)/I_B$. Since S_F^D and I_0 are both constants, sensitivity is inversely proportional to background intensity, as Weber's law predicts. *Equation 1* can also be expressed in the following form (see Ref. 12)

$$\frac{S_F^D}{S_F} - 1 = \frac{I_B}{I_0} \qquad (2)$$

This relationship has been plotted in Figure 2.

The decrease in sensitivity in the presence of steady background light is often referred to as light adaptation or background adaptation. The sensitivity of photoreceptors is also decreased by previous exposure to bright light that bleaches a significant fraction of the visual pigment. It is a common experience that bright light produces a long-lasting desensitization, called bleaching adaptation, that recovers slowly with a time course of many minutes
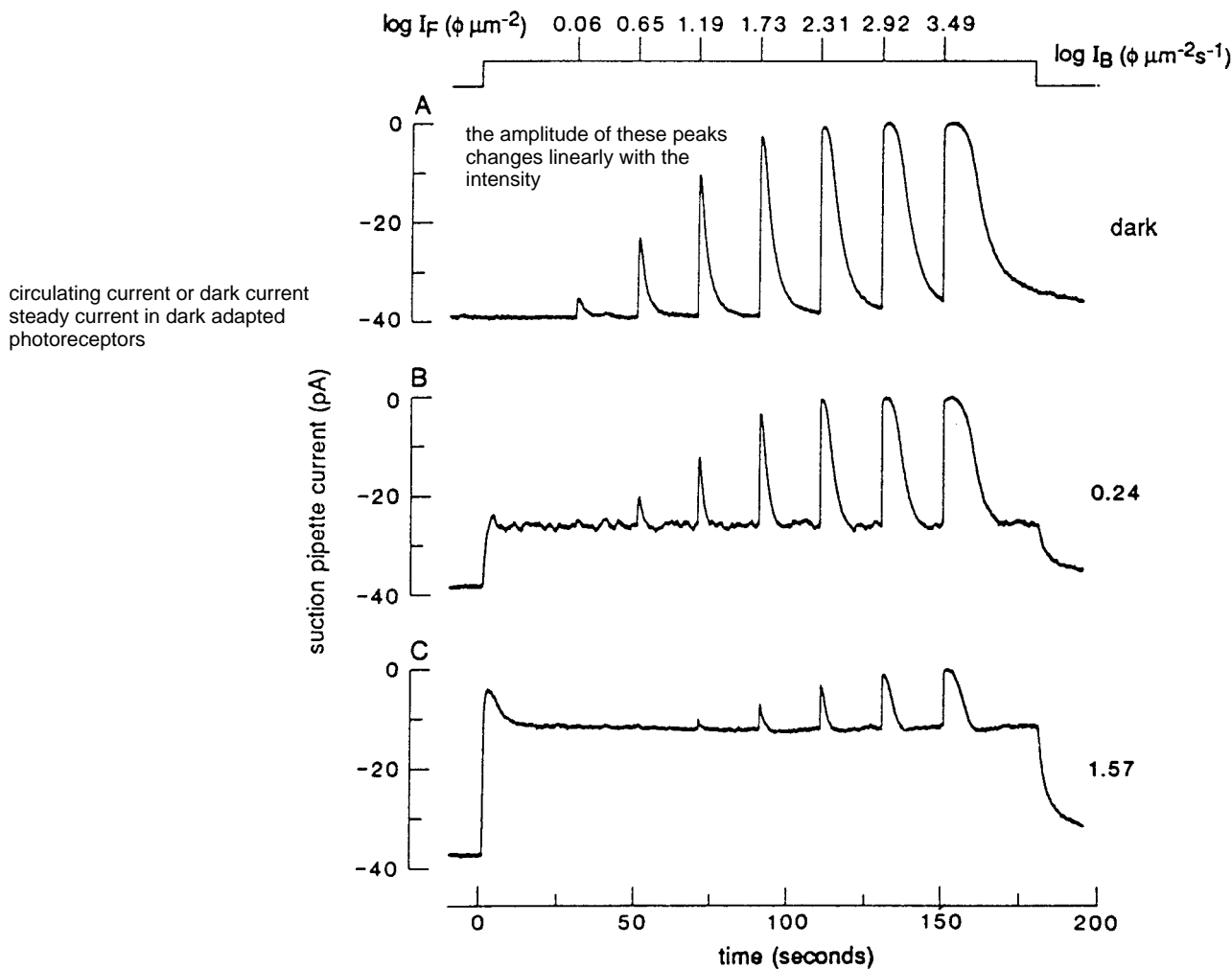
FIG. 1. Light adaptation of responses from a salamander rod recorded with a suction pipette. Timing of flashes and steps of light are indicated by upward deflections in top trace. Intensities of flashes (I_F) are given for the responses in *A-C* by the numbers above each of the flash markers, in units of $\log_{10}$ photons $(\Phi)\cdot\mu m^{-2}$. Flashes increase progressively in intensity with each flash about 4 times as bright as its predecessor, in darkness (*A*) or in the presence of steady backgrounds of intensities 0.24 (*B*) and 1.57 (*C*) $\log_{10}$ photons$\cdot\mu m^{-2}\cdot s^{-1}$. Note decrease in amplitude and speeding of kinetics of the response to any given flash intensity for backgrounds of increasing brightness. [From Matthews (171). Reprinted with the permission of Cambridge University Press.]

during dark adaptation. This process has also been studied in considerable detail by visual psychophysicists (summarized in Refs. 7, 8, 23, 153, 243).

Stiles and Crawford (265) first suggested that light bright enough to produce significant bleaching of the photopigment desensitizes by producing an "equivalent" background light. This equivalent background was later proposed to be produced in some way by bleached pigment and to persist until the pigment was regenerated (see Refs. 7, 8). Although the equivalent background hypothesis was originally proposed without reference to a molecular mechanism, we now know that in single photoreceptors, bleached photopigment can in fact stimulate the visual cascade and produce a steady excitation much like the one produced by steady background light.

In this review, we summarize what is presently known about the molecular mechanisms of both background and bleaching adaptation. We begin by describing visual transduction, since a detailed knowledge of this process is fundamental to our understanding of adaptation. The regulation of key components of the transduction cascade is almost certainly responsible for the changes in sensitivity produced by background light and bleaches. We then describe what we know about adaptation itself, beginning first with the role of Ca^{2+} as a second messenger controlling the sensitivity of the photoreceptor and then presenting recent evidence for possible pathways regulated by Ca^{2+}. Finally, in section IV, we present our current understanding of the mechanism of bleaching adaptation. We describe the evidence for
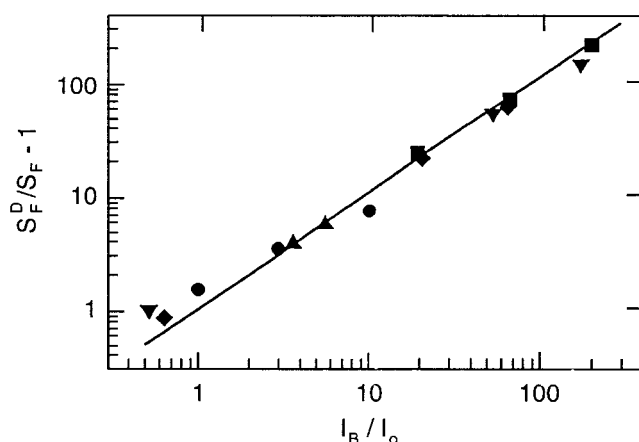
FIG. 2. Flash sensitivity (S_F) of salamander rods as a function of background intensity (I_B). Flash sensitivity has been linearized with *Equation 2*. Each symbol represents data for a different cell. The results have been normalized for each cell to I_o, the background intensity required to decrease the flash sensitivity by a factor of 2. [From Jones et al. (121), by copyright permission of The Rockefeller University Press.]

excitation of the cascade by bleached pigment and then explain how this excitation is believed to occur at a molecular level and how it may be responsible for the changes in photoreceptor sensitivity after exposure to bright light. Some of this material has been the subject of previous articles, which may also be usefully consulted (11, 50, 66, 69, 102, 138, 162, 191, 215, 225, 229–231).

II. VISUAL TRANSDUCTION

A. Rhodopsin

Visual detection begins with the absorption of a photon by the photopigment rhodopsin (summarized in Refs. 101, 102, 215, 233), a member of the G protein-coupled receptor family also including many hormone receptors, odorant receptors, and metabotropic synaptic receptors. These proteins are all integral membrane proteins having seven, mostly α-helical, transmembrane domains (253, 261, 273). Rhodopsin is unusual among the G protein-coupled receptors in that it is bound in darkness (in its inactive conformation) to a small-molecular-weight chromophore, 11-*cis*-retinal, that regulates its activity. The 11-*cis*-retinal is covalently bound via a Schiff base linkage to the terminal (ϵ) amino group of a lysine (Lys-296 in bovine rhodopsin), and this Schiff base linkage is protonated (see Refs. 32, 233). The absorption of a photon by the 11-*cis*-retinal chromophore produces a photoisomerization to all-*trans*-retinal, resulting in a subtle change in the conformation of rhodopsin (73, 75, 261).

The active form of the photopigment is a spectrally distinct intermediate called metarhodopsin II or Rh* (61), and it is this form of the pigment that triggers the transduction cascade (see Fig. 3). Rh* is inactivated by two sequential processes: first the phosphorylation of rhodopsin (21, 146) and then the binding of arrestin to the
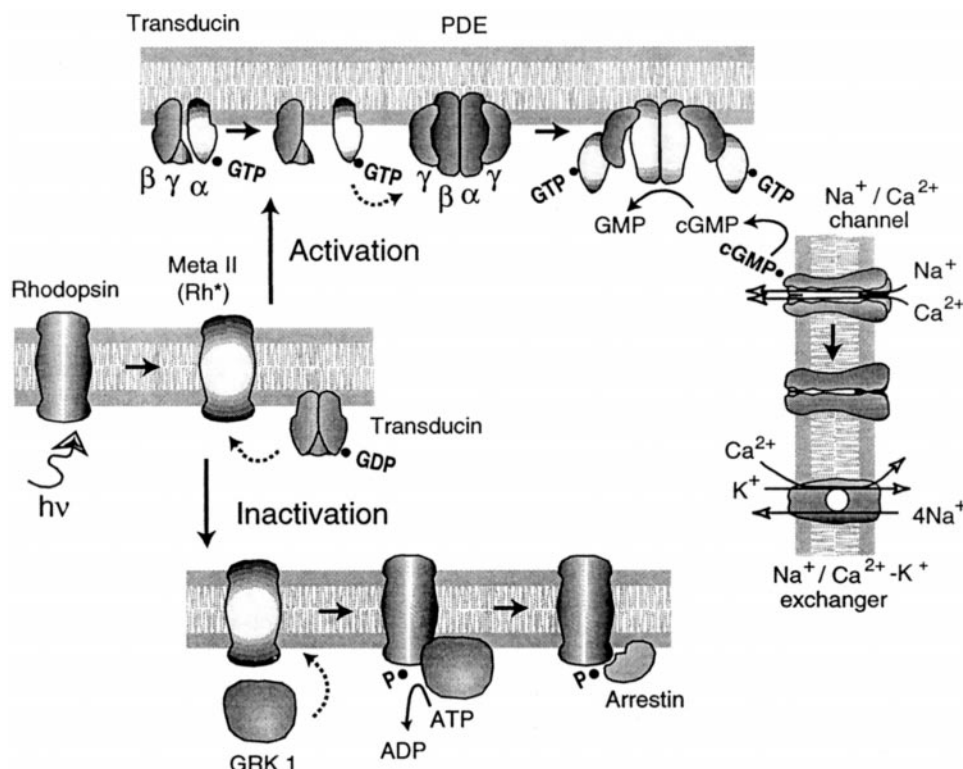

FIG. 3. Schematic diagram illustrating mechanisms of activation and inactivation in vertebrate photoreceptors. [From Fain (65). Copyright © 1999 by The President and Fellows of Harvard College. Reprinted by permission of Harvard University Press.]

phosphorylated photopigment. The COOH terminus of rhodopsin contains seven serines and threonines that are capable of being phosphorylated, and in rod outer segments in vitro, an average of seven phosphorylations per rhodopsin has been observed (281). Nevertheless, it is likely in vivo that the phosphorylation of fewer amino acids, and possibly only of one, at (in the bovine pigment) Ser-334 or Ser-338, normally contributes to rhodopsin turnoff (207). In mouse rods containing COOH-terminal-truncated rhodopsin and lacking Ser-334 and Ser-338, the termination of transduction is greatly retarded (31).

Phosphorylation of rhodopsin can, of itself, reduce the ability of Rh[*] to activate the transduction cascade (see Refs. 158, 284). A complete inactivation of Rh[*], however, requires in addition the binding of either arrestin (143) or perhaps its splice variant p44 (263). Both p44 and arrestin have been shown to bind preferentially to phosphorylated rhodopsin, although p44 also will bind to nonphosphorylated rhodopsin. The binding to rhodopsin turns off the transduction cascade, probably by sterically hindering the binding of the G protein transducin (141, 147, 232). In mouse rods lacking the arrestin gene (and therefore both arrestin and p44), the decay of the photoresponse is greatly prolonged (284). Furthermore, phytic acid, which inhibits the interaction of arrestin and phosphorylated Rh[*], also greatly prolongs the light response (214, 177).

The time course of phosphorylation and arrestin binding determines the lifetime of Rh[*], which probably varies considerably for rods from different species and between rods and cones. Pepperberg et al. (222) have argued from an analysis of the time course of decay of the photoresponse that the lifetime of Rh[*] in tiger salamander rods is on the order of 1–2 s and is rate limiting for the recovery of the photoresponse. A similar value was obtained by Rieke and Baylor (236) from dialyzed rod outer segments; they used the clever technique of abruptly increasing the gain of transduction by presenting elevated GTP concentrations at fixed times after the presentation of a light flash. Other experiments, however, suggest that the lifetime of Rh[*] may be much shorter than this and not rate limiting (176, 249). We shall return to this subject in section III*D*, since regulation of the lifetime of Rh[*] has been proposed to be an important mechanism of sensitivity regulation.

After the inactivation of Rh[*] by phosphorylation and arrestin binding, the photopigment must be regenerated before a new photon can be absorbed. This is a multistep process; the protein moiety (opsin) must be dephosphorylated, and the chromophore must be converted from all-*trans*-retinal back to 11-*cis*-retinal. The retinal in the outer segment is first reduced to all-*trans*-retinol by a membrane-associated all-*trans*-retinol dehydrogenase within the outer segment (99). The chromophore then leaves the photoreceptor and is transported to an adjacent layer of epithelium, called the retinal pigment epi-thelium (or RPE; see Ref. 215). There it is isomerized to the 11-*cis*-isomer and then retransported back to the photoreceptor (see Ref. 44), where it recombines with dephosphorylated opsin. This process of pigment regeneration is often referred to as the visual cycle, and we shall describe some of its features in more detail in section IV*D*.

B. Transducin and Phosphodiesterase

The change in the conformation of rhodopsin exposes groups of amino acids on the cytoplasmic side of the protein that form a binding site for the heterotrimeric G protein transducin. The binding of transducin facilitates an exchange of GTP for GDP on the guanosine nucleotide binding site of the transducin α-subunit, producing the active form T_α·GTP, which binds to and activates a membrane-bound cyclic nucleotide phosphodiesterase (PDE). This enzyme hydrolyzes cGMP and effectively reduces the concentration of this nucleotide in the cytoplasm of the outer segment, decreasing the probability of the opening of cyclic nucleotide-gated channels. The closing of these channels generates the electrical response of the photoreceptor (see Fig. 3).

Transducin resembles other heterotrimeric G proteins in structure and function (see Refs. 17, 20, 96, 100, 202). There are three subunits, called T_α, T_β, and T_γ (or just α, β, and γ), with a stoichiometry of 1:1:1. The rod and cone T_α subunits are distinct molecules but share 80% sequence identity; both are members of the α_i/α_o subfamily of G protein α-subunits. Rods and cones also contain different β-subunits. Inactive $T_{\alpha\beta\gamma}$ is normally membrane bound; after the exchange of GTP for GDP on the α-subunit, the $T_{\beta\gamma}$ remains membrane bound, but the T_α·GTP appears to be less tightly associated with the membrane and may very well be released into the space between adjacent disks (6, 144, 275).

The T_α·GTP then diffuses and binds to a membrane-associated photoreceptor-specific PDE. This protein consists of four subunits (see Refs. 15, 74, 102), two catalytic and two inhibitory. In rods the two catalytic subunits of the PDE are different and are called α and β; in cones, they are apparently identical and called α'. Each catalytic subunit is associated with an inhibitory subunit called γ, but there appear to be different γ in rods and cones (210). In addition, a subunit called δ found in rod but not cone outer segments can bind to the rod $\alpha\beta\gamma_2$ complex and solubilize it, disrupting its normally close association with the disk membrane (78). It is possible that δ somehow regulates PDE activity by removing it from its normal site of interaction with T_α·GTP.

Because the PDE has two inhibitory subunits each of which can bind T_α·GTP (49), and because the catalytic activities of the PDE α- and β-subunits are approximately the same, the gain of the activation of the PDE by T_α·GTP

is 0.5. The PDE then hydrolyzes cGMP with high velocity so that the total gain from Rh* to cGMP in a dark-adapted rod is of the order of 10^5-10^6 molecules hydrolyzed during the lifetime of a single activated pigment molecule (294). This reaction is thought to be terminated as for other heterotrimeric G proteins, when the GTP of $T_\alpha \cdot$GTP is hydrolyzed to GDP. Alternative mechanisms of termination have also been suggested, including inactivation of PDE by excess PDEγ (63) and phosphorylation of PDEγ by a specific kinase, leading to inactivation of PDE even without GTP hydrolysis (271, 272). The significance of these mechanisms is not yet clear.

The rate of hydrolysis of GTP to GDP may be critically important in determining the time course of the photoresponse and may be the rate-limiting reaction that determines the decline of the photocurrent back to the dark-adapted level (249). The rate of hydrolysis of bound GTP is quite slow in vitro, taking many seconds (see, for example, Ref. 3). This is too slow to account for the turn off of transduction, and it is likely that hydrolysis is much more rapid in the rod outer segment (274). Recent experiments indicate that hydrolysis is accelerated in vivo by a membrane-bound factor (1) that acts as a GTPase accelerating protein, or GAP. The most important GAP in photoreceptors seems to be the membrane-associated protein RGS9 (104), which is expressed in rods and at even higher levels in cones (42). RGS9 can by itself produce a large acceleration of the rate of the GTPase hydrolysis, although the rate of hydrolysis is further accelerated by the addition of PDEγ, which is also known to function as a photoreceptor GAP (4, 270). Other RGS proteins called RGS4 (203) and RGS-r or RGS16 (30, 280) have also been reported to be found in retina, but their role in transducin GTPase modulation is less clear.

It is possible that the rate of GTP hydrolysis by $T_\alpha \cdot$GTP is also regulated by noncatalytic binding sites for cGMP on the PDE molecule (286). Both the α- and β-subunits have noncatalytic sites for cGMP binding, and the affinity of these sites for cGMP decreases after PDE activation (41, 285). Because the rate of GTP hydrolysis by $T_\alpha \cdot$GTP is accelerated when cGMP dissociates from these noncatalytic sites, it would be possible to imagine that activation of the PDE by the binding of $T_\alpha \cdot$GTP to PDEγ would decrease the concentration of cGMP in the outer segment, leading to a decrease in the binding of cGMP to noncatalytic sites on the PDE α- and β-subunits and an increase in the rate of hydrolysis of GTP bound to T_α. This would in effect modulate the duration of the light response. Recent evidence suggests, however, that the affinity of the noncatalytic sites for cGMP is so great (the off rate constant is so slow) that release of cGMP from the noncatalytic binding sites would be unlikely to participate either in the photoresponse or in short-term exposure to background light (26). On the other hand, a role for this mechanism during longer exposures to backgrounds or

after bleaches remains a possible mechanism of long-term modulation of sensitivity.

C. Guanylyl Cyclase

The total concentration of cGMP in an amphibian rod is of the order of 30–60 μM (see, for example, Ref. 282), but the free concentration is much smaller than this, of the order of 6 μM (199). Some of the cGMP in the outer segment is bound to noncatalytic sites on the PDE, as we have seen, and some may be bound to other proteins, including the cyclic nucleotide-gated channels. Activation of the PDE hydrolyzes free cGMP, and the free concentration of cGMP declines rapidly. If the rod is to generate a maintained electrical response to steady illumination (see Fig. 1), then the cGMP must be resynthesized as it is hydrolyzed by the PDE so that the free cGMP concentration can reach a steady-state concentration to produce a steady-state probability of opening of the cGMP-gated channels.

The synthesis of cGMP in the rod is largely produced by specialized photoreceptor guanylyl cyclases, present in the rod and cone outer segments. These enzymes are members of a family of membrane-bound cyclases that includes proteins that act as receptors for hormones and peptides, for example, atrial natriuretic peptide (see Refs. 85, 297). Two cyclases have been cloned and identified in photoreceptors: GC-E (retGC-1 or GC-1) and GC-F (retGC-2 or GC-2; see Refs. 166, 262, 287). The two forms of cyclase appear to be present in both rods and cones, but GC-E is apparently more abundant in cones (52, 124); both forms of cyclase can be found in the same cell (288).

One of the most interesting features of the photoreceptor guanylyl cyclase is its dependence on Ca^{2+} concentration. First discovered by Lolley and Racz (165), this dependence was subsequently shown to be a quite steep function of cytoplasmic Ca^{2+}, probably as a result of a cooperative interaction of Ca^{2+} with the enzyme (134). The cyclase activity increases as the Ca^{2+} concentration declines, with a half-maximal activity at ~100–200 nM free Ca^{2+} (52, 82, 86, 134, 136).

There is now excellent evidence that the Ca^{2+} regulation of photoreceptor guanylyl cyclase is produced by novel, small-molecular-weight Ca^{2+}-binding proteins (see Ref. 228). At least two such proteins, called guanylyl cyclase activating proteins or GCAPs, are present in photoreceptor outer segments (see, for example, Refs. 46, 124) and have three Ca^{2+}-binding EF-hand regions (see, for example, Ref. 216), which appear all to be necessary for the proper functioning of the protein (51). The number of Ca^{2+} binding sites of the GCAPs is thought to be responsible at least in part for the high cooperativity of Ca^{2+} regulation of the cyclase. In low free Ca^{2+}, these sites are unoccupied, and GCAP in this form can stimulate

the cyclase. The Ca^{2+}-loaded form, on the other hand, inhibits the cyclase. As we shall see shortly, the Ca^{2+} concentration of the photoreceptor is relatively high in darkness and decreases in the light. Thus the rate of synthesis of cGMP would be expected to be low in darkness and to increase in light, in the same direction as the change in the rate of PDE activity produced by light stimulation.

In addition to GCAPs, a newly discovered protein in amphibians, called guanylyl cyclase inhibitory protein (GCIP), may also participate in cyclase regulation (163). This protein shares conserved sequences with the GCAPs and is of a similar molecular weight, but GCIP does not stimulate guanylate cyclase in low Ca^{2+} as the GCAPs do, although it does inhibit the cyclase when the Ca^{2+} increases. The role of this protein in cyclase regulation is presently unclear. We shall return to the subject of the Ca^{2+} regulation of the cyclase in section III*D*.

D. Cyclic Nucleotide-Gated Channels

Since the discovery of cyclic nucleotide-gated channels (76), many studies have carefully investigated their molecular biology and physiology in photoreceptors and in other cell types (notably olfactory receptor neurons). We give only a brief summary of the most recent experiments, focusing on the modulation of the channels. Excellent summaries of earlier literature are given in Yau and Baylor (289) and McNaughton (184). Comprehensive treatments of the molecular biology and structure/function of cyclic nucleotide-gated channels can be found in Finn et al. (77), Zagotta and Siegelbaum (298), Frings (81), and Molday (192).

Cyclic nucleotide-gated channels in rods may be tetramers, perhaps consisting both of α-subunits and of β-subunits, having different structures. The α-subunit can be expressed by itself to form homomeric channels, whose physiological properties are somewhat different from native channels. Coexpression of both α and β produces channels more closely resembling those in rod outer segments. Cones contain different α-channel proteins (19), and some as yet indirect evidence suggests that they may also have β-subunits different from the ones in rods (97, 235).

Both the α- and β-subunits have binding sites for cyclic nucleotides, and binding is cooperative with a mean affinity constant ($K_{0.5}$) variously reported but of the order of 50 μM and a Hill coefficient also variously reported to be of the order of 2–3 (see, for example, Ref. 199). Recent evidence indicates that the cooperativity suggested by the Hill coefficient is the result of changes in the probability of channel opening as successive cGMPs bind to the channel (239). In homomeric rod α-channels expressed in *Xenopus* oocytes, photoaffinity analogs of cGMP have been used to produce channels with persistently bound nucleotide. These experiments (239) show that channel opening probability increases as successive nucleotide binding sites are filled, but in a way not previously predicted. Each liganded state can produce channel openings to more than one conductance level; however, for each channel open state, the binding equilibrium constant does not increase by a constant factor as each nucleotide binds. This has the consequence that the subunits interact in some way not presently understood to favor openings to smaller conductance levels for partially liganded channels, but to favor openings to the highest conductance state for the fully liganded channel.

The cyclic nucleotide-gated channels of both rods and cones are permeable to a variety of monovalent cations, including Na^+, K^+, and Li^+. In addition, the channels in both rods and cones are permeable to divalent cations, including Ca^{2+}. This was first shown for rods (107, 291), for which the influx of Ca^{2+} through the channels is estimated to be of the order of 10^7 ions per outer segment per second in toad, or ~10–15% of the circulating current entering the outer segment in darkness (198, 293). The channels in cones are also permeable to Ca^{2+} (226, 190) and contribute an even larger fraction of the dark current entering the outer segment (226). Measurements from membrane patches indicate that the ratio of permeabilities for Ca^{2+} and Na^+ (P_{Ca}/P_{Na}) is considerably larger for cone than for rod channels (227) and may vary with the gating of the channel (98).

There is considerable evidence that the channels can be modulated, although the details are still controversial (299). Hsu and Molday (111) first reported that the apparent affinity of the channels for cGMP in isolated rod outer segments was decreased upon addition of exogenous Ca^{2+}/calmodulin. Although this report has been confirmed in several subsequent studies (10, 89, 112), there is no agreement about whether calmodulin is actually the molecule responsible for the modulation in rods, or what role this modulation plays in functioning photoreceptors. Bauer (10) has argued that the affinity of the channels for Ca^{2+}/calmodulin is so high that calmodulin, or a similar small-molecular-weight protein, is likely to remain bound to the channels in the rods except at very low intracellular Ca^{2+} concentration. Changes in Ca^{2+} concentration have been shown to produce changes in the apparent affinity of the channels for cGMP in permeabilized rods, with affinity increasing for decreased Ca^{2+}, but the EC_{50} for Ca^{2+} is ~50 nM for bullfrog (195) and salamander (248). As we shall see in section II*E*, this is very near the limit of the range of Ca^{2+} concentration in the rod cytoplasm and is only reached in saturating bright light.

Quite different results have recently been obtained for cones. Exogenous calmodulin seems to have little or no effect on the cone channels in isolated membrane patches (97, 103), suggesting that different proteins may

mediate modulation in the two kinds of photoreceptors. Furthermore, the EC_{50} for Ca^{2+} modulation in permeabilized cones has been reported to be quite different from that in rods (235); it is apparently of the order of 250–300 nM, within the physiological range of outer segment Ca^{2+} in cones (see sect. IIE and Ref. 250). This would suggest that channel modulation produced by a change in outer segment Ca^{2+} may have greater importance for the normal physiology of cones than of rods.

In addition to modulation by Ca^{2+}, channels in isolated patches have been reported to be affected by diacylglycerol (DAG) analogs (88) and by phosphorylation (87). This could in principle be quite important, but at present, there is no other evidence in support of either DAG or channel phosphorylation as a mechanism in the response or adaptation of photoreceptors (see for example Ref. 283).

E. Calcium

Because the channels in the outer segments of both rods and cones are permeable to Ca^{2+}, and because these channels are partially open in darkness, there is a steady influx of Ca^{2+} into the photoreceptor that is reduced by illumination. Ca^{2+} is transported out of the outer segment by a Na^+/Ca^{2+}-K^+ exchange protein, which is located almost exclusively in the membrane of the outer segment (131, 140). The physiology of this exchanger has been studied in some detail (see Refs. 184, 185, 255). The stoichiometry of exchange is four Na^+ (normally moving inward) for one Ca^{2+} and one K^+ (both normally moving outward) (28). This has the consequence that the exchange is electrogenic, and the extrusion of Ca^{2+} produces an inward current that can be easily recorded from both rods (108, 292) and cones (200, 226).

When the probability of opening of the cGMP-gated channels decreases in the light, the influx of Ca^{2+} into the outer segment also decreases. Na^+/Ca^{2+}-K^+ exchange does not appear to be affected directly by illumination (137, 198) so that Ca^{2+} continues to be transported out of the cell, resulting in a decrease in the intracellular Ca^{2+} (293). The Michaelis constant (K_m) for the exchanger for Ca^{2+} in salamander is of the order of 1–2 μM (150), severalfold higher than the dark resting Ca^{2+} concentration in a rod or cone (see below). This has the consequence that the rate of Ca^{2+} extrusion is nearly proportional to the Ca^{2+} concentration over most of the physiological range, and Ca^{2+} efflux continues in the presence of a steady light until influx and efflux are equal and the Ca^{2+} concentration reaches a new steady level, lower than its value in darkness.

The light-induced decrease in Ca^{2+} has been demonstrated directly, first in isolated salamander rods with aequorin (150, 186) and then in populations of photoreceptors with fluorometric dyes in the isolated retina of toad (135) and bullfrog (182, 183, 234, 296). Subsequently, outer segment Ca^{2+} was measured from isolated outer segments during simultaneous current recording with indo 1 dextran from *Gekko* rods (93) and from isolated intact salamander rods with fluo 3 (251). These experiments indicate that Ca^{2+} declines from ~500–700 nM in darkness to ~30–50 nM in saturating light, corresponding to a change in the free Ca^{2+} concentration of ~10- to 20-fold.

Upon the presentation of saturating light and sudden closure of all of the cGMP-gated channels, the Ca^{2+} concentration in the outer segment declines with two exponential components, which in both *Gekko* and salamander have time constants of several tenths of a second and several seconds. The monotonic decline of Ca^{2+} in the light, together with electrical measurements of channel and transport activity, suggest that the major mechanisms responsible for determining the Ca^{2+} concentration in the rod are influx through the cGMP-gated channels and efflux from Na^+/Ca^{2+}-K^+ exchange.

There is, however, some reason to suppose that there may be other mechanisms of Ca^{2+} homeostasis in photoreceptors, although their relationship to transduction remains unclear. Rod outer segments have been reported to contain large amounts of Ca^{2+} (70, 257, 260), although this claim has been disputed (264). This Ca^{2+} has been shown to behave as if it were bound very tightly within the cytosol or held within a sequestered pool (70, 71, 256, 257), from which it can be released by light (72, 260). This light-induced release is controversial, and its mechanism is unclear. Although rod outer segments appear to contain one of the isoforms of PLC-β (PLC-β4) (see Ref. 219), the enzyme that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and DAG and thus might evoke Ca^{2+} release, there are few if any IP_3 receptors in rods (48), and the knocking out of PLC-β4 has been reported to have little effect on the receptor light response (117). On the other hand, a light-induced release of Ca^{2+} would be consistent with Ca^{2+} electrode measurements of the Ca^{2+} concentration in the extracellular spaces around the rods in isolated rat retina (133). These measurements show that light bright enough to close all the channels in the rods produces a steady increase in the extracellular Ca^{2+} that persists even 5 min after the beginning of a saturating stimulus. This increase disappears after the light is turned off and Ca^{2+} reenters the rod, but less Ca^{2+} reenters than was extruded, resulting in a decrease in total rod outer segment Ca^{2+} (as in Ref. 260). Finally, recent experiments indicate that bright bleaching lights can produce a release of Ca^{2+} within the photoreceptor outer segment (68). The function of this Ca^{2+} release is not yet clear.

In rods in bright light, Ca^{2+} declines to about 30–50 nM (93, 182, 251). This in itself is surprising, since the

exchanger could theoretically reduce Ca^{2+} below 1 nM (28). One explanation for this difference is that the exchanger is inhibited at low Ca^{2+} concentrations (259), but it is also possible that there is a steady release of Ca^{2+} from some internal compartment in the presence of bright light (258, 260).

Although Na^{+}/Ca^{2+}-K^{+} exchange is apparently the dominant mechanism of Ca^{2+} transport in the rod outer segment plasma membrane, the inner segment plasma membrane appears not to contain the Na^{+}/Ca^{2+}-K^{+} exchange protein (131) and instead extrudes Ca^{2+} with a Ca^{2+}-ATPase (140, 193). A Ca^{2+}-ATPase protein has been localized by immunohistochemistry to the inner segment and synaptic terminal but is apparently absent from the plasma membrane of the rod outer segment (140, 193). It would appear therefore that the inner and outer segments of rods regulate Ca^{2+} through different mechanisms and represent functionally distinct pools of Ca^{2+}, since there may be little movement of Ca^{2+} between these two parts of the cell (140).

Much less is known about Ca^{2+} in cones, in part because their much smaller outer segments make optical measurements with fluorometric dyes more difficult. The first direct measurements of Ca^{2+} in cones have recently been made from salamander by Sampath et al. (250). Some interesting differences between rods and cones have emerged from this work. *1*) The Ca^{2+} concentration is less than in rods (mean values were 410 nM in the dark, 5.5 nM in saturating illumination). Because the Ca^{2+} concentration falls to a lower level in the cone than in the rod, either the exchange proteins in the two cells are different, and/or there is some difference in the release of Ca^{2+} from buffers or internal stores in the two cell types. *2*) The Ca^{2+} concentration in salamander cones changes by nearly 100-fold from darkness to saturating light, a greater range than for rods from the same species. This means that any transduction reactions regulated by Ca^{2+} could in theory be modulated to a greater extent in cones than in rods. *3*) The time constants of Ca^{2+} decay are biexponential in cones as in rods but are much faster in cones, averaging 43 and 640 ms for red-sensitive cone photoreceptors. The amplitude of the fast time constant (the coefficient multiplying the exponential term) is significantly larger than that of the slow one (the amplitudes of these two time constants are nearly equal for rods). Blue-sensitive cones have somewhat slower time constants of decline, corresponding to the slower kinetics of their photoresponses (226), but the time constants of decay of Ca^{2+} in blue-sensitive cones were still a factor of two faster than in rods. The reason for the faster decay of Ca^{2+} in cones is unknown but may be the result of the greater surface-to-volume ratio of cone outer segments, or some difference in the properties of the exchange proteins or Ca^{2+} buffering or sequestration in the two kinds of photoreceptors (see Ref. 190).

One difficulty of making Ca^{2+} measurements from photoreceptors with fluorometric dyes is that light bright enough to elicit dye fluorescence will also produce considerable bleaching of the photopigment. This makes repeated measurements from the same rod almost impossible with present methods. For cones, however, the photopigment can be regenerated with exogenous 11-*cis*-retinal rapidly enough to measure the Ca^{2+} concentration from the same cell repeatedly, for example, in the presence of steady backgrounds over a range of light intensities. This has made it possible to make a direct correlation between circulating current and Ca^{2+} concentration in cones (250). Within the limits of experimental measurement, these two appear to be linearly related, with the free Ca^{2+} concentration declining in parallel with the circulating current. There seems therefore to be no nonlinearity in the relationship between photocurrent and Ca^{2+} in salamander cones like the one reported by Gray-Keller and Detwiler for *Gekko* rods (93), and it is possible that the homeostasis of Ca^{2+} in cones is simpler than that in rods, for reasons that are still unclear.

III. LIGHT ADAPTATION

A. Requirement for an Internal Messenger

Background adaptation in rods is mediated by a cytoplasmic messenger. Although it would be possible to imagine a mechanism for desensitization that was very local and restricted to single disks or even to small subregions of the disk membrane, several observations indicate that this does not occur. In the first place, rods are desensitized by very dim steady illumination; in amphibians the intensity of background light necessary to reduce the sensitivity of the receptor by one-half (I_0; see *Eq. 1*) is of the order of only 5–10 rhodopsins bleached per second (9, 14, 57, 64, 156). Because sensitivity reaches steady state after a few seconds of steady light, only a few tens of pigment molecules need be excited to reduce sensitivity by a factor of two, and because amphibian rods have of the order of 1,000–2,000 disks, some molecule must diffuse between the disks so that a pigment molecule bleached in one disk can affect the response produced by subsequent pigment molecules bleached in other disks (57, 9). For mammalian rods, I_0 is larger: 35 Rh^{*} s^{-1} in cats (267), 20–50 Rh^{*} s^{-1} in guinea pigs (172), and 30–50 Rh^{*} s^{-1} in primates (268). However, because measurements from mammalian rods are made at a higher temperature, the integration time of the receptor is considerably less, and steady state is reached more quickly. The same arguments used for the spread of desensitization in amphibian rods would therefore also apply to mammalian photoreceptors.

The spread of light adaptation has been measured

directly by illuminating the outer segment either at the wavelength of peak sensitivity with narrow slits of light (156, 36) or at a much longer wavelength using two-photon excitation (92). These measurements give a space constant for the spatial spread of desensitization of ~5–10 μm in amphibian rods, larger than can be accounted for by light scatter from the slit. Using a different technique, Hemila and Reuter (106) illuminated rods obliquely to produce a preferential absorption of light by the distal end of the outer segments. They then compared adaptation produced by this stimulus with adaptation produced by homogeneous illumination and estimated that desensitization spreads 5–20 μm from the site of photon absorption. These experiments all support the requirement for an internal messenger in light adaptation, at least in rods. Comparable measurements have not been attempted in cones, but the similarity of the effects of background illumination on the two kinds of photoreceptors suggests that desensitization in cones may occur by a similar mechanism.

B. A Change in Intracellular Ca^{2+} Is Necessary for Background Adaptation in Both Rods and Cones

Bownds (22) first proposed that steady light might produce a maintained decrease in the intracellular Ca^{2+} concentration that in some way regulates the transduction cascade to produce desensitization of the photoreceptor response. A role for Ca^{2+} in light adaptation was subsequently indicated by the experiments of Torre et al. (269), who used whole cell patch recording to incorporate the Ca^{2+} buffer BAPTA into salamander photoreceptors. BAPTA incorporation produces an increase in the maximum amplitude of the light response and a marked retardation of its decay without affecting the initial time course of the response. These changes in amplitude and kinetics are effectively the inverse of the effects of background light on the response waveform (12, 14). It is therefore plausible that, by slowing the decrease in internal Ca^{2+} concentration, BAPTA retards the changes in sensitivity and response waveform that are normally responsible for desensitization. Similar experiments on amphibian cones (180) and mammalian rods (172) gave comparable results, thus indicating that Ca^{2+} is also likely to play a role in the control of response sensitivity and kinetics in these photoreceptors.

Clear evidence for Ca^{2+} as an internal messenger for light adaptation emerged from experiments which attempted to prevent or greatly reduce both the influx and efflux of Ca^{2+} from the photoreceptor outer segment and thereby minimize light-induced changes in intracellular free Ca^{2+} concentration. The outer segment was exposed to a solution that contained a lowered concentration of Ca^{2+} (to reduce Ca^{2+} entry) and no Na^+ (181, 197).

Because Na^+/Ca^{2+}-K^+ transport requires external Na^+, the absence of Na^+ should prevent or greatly reduce Ca^{2+} efflux. Control experiments showed that when rods (67) and cones (180) are exposed to such a low-Ca^{2+}/zero-Na^+ solution with either Li^+ or guanidinium$^+$ as a Na^+ substitute, a circulating current can be recorded that is reasonably stable over a 5- to 10-s interval. This contrasts with the rapid increase in circulating current that ensues when the outer segment is exposed to zero-Ca^{2+} solution in the presence of Na^+, the majority of which originates from a reduction in cytoplasmic Ca^{2+} concentration via Na^+/Ca^{2+}-K^+ transport (154). Thus the relative stability of circulating current in low-Ca^{2+}/zero-Na^+ solution is a good indication that Ca^{2+} is remaining nearly constant with time, since stability of photocurrent implies stability of cGMP concentration, which in turn is an excellent indication of stability of Ca^{2+} because of the strong dependence of the cyclase rate on intracellular Ca^{2+} (see sect. II*C*).

Even greater stability of photocurrent and Ca^{2+} can be achieved by using solutions containing the impermeant ion choline$^+$ instead of Li^+ or guanidinium$^+$, and by removing Mg^{2+} from the bathing solution (168, 173–175). Under these conditions, there appears again to be little influx or efflux of Ca^{2+} and also Mg^{2+}, which may, like Ca^{2+}, affect the enzymes of the transduction cascade (see, for example, Ref. 136). With the impermeant ion choline$^+$ substituted for Na^+, the current through the cGMP-gated channels is outward instead of inward, presumably carried by K^+ (290). Cells seem to survive longer in this solution than in Li^+- or guanidinium$^+$-substituted solution, perhaps because Li^+ or guanidinium$^+$ enters the outer segments and accumulates to toxic levels, since the photoreceptors may lack a mechanism for specifically removing them.

In low-Ca^{2+}/zero-Na^+ solution, the adaptation of both rods and cones to background light is eliminated (67, 180, 181, 197, 268). One way of demonstrating this is shown in Figure 4 (67). Figure 4*A* compares the responses of a rod to a brief flash in Ringer solution (labeled R) and in low-Ca^{2+}/zero-Na^+ solution (larger response). Exposure to the low-Ca^{2+}/zero-Na^+ solution produced an increase in the peak amplitude of the light response and a marked prolongation of the time to peak and slowing of the decay phase, much as after BAPTA incorporation (172, 180, 269). A similar prolongation of the light response is also observed in mammalian rods exposed to low-Ca^{2+}/zero-Na^+ solution (268). If the alteration in the waveform reflects the removal of a component that desensitizes the transduction cascade, then responses in low-Ca^{2+}/zero-Na^+ solution should sum linearly with no evidence of gain adjustment other than through response compression. Response compression is the decrease in response amplitude and sensitivity, produced simply by the instantaneous reduction in the probability of channel
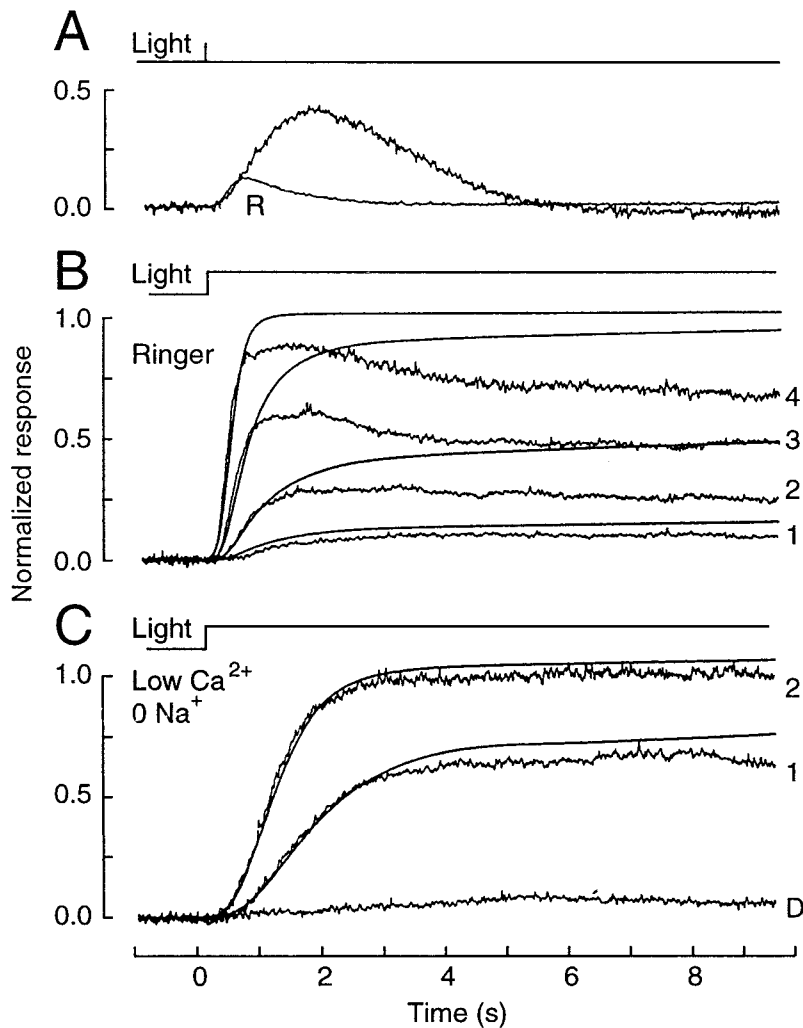
FIG. 4. Prediction of step responses from flash responses for a salamander rod. *A*: flash responses in Ringer solution (labeled R) and in low-Ca^{2+}/zero-Na^+ solution. Flash intensity for both responses was 2.8 photons$\cdot\mu m^{-2}$. *B* and *C*: responses to steps (noisy traces) and predictions (smooth traces) in Ringer solution (*B*) and in low-Ca^{2+}/zero-Na^+ solution (*C*). Predictions were calculated from the flash responses in *A* by integration (after scaling according to the measured step intensity) and compression (according to the measured response-intensity relation) (see text and Ref. 67). Intensities of steps for *traces D* and *1–4* were 0 (dark), 2.4, 9.1, 37, and 140 photons$\cdot\mu m^{-2}\cdot s^{-1}$. Traces have been normalized according to the circulating current in darkness. [From Fain et al. (67).]

closing, as the response-intensity curve saturates and progressively fewer channels remain open and available to close.

The notion that exposure to low-Ca^{2+}/zero-Na^+ solution eliminates adaptation is tested in Figure 4, *B* and *C*, in the following way. The responses to flashes in Figure 4*A* were corrected for response compression, scaled according to the flash and step intensities, integrated with respect to time, and then compressed according to the exponential saturation relation. These predicted step responses in the absence of adaptation (smooth traces) were then compared with responses to steady light (noisy traces). In normal Ringer solution (Fig. 4*B*), the recorded responses initially rose according to the predicted curves but then sagged below them at later times, corresponding to the onset of light adaptation (12). In contrast, when exposed to low-Ca^{2+}/zero-Na^+ solution (Fig. 4*C*), the compressed integral of the flash response came close to predicting the responses to steps of light, as if the adaptation to the steady light produced in normal Ringer so-

lution was essentially eliminated. Similar results have also been obtained for cones (180).

The relaxation in the response to steps in normal Ringer solution is a well-established manifestation of light adaptation (12) that results in the characteristically shallow response-intensity relation for steady light, illustrated for salamander rods in Figure 5*A* by the solid symbols. In contrast, when steady light is presented in low-Ca^{2+}/zero-Na^+ solution, and this relaxation is abolished, the response rises more steeply as a function of intensity (Fig. 5*A*, open symbols) and can be fitted by the exponential saturation relation (181), which in Ringer solution is appropriate only for responses to flashes or to steps during the early rising phase before the onset of adaptation (157).

Another way to investigate the effect of superfusion with low-Ca^{2+}/zero-Na^+ solution is to measure changes in sensitivity from flashes delivered in darkness and during exposure to backgrounds of increasing intensity (181). The curves in Figure 5*B* compare the normalized flash sensitivity (S_F/S_F^D) as a function of steady intensity for
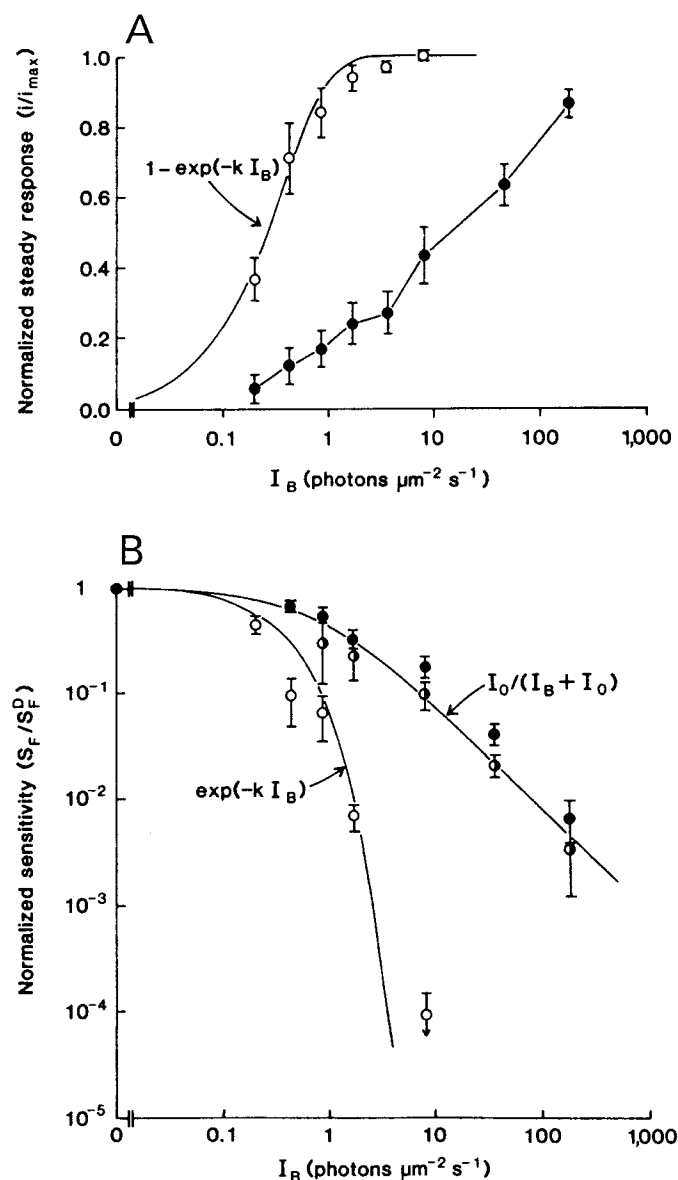
FIG. 5. Normalized response-intensity curves for steady responses (*A*) and incremental sensitivity (*B*) as functions of background intensity (photons·μm^{-2}·s^{-1}) for salamander rods. Solid circles are from cells in Ringer solution, and open circles are from cells superfused with low-Ca^{2+}/zero-Na$^+$ solution before presentation of the background light. Half-filled circles (in *B*) are control experiments, for which rods were exposed to the background light before superfusion with low-Ca^{2+}/zero-Na$^+$ solution. In *A*, steady photocurrent response (i) has been normalized to the maximal current response (i_{max}) obtained with a saturating bright steady light, and in *B*, flash sensitivity (S_F) has been normalized to the sensitivity in the appropriate solution in darkness (S_F^D). The curve in *A* for the open circles is $1 - \exp(-kI_B)$, with k^{-1} equal to 0.38 photons·μm^{-2}·s^{-1}. The curves in *B* are as follows: for the open circles, $\exp(-kI_B)$ with the same value of k as for the open circles in *A*; and for the half-filled and closed circles, $I_0/(I_B + I_0)$ with I_0 equal to 0.8 photons·μm^{-2}·s^{-1}. Error bars give ±SD, and downward arrow indicates upper limit for responses too small to be measured above the noise of the recording. [From Matthews et al. (181). Copyright (1988) Macmillan Magazines Limited.]

rods in normal Ringer solution (solid symbols) and in low-Ca^{2+}/zero-Na$^+$ solution (open symbols). In normal bathing solution, the sensitivity decreases with increasing

steady intensity according to Weber's law (*Eq. 1* in sect. I). In low-Ca^{2+}/zero-Na$^+$ solution, on the other hand, sensitivity declines much more steeply. The change in sensitivity can be adequately fit by the derivative of the response-intensity curve in low-Ca^{2+}/zero-Na$^+$ solution from Figure 5*A*. Thus the precipitous decline in sensitivity that is seen under these conditions is not the result of any feedback modulation of the transduction cascade but instead is caused simply by response compression. Background light closes a fraction of the channels in the outer segment, and this fraction increases as the background intensity increases. This leaves fewer channels available to close. It is of some interest that a relatively dim light in low-Ca^{2+}/zero-Na$^+$ solution is sufficient to close all of the channels and completely saturate the rod, whereas in normal bathing solution the dynamic range of the photoreceptor is greatly extended by mechanisms that the results in Figure 5 show to be dependent on a change in the Ca^{2+} concentration in the outer segment. Similar results have also been obtained for cones (180, 197), indicating that Ca^{2+} plays a broadly similar role in the adaptation of both types of photoreceptor.

If exposure to low-Ca^{2+}/zero-Na$^+$ solution can be assumed greatly to retard changes in outer segment Ca^{2+} concentration, as is argued above, then the results in Figures 4 and 5 show that a change in Ca^{2+} is necessary for light adaptation to occur. In the absence of a change in Ca^{2+}, the photoreceptors simply sum single photon responses with no modulation of the transduction mechanism until all of the cGMP-gated channels are closed and the photoreceptor is saturated. The only change in sensitivity that the photoreceptors show under these conditions is due to response compression, that is, to the nonlinearity of the response-intensity curve. Thus Ca^{2+} appears to function as an internal messenger that regulates the sensitivity of the transduction mechanism.

C. Is a Change in Ca^{2+} Sufficient?

Is Ca^{2+} the only messenger responsible for adaptation? One way of asking this question is to ask whether changes in Ca^{2+} are sufficient to produce light adaptation in a photoreceptor. This question would seem fairly easy to answer but is in fact more complicated than it initially appears. The principal reason for this difficulty is that the transduction mechanism is influenced by Ca^{2+} via a number of powerful feedback mechanisms that influence cGMP homeostasis and that are described in detail in section III*D*. Any reduction in Ca^{2+} imposed in darkness leads to an acceleration in the rate of cGMP synthesis by guanylyl cyclase (see sect. II*C*), and this produces an increase in cGMP concentration and a corresponding elevation in the circulating current. If the extracellular (9, 164) or cytoplasmic (67, 174, 205) Ca^{2+} concentration is

maintained in darkness at a substantially reduced level corresponding to exposure to steady light of moderate to high intensity, then a considerable increase in circulating current results (67), which is accompanied by supralinearity of the response to light (9, 174). These changes, which are presumably due to electrical and biochemical nonlinearities, alter the waveform and sensitivity of the flash response in ways that are difficult to interpret. These difficulties can be avoided in part by recording under voltage clamp and by making only a modest reduction in external (and hence internal) Ca^{2+} concentration (94). Nevertheless, any direct comparison between the effects of Ca^{2+} and steady light must inevitably be restricted to a fairly small reduction in Ca^{2+} concentration, corresponding to relatively dim intensities of steady light.

An approach that avoids this complication and that allows the entire range of adapting intensities to be addressed is to use the time spent in saturation by the response to a bright flash of constant intensity to probe the sensitivity of the transduction mechanism and to examine its dependence on Ca^{2+} and light. The way in which this can be done is illustrated by the recordings in Figure 6*A*, made in normal Ringer solution. First, the rod is allowed to adapt to steady light, then a bright flash is delivered, sufficient to hold the response in saturation for several seconds. As the intensity of the background is increased, the time spent in saturation by the bright flash response progressively decreases as the cell adapts (67, 174, 223). However, if the rod is maintained in low-Ca^{2+}/ zero-Na^+ solution throughout the time that the steady light is present, then this shortening of the time spent in saturation is abolished (67). This observation demonstrates that a change in Ca^{2+} concentration is necessary for this particular manifestation of adaptation over the full range of background intensities, instead of only the rather modest intensity that is able to saturate the electrical response when the outer segment is stepped to low-Ca^{2+}/zero-Na^+ solution in darkness (see Fig. 4*C*).

The progressively earlier onset of the recovery from saturation of the response in Ringer solution during such a step-flash protocol seems likely to represent actions of Ca^{2+} principally on early stages in the transduction cascade which sense the Ca^{2+} concentration at or near the time of the flash (174–176). The reason for this is that the kinetics of Ca^{2+} extrusion are sufficiently rapid in normal Ringer solution that by the time the response to the flash begins to recover from saturation, the cytoplasmic Ca^{2+} concentration will have fallen greatly from its value in darkness or moderate background illumination to levels approaching those attained during steady saturating light (93, 108, 251, 293). Hence, any process (such as modulation of the rate of guanylyl cyclase) that can also be influenced by Ca^{2+} at later times during the response will be maximally affected by the time the response begins to recover from saturation, and thus would have essentially
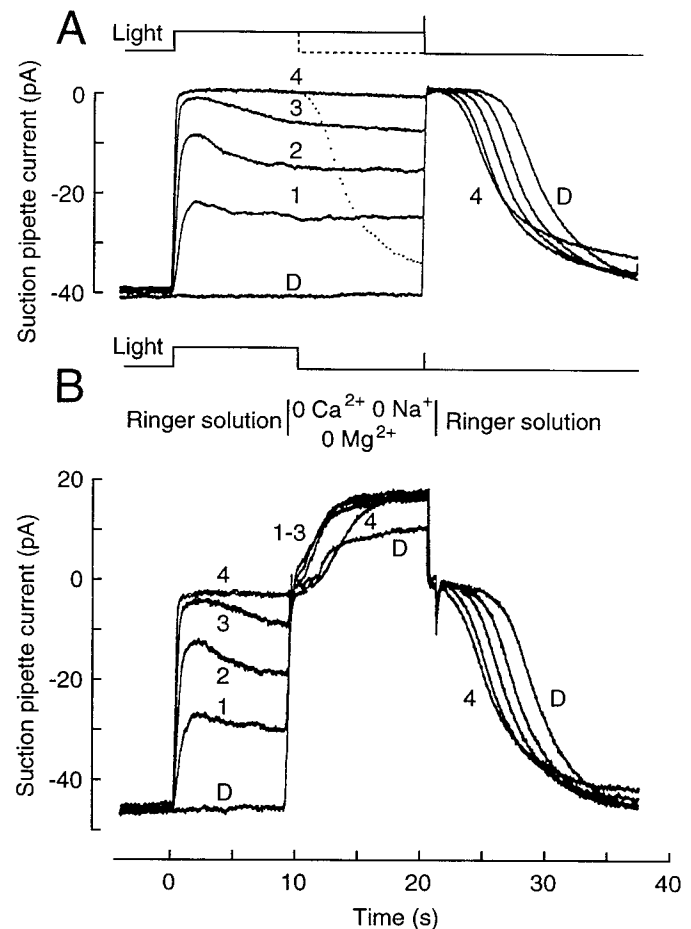

FIG. 6. Response recovery for salamander rods in Ringer solution and in low-Ca^{2+}/zero-Na^+ solution with increasing steady background intensity. Each trace is the average of two responses obtained at the start and two at the end of each experiment. Light monitors above traces indicate timing of 20-ms saturating flashes, of intensity 1,600 photons·μm^{-2}, and of steady backgrounds. For *traces 1–4*, background intensities were 4.36, 20.6, 93.7, and 473 photons·μm^{-2}·s^{-1}; for *trace D*, there was no background exposure. *A*: responses recorded in Ringer solution. *B*: recordings begun in Ringer solution; at the time indicated, the rod was perfused with a zero-Ca^{2+}/zero-Mg^{2+}/zero-Na^+ solution in which choline was substituted for Na^+. This would have the effect of holding the intracellular free Ca^{2+} near to its level in darkness (*trace D*) or its presumably lower level in each of the backgrounds for the duration of exposure to the solution. Traces have been corrected by subtraction of junction currents. [From Matthews (174).]

the same effect on response recovery regardless of the intensity of the preexisting background. Consequently, only events occurring near the time of the flash would be likely to affect the time at which recovery from saturation commences.

To assess whether a reduction in Ca^{2+} concentration alone is sufficient to evoke this change in the time course of recovery to a saturating flash, the procedure illustrated in Figure 6*B* was adopted (174). First the rod was allowed to adapt to the steady light and then the outer segment was exposed to zero-Ca^{2+}/zero-Na^+ solution, thereby holding the Ca^{2+} concentration near the appropriate light-

==highlight==induced level for that particular intensity. The steady light was then extinguished and the rod remained in darkness for a period that would have been sufficient for substantial recovery from even the brightest background (interrupted curve, Fig. 6*A*). Finally, a bright flash was delivered and the outer segment was returned to normal Ringer solution before the response began to recover. Comparison of the two panels in Figure 6 reveals the striking observation that the onset of response recovery was speeded to an equivalent degree by each steady intensity irrespective of whether the background was actually present at the time of the bright flash. In other words, the decrease in Ca^{2+} concentration induced by the background is sufficient to evoke this particular manifestation of adaptation without a requirement for light itself (174).==

Another manifestation of light adaptation is the alteration of the time course of responses to dim flashes (12). When dim flashes are presented during steady background light, the response kinetics are considerably accelerated, corresponding to both a shortening of the time to peak of the response and a speeding of the subsequent recovery (12, 14, 64). Is this characteristic change in the dim flash response solely due to the change in Ca^{2+} concentration and the cyclic nucleotide economy of the photoreceptor, or might some further feedback messenger also be involved?

One way to address this question is to illuminate a photoreceptor with background light while preventing changes in Ca^{2+} concentration and in cyclic nucleotide economy, and to examine the consequences for the dim flash response. Changes in Ca^{2+} can be minimized by superfusing the outer segment with zero-Ca^{2+}/zero-Na^+ solution. The light-induced changes in cyclic nucleotide economy can be counteracted by reducing the activity of the PDE (which is accelerated by the background light) to close to the initial dark-adapted level with the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX).

An example of such an experiment is shown in Figure 7. In Figure 7, *trace DI*, both Ca^{2+} concentration and PDE activity were maintained near their original dark-adapted levels by first stepping the outer segment to zero-Ca^{2+}/zero-Na^+ solution and then presenting bright steady light. The outer segment was then exposed to zero-Ca^{2+}/zero-Na^+ solution containing IBMX at a concentration sufficient to return the circulating current, and hence, the cGMP concentration, to approximately its original level in darkness. During the exposure to this solution, only relatively modest and gradual changes in circulating current were observed (174), indicating that Ca^{2+} concentration and the cyclic nucleotide economy remained relatively stable during this period. Because the Ca^{2+} concentration was maintained near its dark-adapted level, the rate of cGMP synthesis by guanylyl cyclase will also have remained unchanged. Consequently, the restoration of the dark current implies that PDE activity was reduced by
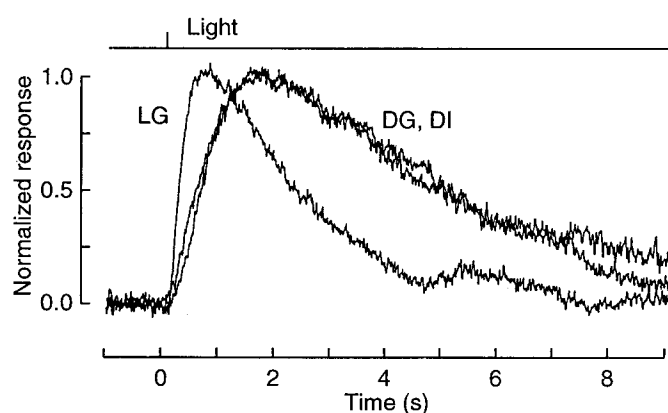

FIG. 7. Comparison of flash response waveform in darkness and in steady background light for a salamander rod. For *trace DG*, response was recorded in darkness after exposure to zero-Ca^{2+}/zero-Mg^{2+}/zero-Na^+ solution, to hold the intracellular free Ca^{2+} concentration near its dark-adapted level. For *trace DI*, the rod was also first exposed to zero-Ca^{2+}/zero-Mg^{2+}/zero-Na^+ solution to hold the intracellular free Ca^{2+} concentration near its dark-adapted level; the rod was then exposed to background light in the presence of 500 μM 3-isobutyl-1-methylxanthine (IBMX; *trace DI*) to inhibit the phosphodiesterase (which was stimulated by the steady light) to bring its velocity near to that in darkness. For *trace LG*, the perfusion with the zero-Ca^{2+}/zero-Mg^{2+}/zero-Na^+ solution was begun after turning on the background light so that the intracellular free Ca^{2+} concentration would have been held at a lower level than for *traces DG* and *DI*. Steady light delivered 70.7 photons$\cdot\mu m^{-2}\cdot s^{-1}$ for *trace DI* and 33.2 photons$\cdot\mu m^{-2}\cdot s^{-1}$ for *trace LG*. Flashes suppressed around one-third of the circulating current in each case and delivered 1.42 photons$\cdot\mu m^{-2}$ for *trace DG*, 121 photons$\cdot\mu m^{-2}$ for *trace DI*, and 31.5 photons$\cdot\mu m^{-2}$ for *trace LG*. In each case, a record obtained under the same conditions in the absence of the flash has been subtracted from the flash response to correct for slow changes in circulating current. Responses have been normalized to the same peak amplitude. [From Matthews et al. (174).]

IBMX to around its dark-adapted level. Under these conditions, the waveform of the dim flash response was similar to *trace DG* in Figure 7, which was obtained in zero-Ca^{2+}/zero-Na^+ solution in darkness, even though for *trace DI* the rod was continuously exposed to light. This is despite the fact that in Figure 7, *trace DI*, a considerably brighter flash was required to evoke a criterion response than in *trace DG*, presumably because of the inhibition by IBMX of the increase in PDE activity evoked by the flash. This constancy of response kinetics contrasts with the acceleration of both time to peak and recovery seen in Figure 7, *trace LG*, in which the outer segment was exposed to zero-Ca^{2+}/zero-Na^+ solution after adaptation to steady light so that the free Ca^{2+} concentration was permitted to decline before it was maintained at a reduced level. The preservation of dark-adapted response kinetics even in the presence of bright background illumination implies that steady light per se is unable to alter the kinetics of the dim flash response, provided there is no change in Ca^{2+} concentration or cGMP economy (174).

An alternative approach to investigate this same question was adopted by Gray-Keller and Detwiler (94).

They compared the dim flash response in isolated voltage-clamped outer segments after adaptation to steady light with that recorded from outer segments in which the cytoplasmic Ca^{2+} concentration had been reduced artificially in darkness. In each case, the mean Ca^{2+} concentration was carefully measured with the fluorescent dye indo 1 dextran, so as to allow the responses to dim flashes to be compared for an equivalent reduction in Ca^{2+} concentration in the presence and absence of steady light. They found that while steady light both decreased the activation gain of the transduction process and speeded the recovery of the dim flash response, similar reductions in Ca^{2+} concentration in darkness only desensitized the rod without accelerating recovery. They interpreted these results as indicating that adaptational changes in the response are not due purely to feedback effects on recovery (94) and that light is needed over and above any change in Ca^{2+} for the speeding of the response recovery, suggesting that Ca^{2+} might not be the only messenger of adaptation (92).

How might this apparent discrepancy between these two approaches arise? We propose the following explanation. In the experiments of Gray-Keller and Detwiler (94), the intracellular free Ca^{2+} concentration was lowered artificially in darkness, which will have stimulated the synthesis of cGMP by guanylyl cyclase. Although this will have resulted in an increase in cGMP concentration and a corresponding increase in cGMP turnover, there will have been no change in the steady activity of the PDE itself, since background light was not present. This is an important distinction, since the increase in PDE activity induced by steady light turns out to be crucial for the acceleration of the recovery of the responses to dim flashes during background adaptation. The mathematical basis for this assertion is given in section VI (see in particular *Eqs. A14* and *A17*) but is summarized here in qualitative terms.

The steady increase in PDE activity induced by the background (β_b) speeds the rate constant which governs the delay between the transient increase in PDE activity induced by the flash and the resulting fall in cGMP concentration (see sect. VI, *Eq. A14*). This rate constant is accelerated further in absolute terms when the Ca^{2+} concentration is allowed to change during the response to the flash itself, through the actions of the dynamic fall in Ca^{2+} on guanylyl cyclase (see sect. VI, *Eq. A17*). Thus the steady increase in PDE activity allows the cGMP concentration, and hence the photocurrent, to follow ever more closely the flash-induced waveform of PDE activity (β^*) as the background intensity is increased. Consequently, although the failure to observe an acceleration of response recovery when Ca^{2+} concentration is lowered in darkness can be taken to show that light plays a role in light adaptation (94), it appears to do so only in the relatively trivial sense that light increases PDE activity. There is no necessity that the transduction mechanism be modulated by an additional, Ca^{2+}-independent mechanism. In summary, in our view, there is at present no compelling reason to believe that sensitivity in the photoreceptor is modulated during light adaptation by any feedback messenger other than Ca^{2+}.

D. Ca^{2+}-Dependent Mechanisms of Transduction Modulation

In section II, *C* and *D*, we described two Ca^{2+}-dependent mechanisms of regulation of the transduction cascade: *1*) the modulation of the guanylyl cyclase by the binding of Ca^{2+} to small-molecular-weight Ca^{2+}-binding proteins, called GCAPs, which in turn bind to and regulate the cyclase; and *2*) the Ca^{2+}-dependent modulation of the cGMP-gated channels. Both of these mechanisms could in principle contribute to light adaptation. Because background light produces a maintained decrease in the outer segment Ca^{2+} concentration, the cyclase would be stimulated (see sect. II*C*), and the increased synthesis of cGMP could contribute to the time-dependent increase in photocurrent that prevents the light response from reaching saturation (see Fig. 1). The effect of Ca^{2+} on the cGMP-gated channels could also contribute to the time-dependent "sag" of the photocurrent, since a decrease in Ca^{2+} has been shown to produce an increase in the affinity of the channels for cGMP (see sect. II*D*). This would have the effect of increasing the probability of channel opening in steady backgrounds, since more channels would be open at any given cGMP concentration.

In addition to the Ca^{2+} regulation of the cyclase and the channels, Ca^{2+} has also been proposed to modulate an early step in transduction (175, 194), within the first second or so of the activation of rhodopsin (176). The nature of this step is still unclear, but it appears to be kinetically distinct from the rate-limiting step in response inactivation (175, 176, 194, 206). Lagnado and Baylor (149) have shown that decreases in Ca^{2+} can decrease the sensitivity of the photoresponse of salamander rods by reducing the amplification of the transduction cascade (see also Ref. 119). This effect occurs only within the first second or so of light stimulation, suggesting that the action is on an early stage in transduction. Lagnado and Baylor (149) suggested that low Ca^{2+} decreases the catalytic activity of Rh^* by decreasing the gain of Rh^* activation of the PDE. The importance of this phenomenon is unclear, since sensitivity is altered only by a factor of 3–5 (as compared with the 100-fold change that can occur in background light), and this regulation of Rh^* activity appears to have a $K_{0.5}$ for Ca^{2+} of the order of 35 nM, at the extreme end of the range of physiological Ca^{2+} concentration in an amphibian rod.

Ca^{2+} has also been reported to alter the activity of

the PDE (128, 137), probably not directly (137) but rather by regulating some initial step in the transduction cascade. Kawamura (126) has proposed that this regulation occurs because Ca^{2+} alters the lifetime of Rh^* by modulating the activity of rhodopsin kinase, the enzyme that phosphorylates rhodopsin and initiates rhodopsin turnoff. Furthermore, he identified a small-molecular-weight binding protein in amphibians that he termed S-modulin, which mediates the Ca^{2+}-dependent regulation of kinase activity. Increases in Ca^{2+} were shown to produce an inhibition of rhodopsin phosphorylation, and Kawamura (126) argued that a decrease in Ca^{2+} may stimulate the kinase and increase the rate of phosphorylation, effectively decreasing the lifetime of Rh^*. Kawamura et al. (127) also showed that the activity of S-modulin can be mimicked by the protein recoverin, first identified in bovine retina (53) and that the amino acid sequences of amphibian S-modulin and bovine recoverin are similar.

The roles of recoverin and Ca^{2+}-dependent regulation of Rh^* phosphorylation have not been resolved and continue to be controversial. There is evidence that recoverin can mediate Ca^{2+}-dependent modulation of rhodopsin kinase activity in vitro (29), although the EC_{50} for Ca^{2+} is rather high, of the order of several micromolar. Recent experiments on permeabilized rods (211) and on intact retina (114) seem, however, to give an opposite result: rhodopsin phosphorylation in these studies was insensitive to changes in Ca^{2+}. Dialysis of recoverin into *Gekko* photoreceptors with whole cell patch recording produces an increase in the peak amplitude of the light response and a prolongation of response decay, although these effects may be partially due to an increase in Ca^{2+} buffering (95). Recoverin has also been shown to produce a prolongation of the response to bright flashes when introduced into the outer segments of truncated salamander rods (62), and there is evidence that this effect may be due to a slowing of the rate of rhodopsin deactivation. This phenomenon has however a dissociation constant (K_d) for Ca^{2+} of 13 μM, much higher than the physiological range of Ca^{2+} concentration in amphibian rods. This high K_d does not necessarily exclude a role for recoverin under physiological conditions, since the recombinant recoverin used in the experiments of Erickson et al. (62) was from bovine retina and used at a concentration that may have been lower than physiological. Furthermore, the high value for the K_d may not necessarily reflect the K_d of native recoverin in vivo.

Perhaps the most interesting experiments probing a possible role for recoverin in photoreceptor function and adaptation are those of R. L. Dodd on recoverin-knockout ($-/-$) mice (54), in collaboration with D. A. Baylor, J. Chen, J. Xu, and M. I. Simon. Dodd (54) showed that the decay of the response waveform is considerably accelerated in the photoreceptors of recoverin-knockout mice compared with those of normal mice, and this accelera-

tion is seen not only for bright flashes but for responses over the entire physiological range of the rod. Dodd also examined the responses of mouse rods to a step-flash protocol (see sect. IIIC and Fig. 6A), a stimulus consisting of a steady background light (or light step) followed immediately by a brief saturating flash. Dodd showed that in normal mice, a preceding steady light produces an acceleration of the recovery of the response to the flash, and in amphibian photoreceptors this effect has been shown to be mediated by a change in Ca^{2+} concentration (67, 174). In recoverin-knockout mice, however, this acceleration of recovery is completely lacking. This would seem to indicate that recoverin plays some role in mediating the Ca^{2+}-dependent regulation of the transduction cascade during light adaptation.

Curiously, however, when Dodd (54) examined the adaptation of the photoreceptors directly by measuring increment sensitivity as a function of background intensity (as in Figs. 1 and 5B), he could detect no difference between normal and recoverin $-/-$ mice. Two conclusions seem possible. On the one hand, the change in the time course of response decay and the results from the step-flash protocol can be taken to show that recoverin affects the adaptation of rods, and the measurements of increment sensitivity can be assumed not to have been sufficiently accurate to reveal this effect. On the other hand, the lack of effect on the increment-sensitivity curve can be taken to show that recoverin plays little or no role in light adaptation, and the results from response waveform comparisons and other measures of rod function can be thought not to be as important as is usually assumed for understanding the regulation of sensitivity. Regardless of which conclusion is the correct one, the contribution of recoverin to sensitivity modulation would appear to be relatively small.

E. How Do Photoreceptors Adapt to Backgrounds?

One way of evaluating the importance of the various proposed mechanisms of light adaptation would be to measure the Ca^{2+} dependence of each mechanism and then to calculate the effect of each on sensitivity with a mathematical model of the transduction cascade. Results like this for amphibian rods (from Ref. 137) are given in Figure 8. These calculations are based on electrophysiological measurements from truncated salamander rods of the Ca^{2+} dependence of the cyclase (136), of the Ca^{2+} dependence of the cGMP-gated channels (195), and of the Ca^{2+} dependence of PDE rate, presumably reflecting some early stage in phototransduction such as regulation of Rh^* gain or lifetime (137). Figure 8 compares the fractional contribution to threshold, defined as the reciprocal of step sensitivity, of each of the mechanisms that regulate rod sensitivity. The calculations assume a dark-rest-
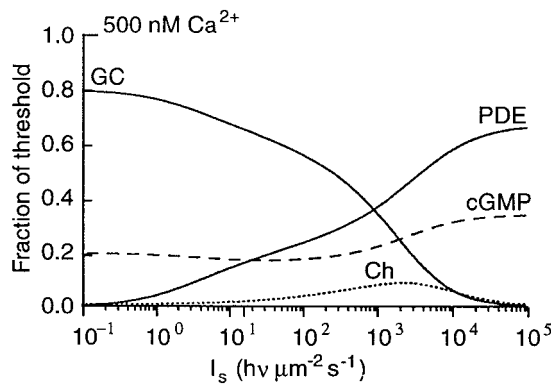
ing Ca^{2+} concentration in the salamander outer segment of 500 nM (not too different from that actually measured in the intact rod by Sampath et al., Ref. 251). The free Ca^{2+} in bright light is assumed to go to 0 nM. Because the free Ca^{2+} in the outer segment appears to go to 30–50 nM in a rod in bright light (93, 251), the contributions of the various mechanisms may not be accurately estimated at the brighter background intensities, but this effect is likely to be small, and the principal conclusions drawn from this figure would not be affected.

Over most of the range of background intensities, the most important mechanism that regulates the response to steady light is the Ca^{2+}-dependent modulation of the guanylyl cyclase. The direct effect of the cyclase is labeled GC in Figure 8. The principal function of this effect of Ca^{2+} on the cyclase is to prevent saturation of the photoresponse and extend the range of intensities over which the rod can operate (see Fig. 5*B*). A second indirect effect of cyclase modulation is labeled cGMP and reflects the increase in the stimulation of the PDE required to produce the same (threshold) decrease in the fractional cGMP concentration. Because background light produces a maintained stimulation of the PDE, an increment superimposed on the background must be sufficiently bright to produce enough additional stimulation of the PDE so that there will be a criterion reduction in cGMP concentration to close to a threshold number of the cGMP-gated channels. As the intensity of the steady light increases, the stimulation of the PDE by the background will increase

also, and more light will be required to produce the same fractional decrease in cGMP. This effect accounts for a fairly constant 20–30% contribution to the change in sensitivity. It is related indirectly to the Ca^{2+}-dependent stimulation of guanylyl cyclase, since if the cyclase were not accelerated as the Ca^{2+} falls in steady background light, the rod would saturate and no light, however bright, would produce a response to incremental flashes. The increase in cyclase rate allows the photoreceptor to reach a steady state in which both the PDE and cyclase are accelerated and in which a proportion of the cGMP-gated channels is still open, and thus available to be closed.

It is of some interest that the contribution of early stages in transduction (labeled in Fig. 8 as PDE) is small for dim backgrounds but becomes progressively more important as the background intensity is increased. This emphasizes the point made in the previous section that a contribution of recoverin and rhodopsin lifetime to increment sensitivity may be difficult to reveal, since it may be largest at the brightest background intensities, where increment sensitivity is the most difficult to measure because the rod is nearly saturated and light responses are small. The contribution of channel modulation in rods is predicted to be small over the entire range of background intensities and would have been even smaller had the rod Ca^{2+} concentration been assumed to go to 30–50 nM in bright light instead of to zero. The small influence of channel modulation is not surprising, since this effect is rather modest (see sect. II*D*) and only becomes significant in rods at very low Ca^{2+} concentrations, near the extreme limit of the Ca^{2+} concentration reached in bright light. In cones, however, the EC$_{50}$ for free Ca^{2+} is of the order of 250–300 nM (235), near the middle of the physiological range of Ca^{2+} (see Ref. 250). It is therefore possible that channel modulation plays a much more important role in adaptation in cones than in rods. Calculations like those in Figure 8 but for cones instead of rods have not yet been reported but will clearly be of considerable interest.

During adaptation, the changes in circulating current and sensitivity are accompanied by a progressive acceleration of both the time to peak and the recovery phase of the flash response (see Fig. 1). As discussed in section III*C* and modeled in detail in section VI, the principal mechanism responsible for these effects appears to be the increase in PDE activity induced by the background itself, which allows the cGMP concentration, and hence the photocurrent, to track the flash-induced waveform of PDE activity more closely as the background intensity is increased. In contrast, the steady increase in guanylyl cyclase rate that results from the reduction in Ca^{2+} induced by the background does not, of itself, lead to acceleration of dim flash response kinetics during steady light, but serves instead to rescue the photoreceptor from the premature saturation of the response which would otherwise ensue (see Fig. 5*B* and Refs. 137, 181, 197, 231).

IV. BLEACHING ADAPTATION

Exposure of the eye to bright light results in profound desensitization to subsequent light stimulation. This depressed adaptational state and the recovery of sensitivity that occurs subsequently in darkness, often referred to as bleaching adaptation and dark adaptation, have been extensively investigated. Stiles and Crawford (265) first showed that there is a strong similarity or even equivalence of bleaching adaptation and background adaptation in human visual threshold. Their work and many subsequent studies (7, 8, 18, 43) have shown that the desensitization due to bleaching behaves very similarly to that due to background light; that is, during recovery of sensitivity following bleaching, the retina behaves as if it is being illuminated by a steady light of gradually decreasing intensity.

Hecht et al. (105) provided one of the first clues about the nature of bleaching desensitization. They showed that the human visual system recovers from strong bleaching light in two phases with different time courses, a fast and a slow, and that these separate limbs of the curve could be attributed to the cones and the rods. Many experiments on whole retina as well as on single isolated rods and cones have now established that bleaching adaptation resides in large part within the photoreceptors themselves (90, 224, 279) and that the rate of recovery of sensitivity following bleaching is substantially slower in rods than in cones (35, 122, 241).

The relationship between sensitivity and photopigment was further clarified by Wald (276), who demonstrated that visual pigment is composed of a protein (opsin) covalently attached to a chromophore (11-*cis*-retinal) via a Schiff base linkage. Wald and co-workers (113) subsequently showed that the only action of light is to photoisomerize retinal from the 11-*cis*- to the all-*trans*-form, thus initiating a series of reactions that trigger activation of the transduction cascade. A separate set of reactions, termed the visual cycle, is necessary for regeneration of the pigment. Thus the photoproducts of bleaching (ultimately all-*trans*-retinol*)* must be removed from the bleached photoreceptors, transported to the pigment epithelium to be resynthesized to 11-*cis*-retinal, and finally transported back to the photoreceptors for reattachment to opsin before dark adaptation is complete.

Early experiments emphasized the importance for desensitization and recovery of a long-lasting product of photopigment bleaching. Rushton (240) and Dowling (59) demonstrated an approximately linear relationship between log threshold and rhodopsin content during recovery of sensitivity from strong bleaches in human and rat eyes, and these results led Dowling (59) to propose that dark adaptation is composed of two phases: a slow "photochemical" phase proportional to the rate of regeneration of visual pigment, and a more rapid "neural" phase. The recovery of sensitivity during the photochemical phase was proposed to be rate limited by the regeneration of the visual pigment, as if the desensitization during recovery were somehow produced by the accumulation of unregenerated visual pigment (7). The exact nature or cellular locus of the neural phase was not specified, but some alternative mechanism seemed to be required to explain the large and prolonged reduction in sensitivity following exposure to lights bleaching only a small fraction of the visual pigment (55, 91, 167, 244).

Later experiments, particularly those of Donner and Reuter (58), emphasized the importance of more transient photoproducts. From measurements of the relationship between sensitivity and the concentration of photopigment intermediates in frog retina, Donner and Reuter (58) hypothesized that the extent of desensitization after bleaching is proportional to the concentration of Meta II, the photoproduct responsible for activation of the visual transduction cascade. After an intense bleach, a large amount of Meta II is transiently formed that requires tens of minutes to decay and may be responsible for the transient desensitization produced by the bleach. Many physiological (56, 151, 160, 161) and biochemical (116, 208) experiments over the past two decades have lent strong support to the notion that transient photoproducts of rhodopsin bleaching play an important role in the decrease in sensitivity following bleaching. Recent physiological experiments have shown, for example, that the desensitization following light estimated to have bleached around 1% of the photopigment is substantially attenuated by administration of hydroxylamine, which is known to accelerate the irreversible hydrolysis of Meta II (160).

Experiments during the last three decades suggest that both transient and longer lived photoproducts of bleaching probably make important contributions to bleaching desensitization. For small bleaches, short-lived photoproducts including Meta II appear to be particularly important. The evidence for this has been recently reviewed (162) and is not described further here. Stronger bleaches are more likely to produce significant concentrations of longer lived photoproducts, including opsin. Biochemical experiments have shown that opsin is capable of producing an equivalent background by activating transducin (33, 187, 266), and isolated rods and cones subjected to large bleaches show a persistent activation of the transduction cascade that is difficult to attribute to transient photoproducts of bleaching and is more likely due to the presence of opsin in some form (34, 38). Experiments describing opsin activation and elucidating its mechanism are delineated in the remainder of this section.

A. Persistent Desensitization of Bleached Photoreceptors

Because in the intact eye pigment regeneration occurs continuously during and after light exposure (see

sect. IV*D*), it is difficult to study the relationship between the photoreceptor response and the bleaching and regeneration of pigment. For this reason, many studies of bleaching adaptation have used photoreceptors isolated from the RPE, for which only a small fraction of the bleached pigment can be regenerated (5). In this way, the steady-state sensitivity of the rod or cone can be measured a suitable time after exposure to a bright bleaching light, and the pigment can be artificially regenerated by addition of exogenous chromophore either in ethanolic solution or in phospholipid vesicles (37, 224, 295).

Experiments using these techniques to study rods and cones that have been subjected to bleaches of up to 99% of the photopigment have demonstrated the existence of a stable component of bleaching desensitization. This persistent desensitization is produced by the presence in the cell of a stable fraction of bleached photopigment. Like the desensitization produced by real light, it is accompanied by a more rapid time course of the flash response, increased steady-state velocities of cGMP PDE and guanylyl cyclase, and a decreased concentration of cytoplasmic Ca^{2+}. Desensitization in the absence of pigment regeneration has been observed to persist for up to days, and recovery of sensitivity to prebleach dark-adapted levels only occurs when new pigment is formed following exogenous administration of 11-*cis*-retinal.

The long-lasting effect of pigment bleaching on an isolated rod is illustrated in Figure 9. In this experiment, the rod was first exposed to a light that bleached 90% of the visual pigment. Immediately after the bleach, the response of the rod was saturated (i.e., all of the cGMP-gated channels were closed), and no responses could be

recorded to subsequent flashes of light. Over time, however, the circulating current slowly recovered, presumably as the result of a slow decline in the rate of the PDE and a gradual increase in the concentration of cGMP in the outer segment. As circulating current recovered, responses could be recorded to bright flashes, although the rod was considerably less sensitive than in the dark before the bleach. After about an hour, the rod reached steady state (Fig. 9, open diamonds), with the dark current about one-half its value before the bleach and the sensitivity about a factor of 100 lower. Furthermore, the waveform of the response to dim flashes (Fig. 9, *inset*) was considerably accelerated after the bleach (Bl) by comparison to the response waveform of the dark-adapted photoreceptor (Dk). When the photoreceptor was then exposed to liposomes containing exogenous 11-*cis*-retinal, the circulating current and sensitivity slowly recovered nearly to their values before the bleach (Fig. 9, solid triangles and diamonds), and the waveform of the light response (Rg) also returned nearly to that recorded from the dark-adapted rod at the beginning of the experiment (Dk).

The results in Figure 9 show that the bleaching of photopigment in an isolated rod produces a steady decrease in circulating current like that produced by a background (see Fig. 1). Figure 9 also shows that bleaches produce a decrease in sensitivity and an acceleration of the waveform of the response (39, 66, 161). The rod is behaving as if bleached photopigment were somehow producing a sustained stimulation of the visual cascade, and similar phenomena are also seen after bleaching in isolated cones (see Ref. 123).
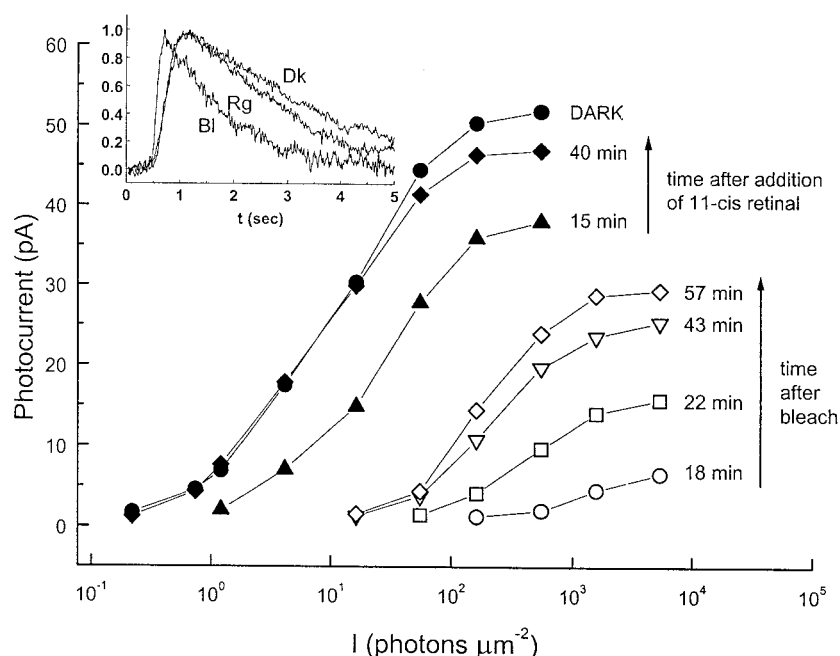
FIG. 9. Effect of bleaching and regeneration of photopigment on the light responses of a salamander rod. Response-intensity curves and flash-response waveforms in darkness, after exposure to a bright bleaching light, and after addition of 11-*cis*-retinal were all recorded with a suction pipette from the same cell. For curves, ordinate gives peak amplitude of the flash response plotted as a function of flash intensity, in photons·μm^{-2}. Solid circles give response amplitudes in darkness, open symbols at the indicated times after exposure to a bright light bleaching 90% of the rod photopigment, and solid triangles and solid diamonds at the indicated times after addition of exogenous 11-*cis*-retinal in phospholipid vesicles. *Inset*: traces show small amplitude responses to brief flashes in darkness at the beginning of the experiment (*trace Dk*), at steady state 55 min after the bleach (*trace Bl*), and 36 min after adding 11-*cis*-retinal (*trace Rg*). Responses have been normalized according to their peak amplitude. [From Fain et al. (69), with permission from Elsevier Science.]

B. Bleached Pigment Activates Transduction

One way to demonstrate that bleached pigment activates the transduction cascade is to show that bleaching produces a maintained increase in the rate of the cGMP PDE (see Fig. 3). A method for doing this was first described by Hodgkin and Nunn (109). They postulated that the cGMP concentration in the photoreceptor is determined by the rates of its synthesis and hydrolysis, with the velocities of the cyclase and PDE given as α and β

$$\text{GTP} \xrightarrow{\alpha} \text{cGMP} \xrightarrow{\beta} \text{GMP}$$

At steady state in the dark or after a bleaching exposure, the rates of synthesis and hydrolysis must be the same. Hodgkin and Nunn (109) used suction pipette recording to estimate the free cGMP concentration in the outer segment from the percentage of open channels and showed that the relative values of α and β could be estimated if either the cyclase or PDE were suddenly inhibited. When the cyclase is inhibited, the PDE hydrolyzes cGMP at a rate $\beta[\text{cGMP}]$, and the cGMP concentration declines exponentially with a rate constant that can be estimated from the decline of outer segment current. Similarly, when the PDE is inhibited, the cGMP concentration increases, and the rate of the cyclase can be estimated from the initial rate of increase of the circulating current, provided the GTP concentration remains constant (2).

Typical results obtained with this method on bleached salamander rods are given in Figure 10. In these experiments (34), the cyclase was inhibited by suddenly exposing the rod to a solution in which the Na^+ was substituted with Li^+. This has the effect of preventing Na^+/Ca^{2+}-K^+ exchange and increasing the outer segment free Ca^{2+} concentration; the Ca^{2+} then binds to GCAP and inhibits the cyclase (see sect. IIC). The current initially increased, because the cGMP-gated channels are somewhat more permeable to Li^+ than to Na^+ (see, for example, Ref. 290). The current then declined according to an approximately exponential time course, as can be seen from the curves to the right in Figure 10 plotted in semilogarithmic coordinates. The time constant of current decline (and therefore the relative rate of the PDE) can be estimated from this exponential decline. Bleaching increases the activity of the PDE, and exposing the rods to exogenous 11-*cis*-retinal (to regenerate the visual pigment) brings the PDE activity back to its value in darkness before the bleach. A corresponding increase in velocity can be seen for the cyclase if the PDE is inhibited with IBMX (34).

The increases in PDE activity and cyclase velocity are linearly proportional to the percentage of pigment bleached (34). Background light also leads to an increase
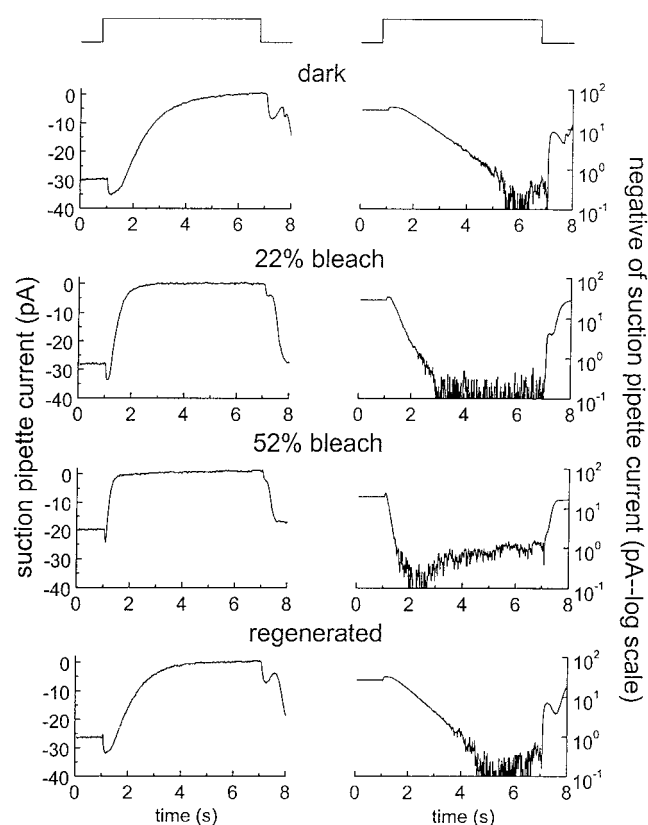

FIG. 10. Bleached pigment activates the photoreceptor cGMP phosphodiesterase. Traces show photocurrent recorded from a salamander rod stepped into an extracellular solution in which Na^+ was replaced with Li^+ to suppress the synthesis of cGMP by guanylyl cyclase (see text). All records are from the same rod. Traces show (from *top* to *bottom*) the suction pipette current recorded in darkness, 43 min after exposure to a light bleaching 22% of the photopigment, 50 min after a second exposure producing a cumulative bleach of 52% of the photopigment, and 99 min after exposure to phospholipid vesicles containing 11-*cis*-retinal. Solution monitor traces at top of figure give time course of exposure to the Li^+-substituted solution. *Left panels* show change in suction pipette current, with initial negative deflection caused by greater permeability of the cGMP-gated channels to Li^+ than to Na^+. *Right panels* show the negative of the currents on the *left*, plotted on a log scale. The nearly linear decline indicates that current declines in Li^+ solution approximately exponentially, with a time course that is speeded by bleaching but restored by pigment regeneration. [From Fain et al. (69), with permission from Elsevier Science; modified from Cornwall and Fain (34).]

in the rates of the destruction and resynthesis of cGMP by PDE and cyclase, but this relationship is not linear and saturates with increasing background intensity (34, 109). This is of some interest, since it suggests that there may be some differences between the effects of Rh* and bleached pigment. It is possible, for example, that mechanisms for background adaptation that occur early in the visual cascade (such as modulation of the gain or lifetime of rhodopsin; see sect. IIID) may not contribute to bleaching adaptation, since the gain or lifetime of bleached pigment may not be modulated in a similar fashion. We return to this subject in section IVC.

From the initial slopes of the relationships between PDE or cyclase rate and bleaching and background intensity, it is possible to estimate the number of stably bleached pigment molecules that produce an increase in PDE activity equivalent to one Rh*. This number is $\sim5 \times 10^6$. Thus a stably bleached pigment molecule is only $\sim10^{-7}$ to 10^{-6} times as effective as Rh* in exciting the visual cascade (see also Ref. 159). Similar measurements have been made in cones, for which bleached pigment also activates the transduction cascade (38). For cones, however, the ratio of activation by Rh* to that by stably bleached pigment is of the order of 10^4 to 10^5, nearly two orders of magnitude smaller than for rods. The reasons for this seem to be that cone Rh* is less effective in stimulating excitation than rod Rh* and that bleached pigment seems to be noisier in cones than in rods (38).

The form of bleached pigment responsible for desensitization in an isolated photoreceptor at steady state is probably opsin, that is, rhodopsin without chromophore. The evidence for this identification stems from a number of sources. *1*) Steady-state desensitization is only reached 45–60 min after exposure to the bleaching light, at a time when spectroscopic measurements from isolated amphibian retinas show that Rh* has decayed completely to opsin (see Ref. 69). If bleached rods are maintained for an additional hour, or even for several days (40), the PDE and cyclase rates remain accelerated, and the level of desensitization does not change. *2*) Measurements of the fluorescence of retinol in isolated rods demonstrate that the reduction of all-*trans*-retinal to all-*trans*-retinol is complete within 45–60 min after bleaching (125). The work of Hofmann et al. (110) has demonstrated that this reaction is essentially irreversible, and once it takes place, there is no longer any all-*trans*-retinal available to form intermediates capable of activating the transduction cascade. *3*) β-Ionone and other retinoid analogs can interact with the binding pocket of opsin in bleached rods and cones to modulate activity of the transduction cascade (118, 129). This interaction is noncovalent and reversible; it seems unlikely that such rapid binding and unbinding would occur if opsin were not free of retinal. In isolated cones, it is possible to relieve at least part of bleaching desensitization by exposing the photoreceptors to analogs of 11-*cis*-retinal such as 9-*cis*-C17 aldehyde and β-ionone,which fill the pocket normally occupied by the chromophore but do not form a Schiff base linkage with opsin (see sect. IV*D*). These analogs do not form visual pigments and cannot mediate transduction or the formation of Rh*. In cones, they nevertheless produce an increase in circulating current and sensitivity and a slowing of the kinetics of the dim flash response as if the pigment were regenerating (118), and they reduce the activities of PDE and cyclase (38). It is remarkable that the effects of these analogs are rapidly reversible, and β-ionone can be perfused onto the cone and removed again several times,

reversibly increasing circulating current and sensitivity (38). It is difficult to see how this could occur unless the form of stably bleached pigment were simply opsin, that is, protein with an empty chromophore binding pocket.

A final reason for believing that opsin can activate the transduction cascade comes from experiments with visual pigment in vitro. Melia et al. (187) have recently shown that isolated bovine opsin can directly activate transducin at densities of opsin and transducin similar to those found in intact photoreceptors. They estimate the effectiveness of opsin to be $\sim10^{-6}$ that of Rh*, very similar to the value obtained from physiological measurements for bleached pigment from isolated salamander rods. These results do not, however, exclude the possibility that in the intact photoreceptor the activity of opsin after a bleach is affected by the continued presence of all-*trans*-retinal (110, 116, 247). Furthermore, the eventual conversion of all-*trans*-retinal to all-*trans*-retinol could produce a concentration of retinol in the outer segment as high as several millimolar, which may not be removed from an isolated rod in the absence of interphotoreceptor matrix retinoid binding protein (IRBP; see sect. IV*D*). The effect of this large concentration of retinol is presently unknown.

C. Mechanism of Transduction Activation by Bleached Pigment

A schematic representation of possible pathways for the activation of the transduction cascade by bleached pigment is summarized in Figure 11. A simplified version of the transduction cascade is represented as a string of reactions starting with the formation by Rh* following photon absorption (Fig. 11, *left*) and culminating in a change in cytoplasmic calcium concentration (Fig. 11, *right*). The feedback pathways represented above this

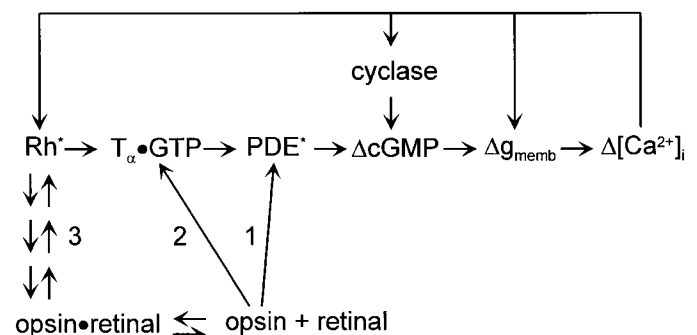FIG. 11. Possible mechanisms of activation of visual transduction cascade by opsin in stably bleached photoreceptors (see text). $T_\alpha \cdot$GTP, activated alpha subunit of transducin; PDE, phosphodiesterase; ΔcGMP, change in free concentration of cGMP; Δg_{memb}, change in outer segment membrane conductance; $\Delta[Ca^{2+}]_i$, change in outer segment free Ca^{2+} concentration.

line show the ways in which Ca^{2+} has been demonstrated to modulate these reactions during background light, and the pathways below this line represent different ways in which bleached pigment might mediate bleaching adaptation.

In stably bleached photoreceptors, bleached pigment might activate transduction by stimulating the PDE directly (*pathway 1*, Fig. 11), but it seems more likely that instead bleached pigment mimics Rh^*, binding to the G protein transducin and facilitating the exchange of GTP for GDP on the T_α guanosine nucleotide binding site (*pathway 2*, Fig. 11). T_α would then stimulate the PDE, reduce the cGMP concentration, close the cGMP-gated channels, decrease the outer segment free Ca^{2+} concentration, and modulate the cascade to produce the changes in sensitivity and response waveform shown in Figure 9.

The possibility of direct activation of T_α by opsin was investigated by dialyzing guanosine 5'-O-(3-thiotriphosphate) (GTPγS) into previously bleached salamander rods (178). GTPγS is known to mimic GTP and to bind to activated transducin at the guanosine nucleotide binding site, but unlike GTP, it is poorly hydrolyzable so that the lifetime of T_α·GTPγS is much longer than that of T_α·GTP. When GTPγS was incorporated into dark-adapted rods, there was a slow decrease in the circulating current that was accelerated by light stimulation (252, 155) and a dramatic prolongation of the response to an intense flash (155). If the GTPγS was introduced into a previously bleached rod, on the other hand, the decrease in circulating current in darkness was considerably accelerated (178). The simplest interpretation of this experiment is that the decline in circulating current is caused by persistent activation of transducin that has bound GTPγS instead of GTP and that transducin is activated much more rapidly by bleached pigment than by dark-adapted rhodopsin. How much more rapidly is difficult to say, since the time course of activation in these experiments was probably limited by the rate of dialysis of GTPγS into the rod outer segment. These results support the notion that bleached pigment activates the cascade by binding to transducin and stimulating guanosine nucleotide exchange, much like Rh^*.

It is also possible to make some inferences about the mechanism of transducin stimulation by examining the electrical noise in bleached photoreceptors. If rods are exposed to dim steady background light, they show an increase in electrical noise produced by randomly occurring single photon responses (13, 120). As the background light is made brighter, the noise amplitude increases, but in very bright backgrounds it decreases again, as the sensitivity of the receptor declines and the single photon responses become very small.

If bleached pigment activated the transduction cascade directly, in a manner identical to Rh^* following large bleaches, a similar increase in noise should be observed.

This possibility was examined in rods at steady state after exposure to illumination sufficiently intense to bleach a significant fraction of the total photopigment, and no increase in noise was detected (66, 120). If the form of bleached pigment that stimulates the cascade in stably bleached rods is in fact opsin (see sect. IV*B*), then opsin appears not to stimulate the cascade exactly as light does, for example, by a reversion to a conformation identical to Rh^* (152). It seems more likely that opsin itself can assume an active conformation with a low probability and short lifetime. A single Rh* can produce as many as several hundred activated T_α·GTP molecules (84), but the gain at this step in the cascade must be much lower for opsin, perhaps as low as a single T_α·GTP per activated opsin. The reason for this may be either that the lifetime of the active conformation of opsin is much shorter than for Rh^* or that the coupling between opsin and transducin activation is much less efficient. The probability of opsin reaching an active conformation may also be rather low.

Somewhat different results are obtained when isolated rods are stimulated with a light that bleaches only a few percent or less of the visual pigment. Under these conditions, complete recovery of sensitivity can be observed (5), and rods show an increase in photonlike events in darkness during the subsequent recovery (151). These events are probably caused by a reversion of some photoproduct (perhaps Meta II) to Rh^* (160), indicated in Figure 11 by *pathway 3*. Although originally supposed to be responsible for the decrease and recovery of sensitivity in rods subjected to small bleaches (151), it is now clear that the effect of these photonlike events on bleaching desensitization is rather small (161). Most of the desensitization even for small bleaches is produced by events having low noise, and the predominant effect of bleached pigment seems not to be mediated via a reversion to Rh^* but rather via a low-gain stimulation by photoproducts of the visual cascade, similar in at least some respects to that in rods at steady state after large bleaches (34, 66, 120).

On the basis of the experiments outlined above, the stimulation of T_α by opsin and other forms of bleached pigment produces an activation of the PDE that lowers the free cGMP concentration and is responsible for the maintained decrease in circulating current in a photoreceptor at steady state after a bleach (Fig. 9). It is of some interest that, in an isolated rod exposed to bright light, steady state is reached only after as much as 30–60 min. Presumably all of the photoproducts of bleaching (Meta II, Meta III, opsin) can activate transducin with greater or lesser efficiency, and only after 30–60 min have the concentrations of these photoproducts reached stable levels. The decrease in circulating current caused by transducin activation would then be expected to produce a corresponding decrease in Ca^{2+} concentration, and this has been demonstrated to be the case both for rods (251) and

cones (250). Ca^{2+} measurements from photoreceptors loaded with the fluorometric dye fluo 3 show a maintained decrease in outer segment free Ca^{2+} after bleaching, which recovers to the dark-adapted level after regeneration of the photopigment with 11-*cis*-retinal.

The decrease in Ca^{2+} in a stably bleached photoreceptor is probably responsible for the maintained activation of the guanylyl cyclase and the reduction in sensitivity. A change in free Ca^{2+} concentration has been shown to be necessary for bleaching desensitization, at least in cones (179). This was demonstrated by bleaching photopigment in an isolated salamander cone whose outer segment was perfused with low-Ca^{2+}/zero-Na^+ solution to minimize changes in intracellular free Ca^{2+} concentration (see sect. III*B*). After the bleach, the cone in this solution remained saturated, presumably as the result of continued stimulation of PDE, but responses in low-Ca^{2+}/zero-Na^+ solution could be recorded after partially inhibiting the PDE with IBMX (much as in Fig. 7 during background illumination; see sect. III*C*). In this way, it was possible to show that bleaching has little effect on response waveform or sensitivity under conditions in which changes in the free Ca^{2+} concentration are minimized and PDE activity is maintained near its dark-adapted level.

How does the change in free Ca^{2+} produce bleaching desensitization? Because stably bleached rods show both a decrease in Ca^{2+} and an increase in the rate of the guanylyl cyclase, it seems likely that much of the desensitization is produced by the cyclase either directly (Fig. 8, curve labeled GC) or indirectly as a consequence of the increase in PDE stimulation required to produce a threshold decrease in cGMP concentration (Fig. 8, curve labeled cGMP). Effects of the change in Ca^{2+} concentration on the probability of opening of cGMP-gated channels may also make a significant contribution to the change in sensitivity in cones (235), but the contribution of channel modulation in rods is likely to be small (Fig. 8, curve labeled Ch).

The effects of backgrounds and bleaches in rods were explicitly compared by Leibovic et al. (159) and by Jones et al. (121). No differences were detected for the two kinds of adaptation in the relationships between sensitivity and either circulating current or cyclase or PDE activation. This is not to say, however, that the mechanism of these two forms of adaptation is identical. A comparison between background adaptation and bleaching adaptation in stably bleached photoreceptors has been possible over only a restricted range of background intensities, because stably bleached photopigment is much less effective than Rh^* in stimulating the transduction cascade. This has the result that, even for the brightest bleach, the desensitization is equivalent to a background of only moderate intensity. Over a range of backgrounds extending from dim to moderate intensities, contributions from early stages in the transduction cas-

cade appear to be small (see Fig. 8, curve labeled PDE). It is therefore not at present possible to say whether modulation of early stages plays a role in bleaching desensitization. Stably bleached photoreceptors may in any case be a poor preparation for investigating this question, since early-stage modulation may play little role in desensitization. By these late times after bleaching, photoproducts such as Meta II and Meta III are presumably already phosphorylated and present at low concentration, and the phosphorylation of opsin may not be regulated in a way that would contribute to sensitivity modulation.

D. Pigment Regeneration

For the photoreceptor to recover fully from previous light exposure, bleached photopigment must be regenerated to a form containing 11-*cis*-retinal. In the vertebrate retina, pigment regeneration is a complex process requiring the participation of both the photoreceptors and the pigment epithelium (see Ref. 44). Furthermore, it occurs at different rates for rods and cones. Cone pigment regeneration (as measured in humans by reflection densitometry) is complete in <10 min (242, 245, 277), whereas rod pigment regeneration requires at least 20–30 min (27, 237, 238). These differences appear to correspond to the differences in the rate of recovery of rod and cone vision (105).

The rates of deactivation of rhodopsin and pigment regeneration appear to play pivotal roles in the mechanism of bleaching adaptation. It is now generally accepted that the ensemble of steps that results ultimately in the full recovery of sensitivity is complicated and involves reactions that take place in both the retina and the pigment epithelium. After *cis* to *trans* photoisomerization of the retinal chromophore and the subsequent activation of the transduction cascade, Rh^* must first be quenched. The first step in this inactivation involves rhodopsin phosphorylation by a specific kinase, rhodopsin kinase (25, 80, 189, 218), and possibly by protein kinase C (145, 204). Phosphorylation of rhodopsin increases its affinity for a regulatory protein, arrestin, which in association with the phosphorylated visual pigment accelerates deactivation, thereby terminating the light response (see sect. II*A*).

The first suggestion of a physiological role for phosphorylation came from the work of Kühn and co-workers (142, 148) and Bownds and co-workers (24, 188). Together, these studies established that there is a correlation between phosphorylation and recovery of sensitivity during dark adaptation. The importance of this relationship was demonstrated in voltage-clamp studies examining the effects of sangivamycin, an inhibitor of rhodopsin kinase, and phytic acid, an inhibitor of arrestin binding to phosphorylated rhodopsin (214). Both agents dialyzed into photoreceptor outer segments produced a prolonga-

tion of the light response, indicating some delay in deactivation of transduction. Furthermore, in transgenic mice for which all of the phosphorylation sites from the COOH-terminal tail were removed by truncation at Ser-334 (31), dim flash responses measured electrophysiologically from affected cells were significantly prolonged, suggesting that termination of the light response was impaired. Protein phosphatase has been detected in the outer segments of rod photoreceptors and has been shown to dephosphorylate rhodopsin (79, 212).

Numerous studies have established that there are as many as seven possible sites for phophorylation of rhodopsin (115). The major sites of phosphorylation in in vitro experiments appear to be different from those in in vivo studies, and phosphorylation and dephosphorylation of these different sites have been shown to occur at different rates. Of particular interest are the studies of Ohguro et al. (207), which show that Ser-338 is phosphorylated primarily after flashes whereas Ser-334 is phosphorylated primarily after continuous illumination that bleached larger fractions of the visual pigment. In addition, Ser-334 is dephosphorylated faster than Ser-338, and the rate of Ser-334 dephosphorylation is correlated with the regeneration of rhodopsin.

Visual pigment regeneration is known to begin with the reduction of all-*trans*-retinal to all-*trans*-retinol and the removal of bound arrestin. Both must occur before the opsin binding site becomes accessible for reconjugation with new 11-*cis*-retinal (110). All-*trans*-retinol is removed from the outer segment by an unknown mechanism and is thought to be then taken up by the interphotoreceptor matrix retinoid binding protein (IRBP) for translocation to the pigment epithelium (209); however, animals completely lacking IRBP show normal rates of pigment regeneration (217), suggesting that other mechanisms for retinoid transport may also exist. In the pigment epithelium, all-*trans*-retinol is stored in a detoxified form as a retinyl ester following conversion via retinol ester synthetase (246). As Bernstein and Rando (16) showed, an isomerization reaction then converts the all-*trans*-retinyl ester directly into 11-*cis*-retinol by an isomerohydrolase that uses the energy from the hydrolysis of the ester to drive the energetically unfavorable isomerization of the retinal from the all-*trans*- to 11-*cis*-isomer. The 11-*cis*-retinol is then either oxidized to 11-*cis*-retinal and transported back to the receptor outer segments, where it conjugates with opsin to form visual pigment, or it is converted to an 11-*cis*-ester for storage.

The conjugation of 11-*cis*-retinal to opsin occurs in at least two steps. The first requires the binding of the 11-*cis*-isomer of retinal into a binding pocket located deep in the hydrophobic core of opsin, so as to bring the terminus of the polyene chain of retinal close to the NH_2 terminus of a lysine (lysine-296 in bovine rhodopsin), located in the seventh transmembrane α-helix of the opsin

protein. The second step is the formation of a protonated Schiff base between the lysine and the 11-*cis*-retinal chromophore, resulting in a complete inactivation of the transduction cascade and full recovery of sensitivity. The high degree of identity of amino acid sequence between rod and cone pigments, as well as the similarity in the position of a lysine residue in the seventh transmembrane α-helix of rod and cone pigments, suggests that similar mechanisms may be involved in both kinds of photoreceptors (201).

In rod opsin, the first step in the conjugation of 11-*cis*-retinal requires a noncovalent interaction between the ring end of the chromophore and an anchoring site within the hydrophobic binding pocket of opsin (170). The exact conformation of this site is unknown. Binding studies involving retinal analogs, however, indicate that two methyl groups at positions 1 and 5 on the ionone ring are required, but an integral ring structure is not (45). An additional methyl group at position 1 on the ionone ring and a lengthening of the side chain of the retinal can both increase the strength of the initial binding (45, 47). This binding site on the opsin molecule appears to have only moderate stereospecificity, since retinal analogs having this ring structure and a side chain of less than ~10 (e.g., 11-*cis*-retinal, 9-*cis*-retinal, 9-demethylretinal, 13-demethylretinal, 10 methyl-retinal) can all be accommodated to form a visual pigment.

The strength of this initial binding step is rather weak. The 11-*cis*-retinal can be shown to compete for binding with noncovalently bound retinal analogs for all analogs with a structure identical to retinal up to carbon 11. In addition, physiological studies on salamander rods and cones have shown that those analogs that bind to this site but do not form a Schiff base, such as β-ionone, can be displaced by bathing an isolated photoreceptor with IRBP or simply by superfusing with salamander Ringer solution not containing the retinoid (38, 118).

Analogs binding to this site can produce significant physiological effects that can be quite different for rods and cones. Recent physiological experiments on rod photoreceptors have demonstrated that entry of β-ionone into the binding site of bleached salamander rod photoreceptors resulted in an excitation of the transduction cascade as evidenced by a decrease in sensitivity and dark current as well as increases in the velocities of PDE and guanylyl cyclase (129). These results suggest that occupancy of the binding site by the chromophore produces a steric change in opsin that resembles the effect of light. These data are consistent with other biochemical studies that have shown that all-*trans*-retinal as well as a number of other short-chain analogs of retinal activate transducin (83) and promote the phosphorylation of rhodopsin by rhodopsin kinase (110, 213) and the binding of arrestin (116). All of these biochemical effects can occur in the absence of Schiff base formation (25, 116).

Interestingly, when isolated bleached cones are treated with a retinal analog that cannot form a Schiff base linkage, the effects are opposite to those in rods. The best-studied example is β-ionone which, when superfused onto a stably bleached salamander cone (see sect. IV*B*), produces a rapid increase in sensitivity, a decrease in the rate of guanylyl cyclase, and an increase dark current (38, 118, 129). Similar effects on sensitivity and dark current for cones have been observed with 11-*cis*-retinol and 9-*cis*-C17-retinal (118, 122), which are also unable to form a Schiff base linkage with opsin. Taken together, the opposite effects of analog-mediated regulation of the transduction cascade in rods and cones demonstrate a fundamental difference in the way in which noncovalent retinoid binding affects the physiology of these different receptor types. The difference in the binding of retinal and its analogs to rod and cone opsins may provide an important clue to the difference in the rate of recovery of rods and cones after exposure to bright bleaching light (130).

E. Decline and Recovery of Sensitivity During Dark Adaptation

For stably bleached photoreceptors, we now have a fairly clear notion of how bleaching desensitization is occurring (see Fig. 11). The bleached photopigment, probably as opsin, directly activates transducin with low probability and gain, and $T_\alpha \cdot$GTP then stimulates the PDE, which reduces the cGMP concentration. As a consequence, the cGMP-gated channels close, outer segment free Ca^{2+} concentration decreases, guanylyl cyclase is stimulated, and the sensitivity and kinetics of the light response are altered by a mechanism similar to that for a rod exposed to dim or moderate background illumination (see sect. III*E*).

Because bleached pigment directly activates the transduction cascade at a rate that is proportional to the amount of pigment bleached, bleaching will produce an equivalent background light whose intensity is directly proportional to the fraction of bleached pigment. The term I_B in *Equations 1* and *2* for the Weber relation for background adaptation can therefore be substituted with a term for an equivalent background, proportional to the fraction of pigment bleached. A further correction to these equations must be made, since bleaching has two effects on sensitivity: one due to the activation of the transduction cascade and one due trivially to the reduction in the probability of the absorption of a photon in the outer segment, as a result of the decrease in the concentration of photoexcitable pigment.

Figure 12 shows the decrease in sensitivity for a number of rods at steady state, after bleaches whose magnitudes (expressed as a fraction of total pigment) are
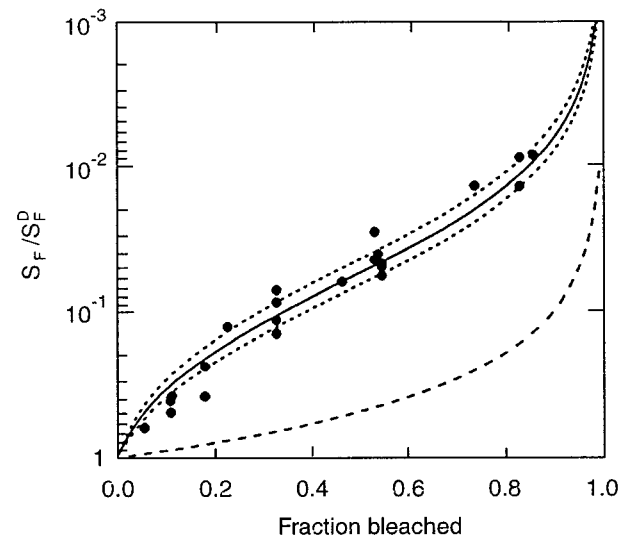

FIG. 12. Decrease in flash sensitivity for salamander rods at steady state after exposure to light bleaching a significant fraction of the photopigment. Each point represents the effect of a single bleach on one cell. The ordinate gives the fractional decline in flash sensitivity (S_F^D) plotted as a function of the fraction of photopigment bleached (S_F). The solid line gives the sum of the sensitivity loss expected from the decrease in quantum catch due to bleaching of the photopigment, and from adaptation of the transduction mechanism, on the assumption that bleached pigment produces an equivalent background light whose intensity is proportional to the fraction bleached. The dotted curves are similar relationships produced by increasing or decreasing the photosensitivity by 15%. The dashed curve shows the loss in sensitivity expected from the decrease in quantum catch alone. [From Jones et al. (121), by copyright permission of The Rockefeller University Press.]

given on the abscissa. In Figure 12, the line with large dashes shows the decrease in sensitivity expected from the decrease in quantum catch alone, that is, from the decrease in the probability of photon absorption due to the decrease in the concentration of pigment. The actual sensitivity decrease recorded from these receptors (solid circles) is much larger, since in addition to the loss of quantum catch there is also a Ca^{2+}-dependent modulation of the transduction cascade. The data are well fitted by the sum of the sensitivity loss expected from quantum catch and adaptation of the transduction mechanism, on the assumption that bleached pigment produces an equivalent background light whose intensity is proportional to the amount of bleached pigment (see also Refs. 152, 221). If the intensity of the equivalent background is linearly related to the fraction of pigment bleached, then each opsin molecule must be activating the cascade with the same probability and gain. Furthermore, the close correspondence of this model with the data in Figure 12 indicates that there is no reason to suppose that the mechanism of adaptation in a stably bleached rod differs greatly from that of a rod exposed to a background light, at least for the range of background intensities for which this comparison can be made.

V. CONCLUSIONS

When a vertebrate rod or cone is exposed to steady background light, the sensitivity to a brief flash is decreased, and the kinetics of the response waveform are accelerated. These changes, collectively referred to as light adaptation, have been shown to depend on a change in the free Ca^{2+} in the cytoplasm of the receptor. Results from many experiments indicate that Ca^{2+} functions as a second messenger in the regulation of photoreceptor sensitivity during light adaptation, and there is at present no compelling reason to believe that additional messenger substances play a significant role. Although many effects of Ca^{2+} on the transduction cascade have been discovered, the most significant (at least for rods) seem to be those that are produced directly or indirectly by the Ca^{2+}-dependent modulation of the rate of guanylyl cyclase. Cyclase modulation rescues the photoreceptor photocurrent from saturation, extends the operating range of the light response, and makes it possible for the cell to respond to incremental changes in light intensity despite a maintained increase in the activity of the PDE produced by the steady light. The acceleration of the PDE makes it possible for the photoreceptor to follow more closely incremental changes in light intensity and is mostly responsible for the quickening of the kinetics of the light response. Effects of Ca^{2+} on early stages in the transduction pathway, for example, on the gain or lifetime of rhodopsin, have less of an effect on response sensitivity and waveform but become increasingly important in bright background light. A Ca^{2+}-dependent modulation of the cGMP-gated channels seems to contribute little to adaptation in rods but may play a more important role in cones.

Exposure of the photoreceptor to light bright enough to bleach a significant proportion of the photopigment produces a decrease in sensitivity, called bleaching desensitization, followed by a slow recovery of the photoresponse, called dark adaptation. The desensitization of the photoreceptor is now known to be produced by photoproducts of bleaching, such as Meta II and opsin, that accumulate after the bleach and stimulate the transduction cascade as would an equivalent background light. These photoproducts appear to interact with the G protein transducin in much the same way as light-activated rhodopsin, although with reduced gain. As a result, they produce a maintained elevation in the activity of the PDE and a decrease in circulating current, leading to a fall in Ca^{2+} concentration and an acceleration in guanylyl cyclase velocity. In a stably bleached photoreceptor, the extent of the desensitization can be adequately predicted from a simple model, for which bleached pigment (probably as opsin) produces an equivalent background light proportional to its concentration. The gradual recovery of sensitivity during dark adaptation is produced by the regeneration of the photopigment and the gradual disappearance of this equivalent background stimulation.

VI. APPENDIX

Adaptation of a rod photoreceptor to background light affects both the amplitude and the kinetics of the response to a dim flash of light. Background light changes the steady-state PDE activity as well as the steady-state Ca^{2+} concentration. Because the light-sensitive current follows the cGMP concentration, the effects of adaptation on the light response can be traced primarily to changes in the guanylyl cyclase and the PDE activities. Nevertheless, it has not been clear which manifestations of light adaptation can be attributed to which biochemical pathways. This appendix presents a model of the light response for dim flashes, which shows that the change in kinetics can be attributed principally to the change in background PDE activity, while the change in normalized amplitude is the result of the Ca^{2+}-mediated reduction in the amplitude of the flash-activated PDE activity. Broadly similar conclusions are also implicit in the recent study of Nikonov et al. (206).

We consider a rod photoreceptor in the presence of a background light of intensity I_b, with a steady-state circulating current J_b. The steady-state cGMP and Ca^{2+} concentrations are G_b and C_b, respectively, while the steady-state cyclase and PDE activities are α_b and β_b. The steady-state PDE activity β_b is the sum of the basal, light-independent activity β_d and the activity stimulated by the background light. To analyze light adaptation, we consider the light response to a dim flash superimposed on the background. The flash will give rise to a time- and Ca^{2+}-dependent PDE activity β^*. If G and C are the cGMP and Ca^{2+} concentrations during the flash response, J is the circulating current, α is the cyclase activity, and β is the PDE activity due to the background light (this activity will change due to flash-induced changes in Ca^{2+} concentration), we can write

$$\frac{dG}{dt} = \alpha - (\beta + \beta^*) \cdot G \quad (A1)$$

$$\frac{dC}{dt} = F \cdot J - K_{ex} \cdot C \quad (A2)$$

where $F \cdot J$ is the Ca^{2+} influx through the cGMP-gated channels, with F being a proportionality constant, and K_{ex} is the clearing rate for the Na^+/Ca^{2+}-K^+ exchanger, since the exchanger is operating in the linear range. *Equation A2* ignores Ca^{2+} buffering, a simplification that does not affect the final conclusions regarding light adaptation. We shall also ignore the Ca^{2+} effect on the cGMP-gated channels, since it has been shown to make only a relatively

minor contribution to light adaptation (see Fig. 8 and Ref. 137). For dim flashes, we can write

$$G = G_b - g \qquad (A3)$$

$$C = C_b - c \qquad (A4)$$

$$J = J_b - j \qquad (A5)$$

Linearizing about the operating point during background illumination for flash-induced changes in Ca^{2+} concentration

$$\alpha \approx \alpha_b + a \cdot c \qquad (A6)$$

$$\beta \approx \beta_b - u \cdot c \qquad (A7)$$

where

$$a = - \left. \frac{d\alpha}{dC} \right|_{C=C_b} \qquad (A8)$$

and

$$u = \left. \frac{d\beta}{dC} \right|_{C=C_b} \qquad (A9)$$

Also, $J/J_b = G^n/G_b^n$ and therefore the light response j is given by

$$j \approx n \cdot \frac{J_b}{G_b} \cdot g \qquad (A10)$$

Furthermore, at steady state

$$\alpha_b = G_b \cdot \beta_b \qquad (A11)$$

$$F \cdot J_b = K_{ex} \cdot C_b \qquad (A12)$$

Substituting *Equations A3–A7* and *A10–A12* into *Equations A1* and *A2*, and ignoring second-order terms, we obtain for the deviations g and c

$$\frac{dg}{dt} + (a + u \cdot G_b) \cdot c + \beta_b \cdot g = \beta* \cdot G_b \qquad (A1')$$

$$\frac{dc}{dt} + K_{ex} \cdot c = \frac{n \cdot F \cdot J_b}{G_b} \cdot g \qquad (A2')$$

Because second-order terms have been ignored, the PDE activity elicited by the dim flash β^* is taken to be at the steady-state Ca^{2+} concentration C_b.

A. Ca^{2+} Prevented From Changing During the Flash Response

Equations A1′, A2′, and *A10* provide a model of light adaptation. We use this model first to consider the simpler case in which the intracellular Ca^{2+} has, through experimental manipulations, been prevented from changing during the flash response.

In this case, c = 0, *Equation A2′* is irrelevant, and *Equation A1′* becomes

$$\frac{dg}{dt} + \beta_b \cdot g = \beta* \cdot G_b \qquad (A13)$$

Substituting *Equation A10* into *Equation A13*, we obtain for the light response

$$\frac{dj}{dt} + \beta_b \cdot j = n \cdot J_b \cdot \beta* \qquad (A14)$$

Background light affects the light response by increasing the background PDE activity β_b and by reducing the steady-state Ca^{2+} concentration. *Equation A14* shows that the increase in the background PDE activity β_b speeds up the kinetics of the flash response. It does so by serving as the "rate constant" that governs the delay between a change in PDE activity and the resulting change in cGMP concentration. One way of seeing this is to imagine a step increase in β^*; the response predicted by *Equation A14* is an exponential decay in j with rate constant β_b to a new and reduced steady level of circulating current. In a similar way β_b will govern the delay between a change in PDE activity and the consequent change in circulating current for more physiologically realistic waveforms for the flash-evoked PDE activity. Thus an increase in β_b allows the cGMP concentration, and hence the photocurrent j, to track more closely the underlying change in PDE activity β^* induced by the flash. Although the background leads to a reduction in the steady-state Ca^{2+} concentration, and a consequent increase in cyclase activity, it can be seen from *Equation A14* that this does not itself affect the kinetics of the response. Instead, it leads to an increase in the magnitude of J_b, the circulating current during background illumination, which depends on the balance between cyclase and background PDE activity.

The Ca^{2+}-mediated increase in cyclase activity thereby serves to increase the amplitude of the light response; were it not to take place, the rod would saturate

abruptly at a relatively low background intensity, as for rods in low-Ca^{2+}/zero-Na^+ solution (see Fig. 5, open circles). If we divide the dim flash response by the magnitude of the circulating current J_b (as is frequently done in the analysis and presentation of light-adapted responses), then the restoration of the circulating current by this shift in the balance between cyclase and background PDE activities will not affect the normalized amplitude of the dim flash response. However, in the presence of the background, the flash-induced increment represents a smaller fraction of the total PDE activity, leading to a corresponding decrease in the normalized response amplitude. The only physiologically relevant action of Ca^{2+} concentration on β^* that has been reported is a scaling effect that leaves the kinetics unaffected (94, 119, 149). This reduction in the magnitude of the flash-stimulated increment in PDE activity will lead to a decrease in the amplitude of the normalized dim flash response in the presence of background light. If there were an additional effect of Ca^{2+} on the kinetics of β^*, this would provide a further mechanism through which background light could alter the kinetics of the dim flash response.

B. Ca^{2+} Free to Change During the Flash Response

In the more general case where Ca^{2+} is allowed to change during the light response, we have to solve the system of *Equations A1'* and *A2'*. In the case of dim backgrounds, *Equation A1'* can be simplified. In this case, $K_{ex} > \beta_b$, since $K_{ex} \sim 140$ s^{-1} (93) and $\beta_b \sim 1$ s^{-1}, only slightly higher than the light-independent PDE activity β_d of ~ 0.3 s^{-1} (see Ref. 137). This large disparity between the Ca^{2+} and the cGMP kinetics would hold even if we took native Ca^{2+} buffering into account (150). Because the Ca^{2+} kinetics are much faster than the kinetics of cGMP, we can assume that Ca^{2+} is close to equilibrium at every point during the light response. *Equation A2'* therefore reduces to

$$c \approx \frac{n \cdot F \cdot J_b}{K_{ex} \cdot G_b} \cdot g \qquad (A15)$$

Substituting *Equations A10* and *A15* into *Equation A1'*, we obtain for the light response in the general case

$$\frac{dj}{dt} + \left[(a + u \cdot G_b) \cdot \frac{n \cdot F \cdot J_b}{K_{ex} \cdot G_b} + \beta_b \right] \cdot j = n \cdot J_b \cdot \beta^* \qquad (A16)$$

In the case of dim backgrounds, we can further simplify *Equation A16*, since we can ignore the contribution of the Ca^{2+} effect on the background PDE. The reason for this is that, at dim backgrounds, Ca^{2+}-dependent modulation of the PDE via modulation of early stages in transduction

does not contribute significantly to adaptation (see Fig. 8). Therefore $a + u \cdot G_b \sim a$. We can also assume that α is proportional to C^{-m}, where m is the cooperativity for the inhibition of the cyclase by Ca^{2+}. Then, $\alpha = \alpha_b \cdot C_b^m \cdot C^{-m}$, and from the definition given in *Equation A8*, we have

$$a = m \cdot \frac{\alpha_b}{C_b} \qquad (A8')$$

Substituting *Equations A11, A12*, and *A8'* into *Equation A16*, we finally obtain

$$\frac{dj}{dt} + (m \cdot n + 1) \cdot \beta_b \cdot j = n \cdot J_b \cdot \beta^* \qquad (A17)$$

Equation A17 has the same form as *Equation A14*, which described the dim flash response when the Ca^{2+} concentration was not allowed to change. Therefore, inferences regarding light adaptation in the general case (at least for dim backgrounds) are essentially the same as in the case of constant Ca^{2+}. However, comparison of *Equations A14* and *A17* reveals that allowing Ca^{2+} to change during the light response substantially increases the rate constant that links changes in PDE activity to changes in cGMP concentration by a factor of $(m \cdot n + 1)$, which ranges from ~ 5 to 7. This effect arises through the ability of this dynamic fall in Ca^{2+} to relieve the inhibition of guanylyl cyclase during the light response, allowing the cGMP concentration to track the flash-induced increment in PDE activity more closely. However, it is important to note that this dynamic modulation of guanylyl cyclase will also take place during the response in darkness and that the acceleration of the response kinetics relative to this dark-adapted state will depend on the PDE activity evoked by the background.

Thus the principal mechanism by which the kinetics of the dim flash response are accelerated during light adaptation is the increase in PDE activity induced by the background. The dynamic fall in Ca^{2+} concentration that takes place during the flash response results in a speeding of response kinetics both in darkness and during steady light, a process which has recently been modeled quantitatively by Nikonov et al. (206). In contrast, the steady acceleration of guanylyl cyclase that results from the reduction in Ca^{2+} induced by the background prevents premature saturation of the response to the background itself, but it does not contribute directly to the changes in the kinetics or amplitude of the normalized dim flash response evoked by steady light.

C. Effects of Backgrounds on the Response Waveform

To examine the effect of these changes in background-evoked PDE activity on the light response, it is

necessary to assume a waveform for β^*, the flash-induced rise and decay of PDE activity. For the purposes of illustration, we have used the formalism and parameters proposed by Lyubarsky et al. (168) for the inactivation kinetics of the rod phototransduction cascade. This approach ignores any effects of Ca^{2+} on the magnitude or kinetics of β^*, since their model was obtained under conditions of constant calcium concentration. Nevertheless, it provides a means of assessing the qualitative changes in the waveform of the flash response that would result from the increase in PDE activity evoked by steady light.

Figure 13 illustrates the predicted responses to dim flashes when Ca^{2+} was either prevented from changing (*A*) or was free to change (*B*) during the flash response.
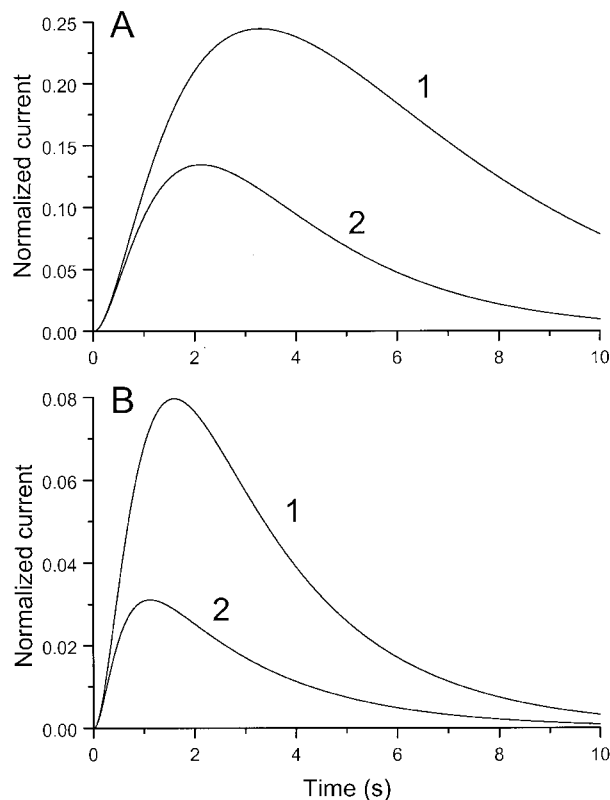

FIG. 13. Simulation of the effect of steady background light on the response to a dim flash according to the calculations described in section VI. *A:* Ca^{2+} prevented from changing during the flash response (see sect. VI, *Eq. A14*). *B:* Ca^{2+} free to change during the flash response (see sect. VI, *Eq. A17*). The increase in phosphodiesterase activity evoked by the flash (β^*) is assumed to follow the waveform given by the model of Lyubarsky et al. (168) with an amplification constant (symbol A in Table 1 of Ref. 168) of $0.06 \ s^{-2} \cdot Rh^{*-1}$ and two deactivation steps with time constants of 0.4 s and 2.4 s (*Eq. 4* in Ref. 168; values of other parameters are as given by Table 1 of Ref. 168). The cooperativity for channel opening by cGMP is assumed to be $n = 2.5$ (as in Ref. 168), and the cooperativity for the inhibition of the cyclase by Ca^{2+} is assumed to be $m = 2$. In each panel, *trace 1* was obtained with a dark-adapted phosphodiesterase activity of $0.3 \ s^{-1}$, and *trace 2* with an increased phosphodiesterase activity of $0.9 \ s^{-1}$ to simulate a background light of $10 \ Rh^* \ s^{-1}$; dim flashes delivered $10 \ Rh^*$. In each case, the flash responses (*j*) have been normalized according to the circulating current (J_b) before the flash.

Each panel in Figure 13 shows a modeled response in darkness (*1*) and during dim steady light (*2*), expressed in each case as a fraction of the total circulating current. It can be seen that the effect of the increase in the steady PDE activity evoked by the background (β_b) was in both cases to reduce the normalized response amplitude, to shorten the time to peak of the response, and to speed the final recovery of circulating current, all these changes parallelling light adaptation. The faster response kinetics result from the ability of the photocurrent to follow the underlying waveform of PDE activity induced by the flash (β^*) more closely in the presence of the elevated steady PDE activity evoked by the background than in darkness. In this simplified model, the normalized response amplitude is reduced because this flash-induced increment in PDE represents a smaller fraction of the total PDE activity in the presence of the background. The amplitude of the adapted rod response is likely to be smaller still through actions of Ca^{2+} on the magnitude of the increase in PDE activity evoked by the flash. Even though both dark-adapted and light-adapted responses are faster when Ca^{2+} was allowed to fall after the flash, it should be noted that the relative degree of response acceleration was comparable in both cases. It is important to note that these qualitative changes in response kinetics and amplitude do not depend strongly on the precise waveform assumed for the increase in PDE activity induced by the flash, but instead are robust consequences of the steady increase in PDE activity evoked by the background.

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