Journal of Statistical Physics, Vol. 39, Nos. 5/6, 1985

Molecular Mechanisms of Visual Excitation: A Concatenation of Nonlinear Cellular Processes

M. W. Bitensky,[1] J. S. George,[1] M. Whalen,[1] and A. Yamazaki[1]

The mechanism of transduction in the photoreceptor remains the least understood link in the chain of molecular and cellular processes that are responsible for vision. This paper reviews the molecular species and processes involved in the absorption of light and subsequent biochemical responses of the photoreceptor cell. The cells' electrical response and the probable ionic basis of the response are described. We develop the outlines of a mechanistic model that attempts to link biochemical and electrophysiological responses of photoreceptors of the vertebrate retina.

KEY WORDS: Vision; retinal pigments; photoreceptors; visual transduction.

1. INTRODUCTION

The vertebrate visual system can be described as an optical system linked to a complex neural network. The optical functions are readily explained by analogy with the camera; the eye has a protein lens, a light aperture (the muscular iris), and a mosaic of photoreceptors arrayed in the focal plane of the retina (Fig. 1). The neural network integrates visual input and transmits visual information along parallel channels through relay neurons in the retina, optic nerve, and lateral geniculate nuclei, finally into the visual cortex. The functional organization of processing elements and at least some of the information processing activities of the visual pathways from photoreceptor to cortex are understood in cellular, molecular, and electrophysiological terms.

The signal-transducing elements at the front end of the neural network are photosensitive cells which are called rods and cones. The highly specialized light-capturing outer segments of rod and cone cells contain a visual pigment called rhodopsin. When rhodopsin absorbs a photon it

[1] Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545.

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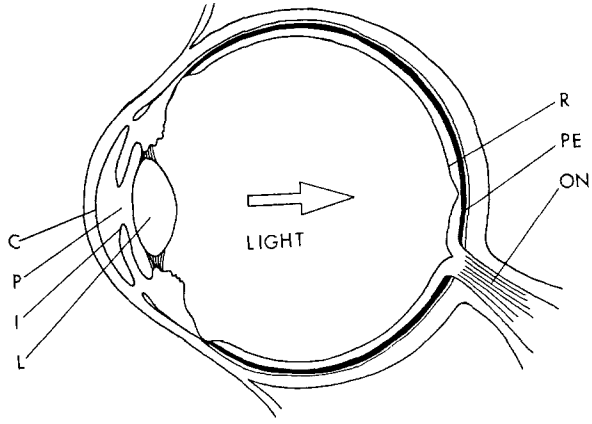


Fig. 1. Schematic cross section of the vertebrate eye. C is the cornea; P is the pupil; I is the iris; L is the lens; R is the retina; PE is the pigment epithelium; ON is the optic nerve.

undergoes a series of spectroscopic and conformational changes that cause a loss of color or bleaching of the pigment. The photon's energy is transduced and enzymatically amplified to produce a cytoplasmic signal which reduces the flow of sodium ions (lowers the Na^+ conductance) of the outer segment plasma membrane. In the dark a steady current is generated by Na^+ extrusion from the photoreceptor inner segment. The Na^+ returns across the outer segment membrane; light suppresses this "dark current," initially causing the outer segment cell membrane to hyperpolarize. The photoreceptor outer and inner segment membranes behave like cables, so that the electrical response of the outer segment changes the membrane potential at the base of the cell, and appears to inhibit the release of an inhibitory neurotransmitter. Successive neural layers are less specialized; the retinal ganglion cells whose axons constitute the fibers of the optic nerve are typical neurons that encode information as frequency-modulated trains of action potentials.

The photochemistry of rhodopsin bleaching is now reasonably well characterized, and the basic electrophysiological mechanism of the photoreceptor response is also understood. The most significant gap in our understanding of the peripheral processes of vision is our lack of knowledge of the molecular mechanisms of visual transduction. How does the photon-initiated bleaching of rhodopsin control the sodium conductance of the photoreceptor plasma membrane to produce the cellular response? The anatomical structure of the photoreceptor with physically separate disk and plasma membranes and the kinetics of the photoreceptor response suggest that a diffusible cytoplasmic transmitter, produced by rhodopsin bleaching, is somehow responsible for the subsequent reduction

in the dark current. Two substances, calcium ion and cyclic guanosine monophosphate, are regarded as attractive candidates for the transmitter role, but the identity of the transmitter is not yet clear. While light-sensitive, biochemical systems have been described in the vertebrate rod, their relationship to the physiological response has not been determined.

The purpose of this paper is to describe those molecular species and processes that appear to support the process of visual transduction. We will also develop the outlines of a mechanistic model that attempts to link biochemical and electrophysiological responses of photoreceptors in the vertebrate retina and discuss the complex yet inevitable goal of deriving physiological insight from biochemistry.

2. RETINAL, RHODOPSIN, AND THE PRIMARY PHOTOCHEMICAL EVENTS

The absorption of a photon by rhodopsin triggers a chain of molecular and cellular processes that constitute the visual process. Rhodopsin is a complex of two covalently linked molecular species: retinal and opsin.[1] Retinal, the aldehyde of vitamin A, is the chromophore, and contributes the primary absorbance function and spectral characteristics of the complex. Opsin is an integral membrane protein that includes a hydrophobic domain that houses retinal; the interaction of opsin with retinal shifts the peak of the retinal absorption spectrum. Different forms of opsin combine with the same chromophore to produce the rod pigment rhodopsin, and the red-, green-, or blue-sensitive cone pigments.

2.1. Retinal

Retinal incorporates an extended conjugated electronic structure that stretches across a nine-carbon polyisoprenoid side chain and into a six-member β-ionone ring (Ref. 2; Fig. 2a). With four double bonds in the side chain, at least 16 stereoisomers can potentially exist. Early analysis suggested that some forms were less probable because of steric hindrance. A number of these improbable forms have been found in nature, including the 11-*cis* isomer that serves as the physiological chromophore. Apparently the retinal polyene system is not rigidly planar. Positions of the double bonds are relatively stable, limiting resonance stabilization of any particular isomer, and allowing twisting about single bonds adjacent to an isomerized double bond, thereby relieving intramolecular hindrance.[3]

11-*cis* retinal combines with opsin to form rhodopsin. Photosensitive pigments can also be produced with 9-*cis* retinal as well as an array of synthetic retinal analogs. The end products of the bleaching of 11-*cis* or 9-*cis*

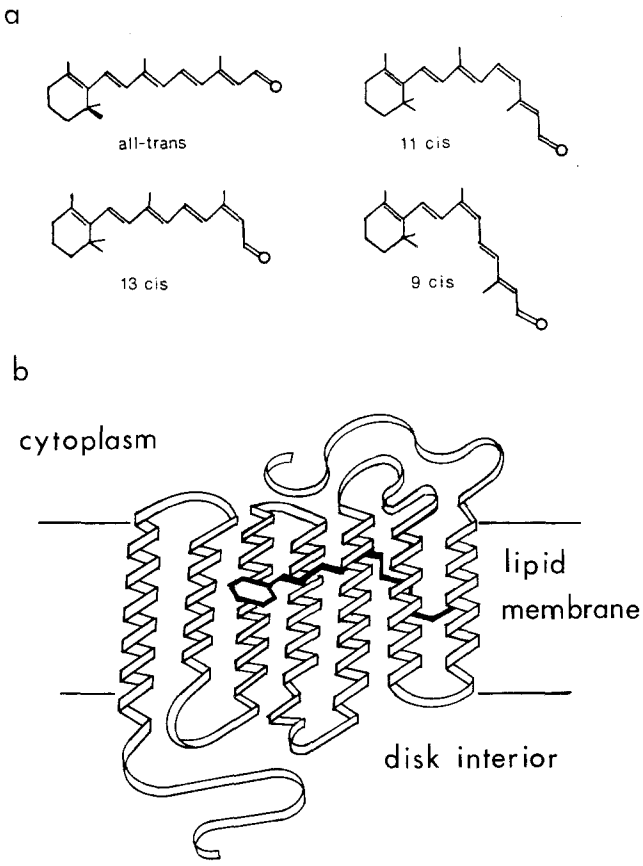


Fig. 2. Retinal and rhodopsin. (a) Structural formulae of some naturally occurring isomers of retinal. The visual pigment chromophore is 11-*cis* retinal. Conformers produced by rotation around single bonds are not indicated. (b) Diagram showing the organization of rhodopsin in the disk membrane. Seven alpha helical peptide sequences span the lipid bilayer; the chromophore is located roughly in the plane of the membrane.

pigments are free all-*trans* retinal and opsin.[4] There are other compelling data that support the basic concept that the primary photochemical event of vertebrate and invertebrate vision is the *cis/trans* isomerization of the retinal chromophore.

2.2. Opsin and Rhodopsin

Once Wald and Hubbard realized the critical importance of retinal conformation in the reconstitution of the visual pigment complex from retinal and opsin, they guessed that the structure and the function of the

complex depended on the "gestalt of protein–chromophore interaction." The smaller molecule must "fit" the larger facilitating hydrogen and electrostatic bonding and short-range van der Waal's interaction with opsin.[1,5]

The amino acid sequence of bovine opsin[6,7] as well as the nucleotide sequence of the encoding gene[8] have been determined. Opsin is an integral membrane protein (Fig. 2b). Much of the molecule is hydrophobic, forming seven alpha helical domains of the protein which span the disk membrane. The amino terminus of opsin is decorated with short carbohydrate chains that protrude into the aqueous space contained within the flattened saccular disk membranes that bear rhodopsin. The carboxyl terminus is relatively hydrophilic, extending into the interdiskal aqueous milieu or rod cytoplasm. The terminal peptide sequence incorporates a number of sites for the covalent attachment of phosphate which become accessible to cytoplasmic phosphorylating enzymes only after bleaching.

The protein portion of rhodopsin effectively orients its retinal chromophore for efficient photon capture. The conjugated carbon chain is located in a plane roughly perpendicular to the long axis of the rod and therefore perpendicular to the normal path of incident photons.[9] Rhodopsin can freely rotate within the plane of the membrane, an arrangement which may also contribute to the efficiency of photon capture.

Retinal is bound to opsin in a hydrophobic pocket buried in the membrane. The range of molecular analogs that can form a complex with opsin suggests that the molecular fit is not tight, but the length of the chromophore is restricted. All-*trans* retinal and other isomers longer than approximately 11.2 Å do not fit the pocket, i.e., do not readily regenerate rhodopsin when combined with opsin.[10]

Retinal is attached to opsin via a Shiff's base linkage to the ε-amino function of a lysine residue at position 296 in the opsin sequence. The amino nitrogen is probably in a quarternary, protonated state in the dark; the proton is lost during bleaching.[9] The positive charge on the nitrogen is probably coupled to one or more counterions on adjacent amino acids. Variation in placement of such counterions could account for the range of photopigment spectra observed in the rhodopsins of different species. This salt bridge is broken by the conformational changes in the complex following photoisomerization of retinal.[2,12] The absorption spectra of later photocomformers of bleached rhodopsin are similar to free retinal.

2.3. Photoproducts

A single photon is sufficient to produce excitation. This has been demonstrated by psychophysical measurements[13] of the threshold of per-

ception and the direct measurements of photoreceptor response.[14] Not every incident photon need be effective to account for perception at the lower threshold of visual activity, but one of every two must be. Moreover, every absorbed photon does not cause bleaching. Exhaustive illumination at very low temperatures, followed by warming of rhodopsin, produces thermal bleaching of about 50%.[15] *In vivo* studies also indicate that only about 1/2 of the photons absorbed cause rhodopsin bleaching.[16] Molecular dynamics calculations indicate that the excited state potential surface of rhodopsin is barrierless[2]; after the high-energy form of the opsin retinal complex is created by the absorption of a photon, relaxation to the original state is only slightly less probable than evolution to metarhodopsin II, the photoconformer which has been linked to the physiological processes of visual excitation.

Following absorption of a photon, rhodopsin proceeds through a sequence of intermediates before the breakdown of the complex and release

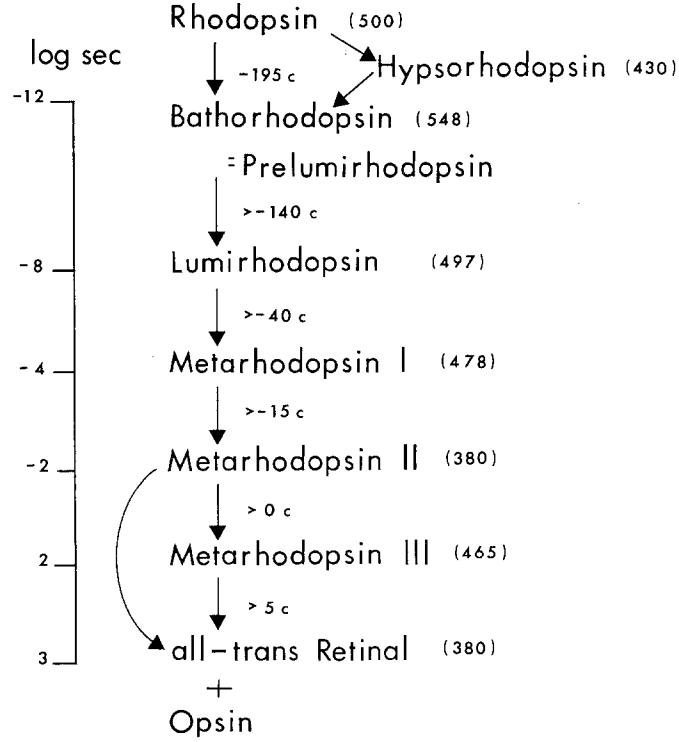


Fig. 3. The sequence of bleaching intermediates of vertebrate rhodopsin. Numbers in parentheses indicate absorption maxima (in nanometers). Temperature notations indicate limits of thermal stability. The time scale indicates approximate transition times at 20°C.

of opsin and free retinal. The intermediates are spectrally defined. Some of the molecular species are so transient that they are only revealed in glycerol water dispersions at liquid nitrogen temperatures or by picosecond spectroscopy.[1–3] The central pathway of pigmant bleaching (*in vitro*) is outlined in Fig. 3.

The 11-*cis*/all-*trans* isomerization of retinal was to be the only photodependent step in the bleaching process.[4,5] The initial photoproduct, forming in 2–8 psec at physiological temperatures, is bathorhodopsin. (An alternative product called hypsorhodopsin can be formed under experimental conditions using very intense illumination.) Bathorhodopsin is stable at liquid-nitrogen temperature or below, and is detected by shift of the absorption spectrum toward a longer wavelength. Irradiation with orange light readily drives bathorhodopsin back to rhodopsin. Bathorhodopsin is obtained by bleaching either 11-*cis* or 9-*cis* pigment complexes; all-*trans* retinal is the only single-step derivative common to both chromophores. Alternative structures for the batho-chromophore have been proposed based on proton translocation along the polyene chain or electron "tunneling." Biophysical tests of these ideas have not supported the alternative structures, but they suggest that the all-*trans* chromophore of bathorhodopsin is deformed within its opsin binding site.[11,17]

Photocalorimetry has shown that the ground state of bathorhodopsin lies 35 kcal/mole above rhodopsin; more that 60% of the absorbed photon's energy is stored in the tension between isomerized retinal and its binding site.[18] Thus bathorhodopsin is an evanescent state, with chromophore and opsin in a distorted geometry. The high-energy excitation step would appear to limit rhodopsin's utility as an energy transducer, but it appears to represent an effective tactical defense against the ambiguities of thermal noise in a biological photon detector.[2]

At higher temperatures, bathorhodopsin undergoes minor conformational changes to produce lumirhodopsin. Lumirhodopsin is characterized by a shift in the absorption maximum back to shorter wavelengths, although in most species the λ_{max} of lumirhodopsin remains higher than that of the native pigment. At higher temperatures, greater conformational changes occur to produce the metarhodopsins. Once again there is a shift to shorter wavelength absorbance maxima.

There are clear indications that concomitant with the appearance of Meta-II, significant conformational changes have occurred in the opsin moiety.[4] At physiological temperatures this photoproduct is formed within approximately 10 msec, and is accompanied by appearance of 2-3 titratable sulfhydryl groups as well as a proton binding group (perhaps imidazole) with a pK_a of approximately 6.6. The transition is also associated with deprotonation of the Schiffs base linkage between

chromophore[19] and protein. Although rhodopsin may be reformed through irradiation of the metarhodopsins with light of the appropriate wavelength, this regeneration may be prevented by first cooling the meta-products to $-60°C$. This presumably locks the protein into a conformation unsuitable for rhodopsin regeneration. Earlier products remain reversible at much lower temperatures. Optical rotary dispersion studies and far uv spectroscopy indicate that free opsin is conformationally different from the complexed form; there are also changes in the mechanical and electrical properties of the molecule as a function of Schiffs base formation.[2,4,11] Meta II is the most likely intermediate to actively participate in visual tranduction; subsequent products are formed much too slowly. Kinetic observations and biochemical trapping experiments suggest that meta II is fact the bleaching intermediate that signals the absorption of a photon to subsequent elements of the excitation cascade.[20,21]

3. PHOTORECEPTOR ANATOMY AND ELECTROPHYSIOLOGY OF THE PHOTORECEPTORS

3.1. Architecture of the Retina

The retina is an ordered neuronal assemblage consisting of six cell types in a layered matrix (Fig. 4; Ref. 22). The layers reflect the anatomical positions of the cell bodies of the six neuronal variants. Photoreceptor cells (rods and cones) are found in the deepest layer of the retina, farthest away from incoming light. Photoreceptors are backed by pigment epithelium which absorbs stray photons and digests the tips of the photoreceptors, which grow continuously.[3,23] The rods and cones are quite distinct morphologically; the name describes the shape. The rods are active primarily at low light intensities; the rhodopsin which characterizes them has a relatively broad absorption band in the blue-green region of the spectrum (λ_{max} at 498 nm).

Cones are primarily active in daylight and support color vision. They are quite inefficient at low light intensities, so that the moonlit world is perceived almost entirely with the coarse resolution and shades of gray which are characteristic of rod-mediated vision. Three types of cone may be defined on the basis of the absorbance maximum of their photosensitive pigments.

The photoreceptors are synaptically connected to the bipolar cells, which in turn drive the ganglion cells. The axonal processes of the ganglion cells constitute the fibers of the optic nerve. These connections form the main through-path for visual impulse propagation; there are, in additon, lateral connections provided by the amacrine and horizontal cells which

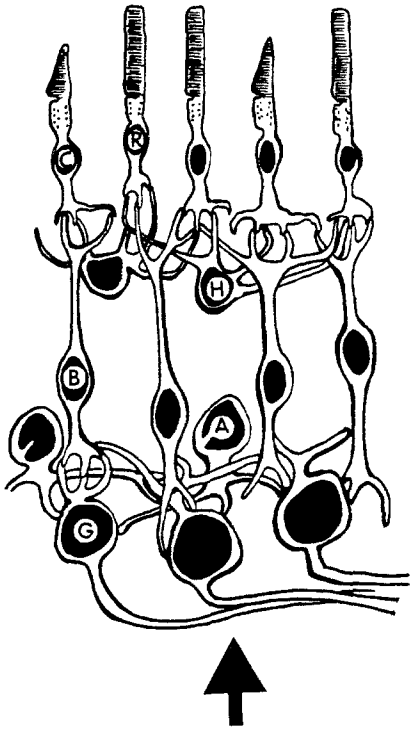


Fig. 4. Diagram of the retina, showing cell types and functional connections. R is a rod; C is a cone; H is a horizontal cell; B is a bipolar cell; A is an amacrine cell; G is a ganglion cell. The arrow indicates the path of incident light.

are involved in retinal data processing of photoreceptor inputs.[22,24] These horizontal connections increase the lateral area over which information can be integrated by the retina, and provide a mechanism for dynamic interaction of photoreceptors. The second function is not exclusive; direct electrical coupling between contiguous photoreceptors has been observed electrophysiologically, and gap junctions have been microscopically demonstrated between rods.[25]

Electrical activity in the photoreceptor, bipolar, and horizontal cells is graded, in contrast to the all-or-none firing of ganglion cells and other neurons. The graded electrical response of these cells is probably reflected in the graded release of transmitter. However, in the optic nerve, stimulus amplitude is encoded by firing frequency.

3.2. Photoreceptor Structure

The absorption of incident photons by rhodopsin occurs in the outer segments of rods and cones (Fig. 5; Ref. 4). The rod outer segment is a modified cilium filled with a free-floating stack of double-membrane closed disks which resemble flattened sacks, all enveloped by the plasma membrane of the cell. Disks are continuously formed at the base of the outer segment by infolding and pinching off of sections of the plasma membrane (Fig. 5). In the cone outer segment, most membrane invaginations do not entirely seal; the interior of such disks remains open to the extracellular cytoplasmic space.[3,26]

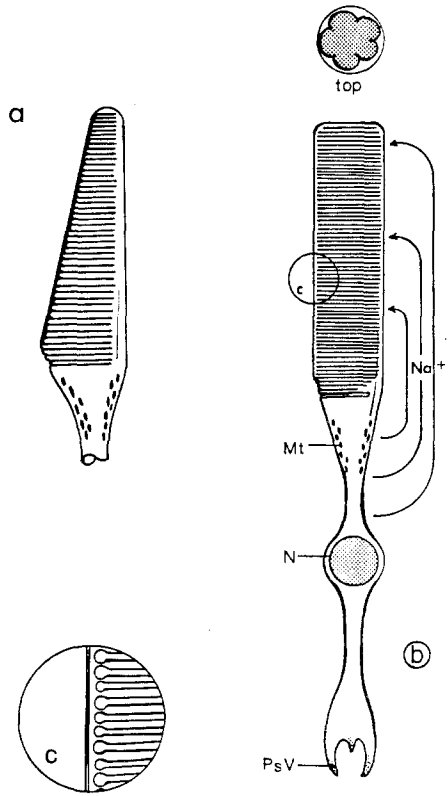


Fig. 5. Structure of vertebrate photoreceptors. (a) Diagram of a cone outer segment showing infolding of the plasma membrane. (b) Diagram of a rod, illustrating flow of the Na^+ "dark current." Mt are mitochondria; N is the nucleus; PsV are presynaptic vesicles containing neurotransmitter. (c) Structure of rod outer segment disks. These "free-floating" organelles containing most of the cell's rhodopsin are formed by infolding and pinching off of the plasma membrane.

The rod disk lumen contains the glycopeptide amino terminus of rhodopsin and is probably filled with an aqueous ionic fluid.[27] 100 Å is a generally accepted value for the thickness of the disks, which implies that the shortest dimension across the flattened lumenal space is 15–20 Å. The long external diameter of the disk ranges from 400 Å in the rat to 4000 Å in the frog. The disks are estimated to contain 10% of total outer segment fluid volume.[27]

Rhodopsin contributes as much as 30% of the rod outer segment mass; 3×10^7 rhodopsin molecules are present in the typical rat rod,[27] 1.5×10^9 in a larger amphibian rod. These molecules are distributed in what has been termed a "quasicrystalline" or "solid-state" array in the disk membranes.[9] A number of other peripheral and integral membrane proteins (besides rhodopsin) have been demonstrated in disks.

Dense clusters of mitochrondria are found along stretches of the inner segment plasma membranes in the region of the cell's major metabolic activity. The base of the cells is similar to that of other neurons. Photoreceptor synaptic function appears inhibitory with light-induced hyperpolarization of the photoreceptor inhibiting the release of a putative inhibitory neurotransmitter. In contrast, excitation depolarizes most of the cells comprising the rest of the visual pathway.

3.3. Electrical Activity

Stimulation of most electrically excitable cells—including the cells of the neural retina—is associated with relative membrane depolarization.[22] This is due to an influx of Na^+ into the electrically negative cytoplasm of the cell. The cell interior may remain negative throughout firing due to fixed anions, but the influx of positive charge decreases the potential difference across the membrane. Electrophysiologically, this is characterized as increased conductance to sodium ions along an established potential gradient. Although typical neurons may hyperpolarize in the late course of an action potential, the initial (and characteristic) behavior during nerve firing is depolarization. Depolarization triggers the release of neurotransmitter from these cells.

In contrast, the photoreceptor cells of vertebrate eyes respond to incident radiation with membrane hyperpolarization; a larger potential difference is established across the cell membrane. In comparison with typical excitable cells, they behave as though dark were the stimulus and light represented an absence of stimulus. Yet many optic nerve fibers respond to receptive field illumination by increased firing. How is the stimulus-negative response of the photoreceptors translated into the stimulus-positive response found in the rest of the sensory chain?

The most likely possibility is that inhibitory transmitter is continually released in the dark by the depolarized photoreceptor synapse: i.e., dark photoreceptors continuously inhibit connected neurons. An inhibitory transmitter functions as a logical "not" in an analog neuron such as the bipolar cell. Light-induced slowdown of photoreceptor release of an inhibitory transmitter would depolarize the bipolar cell and cause increased stimulatory transmitter release at the bipolar/ganglion synapse. Many cone-driven bipolar cells are noisier in light than dark, suggesting that in light, smaller quantitities of inhibitory transmitter are released by cones.[28]

With intracellular microelectrodes cones were demonstrated to have resting membrane potentials of 30–40 mV in darkness; bright light causes a prompt and reversible hyperpolarization to negative 40–60 mV.[29] Rods also hyperpolarize in response to light, though sensitivity and response kinetics are different. During the light response, recording electrode resistance increases by several megohms, probably due to an increase in membrane resistance in some part of the cell.[30] Measurements of the voltage distribution around the photoreceptor indicate that the relatively low membrane potential of the unilluminated photoreceptor is due to a cationic membrane current which enters the outer segment. This "dark current" is generated by inner segment plasma membrane ionic pumps which extrude Na^+. Na^+ reenters the rod outer segment rod via channels or carriers in the outer segment membrane. The outer segment is connected to the inner segment by a microtubular assembly called the axoneme or connecting cilium. It is the Na^+ dark current which is transiently reduced by light.[31] Absorption of a single photon by the rod receptor outer segment reduces the dark current by 3%–5%. Absorption of 30 photons halves the rod dark current.[14] The relation between stimulus intensity and current suppression is hyperbolic. As responses saturate in amplitude they increase in duration.

When $Tris^+$ or Li^+ is substituted for Na^+ in the perfusion fluid of an isolated retina, the dark current and photoresponse are entirely suppressed. Ouabain, a Na^+ K^+ ATPase pump inhibitor also suppresses the dark current. Washing the ouabain-inhibited retina in low Na^+ and then switching to high Na^+ reestablishes a driving force for the Na^+ current and temporarily restores it.[31] Measurements of outer segment ionic composition suggest that ionic gradients comparable with those in nerve cells drive the electrical activity of the receptor membrane. Thus the dark current probably enters the outer segment plasma membrane as a Na^+ influx. The sodium conductance of the plasma membrane, estimated to be 400–1200 mho in the dark, falls rapidly with illumination.

This explanation of photoreceptor electrical activity is well supported by experiment and has proven durable.[32] The mechanism by which

rhodopsin photochemistry is actively coupled to outer segment plasma membrane electrical response is less well understood.

4. VISUAL TRANSDUCTION

4.1. Diffusible Transmitter

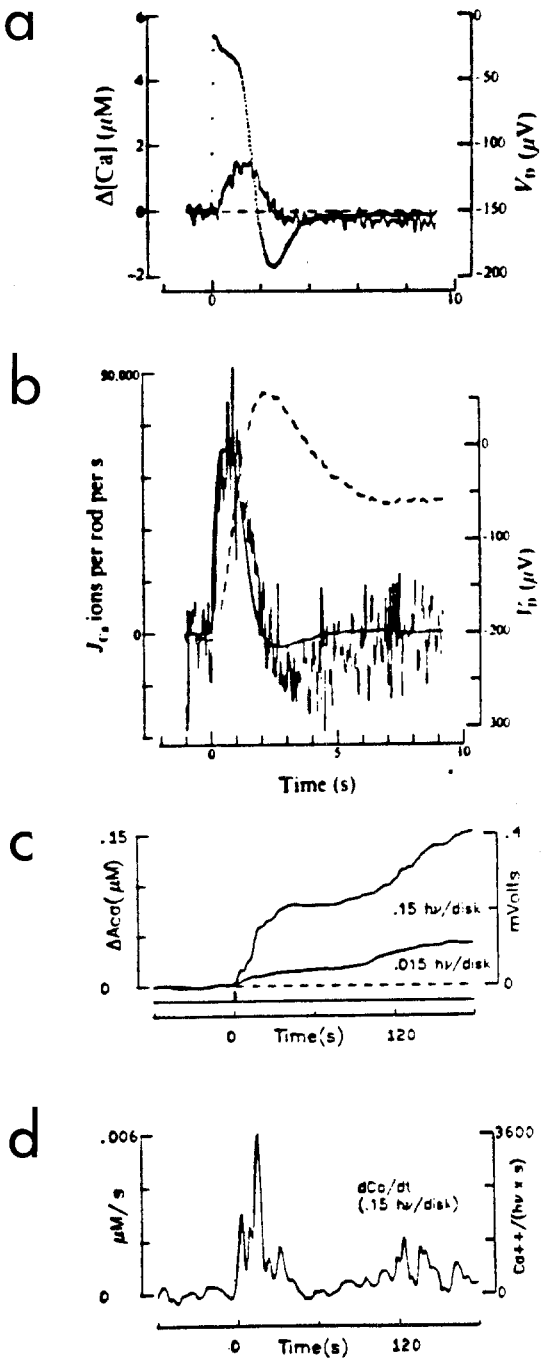
How might rhodopsin bleaching suppress sodium influx through the plasma membrane? There are two important considerations, First, ultrastructural, electrical, and dye-staining analyses of rod outer segments indicate that the rhodopsin-bearing disks are not continuous with the cell plasma membrane. Thus, on purely morphological grounds, diffusion of a substance might be required for communication between the disk and plasma membrane. There is also a profound need for signal amplification within the visual system. The absorption of a single photon by rhodopsin must account for a 5% change in a Na^+ flux which may be as high as 3×10^8 ions/sec and may involve hundreds to thousands of Na^+ channels or carriers.[31]

Baylor and Fuortes suggested an internal transmitter to explain the saturable kinetics and the shape of the vertebrate cone response.[33] Yoshikami and Hagins, in the course of their investigation of the dark current, concluded that the two essential requirements of a transduction link, diffusion and amplification, could be met by postulating that a photon releases many transmitter molecules from the outer segment disks.[27,34] The transmitters would diffuse through the cytoplasm and bind at sites along the plasma membrane. Binding of the transmitter would interfere with the entry of sodium ions.

4.2. Ca^{2+} Transmitter Hypothesis

Yoshikami and Hagins observed that increasing the calcium concentration in the solution perfusing an intact retina rapidly and reversibly suppressed the dark current, mimicking the effect of light.[34] When the cells were rendered permeable to Ca^{2+} by ionophores, a lower concentration of Ca^{2+} was required, suggesting that internal (cytoplasmic) (Ca^{2+} is more effective than external in this function. These observations lead to the suggestion that Ca^{2+} is the internal transmitter mediating visual excitation. There is ample biological precedent for such an idea; in other "excitable" systems cytoplasmic Ca^{2+} appears to mediate cellular response, including muscle contraction and glandular secretion.

The model proposed that Ca^{2+} is actively accumulated and sequestered by disks: that a Ca^{2+} concentration gradient is established and


a
Δ[Ca] (μM)
V_{ip} (μV)
b
J_{ca}, ions per rod per s
I_{ip} (μV)
Time (s)
c
ΔAcσ (μM)
mVolts
.15 hν/disk
.015 hν/disk
Time(s)
d
μM/s
dCa/dt
(.15 hν/disk)
Ca++/(hν s)
Time(s)

maintained by expenditure of metabolic energy. Ca^{2+} might be released in such a system by simply opening a gate. Several approaches have been used to calculate the quantity of Ca^{2+} that should be released by a photon in order to satisfy model constraints. Theoretical answers range between 20 and 1000 Ca^{2+} per photon.[27,35] Although the disks could accommodate the requisite stores of Ca^{2+}, the experimental demonstration of light-stimulated release of appropriate quantities of Ca^{2+} has required almost a decade.

4.3. Ca^{2+} Release

Evidence for light-induced Ca^{2+} release from rods which appears to satisfy both the quantitative and kinetic criteria demanded by measurements of photoresponse has come from several laboratories using different but related approaches (Figs. 6a and 6b). If a Ca^{2+} selective microelectrode is inserted into the space between photoreceptors in the outer segment layer of an isolated retina, or a planar Ca^{2+} selective electrode is placed against the retina, an efflux of Ca^{2+} from the outer segment layer is observed almost simultaneously with the light response.[36,37] By taking into account diffusion limits and the volume and geometry of the rod and the space between outer segments and the kinetics of perfusion, it is possible to calculate the Ca^{2+} efflux which produced the observed changes in interstitial Ca^{2+} activity. The calculated Ca^{2+} flux agreed well with observed changes in the dark current.

However, these were extracellular measurements, and the cellular or molecular mechanisms for Ca^{2+} extrusion were not well characterized.

Fig. 6. Ca^{2+} efflux from rods. (a) Ca^{2+} fluxes from the retina measured with Ca^{2+} selective microelectrodes (from 36). Changes in interstitial dark voltage gradient (Vd or V_D) and Ca^{2+} activity (Aca) in the rod layer of an albino rat retina. 2 ms flashes at 560 ± 20 nm at $T=0$ delivered 110 photons/rod. Noisy curve is Aca. Dashed curve is change in Vd. Dotted line is drawn at preflash level. Each curve is the mean of 16 responses. (b) Time course of the light-induced Ca^{2+} flux from the rod layer compared with the light-induced reduction in Vd. Smooth line: Vd response convolved with exponential response function of time-constant 130 ms to simulate time lag of Ca^{2+} electrode. Noisy line: Ca^{2+} flux from rods (J_{Ca}), computed as described.[36] Dashes: time integral of calculated Ca^{2+} flux. Data are averages of four responses to stimulus as in a. Time course of the Ca^{2+} efflux and changes in Vd are similar. About half of the Ca^{2+} released by the flash is reaccumulated within 10 sec in this example. (c) Light-induced rise of Aca measured with a Ca^{2+}-selective electrode in a medium containing broken rod outer segments (from Ref. 39). Response of a single suspension to 2 sec of illumination delivering 0.15 or 0.015 *hv*-absorbed/disk. Curves were corrected for small drifts in the baseline (<0.01 mV/min). (d) Numerical derivative of 0.15 *hv*/disk response from c. Ca^{2+} fluxes indicated are lower limits of actual release since no correction was made for the electrode response time or for any simultaneous Ca^{2+} removal.

Ca^{2+} efflux appeared to depend at least partially on Na^{+}/Ca^{2+} exchange.[36,38] Since photoreceptor membrane hyperpolarization in the light increases the electrochemical potential driving such an exchange reaction, it might be possible that the observed Ca^{2+} efflux was actually a consequence of hyperpolarization rather than the cause; i.e., cytoplasmic Ca^{2+} might actually decrease in response to light.

Subsequent experiments have utilized suspensions of isolated rod outer segments with leaky plasma membranes. These experiments indicate that Ca^{2+} is released from the disks by light. Given an appropriate metabolic energy source, disks take up Ca^{2+} in the dark. Depending on the intensity of a light stimulus, less than one to more than 100,000 Ca^{2+} per photon may be rapidly released by light (Figs. 6c, 6d; Ref. 39). Electrode response and chamber mixing times limit the capability to resolve Ca^{2+} release kinetics, which are in some cases clearly faster than the recording system response.

Even considering the limitations of these experimental approaches, the data certainly suggest that Ca^{2+} is released from disks by light: the amount of Ca^{2+} is sufficiently large and the kinetics of release sufficiently fast to support visual transduction. The precise molecular mechanisms supporting Ca^{2+} uptake and release are less certain, but recent studies have suggested some appealing models (see below).

4.4. cGMP as Transmitter

In recent years cyclic guanosine monophosphate (cGMP) has received increasing attention as a possible modulator or participant in the process of visual excitation. cGMP is a derivative of guanosine triphosphate (GTP), the high-energy phosphate form of one of the four nucleotide building blocks of genetic material. Cyclic nucleotides, particularly cAMP and cGMP, are also involved in a variety of intracellular signaling systems. In most of the well-characterized examples the cyclic nucleotides appear to regulate cyclic nucleotide dependent protein kinases.[40] The kinases are a class of enzymes which specifically phosphorylate target proteins, often in direct response to cAMP or cGMP.

A fast, light-activated enzyme that hydrolyzes cGMP is found in the outer segment disk membranes.[41] The enzyme, phosphodiesterase (PDE), is rapidly activated as a consequence of rhodopsin bleaching,[42,43] and then spontaneously inactivated in the presence of physiological levels of GTP and ATP.[44] Light-sensitive cGMP metabolism involves at least two levels of amplification: as many as 300–500 PDE molecules can be activated by a single bleached rhodopsin, and 2000 cGMP molecules can be hydrolyzed per second before PDE is inactivated.[45,46] Coupled with physiological

evidence to be discussed, these observations have raised the suspicion that cGMP also participates in the process of phototransduction.

4.5. PDE Activation

The biochemical cascade of PDE activation has been extensively studied (Fig. 7; Refs. 42, 43). It involves at least a dozen identified gene products which have been characterized to varying degrees. The outline of the control algorithm is reasonably well established, though new mechanistic details are still being added.

The molecular machinery regulating cGMP hydrolysis may be logically divided into three segments. The first consists of rhodopsin and an array of proteins that can modify its biochemical interactions following absorption of a photon. The second is a GTP-binding protein that modulates the activation of PDE. The third functional domain is the PDE complex itself. Herein lies the catalytic activity that hydrolyzes cGMP.

The Meta II form of bleached rhodopsin can specifically activate about 400 GTP-binding protein complexes by promoting the exchange of GTP for GDP bound to the α subunit of the GTP-binding protein.[47,48]

The other GTP-binding protein subunits (β and γ) appear to potentiate GTP-binding[49] and γ may be a locus at which the complex interacts with the disk membrane.[50]

In the dark the two major subunits of phosphodiesterase are associated with a heat stable inhibitory subunit. After formation of the complex Gα GTP, the inhibitor is released from PDE into the cytoplasm, turning on PDE catalytic activity.[51]

Within 100 msec,[52] GTP bound to Gα is hydrolyzed by an intrinsic enzymatic activity forming the inactivated Gα GDP complex. Gα GDP can be cyclically reactivated by GTP and bleached rhodopsin until rhodopsin is itself inactivated, presumably by phosphorylation and/or binding of 48K.[53]

Metarhodopsin II can be inactivated by the regeneration of the "dark" form of the pigment. However, this process, operating via the normal physiological pathways, is much too slow to account for the physiological turn off of PDE. Apparently Meta II is rapidly inactivated by multiple serine and or threonine phosphorylations catalyzed by the enzyme opsin Kinase.[44] Another auxilliary protein called "48K" appears to have a high affinity for phosphorylated metarhodopsin and forms a complex with it, thereby limiting access of the GTP binding protein sununits.[54] It is not clearly known whether the inactivation of rhodopsin reflects its phosphorylation or the binding of 48K which may be facilitated by this phosphorylation. The phosphates on rhodopsin are removed during or

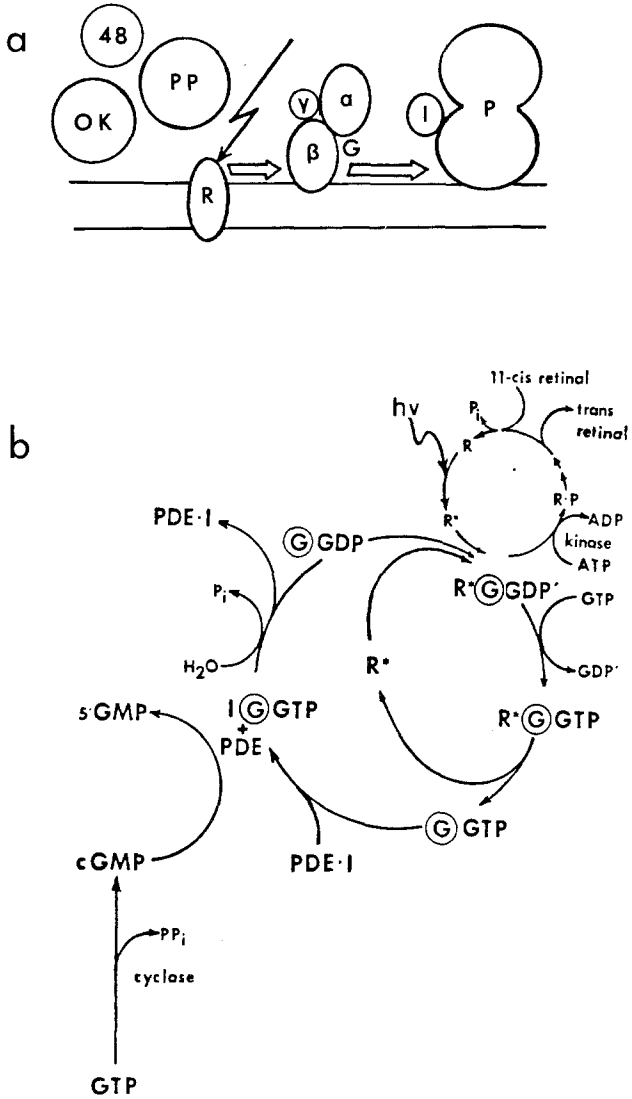


Fig. 7. Cyclic GMP metabolism in the photoreceptor. (a) Major protein components in the activation cascade of phosphodiesterase. R is rhodopsin; G is the GTP binding protein complex; P is phosophodiesterase; I is the phosphodiesterase inhibitor; OK is opsin kinase; PP is a protein phosphatase; and 48 is a 48K dalton protein. (b) Cyclic biochemical pathways mediating the activation and subsequent shutdown of cGMP hydrolysis in response to transient illumination. A bleached rhodopsin molecule (R*) serially activates many G protein complexes by catalyzing the exchange of GTP for GDP bound to the α subunit. G activates P by releasing the inhibitory subunit. G_α is inactivated by hydrolysis of bound GTP. R* continues to activate G until it is phosphorylated or regenerated.

after rhodopsin regeneration by another uncharacterized enzyme, phosphoopsin phosphatase.

4.6. cGMP as Transmitter

The purpose of so much molecular machinery is not yet clear since the function of cGMP in the photoreceptor has not yet been clearly established. This is opposite to the case for the ROS Ca^{2+} regulatory system where the putative function is clear but the molecular mechanisms and participating gene products are not well defined.

Nevertheless, models have been proposed to explain how a change in cGMP levels in the photoreceptor cytoplasm could control the cell's physiological response by modulating the activity of a protein kinase. There is no compelling evidence that such a system (specifically sensitive to cGMP) exists in the rods. Moreover, while phosphorylation and dephosphorylation of some proteins is found, the on/off kinetics are far too slow for visual excitation. A second possibility is that cGMP levels might directly control the Na conductance channels. While there is evidence that cGMP can influence disk membrane ionic conductance,[55] the physiological significance of these observations is not yet clearly understood.

There are important theoretical and experimental difficulties with the idea that a light-mediated decline in cGMP concentration can modulate the dark current. The design of such a system is not well suited for a high-sensitivity, high-reliability detection device. A small increase imposed upon a very low background is easier to detect than a small decrease upon a high background level. Also it has proven difficult to experimentally demonstrate a light-induced fall in rod cGMP levels under physiological conditions. Moreover, in the dark, when PDE is inactive, a large fraction of the cGMP may be bound to noncatalytic, high-affinity, cGMP-specific sites on PDE.[56] On the other hand, there are very suggestive data that light stimulates a large amount of cGMP hydrolysis without a substantial fall in cGMP concentrations.[57] This suggests that the light-activated catabolism of cGMP must somehow be balanced by increased rates of cGMP synthesis.

It is possible that cGMP hydrolysis might serve as an intracellular signal even in the absence of a significant change in the level of bound cyclic nucleotide. Hydrolysis of cGMP produces 5′-GMP, which has a lower pK_a, so that a proton is generated by hydrolysis of cGMP at physiological pH. pH changes due to this reaction may be monitored and are the basis of a useful kinetic assay of PDE activity.[45] Protons explosively generated by light-mediated cGMP hydrolysis might drive or

modulate other processes in the photoreceptor outer segment, even processes related to Ca^{2+} release!

4.7. Physiological Activity of cGMP

As a clearer picture of light-sensitive cGMP metabolism in photoreceptors emerged, a number of investigators have described physiological effects on photoreceptor function which are related to elevated cGMP. Exogenous application of PDE inhibitors such as methylisobutylxanthine (MIX) increases the dark current and the size of the maximum photoresponse recorded intracellularly. Such treatment slows the kinetics of rise and recovery for subsaturating photoresponses, while speeding recovery of the dark current following a large "adapting" flash.[58,59]

Intracellularly injected cGMP further depolarizes the plasma membrane of the dark rod and increases the latency of a subsequent light response (from <0.1 to >5 sec), as though the injected nucleotide must be hydrolyzed in order to permit a light response.[60] If a flash is not delivered following a cGMP injection, the dark current remains high and relatively stable for 20–40 sec before relaxing to preinjection levels in 5–10 sec.[61] This suggests a saturable locus of cGMP action; above the saturating level, larger quantities of injected cGMP do not affect the amplitude of depolarization but increase the duration. In the dark, small injections of cGMP cause transient outer segment depolarization; the recovery from such injections was accelerated by light, though "apparent PDE activity" estimated from recovery rates did not peak until after the peak of the light response.[62]

The effects of cGMP or analogs, MIX, or protaglandins suggested to Lipton *et al.* that Ca^{2+} and cGMP are interelated messengers in the ROS cytoplasm. They postulated that low Ca^{2+} might lead to increased cGMP levels and/or that high cGMP levels might lower cytoplasmic Ca^{2+}, perhaps by stimulating Ca^{2+}-ATPase pumps in the outer segment.[58,63]

Recent studies of isolated rod outer segments support the idea that cGMP can affect rod electrophysiology through effects on Ca^{2+} metabolism. cGMP stimulates Ca^{2+} uptake by rod disks, apparently by stimulating the hydrolysis of ATP which indirectly drives uptake.[39,64] Such a process would be expected to cause membrane depolarization in the context of the Ca^{2+} model. A second observation is that cGMP hydrolysis releases Ca^{2+} from stores in the disks.[65] The mechanism of this effect is not understood though it is clear that the effect cannot simply be explained by a fall in cGMP levels and resultant slowing of Ca^{2+} uptake.

5. MECHANISM OF TRANSDUCTION

While there remain a number of unanswered questions about the mechanism of visual transduction, the growing body of experimental data allows us to suggest a reasonable model. This is summarized in Fig. 8.

5.1. Control of the Dark Current

In the dark, Na^+ flows down its electrochemical gradient into the outer segment of the photoreceptor. The dark conductance is relatively nonspecific, and will admit a variety of other ions including Ca^{2+} under experimental conditions.[32] The molecule or molecules responsible for accommodating this Na^+ current have not been identified. It is not clear from conductance measurement whether the Na^{2+} conductance results

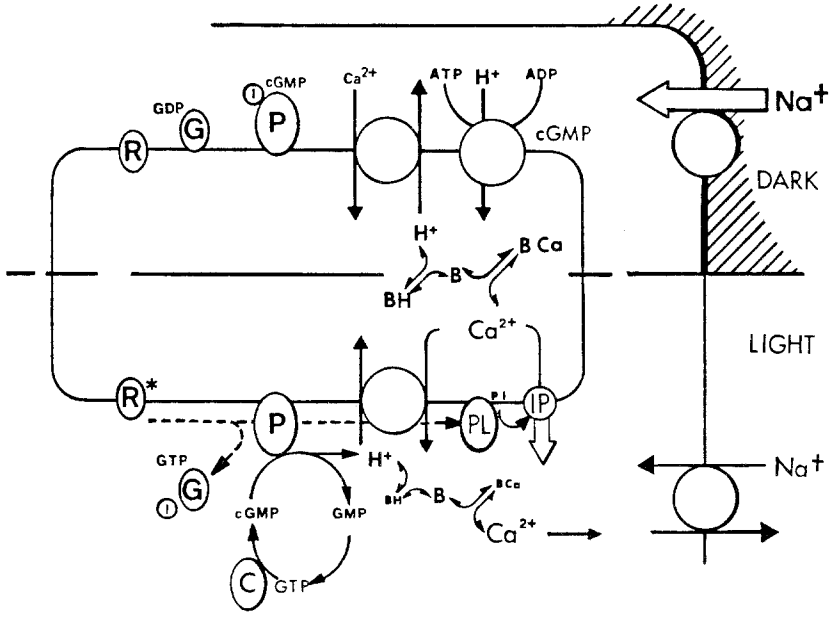


Fig. 8. A model of visual transduction. In the dark (upper half of diagram) the Na^+ current through the plasma membrane is large because cytoplasmic Ca^{2+} is low. Rhodopsin (R), G, and phosphodiesterase (P) are inactive. cGMP levels, ATPase activity, and Ca^{2+} uptake by H^+/Ca^{2+} exchange are high. Light bleaches rhodopsin, activating G and thereby P. H^+ produced by cGMP hydrolysis exchange for disk Ca^{2+} and compete for binding sites (B) in the cytoplasm or disk lumen. cGMP is regenerated by guanylate cyclase (C). Active G might also activate a phospholipase (PL) to produce a lipid-derived Ca^{2+} carrier or inophore such as IP_3 to allow Ca^{2+} to move along its concentration gradient. High cytoplasmic Ca^{2+} inhibits the Na^+ current but is removed by Na^+/Ca^{2+} exchange.

from a carrier shuttling ions across the membrane, a transmembrane channel, or something functionally in between.

A rise in cytoplasmic Ca^{2+} in response to photon capture probably directly triggers the shutdown of the dark current. The measurement of Ca^{2+} release by light constitutes a proof of opportunity, but a number of physiological observations also strongly support this conclusion. In the light, Na^+/Ca^{2+} exchange through the plasma membrane tends to lower cytoplasmic Ca^{2+} and appears to be a primary mechanism of recovery following transient illumination. Hagins has made the interesting suggestion (with some support from experimental data) that Na^+/Ca^{2+} exchange and the Na^+ dark current may be different operational modes of the same molecular carrier.

5.2. Ca^{2+} Metabolism

In the dark, rod outer segment disks accumulate Ca^{2+}. This process is driven by expenditure of metabolic energy in the form of ATP. In certain tissues such as muscle, Ca^{2+} transport is directly coupled to ATP hydrolysis by a specialized ion pumping enzyme. Several lines of evidence suggest that in the disk, the coupling is indirect. ATP hydrolysis appears to drive transport of protons across the disk membrane, alkalinizing the cytoplasm and creating an electrochemical potential. The proton gradient is subsequently exploited to drive Ca^{2+} uptake by H^+/Ca^{2+} exchange.[64] The effect of cGMP to stimulate Ca^{2+} uptake appears to be through stimulation of ATP hydrolysis.[64,66] The rate of Ca^{2+} uptake by disks and the steady state levels of Ca^{2+} inside the disk in part reflect levels of free cytoplasmic cGMP.[39,64]

Ca^{2+} is buffered in the cytoplasm and within the disk lumen. Ca^{2+} is probably bound to the anionic head groups of the lipids (phospholipids and glycolipids) comprising the disk membranes. Certain disk membrane lipids, such as the ganglioside GD_3 (Fig. 9a), have chemical structures particularly suitable for the chelation of divalent cations. The configuration of the carbohydrate moiety of GD_3 could place the two carboxyl groups of the molecule (on adjacent sialic acids) 5–10 Å apart.[67,68] This distance may be an important factor in the Ca^{2+} chelating capacity of GD_3. Ca^{2+} might also bind to charged moieties of membrane lipids or specialized proteins. The affinity of most of these binding sites is a function of pH, with protons competing with Ca^{2+} for common binding sites.[70]

5.3. Cyclic GMP Metabolism

Several processes regulate the level of free cGMP in the cytoplasm. Presumably the most potent is the light-activated phosphodiesterase, which

has been described in some detail. Guanylate cyclase (GC) is the enzyme that synthesizes cGMP from GTP. The rod GC enzyme is one of the most active forms yet studied. The possibility that cyclase may be regulated by Ca^{2+} or by levels of cGMP itself make this a potentially important regulatory site.[69,70] Free cGMP levels are also regulated by binding. Two or more cGMP molecules can be bound to noncatalytic sites on PDE[56]; evidence from biochemical experiments suggests that this bound cGMP is in fact released by the light-induced formation of the complex $G\alpha$.GTP. The function of the bound cGMP is not clear. It might somehow regulate the activity of the enzyme, or serve as a ready supply of substrate to be hydrolyzed. A jump in free cGMP (associated with inhibitor release by light and GTP) might stimulate Ca^{2+} uptake during recovery from a flash. On the other hand, the inhibitor of PDE stimulates cGMP binding to noncatalytic sites and could cause a sudden fall in [cGMP] following inactivation of $G\alpha$.

The activation of phosphodiesterase is one of the fastest biochemical responses known to be mediated by bleached rhodopsin. As previously described, PDE activation involves the formation of a complex of the α subunit of the G protein with GTP. This process is accompanied by the release of a PDE inhibitor, and very rapid PDE activation.

5.4. cGMP/Ca^{2+} Interaction

Our studies suggest that cGMP hydrolysis might elicit an increase in cytoplasmic Ca^{2+} by at least two mechanisms. A fall in cGMP levels would be expected to slow Ca^{2+} uptake and reduce steady state stores of disk Ca^{2+}. The significance of this mechanism for transduction is doubtful, since to rapidly release large amounts of Ca^{2+} would require a large passive leak, which would be energetically inefficient. Also it is not clear that cGMP levels fall rapidly in the illuminated photoreceptor under physiological conditions. This mechanism would seem more suitable for adaptation.

Direct release of Ca^{2+} by cGMP hydrolysis has been observed and is a more likely mechanism for PDE involvement in visual transduction. The process should not depend on a cGMP concentration change, and in fact might work better in the absence of such a change. Kinetics should to some extent reflect the kinetics of PDE activation, which are fast. The possibility that protons generated by cGMP hydrolysis are the basis of the mechanism is attractive, given the apparent importance of transmembrane proton gradients for Ca^{2+} uptake in the rod. Protons produced in the cytoplasm would tend to shunt the disk transmembrane gradient, slowing uptake, and eventually releasing Ca^{2+} by H^+/Ca^{2+} exchange. Also, by competing with

Ca^{2+} for binding sites in the cytoplasm or on membrane surfaces, protons would tend to raise Ca^{2+} activity in the cytoplasm.

Even this mechanism may not be sufficient to account for the release of Ca^{2+} by light observed experimentally and required by models to control the dark current. The mechanism may not be fast enough, particularly in response to a flash. Under some *in vitro* experimental conditions using broken rod preparations, Ca^{2+} release by cGMP hydrolysis appears to have a significant latency. In many light response experiments with isolated rod outer segments a distinctly biphasic release of Ca^{2+} was observed. The first phase was rapid without discernable latency; the second phase was much larger but became apparent only 1–2 min following the flash. This secondary response may be due to cGMP hydrolysis. While the mechanism might be much more rapid in fully intact photoreceptors, the question of what molecular mechanism produces the initial Ca^{2+} response remains of primary interest.

5.5. Lipid Metabolism

One possibility with merit is that light might produce some sort of biochemical ionophore, to allow Ca^{2+} to move down its concentration gradient across the disk membrane. Early studies prompted by Yoshikami and Hagin's model looked for such a function in rhodopsin itself. There has been, however, no convincing demonstration that bleached rhodopsin might serve as a physiological Ca^{2+} ionophore. There is some evidence that bleached rhodopsin may conduct protons,[71] which might collapse the electrochemical gradient driving Ca^{2+} uptake. GD_3 (Fig. 9a) is a candidate Ca^{2+} exchanger in the disk membrane. Gangliosides are known to be capable of passing into a hydrophobic environment when associated with Ca^{2+}.[72,73] The physical compaction and charge neutralization of the GD_3 headgroup upon Ca^{2+} chelation might facilitate transmembrane movement of the complex. The perturbation of the ROS (dark) ionic steady state by light-mediated proton generation (due to cGMP hydrolysis) could trigger GD_3-mediated shuttling of Ca^{2+} across the disk membrane, as GD_3 can interact with both protons and Ca^{2+}.[74] GD_3 is available for such a function as it is the predominant ganglioside in ROS comprising $>50\%$ of the total ganglioside.[75]

In other biochemical systems, Ca^{2+}-dependent processes may be triggered by the breakdown of certain membrane lipids releasing molecules that can transport Ca^{2+}. The breakdown of phosphatidyl inositol (PI) and production of inositol triphosphate (IP_3) is one such documented system (Fig. 9b; Ref. 76). There is inferential evidence that the enzyme catalyzing this reaction could be activated by a GTP binding protein functionally

analogous to that in the rod. Such considerations coupled with recent experimental evidence that PI turnover in retina is stimulated by light,[77] and that intracellularly injected IP_3 can mimic the light response in invertebrate photoreceptors,[78] prompts our speculation that this or a similar mechanism may play a part in the process of visual transduction.

6. CONCLUDING REMARKS

The boundary function of visual transduction suggested by electrical response measurements is clearly nonlinear. The membrane voltage changes exhibit kinetics which in fact integrate a huge array of infrastruc-

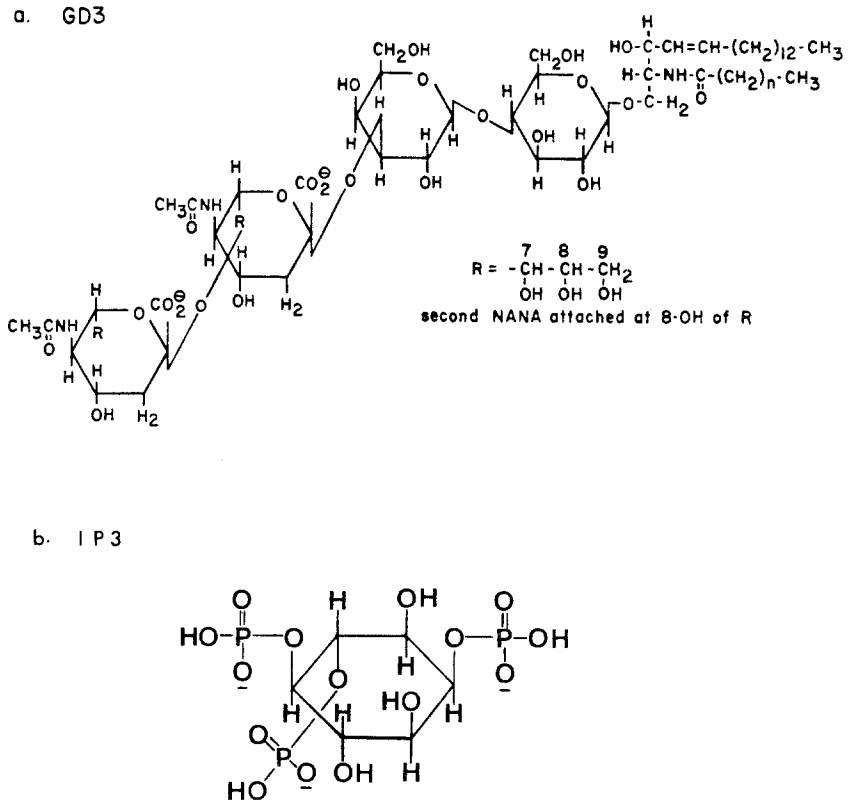


Fig. 9. Membrane lipid materials potentially involved in Ca^{2+} transport. (a) Structural formula of the ganglioside GD_3. Terminal carbohydrate moieties are N-acetyl Neuraminic Acid (NANA). Adjacent carboxyl groups are thought to form the Ca^{2+} binding site. (b) Inositol 1, 4, 5-triphosphate. Inositol is normally attached to lipid by a phosphate ester linkage at carbon 1 which is cleaved by certain phospholipids. Other phosphates may be added before or after carbohydrate liberation.

tural enzymatic and ionic processes which are also nonlinear. Our present understanding of these processes is attended by a level of uncertainty which renders modeling a formidable task which is undertaken with a large burden of ambiguity.

A number of questions need to be addressed in order to solidify the data base upon which a comprehensive model can be assembled:

There is an extraordinary amplification between the processes of photoisomerization of rhodopsin and phosphodiesterase activation. This amplification largely resides in the gain associated by the formation of $G\alpha GTP$ complexes by bleached rhodopsin and the turnover number for PDE. To what extent is this gain counteracted by the kinetics of the turnoff process? To what extent are protons generated by phosphodiesterase activation passively buffered in the cytoplasm? The task of modeling calcium transport and calcium mobilization mechanisms is also complicated by the fact that all of the mechanisms for calcium mobilization by photons are not yet unambigously identified. While it appears clear that phosphodiesterase activation may function as one of the principal mechanisms of calcium release from the disks, the role of phosphotidylinostol metabolism and other potential cellular calcium mobilizing techniques have yet to be defined.

Moreover, there are intriguing data which suggest that there is some hysteresis in the coordination of the turnoff of phosphodiesterase and the recovery of the membrane current. Transmembrane voltage measurements, coupled with small cGMP injections in rods of the gecko suggest that while membrane voltage is returning to the preexcited state, phosphodiesterase has not yet been inactivated.

The role of calmodulin in the modulation of phosphodiesterase, and potentially the modulation of Ca^{2+} uptake or of the sodium channels by calcium, needs further definition. Kinetics of replacement (by synthesis) of cyclic GMP following its hydrolysis by the light-activated phosphodiesterase and mechanisms controlling the overall dynamics of the guanylate cyclase (the cyclic GMP synthesizing enzymes) of rod outer segments are still poorly understood.

The photoreceptor system represents an extremely sensitive, rapidly responding, large gain, finely modulated system which has evolved to an extraordinary degree of specialization and efficiency. It is, in effect, a single quantum counter. However, it is not yet possible to integrate all the component mechanisms into a mathematical construct which adequately predicts both the processes of excitation and adaptation, as well as recovery.

The increasing flow of information concerning the biochemical components of the visual transduction apparatus is an encouraging trend and

suggests that data for the complete modeling of this system will soon be forthcoming. It also appears likely that even in the absence of a complete data base the dialogue between theoretical and experimental investigations will be mutually profitable, as experiments suggest models that in turn suggest new, more sharply focused questions.

NOTE ADDED IN PROOF

Recent results have raised new questions about whether Ca^{2+} is the primary cytoplasmic transmitter modulating the photoreceptor dark current. Voltage clamp studies of isolated membrane patches by Fesenko *et al.* (*Nature* (*London*) **313**:310–313 (1985)) and Nakatani and Yau (*Biophys. J.* **47**:356a (1985)) have demonstrated a conductance in the ROS plasma membrane similar in several respects to the light-sensitive conductance. This conductance appears directly modulated by cGMP—a possibility discussed in this paper. The patch clamp studies found no evidence of regulation of the conductance by Ca^{2+}.

There are significant caveats for interpretation of these studies. Measured conductances are 2 to 4 orders of magnitude higher than expected from electrical measurements of intact cells. This suggests that *free* cGMP may be a minuscule portion ($0.1 - 0.01\%$) of the total ROS pool. This might also reflect loss of other (Ca^{2+} dependent?) modulators of the dark conductance. Soluble proteins (e.g. calmodulin), nucleotides, or other molecules involved in such a hypothetical regulatory system would be lost in the isolated patch experiments.

If Ca^{2+} exerts its effects on the dark current by changing cellular cGMP levels, the relevant control mechanisms are more effective and faster than previously suspected. In any case, the new evidence reinforces the conclusion that the visual response is a product of the interactive molecular systems regulating Ca^{2+} and cGMP in the photoreceptor cytoplasm.

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